Sudan Journal of Medical Sciences
Volume 19, Issue no. 3, DOI 10.18502/sjms.v19i3.16171
Production and Hosting by KnE Publishing

Research Article

The Effect of Disease-modifying Antirheumatic Drugs (DMARDs) on the Expression and Methylation Status of the *FOXO1* gene in Newly Diagnosed Patients with Rheumatoid Arthritis

Shirin Assar[1,†], Seyed Askar Roghani[1,2,3,†], Ramin Lotfi[4,5,6,†], Seyedeh Zahra Shahrokhvand[3], Bahareh Kardideh[2], Kheirollah Yari[2], Bijan Soleymani[2], Fatemeh Khademi[2,*]

[1]Clinical Research Development Center, Imam Reza Hospital, Kermanshah University of Medical Sciences, Kermanshah, Iran
[2]Medical Biology Research Center, Health Technology Institute, Kermanshah University of Medical Sciences, Kermanshah, Iran
[3]Immunology Department, Faculty of Medicine, Kermanshah University of Medical Sciences, Kermanshah, Iran
[4]Blood Transfusion Research Center, High Institute for Research and Education in Transfusion Medicine, Tehran, Iran
[5]Clinical Research Development Center, Tohid Hospital, Kurdistan University of Medical Sciences, Sanandaj, Iran
[6]Lung Diseases and Allergy Research Center, Research Institute for Health Development, Kurdistan University of Medical Sciences, Sanandaj, Iran
[†]The first three authors contributed equally to this work.

Corresponding Author:
Fatemeh Khademi; email:
fkhademi60@gmail.com;
fatemeh.khademi@kums.ac.ir

Received: 23 April 2024
Accepted: 11 June 2024
Published: 30 September 2024

Production and Hosting by KnE Publishing

Editor-in-Chief:
Prof. Nazik Elmalaika Obaid Seid Ahmed Husain, MD, M.Sc, MHPE, PhD.

Abstract

Background: The expression of forkhead box O (FOXO) was found to be connected with developing rheumatoid arthritis (RA), an inflammatory autoimmune disorder. The current study is intended to assess the expression and methylation status of the *FOXO1* gene in individuals with recently diagnosed RA, before and after the administration of customary disease-modifying antirheumatic drugs (DMARDs).

Methods: Twenty participants were investigated in this study. The assessment of the *FOXO1* gene expression in peripheral blood was done by real-time PCR, and the status of *FOXO1* promoter methylation was ascertained via quantitative methylation-specific PCR (Q-MSP) before and after the administration of DMARDs for six months.

Results: Following DMARDs treatment, the study discovered a decrease in *FOXO1* gene expression. However, the decline did not meet the criteria for statistically meaningful ($P = 0.087$). The expression of the *FOXO1* gene was positively correlated with RA disease activity pre- and post-treatment with DMARDs ($P = 0.009$, $r = 0.567$ and $P = 0.001$, $r = 0.656$, respectively). Moreover, the study showed no alterations in the amount of DNA methylation of the *FOXO1* promoter in newly diagnosed RA patients who had not yet received DMARDs, as compared to DMARDs-treated RA patients.

Conclusion: Altogether, this study suggests that DMARDs treatment may reduce *FOXO1* gene expression, potentially helping to alleviate the pro-inflammatory effects associated with this gene.

Keywords: antirheumatic agents, disease activity, *FOXO1*, DNA methylation, rheumatoid arthritis

How to cite this article: Shirin Assar[†], Seyed Askar Roghani[†], Ramin Lotfi[†], Seyedeh Zahra Shahrokhvand, Bahareh Kardideh, Kheirollah Yari, Bijan Soleymani, Fatemeh Khademi* (2024) "The Effect of Disease-modifying Antirheumatic Drugs (DMARDs) on the Expression and Methylation Status of the *FOXO1* gene in Newly Diagnosed Patients with Rheumatoid Arthritis," *Sudan Journal of Medical Sciences*, vol. 19, Issue no. 3, pages 389–400. DOI 10.18502/sjms.v19i3.16171

1. Introduction

Rheumatoid arthritis (RA) is an autoimmune disorder characterized by joint inflammation. Its exact cause is unknown, but it is believed to be influenced by genetic predispositions, immune dysregulation, and environmental factors [1]. Currently, there is no known cure for RA, but disease-modifying antirheumatic drugs (DMARDs) are the mainstay of treatment. DMARDs can suppress the immune system, slow down the progression of RA, and alleviate symptoms. They can also prevent joint damage [2].

Forkhead box O (FOXO) transcription factors, members of the forkhead proteins family, play a crucial role in various biological functions such as cell respiration, organ development, and apoptosis. Insulin and insulin-like growth factors are regulated by these transcription factors [3]. There are several subtypes of FOXO, including FOXO1, FOXO3, FOXO4, and FOXO6 [4]. Research suggests that FOXO factors are important for regulating immune and lymphocyte activity [5]. Studies using *FOXO* gene knockout mice have shown that FOXO1 is necessary for maintaining normal lymphocyte numbers [6, 7]. FOXO1 is highly expressed in B cells, T cells, and ovaries, and it has been linked to various biological activities including cell growth, tumor control, metabolism, and response to oxidative stress [8, 9]. Furthermore, previous studies have indicated that FOXO proteins have a pro-inflammatory effect [10–12]. FOXO1, in particular, has been found to promote the release of the pro-inflammatory cytokine interleukin 1 (IL-1) from macrophages [10].

Epigenetic changes, specifically DNA methylation, have been implicated in the development of RA [13]. DNA methylation is a process that involves adding a methyl group to specific regions of DNA, which can impact gene expression and transcription [14–16]. DNA methyltransferases are the enzymes responsible for this process [17, 18]. Variations in DNA methylation have been associated with various human disorders, including the development of tumors [19]. In RA patients, DNA methylation changes have been observed, with hypomethylation of certain genes, like *STAT3*, *WISP3*, *CHI3L1*, *MAP3K5*, and *CASP1*, leading to overexpression and potentially contributing to the occurrence of RA. Hypermethylation of other genes, like *FOXO1* and *TGFBR2*, has also been reported [20]. Research has shown that RA patients have global DNA hypomethylation in their T-cells and monocytes, and changes in DNA methylation occur in B-cells at the onset of the disease [21, 22].

Methotrexate, a medication commonly used to treat RA, works by blocking enzymes involved in DNA production and purine metabolism [23]. It is postulated that methotrexate inhibits DNA methylation, although global DNA methylation has been detected in methotrexate-treated individuals [24–26]. This study aimed to evaluate the effectiveness of DMARDs therapy on the expression of the *FOXO1* gene and the status of its DNA methylation in newly diagnosed RA patients.

2. Materials and Methods

2.1. Methodology and subjects of the research

Following the 2010 categorization criteria of the American College of Rheumatology (ACR) and European League Against Rheumatism (EULAR), blood samples from 20 RA patients who had just received a diagnosis, as well as those taken six months after starting DMARDs (methotrexate [7.5–15 mg/day], hydroxychloroquine [200 mg/day], and

mPRED [5–15 mg/day]) treatment, were collected. Table 1 displays patient demographics and clinical characteristics.

2.2. Determining anti-CCP levels in plasma

The Medizym anti-CCP enzyme-linked immunosorbent assay (ELISA) Kit (Medipan GmbH, Germany) was utilized for determining the plasma levels of IgG antibodies against cyclic citrullinated peptides (CCPs) following the package recommendations by using the STAT FAX 4200 ELISA reader.

2.3. Isolation of RNA and cDNA synthesis

Following the owner's manual, total RNA was isolated from whole blood samples using an RNA extraction kit (RNX PLUS, SinaClon Bioscience, Iran). A Nanodrop 2000 spectrophotometer was used to measure the amount and purity of the isolated RNA (Thermo Scientific, USA). The Parstous commercial kit (Parstous Biotechnology, Iran) was used to synthesize cDNA following the kit's instructions.

2.4. Real-time PCR

Using the IDT Oligoanalyzer (www.idtdna.com/calc/analyser), primers for *FOXO1* and *GAPDH* (as a housekeeping gene) were created (Table 2). Primers were validated for their precision and specificity using the NCBI's Basic Local Alignment Search Tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). A maximum of 20 µl was used for the real-time PCR analysis, consisting of 1.5 μl of cDNA, 0.8 μl of each primer (forward and reverse), 10 μl of PCR master mix (from Parstous Biotechnology, Iran), and 6.9 μl of distilled water. The different heat treatment variables were 95°C for 30 s, 40 cycles of 5 s at 95°C, 30 s at 60°C, and 62°C for *GAPDH* and *FOXO1*, correspondingly; the melting curve was 5 s at 95°C, 15 s at 65°C, and 5 s at 95°C, and continued melting during the PCR processes on the Light cycler 96 (Roche, Switzerland). The Pfaffl formula [27] was used to conduct the analysis that determined the comparative gene expression. To do this, we determined the concentration of target mRNA in each test related to the quantity of *GAPDH* mRNA transcripts present in each sample to use as a housekeeping gene. Three duplicates of each test were carried out.

2.5. DNA extraction and bisulfite treatment

Twenty patients with a recent RA diagnosis had their whole blood processed with EDTA for DNA isolation purposes, and the samples were analyzed before and after six months of therapy with DMARDs [28]. Purified DNA was evaluated quantitatively and qualitatively using spectrophotometry and agarose gel electrophoresis before being stored at –20°C until use. Following the manufacturer-recommended protocol, we converted a measured volume of each DNA sample using an EpiTect Bisulfite kit (Qiagen GmbH, Germany). For each sample, an equal quantity of genomic DNA (1000 ng) underwent bisulfite treatment. Non-methylated cytosines are converted to uracil with this technique, whereas methylated cytosines were left unaltered. A thermal cycler was used to carry out this treatment, and its settings were 95°C for 5 min, 60°C for 10 min, 95°C for 5 min, and 60°C for 10 min. How much converted DNA was present in the samples was determined using a NanoDrop spectrophotometer. Before performing Q-MSPs, purified DNA was kept

at –20°C. A sensitivity assay was accomplished to evaluate the effectiveness of the bisulfite modification. In this assay, untreated DNA was utilized as a template for PCR amplification using a specific set of MSP primers.

2.6. Quantitative methylation-specific PCR (Q-MSP)

Quantitative methylation-specific PCR, also known as Q-MSP, was carried out with the use of BioFact^{TM} 2X Real-Time PCR Pre-Mix (BioFact, Korea). The NCBI database was applied to locate the base sequence of the human *FOXO1* gene promoter. To confirm the presence of CpG islands in the gene promoter region and generate methylated and unmethylated primers for the FOXO1 promoter (as shown in Figure **1** and Table 3), the MethPrimer software available at http://www.urogene.org/cgibin/methprimer/methprimer.cgi was utilized [29]. Two CpG islands were found in the promoter sequence of the *FOXO1* gene using methprimer's search. The first island was 153 bp in size, while the second one was 724 bp. The target DNA for amplification of the PCR was a 174 bp fragment for the methylated target and 175 bp for the unmethylated target, which was located within CpG island 2 (Figure **1**). The Roche Life Science LightCycler® 96 (Roche, Switzerland) was used to perform real-time PCR in an ultimate volume of 20 μl. The mixture included 25 pmol of each methylated/unmethylated primer (forward and reverse), as well as 50–100 ng of bisulfite-treated DNA. An unaltered DNA and an MSP–PCR reaction that did not contain a DNA template were utilized as negative controls for both sets of methylated and unmethylated primers, respectively. A fully methylated DNA (EpiTect, Qiagen) was regarded as a positive control for the methylated primers. The following real-time PCR cycling parameters were used: 95°C for 60 s, 45 cycles of 20 s at 95°C, 30 s at 55°C, and 12 s at 72°C.

2.7. Statistical analysis

GraphPad Prisms® 6.0 and SPSS software version 24.0 (SPSS, Chicago, IL, USA) were used to conduct data analysis and create graphs (GraphPad Software, La Jolla, California, USA). The Kolmogorov–Smirnov (K–S) test for one sample was utilized to check the distribution of the information. Patient outcomes were compared pre- and post-treatment using the Wilcoxon matched-pairs test. Additionally, statistical significance was assumed for variations with *P*-values < 0.05.

3. Results

3.1. Relative mRNA expression level of the FOXO1 gene

Using *GAPDH* as an internal reference, we examined *FOXO1* transcription between DMARD-naive newly diagnosed RA patients and DMARD-treated care recipients. The statistical study indicates a trend toward lower *FOXO1* gene expression following DMARD therapy, though the change was not statistically meaningful ($P = 0.087$) (Figure **2**).

3.2. The level of FOXO1 methylation in patients with RA

The methylation status of the *FOXO1* gene in patients with RA was assessed using genomic DNA that had undergone bisulfate treatment. Using the Q-MSP technique, we compared RA patients (recently diagnosed naive DMARDs patients and DMARDs-treated patients) with respect to the methylation status of the *FOXO1* promoter in vivo. Twenty patients with a recent RA diagnosis and

Table 1: Participant characteristics and demographics.

Variable	Before treatment	After treatment	P-Value
N	20	20	
Age (Y)	50.8±2.68		
Sex (F/M)	14/6		
BMI (Kg/m^2)	27.4±1.2		
AntiCCP-IgG (U/ml)	686.74±182.72	377.51±109.55	0.332
ESR (mm/h)	33.95±3.67	18.95±2.22	<0.01
DAS-28	4.27±0.88	2.54±0.96	<0.0001
Vitamin D	34.89±3.92	38.5±3.5	0.490
Swollen	5.2±1.03	1.35±0.62	<0.001
Tender	5.85±1.1	1.35±0.65	<0.001

All data were presented as the mean value ± SEM.
BMI: body mass index; ESR: erythrocyte sedimentation rate; DAS-28: disease activity score-28; N: Number.

Table 2: The used primer sequences for real-time PCR amplification of a target and reference gene.

Gene	Forward primer (5'>3')	Reverse primer (5'>3')	PCR product size (bp)
GAPDH	GAAACCTGCCAAGTATGATG	AGGAAATGAGCTTGACAAAG	188
FOXO1	TGGACATGCTCAGCAGACATC	TTGGGTCAGGCGGTTCATAC	97

Table 3: Primers used in quantitative methylation-specific PCR (Q-MSP) and their sequences.

Gene	Forward primer (5'>3')	Reverse primer (5'>3')	PCR product length (bp)
FOXO1	**Methylated**		
	GGTACGGAGAAGGGGTTTTTC	**ACGATATCTTTACTAAACGACGT**	174
	Unmethylated		
	GGTATGGAGAAGGGGTTTTTTG	**CCACAATATCTTTACTAAACAACATA**	175

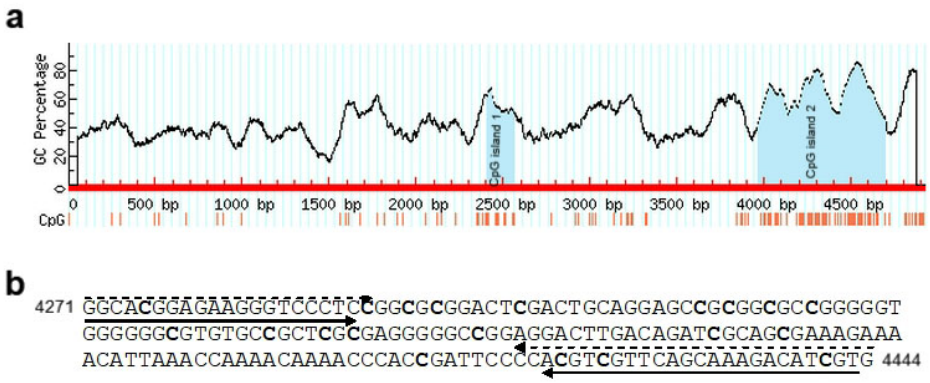

Figure 1: (a) A graphical representation of the CpG islands located in the promoter region of FOXO1, sourced from MethPrimer. The criteria used to identify these islands were a size greater than 100, a GC percentage greater than 50.0, and an Obs/Exp ratio greater than 0.6. (b) A partial sequence of the CpG island 2 in the promoter region of FOXO1 that has been amplified and ranges from 4271 to 4444 (NCBI Reference Sequence: NG_023244.1). The CpG sites that were studied are displayed, with arrows indicating the primer regions (Solid for methylated sequence and dashed for non-methylated sequence).

no prior exposure to DMARDs had unmethylated *FOXO1* promoter regions in their whole blood. The

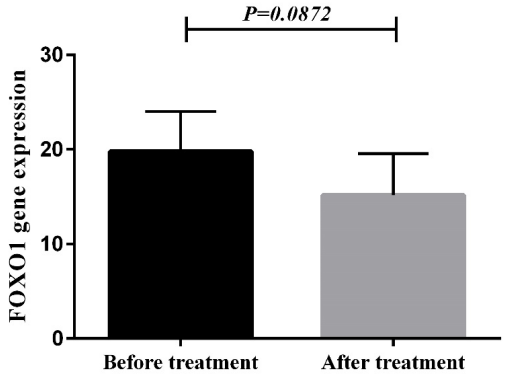

Figure 2: Comparison of baseline and post-treatment levels of *FOXO1* transcription in the peripheral blood of patients with RA. When DMARDs were used, the *FOXO1* gene's transcription level was lower than it was before. Statistical analysis showed that the decline was not significant (*P* = 0.087). Statistical information was displayed using a mean ± standard deviation (SD).

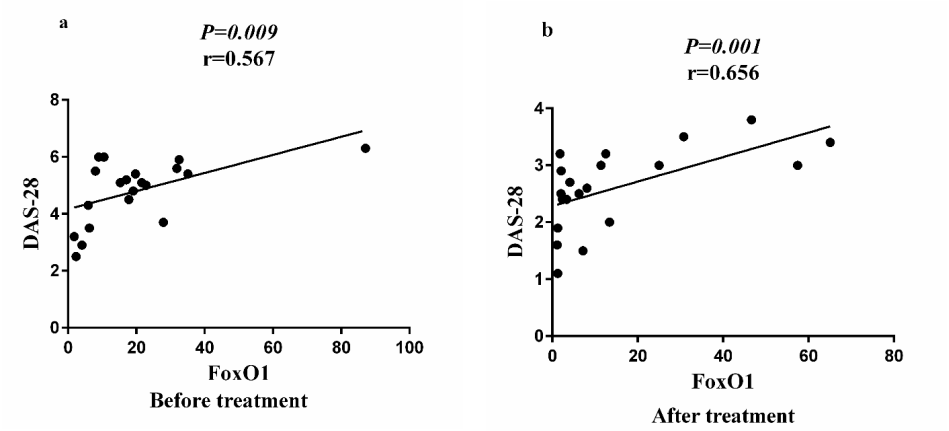

Figure 3: *FOXO1* gene expression positively correlated with disease activity score (DAS)-28 in RA patients both pre- and post-treatment with disease-modifying anti-rheumatic drugs (DMARDs). *P* stands for *P*-value, while r stands for Spearman's rank correlation coefficient.

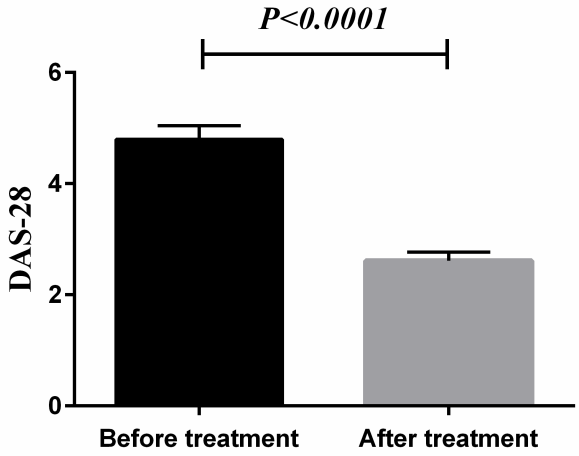

Figure 4: Medication with DMARDs has an impact on RA patients' DAS-28 levels. The rate of DAS-28 decreased significantly in DMARDs-treated RA patients during the six-month follow-up period (*P* < 0.0001). Statistical information was displayed using a mean ± standard deviation (SD).

outcomes of this research likewise revealed that there was no promoter methylation in each of the

20 patients following DMARD therapy. To verify the methylated primers, a fully methylated DNA was applied as a positive control. The amplification of the fully methylated DNA using the methylated primers was successful. As a result, we found no differences in the methylation situation of the *FOXO1* promoter between DMARDs-treated RA patients and recently diagnosed DMARDs-naive RA patients.

3.3. Correlations analysis

FOXO1 gene expression was found to be connected with RA patients' disease activity score (DAS) calculated from a questionnaire measuring disease activity in 28 joints. The findings demonstrated a substantial positive connection between *FOXO1* gene expression and DAS-28 in RA patients both pre- and post-treatment with DMARDs (P = 0.009, r = 0.567 and P = 0.001, r = 0.656, respectively) (Figure **3**). The rate of DAS-28 dropped dramatically in DMARDs-treated RA patients over the six-month follow-up period ($P <$ 0.0001; Figure **4**).

4. Discussion

The engagement of lymphocytes and overall immune system activity are two processes that are heavily influenced by FOXO factors. The expression of the *FOXO* gene is considered to be connected with the development of RA, an autoimmune inflammatory disease [5]. Additional research may be required, though, to determine the precise function that FOXO1 plays in the onset of RA and to determine the impact that DMARDs have on *FOXO1* gene expression and methylation in RA patients. In this study, we ascertained the amount of *FOXO1* gene expression in patients who were DMARDs-naive at the time of diagnosis to those who had been medicated with DMARDs. After receiving DMARD therapy, the *FOXO1* gene expression level decreased. However, this decline was not statistically significant.

FOXOs regulate multiple biological activities, including embryonic development, metabolic activity, modulation of immunological functions, cell growth, tumor suppression, and responses to oxidative stress [30–33]. RA patients' peripheral blood mononuclear cells (PBMCs) have lower *FOXO1* mRNA levels than healthy individuals, according to a small number of investigations, and some have suggested that FOXO proteins may promote inflammation. One analysis revealed that compared to normal controls, PBMC *FOXO1* expression levels were considerably lower in RA patients with current disease activity. They hypothesized that the decreased expression of *FOXO1* would play a role in lowering the activation threshold of lymphocytes, which would contribute to the emergence of RA [5]. Furthermore, the expression of *FOXO1* in RA synovial tissue was found to be inversely linked with disease activity, and mRNA levels of *FOXO1* were found to be decreased in RA peripheral blood, in comparison to healthy donor blood [34]. Contrary to these observations, our investigation demonstrated that *FOXO1* gene expression was positively associated with the disease activity in RA patients pre- and post-therapy with DMARDs. Before DMARDs therapy, the *FOXO1* gene's expression level was higher than after receiving DMARDs therapy, further supporting the notion that FOXO1 plays a pro-inflammatory role in RA before treatment. Prior studies confirm the pro-inflammatory activity of FOXO proteins [10–12]. FOXO4 enhances initial chronic inflammation via endothelial arginase 1, according to Zhu *et al.*'s study [12].

Epigenetic modifications in RA have attracted a lot of attention recently as they relate to the etiology of RA [13, 20]. DNA methylation has been the subject of extensive research due to its importance as an epigenetic process [14, 15]. Recent research has characterized alterations to the DNA methylome in various patient cell types and identified several differentially methylated genes that may be crucial to the etiology of RA [35]. Karouzakis *et al.*'s study revealed worldwide DNA hypomethylation in RA synovial fibroblasts [36]. It has been demonstrated that widespread gene hypomethylation can lead to gene overexpression, which can then lead to RA [20]. Fibroblast-like synoviocytes (FLS) lines from six RA and five osteoarthritis patients were used in research by Kazuhisa Nakano and coworkers [37]. The results showed hypomethylation in important RA genes including *STAT3*, *WISP3*, *CHI3L1*, *MAP3K5*, and *CASP1*, and hypermethylation in *TGFBR2* and *FOXO1* genes [37]. DNA methylation in the five primary subgroups of blood cells (T, B, and NK cells, monocytes, and polymorphonuclear leukocytes) was investigated in the preliminary phase of RA and one month following therapy with methotrexate in a separate study involving 19 RA patients prior therapy commencement and 17 healthy controls. These results demonstrate that patients with RA have global DNA hypomethylation, which is confined to certain subgroups of blood cells and can be reversed by methotrexate therapy. The levels of methylation-related enzymes (DNMT1, DNMT3A, etc.) also changed in tandem with these modifications [22]. Many DNA methylation-related traits in RA, though, are still poorly understood or debatable. For instance, additional studies are needed to see if DNA methylation anomalies in RA promote the onset and course of the disease or whether they are a byproduct of the sickness. Furthermore, it is important to evaluate the DNA methylation patterns in both the early and late phases of the disease [38].

This research intended to assess the effects of DMARDs medication on the status of DNA methylation in the FOXO1 promoter in individuals with a recent RA diagnosis. To learn about the FOXO1 promoter methylation status in vivo, we performed a Q-MSP analysis. Twenty blood samples from recently diagnosed RA patients were analyzed for methylation before and after therapy with DMARDs, and the results indicated that the FOXO1 promoter region was unmethylated in both sets of samples. As a result, we found no differences in the DNA methylation situation of the FOXO1 promoter between DMARDs-treated RA patients and recently diagnosed DMARDs-naïve patients. As seen in some cancers, hypomethylation in the early stage of RA may be accompanied by hypermethylation. Furthermore, methotrexate is hypothesized to impede methylation of DNA through inhibiting the methionine S-adenosyltransferase and SAM [24, 25]. Thus, the reason for the lack of difference in DNA methylation status between RA patients (before and after treatment) is probably due to DMARDs treatment as a result of inhibition of key enzymes responsible for DNA methylation. Notably,, since we used unsorted lymphocytes for analyzing FOXO1 methylation and gene expression, our findings could be impacted by the proportion and absolute numbers of leukocyte subsets in our specimens, which can be regarded as a limitation of the present study.

A previous study showed that higher global DNA methylation in treating naïve RA patients is linked with diminished clinical response following three months of treatment with methotrexate [39]. Here, we hypothesized that early detection of RA patients with *FOXO1* gene hypomethylation could prevent

reduced clinical response or lack of response to DMARDs due to the inhibition of DNA methylation by DMARDs. However, to clarify this hypothesis, more studies should be conducted in early stage RA patients and more advanced RA patients who are not undergoing treatment and after DMARDs treatment.

5. Conclusion

The present study proposes that DMARDs treatment may reduce *FOXO1* gene expression in the blood sample of RA patients who have been recently diagnosed. Additionally, it shows that in the blood sample of RA patients, the *FOXO1* gene is positively linked with clinical symptoms before and after DMARDs treatment, suggesting that FOXO1 may exert a pro-inflammatory role in RA. Lastly, our analysis of FOXO1 promoter methylation revealed no significant differences between newly diagnosed DMARDs-naïve RA patients and DMARDs-treated RA patients. Further investigations with larger sample sizes are required to decipher the specific role of FOXO1 in the inception of RA and the effect of DMARDs on *FOXO1* gene expression and methylation in RA patients.

Declarations

Acknowledgments

The authors are thankful to the Vice Chancellor for Research and Technology, Kermanshah University of Medical Sciences (990929) for supporting this research. They would like to express their gratitude to all members of the Medical Biology Research Center for valuable discussions and expert technical support. They are also particularly indebted to Dr. Kamran Mansouri for the helpful discussions.

Ethical Considerations

The current study was approved by the ethics committee of the Kermanshah University of Medical Sciences (IR.KUMS.REC.1399.403). Samples were recruited based on the 2010 American College of Rheumatology (ACR) and European League Against Rheumatism (EULAR) classification criteria. All persons who participated in this study signed their informed consent in accordance with the principles of the Helsinki Declarations. The authors confirm that human research participants provided informed consent for the participation in the study and the publication of the manuscript results.

Competing Interests

The authors declare that they have no conflicts of interest.

Availability of Data and Material

The datasets generated during and/or analyzed during the present study are available from the corresponding author upon reasonable request.

Funding

This work was supported by the Vice Chancellor for Research and Technology, Kermanshah University of Medical Sciences (Grant number: 990929).

Abbreviations and Symbols

RA: Rheumatoid arthritis

DMARDs: Disease-modifying antirheumatic drugs

FOXO1: Forkhead box O1

ACR: American College of Rheumatology

EULAR: European League Against Rheumatism

Anti-CCP: Anti-cyclic citrullinated peptide antibody

Q-MSP: Quantitative methylation-specific PCR

PBMCs: Peripheral blood mononuclear cells

ELISA: Enzyme-linked immunosorbent assay

SAM: S-adenosylmethionine

DNMTs: DNA methyltransferases

DAS-28: Disease activity score-28

References

[1] Yarwood, A., Huizinga, T. W. J., & Worthington, J. (2016). The genetics of rheumatoid arthritis: Risk and protection in different stages of the evolution of RA. *Rheumatology (Oxford, England), 55*(2), 199–209. https://doi.org/10.1093/rheumatology/keu323

[2] Lin, Y.-J., Anzaghe, M., & Schülke, S. (2020). Update on the pathomechanism, diagnosis, and treatment options for rheumatoid arthritis. *Cells, 9*(4), 880. Advance online publication. https://doi.org/10.3390/cells9040880

[3] Eijkelenboom, A., & Burgering, B. M. T. (2013). FOXOs: Signalling integrators for homeostasis maintenance. *Nature Reviews. Molecular Cell Biology, 14*, 83–97. https://doi.org/10.1038/nrm3507

[4] Wang, M., Zhang, X., Zhao, H., Wang, Q., & Pan, Y. (2009). FoxO gene family evolution in vertebrates. *BMC Evolutionary Biology, 9*, 222. https://doi.org/10.1186/1471-2148-9-222

[5] Kuo, C.-C., & Lin, S.-C. (2007). Altered FOXO1 transcript levels in peripheral blood mononuclear cells of systemic lupus erythematosus and rheumatoid arthritis patients. *Molecular Medicine (Cambridge, Mass.), 13*, 561–566. https://doi.org/10.2119/2007-00021.Kuo

[6] Hosaka, T., Biggs, W. H., III, Tieu, D., Boyer, A. D., Varki, N. M., Cavenee, W. K., & Arden, K. C. (2004). Disruption of forkhead transcription factor (FOXO) family members in mice reveals their functional diversification. *Proceedings of the National Academy of Sciences of the United States of America, 101*(9), 2975–2980. https://doi.org/10.1073/pnas.0400093101

[7] Furuyama, T., Kitayama, K., Shimoda, Y., Ogawa, M., Sone, K., Yoshida-Araki, K., Hisatsune, H., Nishikawa, S., Nakayama, K., Nakayama, K., Ikeda, K., Motoyama, N., & Mori, N. (2004). Abnormal angiogenesis in Foxo1 (Fkhr)-deficient mice. *The Journal of Biological Chemistry, 279*(33), 34741–34749. https://doi.org/10.1074/jbc.M314214200

[8] Accili, D., & Arden, K. C. (2004). FoxOs at the crossroads of cellular metabolism, differentiation, and transformation. *Cell, 117*(4), 421–426. https://doi.org/10.1016/S0092-8674(04)00452-0

[9] Luo, C. T., & Li, M. O. (2018). Foxo transcription factors in T cell biology and tumor immunity. *Seminars in Cancer Biology, 50,* 13–20. Elsevier. https://doi.org/10.1016/j.semcancer.2018.04.006

[10] Su, D., Coudriet, G. M., Hyun Kim, D., Lu, Y., Perdomo, G., Qu, S., Slusher, S., Tse, H. M., Piganelli, J., Giannoukakis, N., Zhang, J., & Dong, H. H. (2009). FoxO1 links insulin resistance to proinflammatory cytokine IL-1β production in macrophages. *Diabetes, 58*(11), 2624–2633. https://doi.org/10.2337/db09-0232

[11] Nwadozi, E., Roudier, E., Rullman, E., Tharmalingam, S., Liu, H. Y., Gustafsson, T., & Haas, T. L. (2016). Endothelial FoxO proteins impair insulin sensitivity and restrain muscle angiogenesis in response to a high-fat diet. *The FASEB Journal, 30*(9), 3039–3052. https://doi.org/10.1096/fj.201600245R

[12] Zhu, M., Goetsch, S. C., Wang, Z., Luo, R., Hill, J. A., Schneider, J., Morris, S. M., Jr., & Liu, Z.-P. (2015). FoxO4 promotes early inflammatory response upon myocardial infarction via endothelial Arg1. *Circulation Research, 117*(11), 967–977. https://doi.org/10.1161/CIRCRESAHA.115.306919

[13] Viatte, S., Plant, D., & Raychaudhuri, S. (2013). Genetics and epigenetics of rheumatoid arthritis. *Nature Reviews. Rheumatology, 9*, 141–153. https://doi.org/10.1038/nrrheum.2012.237

[14] Issa, J.-P. J., Ottaviano, Y. L., Celano, P., Hamilton, S. R., Davidson, N. E., & Baylin, S. B. (1994). Methylation of the oestrogen receptor CpG island links ageing and neoplasia in human colon. *Nature Genetics, 7*, 536–540. https://doi.org/10.1038/ng0894-536

[15] Esteller, M. (2002). CpG island hypermethylation and tumor suppressor genes: A booming present, a brighter future. *Oncogene, 21*, 5427–5440. https://doi.org/10.1038/sj.onc.1205600

[16] Bird, A. (2002). DNA methylation patterns and epigenetic memory. *Genes & Development, 16*, 6–21. https://doi.org/10.1101/gad.947102

[17] Vihinen, M., & Mäntsälä, P. (1989). Microbial amylolytic enzymes. *Critical Reviews in Biochemistry and Molecular Biology, 24*(4), 329–418. https://doi.org/10.3109/10409238909082556

[18] Aslani, S., Mahmoudi, M., Karami, J., Jamshidi, A. R., Malekshahi, Z., & Nicknam, M. H. (2016). Epigenetic alterations underlying autoimmune diseases. *Autoimmunity, 49*(2), 69–83. https://doi.org/10.3109/08916934.2015.1134511

[19] Liu, C.-C., Fang, T.-J., Ou, T.-T., Wu, C.-C., Li, R.-N., Lin, Y.-C., Lin, C.-H., Tsai, W.-C., Liu, H.-W., & Yen, J.-H. (2011). Global DNA methylation, DNMT1, and MBD2 in patients with rheumatoid arthritis. *Immunology Letters, 135*(1–2), 96–99. https://doi.org/10.1016/j.imlet.2010.10.003

[20] Miao, C. G., Yang, Y. Y., He, X., & Li, J. (2013). New advances of DNA methylation and histone modifications in rheumatoid arthritis, with special emphasis on MeCP2. *Cellular Signalling, 25*(4), 875–882. https://doi.org/10.1016/j.cellsig.2012.12.017

[21] Glossop, J. R., Emes, R. D., Nixon, N. B., Packham, J. C., Fryer, A. A., Mattey, D. L., & Farrell, W. E. (2016). Genome-wide profiling in treatment-naive early rheumatoid arthritis reveals DNA methylome changes in T and B lymphocytes. *Epigenomics, 8*(2), 209–224. https://doi.org/10.2217/epi.15.103

[22] de Andres, M. C., Perez-Pampin, E., Calaza, M., Santaclara, F. J., Ortea, I., Gomez-Reino, J. J., & Gonzalez, A. (2015). Assessment of global DNA methylation in peripheral blood cell subpopulations of early rheumatoid arthritis before and after methotrexate. *Arthritis Research & Therapy, 17*, 233. https://doi.org/10.1186/s13075-015-0748-5

[23] Brown, P. M., Pratt, A. G., & Isaacs, J. D. (2016). Mechanism of action of methotrexate in rheumatoid arthritis, and the search for biomarkers. *Nature Reviews. Rheumatology, 12*, 731–742. https://doi.org/10.1038/nrrheum.2016.175

[24] Wang, Y.-C., & Chiang, E.-P. I. (2012). Low-dose methotrexate inhibits methionine S-adenosyltransferase in vitro and in vivo. *Molecular Medicine (Cambridge, Mass.), 18*, 423–432. https://doi.org/10.2119/molmed.2011.00048

[25] Nesher, G., & Moore, T. L. (1990). The in vitro effects of methotrexate on peripheral blood mononuclear cells. Modulation by methyl donors and spermidine. *Arthritis and Rheumatism, 33*(7), 954–959. https://doi.org/10.1002/art.1780330706

[26] Kim, Y. I., Logan, J. W., Mason, J. B., & Roubenoff, R. (1996). DNA hypomethylation in inflammatory arthritis: Reversal with methotrexate. *The Journal of Laboratory and Clinical Medicine, 128*(2), 165–172. https://doi.org/10.1016/S0022-2143(96)90008-6

[27] Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research, 29(9)*, e45. https://doi.org/10.1093/nar/29.9.e45

[28] Miller, S. A., Dykes, D. D., & Polesky, H. F. (1988). A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Research, 16*(3), 1215. https://doi.org/10.1093/nar/16.3.1215

[29] Li, L.-C., & Dahiya, R. (2002). MethPrimer: Designing primers for methylation PCRs. *Bioinformatics (Oxford, England), 18*(11), 1427–1431. https://doi.org/10.1093/bioinformatics/18.11.1427

[30] Carlsson, P., & Mahlapuu, M. (2002). Forkhead transcription factors: Key players in development and metabolism. *Developmental Biology, 250*(1), 1–23. https://doi.org/10.1006/dbio.2002.0780

[31] Kaestner, K. H., Knöchel, W., & Martínez, D. E. (2000). Unified nomenclature for the winged helix/forkhead transcription factors. *Genes & Development, 14*, 142–146. https://doi.org/10.1101/gad.14.2.142

[32] Burgering, B. M. T., & Kops, G. J. P. L. (2002). Cell cycle and death control: Long live Forkheads. *Trends in Biochemical Sciences, 27*(7), 352–360. https://doi.org/10.1016/S0968-0004(02)02113-8

[33] Fontenot, J. D., Gavin, M. A., & Rudensky, A. Y. (2003). Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nature Immunology, 4*, 330–336. https://doi.org/10.1038/ni904

[34] Grabiec, A. M., Angiolilli, C., Hartkamp, L. M., van Baarsen, L. G. M., Tak, P. P., & Reedquist, K. A. (2015). JNK-dependent downregulation of FoxO1 is required to promote the survival of fibroblast-like synoviocytes in rheumatoid arthritis. *Annals of the Rheumatic Diseases, 74*(9), 1763–1771. https://doi.org/10.1136/annrheumdis-2013-203610

[35] Lin, Y., & Luo, Z. (2017). Aberrant methylation patterns affect the molecular pathogenesis of rheumatoid arthritis. *International Immunopharmacology, 46*, 141–145. https://doi.org/10.1016/j.intimp.2017.02.008

[36] Karouzakis, E., Gay, R. E., Michel, B. A., Gay, S., & Neidhart, M. (2009). DNA hypomethylation in rheumatoid arthritis synovial fibroblasts. *Arthritis and Rheumatism, 60*(12), 3613–3622. https://doi.org/10.1002/art.25018

[37] Nakano, K., Whitaker, J. W., Boyle, D. L., Wang, W., & Firestein, G. S. (2013). DNA methylome signature in rheumatoid arthritis. *Annals of the Rheumatic Diseases, 72*(1), 110–117. https://doi.org/10.1136/annrheumdis-2012-201526

[38] Karami, J., Aslani, S., Tahmasebi, M. N., Mousavi, M. J., Sharafat Vaziri, A., Jamshidi, A., Farhadi, E., & Mahmoudi, M. (2020). Epigenetics in rheumatoid arthritis; Fibroblast-like synoviocytes as an emerging paradigm in the pathogenesis of the disease. *Immunology and Cell Biology, 98*(3), 171–186. https://doi.org/10.1111/imcb.12311

[39] Gosselt, H. R., van Zelst, B. D., de Rotte, M. C. F. J., Hazes, J. M. W., de Jonge, R., & Heil, S. G. (2019). Higher baseline global leukocyte DNA methylation is associated with MTX non-response in early RA patients. *Arthritis Research & Therapy, 21*, 157. https://doi.org/10.1186/s13075-019-1936-5