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Investigating the relationship between rheumatoid factor and anti-cyclic citrullinated peptide with FoxP3 gene expression in patients with rheumatoid arthritis

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ABSTRACT

Introduction: Rheumatoid arthritis (RA) is a chronic autoimmune disorder that affect 1-2% of people worldwide. Inflammation is an important factor in the pathogenesis of RA. Anti- Cyclic Citrullinated Peptid (Anti-CCP) antibody and Rheumatoid Factor (RF) are autoantibodies that promotes inflammatory reactions and have a crucial role in RA pathogenesis. T_{reg} cells are necessary for the maintenance of immune homeostasis and prevention of autoimmunity. FoxP3 is essential transcription factor for development of these regulatory cells. In this study, we surveyed the effects of FoxP3 gene expression in peripheral blood on plasma levels of Anti-CCP and RF.

Methods: Peripheral blood samples were collected from 47 patients and 44 healthy subjects. Then plasma levels of Anti-CCP have been evaluated using ELISA method. Also RF was detected with latex agglutination test, and gene expression of FoxP3 analyzed by real time PCR.

Result: The amount of Anti-CCP and RF were significantly higher in our patients in comparison with healthy subjects ($P < 0.001$) and ($P < 0.001$) respectively. Also significant reverse correlation between RF and Anti-CCP with gene expression of FoxP3 have been shown in our study (r : -0.630, r : -0.584) respectively. The sensitivity and specificity of Anti-CCP and RF was (89.1%, 86.95%) and (91.3%, 91.1%) respectively for the diagnosis of RA.

Conclusion: Our data illustrated that FoxP₃ gene expression have reverse significant correlation with plasma concentration of anti-CCP and RF.

Introduction

Rheumatoid arthritis (RA) is one of the chronic inflammatory autoimmune diseases that symmetrically affects the small joints of the individual. About one percent of the world's population is suffering from the disease, with

women having a larger share of it (1-3). Autoimmune diseases, such as rheumatoid arthritis, occur as a result of a collapse in the balance between regulatory and functional T cells of immune system. When reactions occur in the immune system, these reactions intensify by activating inflammatory mediators. Finally, immune regulatory cells act to the point where they

return the body to homeostasis and balance situation (4, 5). The regulatory T cells have different characteristics, including the FoxP3 transcription factor. The expression of this factor leads to the transformation of naive T-cell into regulatory cells (6, 7). In rheumatoid arthritis, inflammation does not return to normal due to the reduction in the number and function of regulatory cells and the person faces with both intra-articular and extra-articular manifestations as complications resulting from it. Thus, cellular and humoral immunity have somehow contributed to this disease, so that the humoral immune system contributes significantly due to the production of autoantibodies (8). Studies have shown that mice with defects in FoxP3 have uncontrollably increased production of the plasma cell and T follicular helper (Tfh) (5, 9).

The diagnosis and identification of rheumatoid arthritis is based on clinical symptoms and laboratory variables, including laboratory factors such as measuring rheumatoid factors (RF) and anti-CCP antibodies (10, 11). Rheumatoid factor is an IgM antibody against individual defective IgGs while anti-CCP antibodies are anti citrullinated proteins. In fact, normal proteins in the body get abnormal by altering the arginine amino acid to citrulline and the immune system recognizes the new citrillinated proteins as foreign substances and produces antibodies against them. Therefore, produced antibodies in the patient's joints accumulate and result in activation of the complement system and inflammation (12, 13). As the disease progresses, destroying the antigen-antibody complexes becomes harder for the immune system and more of them remain in the joints. With the continuity and survival of these immune complexes, regulatory cells will not be able to control these responses, and the body will not return to homeostasis and balance. One of the characteristics of these regulatory cells is the FoxP3 transcription factor, which, in the process of differentiation of T cells, directs a population of these cells to the regulatory T cells. The number and function of regulatory cells have been altered in autoimmune diseases, such as rheumatoid arthritis, and their ability is reduced in relieving inflammation, which will be the cause of the disease's sustainability and survival (14). Regarding the role of regulatory T cells in inhibiting humoral immune response, this study investigates the effect of FoxP3 gene expression on plasma RF and anti-cpp levels in patients with

rheumatoid arthritis. So that finally the effect of the expression of the FoxP3 gene, an important indicator of regulatory T cells, is to be specified on the production of the mentioned autoantibodies produced by self-reactive B lymphocytes.

Methods

Study design and population

This study is a cross-sectional analytical study. The samples were selected convenience-randomly and over time. Our study population in this study is patients with rheumatoid arthritis who were referred to a specialist physician at the Helal Ahmar Clinic in Kermanshah. The inclusion criteria of patients was rheumatoid arthritis disease confirmation according to the diagnosis of rheumatologist. The patient will be excluded from the study if has one of the underlying conditions, such as lupus, scleroderma, polyomyositis, or diseases in which the direction of immune responses is toward Th1 such as MS, diabetes, etc. Because each of these diseases disrupts the measurement of inflammatory factors. Patients approved by the physician based on the American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR). So, after examining these characteristics and criteria, 47 patients with rheumatoid arthritis (40 women, 7 men with an average age of 50.81 ± 12.19), were selected along with 45 healthy subjects that were matched for age and sex. After selecting individuals and obtaining written consent form from them, their blood samples were taken in an amount of 5 ml in EDTA-containing tubes. Finally, the plasma was isolated and stored at -70°C until the reaction time.

Agglutination and ELISA

The plasma level of RF was measured with agglutination kit (Sinagen Co., Iran) and ELISA test for anti-CCP measurements using a kit (Euro Immune Company, Germany) and its results were obtained using the device ELISA reader STAT FAX 4200.

RNA extraction and cDNA synthesis

RNA isolation was performed immediately after sampling due to its sensitivity using RNAXPLUS kit instruction (Sinagen Co., Iran) and each sample was divided to different vials after extraction. It should be noted that the quality and quantity of extracted RNA were measured and confirmed

respectively using gel electrophoresis and Nano drop methods. Then cDNA synthesis was performed using the ROCHE kit protocol.

FoxP3 gene expression assay

Real-time PCR is used to check the expression of the FoxP3 gene expression, so that the primer should be designed for the gene first. (In this study, primers are designed with high accuracy and verified with reputable sites such as <https://eu.idtdna.com/calc/analyzer>, <https://www.ncbi.nlm.nih.gov>

<http://www.cmbn.no/tonjum/oligocalculator3.26/OligoCalc.html>). Housekeeping gene was selected based on the articles on the β -actin gene. samples were tested as duplicates both in the patient group and in healthy subjects, Using the SYBER Green master Mix (TAKARA Co.) and Roche Life Science Light Cycler® 96 device. Finally, with the obtained Cts and the calculations, the comparison was made between the two groups.

Statistical analysis

Statistical analysis of this study was performed using SPSS software version 16. Initially, KS test was used to identify if variables are normal. Also, based on the fact that the desired variables have normal or abnormal distribution, Spearman and Mann-Whitney U tests were used to examine the relationship between variables. A significant level is considered less than 0.05.

Results

A total of 47 patients with rheumatoid arthritis with mean age and standard deviation (50.81 ± 12.19) were selected along with 45 healthy volunteers with mean age and standard deviation (46.34 ± 8.88) by expert physician. In this study, the plasma level of RF and Anti-CCP was significantly correlated with FoxP3 gene expression inversely ($P < 0.001$) and respectively they had correlation coefficient (RF, $r: -0.630$ * Anti-CCP, $r: -0.584$). The inverse relationship means that as the disease progresses and its inflammatory conditions intensifies, the number and function of the regulatory cells, which expression of the FoxP3 gene is their representative in this study, have decreased. This causes the disturbed of balance between the

regulatory and functional cells and the inflammatory conditions remain stable. Thus, in patients whose disease has been confirmed by RF and anti-CCP testing, this reduction in expression occurs in the gene. So, whatever the disease stabilizes, the amount of regulatory cells and expression of their genes will be reduced. According to Table 1, the levels of RF, Anti-CCP and Foxp3 had a significant difference in the patients group compared to the control group ($P < 0.001$). In fact, when the values of these variables are compared to healthy people who have normal level of them, the effect of the disease on these variables is confirmed. As shown in Table 2, there are no significant statistical differences between variables such as ESR : ($P = 0.292$), RF ($P = 0.908$) and Anti-CCP ($P = 0.492$) between men and women. This means that gender does not affect the variables and has not made a significant difference in this study. In Figs. 1 and 2, the relationship between these two factors (RF, Anti-CCP) and the expression level of FoxP3 gene was investigated, which based on it the gradient of the graph is negative and downward. This negative gradient indicates that the higher the amount of gene expression has been increased, the gene expression has been decreased.

Discussion

The present study showed that the plasma level of RF anti-RF antibody and Anti-CCP autoantibodies increased in patients with rheumatoid arthritis compared to healthy subjects. There is also a reverse and significant relationship between these markers and the level of expression of the FoxP3 gene. In fact, these results are consistent with other studies that showed that mice with defective FoxP3 gene have a higher serum level autoantibody such as RF and anti-CCP (8). Reduced expression of FoxP3, which results in downfall of function and differentiation of T cells, can increase the production of RF and anti-CCP in two ways. One is loss of tolerance of the self-reacting T helper (Th) cells and the help of these cells to B-auto reactive cells to produce these two auto anti-body agents and the other one is loss of direct inhibition of T_{reg} cells on specific autoreactive B cells of the citrullinated peptides, and the Fc portion of the IgG molecule that respectively produces anti-CCP and RF autoantibodies.

Table 1: Clinical and laboratory characteristics of the studied subjects

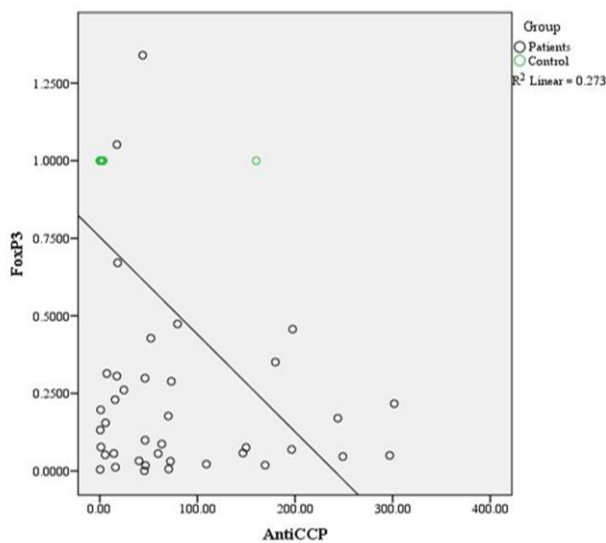
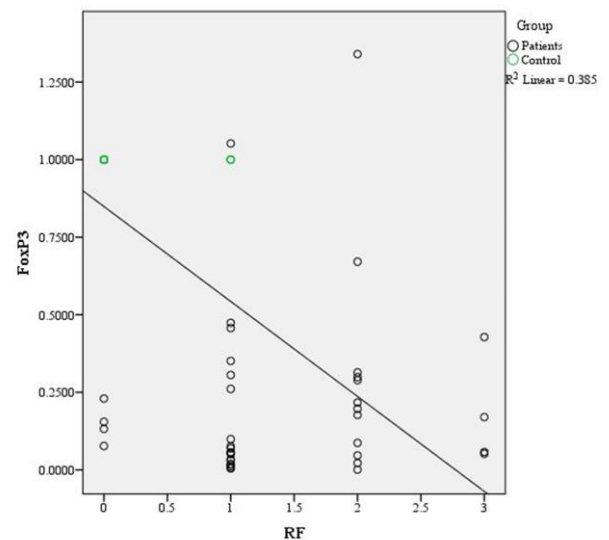
Variable	RA	Control	P value
Age	50.81±12.19	46.34±8.88	0.054 ^a
RF	0(0-1)	1(1-3)	<0.001 ^b
Anti-CCP(RU/mL)	61.71	26.30	<0.001 ^b
Foxp3	22.15	58.50	<0.001 ^b

Mean ± SD; Median (first quartile-third quartile). Data compared by ^aIndependent samples *t*-test; ^bMann-Whitney U

Table 2: Comparison of clinical features based on gender

Variable	Female	Male	P value
Age (year)	49.68±11.63	57.29±14.21	0.129 ^a
Disease duration	8±5.03	7.43±4.96	0.783 ^a
ESR(mm/h)	21.93±15.86	15.14±13.12	0.292 ^a
RF	1(1-2)	1(1-2)	0.908 ^b
Anti-CCP(RU/mL)	46.05	70.6	0.492 ^b

Mean ± SD; Median (first quartile-third quartile). Data compared by ^aIndependent samples *t*-test; ^bMann-Whitney U

**Figure 1:** The relationship between RF plasma level and FoxP3 gene expression**Figure 2:** Relationship between Anti-CCP Plasma Level and FoxP3 Gene

Studies show that in patients with rheumatoid arthritis, not only the number and function of T CD4+ CD25+ FoxP3+ cells are reduced, but also

suppressing potential of them is reduced, which leads to the continuation of the disease and its chronicity. This decrease is not only observed in

rheumatoid arthritis, is also observed in lupus and other autoimmune diseases (15, 16). This reduction is compensated by the treatment of anti-CD4 monoclonal antibodies on mice and has led to the prevention and protection of chronicity of disease and its progress (17). In the present study, in consistent with other studies showed FoxP3 gene expression decreased significantly in the patients group than in the control group. This decrease has been seen in other autoimmune diseases, such as MS (18, 19). Due to the reduction in the number and function of regulatory T cells in autoimmune diseases, especially rheumatoid arthritis, this decrease seems normal, as the inflammation increases in synovial fluid and in other areas, the level of regulatory cells decreases and the disease will continue (20, 21).

Also, the results of this study showed that the sensitivity and specificity of these two factors were 93.1, 91.1 for RF, 89.1 and 86.95 for anti-CCP, respectively. These results, in consistent with other studies, show the diagnostic importance of these two markers in rheumatoid arthritis, which their assessment is necessary as two complementary factors, and each one alone is not sufficient for diagnosis (22). Finally, the present study showed that the level of auto antibodies of RF and Anti-CCP statistically significant and reverse relation with expression of FoxP3. Due to reduced expression of the FoxP3 expression, the differentiation and expansion of the regulated T cells are impaired and with increasing the activity of self-reactive B lymphocytes, the production of RF and Anti-CCP auto antibodies, which are indicators of humoral immunity, has increased that these processes are in line with chronicity of rheumatoid arthritis.

Conclusion

The results of this study showed that plasma levels of RF and Anti-CCP autoantibodies and FoxP3 expression have a statistically significant and inverse relationship in patients with rheumatoid arthritis. In fact, as the disease and its inflammatory conditions intensify, the number and function of the regulatory cells (T_{reg}) decreases, which leads to chronicity and continuation of disease and slowing down the treatment process. In order to help the physician to diagnose and treat the patient faster, it is suggested that more studies be done with a larger sample size and many other methods to help with further certainty and

generalizability to the progression and development of this pathway.

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Ethical disclosure

Before performing the research, it was explained to the participants. An informed consent was obtained from all participants included in the study.

Author Contributions

Nasrin Iranshahi was responsible for all the practical steps, tests and writing. Seyed Mojtaba Amiri performed statistical analysis in this study. Parisa Zafari was involved in the sampling of this project, and Dr. Mahdi Taghaddosi, who was supervisor and leader project.

Conflict of interest

The authors declare that they have no conflict of interest.

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