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Sestrin2, NFATc1, and NRF2 in PBMCs of patients with ankylosing spondylitis treated with etanercept compared to the new case patients

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Abstract

Background: Ankylosing spondylitis (AS) is an inflammatory autoimmune disease characterized by progressive bone destruction and pathological new bone formation. Sestrin2 is activated in inflammatory and oxidative responses and protects cells from injury. Sestrin inhibits reactive oxygen species (ROS) by activating nuclear factor erythroid 2-related factor 2 (NRF2). Nuclear factor of activated T-cell cytoplasmic 1 (NFATc1), a key regulator of osteoclast differentiation, is induced following stimulation of the receptor activator of nuclear factor kappa-B ligand (RANKL) and promotes bone resorption. Emerging evidence suggests that impaired Sestrin2/NRF2 signaling may lead to increased oxidative stress, thereby enhancing NFATc1 activity and exacerbating bone destruction in AS. This study analyzed the expression of these genes in newly diagnosed AS patients and AS patients receiving etanercept, an anti-tumor necrosis factor (Anti-TNF) drug, compared with a control group.

Methods: The expression levels of Sestrin2, NRF2, and NFATc1 genes were analyzed by real-time PCR in 60 peripheral blood mononuclear cell (PBMC) samples, which were divided into three groups: newly diagnosed AS patients, AS patients receiving etanercept (Etanercept group), and healthy control individuals. Statistical analysis was performed using SPSS version 18 software. A P-value < 0.05 was considered statistically significant.

Results: NRF2 gene expression was increased in the newly diagnosed AS group compared with the control group ($P < 0.001$). It was also increased in the etanercept group compared with the control group ($P < 0.01$). The expression levels of the other two genes (SESN2 and NFATc1) in the etanercept group were higher than those in both the newly diagnosed and control groups; however, these differences were not statistically significant ($P > 0.05$).

Conclusion: The expression levels of genes involved in the regulation of inflammation increased following treatment with etanercept. These results suggest that, in addition to its inhibitory effects on the TNF-α pathway, etanercept may also influence the expression of genes involved in the control of inflammatory processes.

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Introduction

Ankylosing spondylitis (AS) is an inflammatory (1) and autoimmune disease (2) that belongs to the group of spondyloarthropathies (2). Men in their third decade of life are more commonly affected by AS (1). The disease primarily affects the axial joints, such as the sacroiliac joints, and adjacent soft tissues (1,2). As the disease progresses, chronic inflammation may lead to fibrosis and calcification, ultimately resulting in loss of spinal flexibility and the development of a rigid "bamboo spine" (2).

Sestrin2, encoded by the SESN2 gene (3) and also known as Hi92 (4), is a highly conserved (4) multifunctional protein. Sestrin is activated in response to DNA damage, oxidative stress, hypoxia (5), and inflammatory responses (3), and has recently been identified as an antioxidant molecule (6). Sestrins protect cells from injury by reducing reactive oxygen species (ROS) activity and regulating mammalian target of rapamycin (mTOR) signaling (7). Sestrin2 reduces oxidative stress by inhibiting nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity. Sestrin also plays a role in immune system regulation (3).

Sestrin reduces ROS levels by activating NRF2 (4). NRF2 is a major transcription factor (8) and an important endogenous antioxidant regulator involved in the oxidative stress response (5). Under normal conditions, NRF2 is inhibited in the cytoplasm by Keap1 (Kelch-like ECH-associated protein 1) (8). When oxidative stress occurs (5), NRF2 dissociates from Keap1 and translocates to the nucleus (5,8), where it binds to the antioxidant response element (ARE) (5). The NRF2/ARE signaling pathway plays an important role in protecting cells from damage (9) and contributes to the antioxidant response in diseases associated with oxidative stress (9).

The interaction between Sestrin2, NFATc1, and NRF2 forms a critical regulatory axis in inflammatory bone diseases such as AS. Sestrin2, a stress-inducible protein, activates NRF2, a master regulator of antioxidant responses, thereby reducing oxidative stress through the neutralization of ROS. In turn, reduced oxidative stress via the Sestrin2/NRF2 pathway may suppress NFATc1, a transcription factor activated by RANKL that promotes osteoclast differentiation and bone resorption. Consequently, impairment of Sestrin2/NRF2 signaling may lead to elevated ROS levels, enhanced NFATc1 activity, and excessive bone destruction. This regulatory axis highlights the protective role of Sestrin2 in maintaining bone homeostasis by linking antioxidant defense (NRF2) to inflammation-mediated bone loss (NFATc1) (4,5,8).

Bone remodeling is a dynamic process involving bone formation by osteoblasts and bone resorption by osteoclasts (10). When this balance is disrupted, bone disease may occur (10). The receptor activator of nuclear factor kappa-B ligand (RANKL) and its receptor RANK are essential for osteoclast differentiation. The interaction between RANKL and RANK activates tumor necrosis factor receptor-associated factor 6 (TRAF6), a member of the TRAF family involved in inflammatory and immune signaling pathways. TRAF6 triggers downstream signaling cascades that induce NFATc1 expression and promote bone resorption (10). NFATc1 is induced by RANKL stimulation, and its activation directly promotes osteoclast formation (11). Sestrin has also been reported to influence RANKL–RANK–TRAF6 signaling (10).

Methods

Patients

In this case-control study, 60 patients aged 20 to 60 years were divided into three groups: Twenty patients with ankylosing spondylitis receiving etanercept; 20 patients with ankylosing spondylitis who did not receive anti-TNF drugs, based on clinical conditions and their physician's decision; and 20 individuals who were considered as the control group. The patients were selected according to the modified New York criteria (1984) (12).

Sample collection and real-time PCR

Five cc of whole blood was collected from each participant into tubes containing anticoagulant. To separate the PBMCs, an equal volume of blood and phosphate-buffered saline (PBS) was mixed and added to a tube containing Ficoll, followed by centrifugation. Total RNA was then extracted using the RNX Plus kit (Cinagen, Iran). The quality and concentration of RNA were assessed using spectrophotometry with a NanoDrop device (DENOVIX, USA). In the next step, 1 µg of total RNA was used for cDNA synthesis (Yekta Tajhiz Azma, Iran). After primer design (Table 1), the expression levels of Sestrin2, NRF2, and NFATc1 genes in all three groups were analyzed by real-time PCR (Applied Biosystems, USA), with the GAPDH gene used as the reference gene.

Statistical analysis

The data were analyzed using SPSS version 18 and GraphPad Prism 9 software. One-way ANOVA and the Mann-Whitney statistical test were applied where appropriate. Results are reported as mean ± SD. Each value represents the average of at least two experimental repetitions. A P-value of less than 0.05 was considered statistically significant

Table 1. The sequences of primers

Gene	Primer sequence (5′→3′)
Sestrin2	F: ACAGCCAAACACGAAGGAGG
	R: GCGAGATCAACAAGTTGCTGG
NRF2	F: TCACACGAGATGAGCTTAGGGCAA
	R: TACAGTTCTGGGCGGCGACTTTAT
NFATc1	F: GCATCACAGGGAAGACCGTGTC
	R: GAAGTTCAATGTCGGAGTTTCTGAG

F: Forward ،R: Reverse

Results

Demographic information

The participants in this study were divided into three groups. The newly diagnosed patient group (20 individuals whose disease was newly diagnosed and who had not received any anti-TNF drugs) had an age range of 24 - 66 years, with a mean age of 41.45 ± 10.97 years. The etanercept-treated group consisted of 20 patients aged 33 - 63 years, with a mean age of 43.2 ± 9.12 years. The control group included 20 individuals with a mean age of 44.35 ± 7.61 years. In both the newly diagnosed group and the etanercept-treated group, 12 participants were men and 8 were women. In the control group, there were 7 men and 13 women.

The relative expression of Sestrin2, NRF2, and NFATc1 genes in PBMCs

Our results in Figure 1 showed that the expression levels of these three genes increased in the etanercept-treated group compared with the newly diagnosed and control groups. Although the expression levels of NFATc1 in the etanercept-treated group increased compared with both the newly diagnosed group ($P < 0.346$) and the control group ($P > 0.294$), these differences were not statistically significant. On the other hand, the newly diagnosed group did not show any significant difference compared with the control group ($P > 0.065$) (Figure 1A).

The expression of the NRF2 gene increased in the newly diagnosed group compared with the control group ($P < 0.001$). It also increased in the etanercept-treated group compared with the control group ($P < 0.01$). In addition, the level of NRF2 gene expression in the newly diagnosed group did not show a significant difference compared with the etanercept-treated group ($P < 0.832$) (Figure 1B).

The expression level of the Sestrin2 gene in the etanercept-treated group was higher than that in the newly diagnosed and control groups; however, these differences were not statistically significant ($P < 0.956$ and $P < 0.629$, respectively). The newly diagnosed group also did not show a significant difference compared with the control group ($P < 0.117$) (Figure 1C).

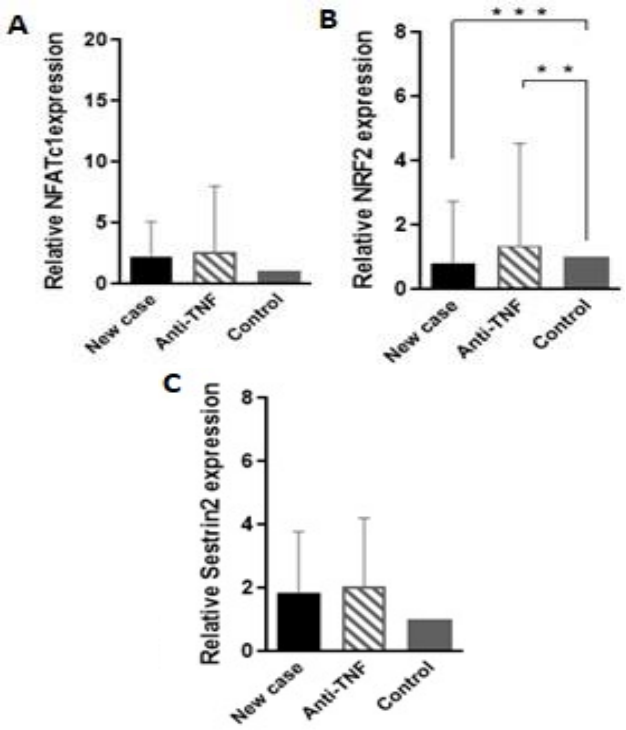

Figure 1. Relative expression levels of NFATc1 (A), NRF2 (B), and Sestrin2 (C) genes in PBMC samples of patients with ankylosing spondylitis (New case and anti-TNF groups) and the control group

Discussion

Ankylosing spondylitis, which belongs to the group of spondyloarthropathies, is an autoimmune disease that mostly affects the joints of the spine, sacroiliac joints (SIJs), and ligaments, as well as tendons (2). Patients may bend forward due to inflammation and ankylosis of the spine. Ossification is the main cause of limitation in vertebral column movement, functional impairment, and long-term consequences of the disease. Spinal stenosis, involvement of peripheral joints, and enthesitis are other features of this disease (13). In more advanced cases, inflammation can cause fibrosis and calcification, leading to loss of flexibility and fusion of the spine (2).

It has been shown that Sestrin2 expression is significantly increased during inflammatory responses to protect against oxidative stress and limit progressive organ damage (3). Conversely, knockout of Sestrin2 is associated with exacerbated inflammatory responses and intensified disease manifestations following oxidative stress (4).

In the present study, we showed that the relative expression of the Sestrin2 gene at the mRNA level was higher in the group receiving etanercept compared with newly diagnosed patients. In a study on patients with septic intestinal dysfunction and in cells induced by lipopolysaccharide (LPS), the expression level of Sestrin2 was decreased. Depletion of Sestrin2 contributed to the inflammatory response in septic intestinal dysfunction by promoting ferroptosis through activation of the AMPK/NRF2 signaling pathway (14). In another study, a novel role of Sestrin2 was shown in modulating the interaction between TRAF6 and p62. This study also revealed that Sestrin2 regulates the activities of NF-κB, MAPKs, and downstream signaling pathways of NFATc1 during osteoclastogenesis, thereby modulating osteoclast differentiation. Accordingly, the expression of NFATc1 in bone marrow monocytes/macrophages was reduced following inhibition of Sestrin2 (10). In a study conducted on patients with atherosclerosis, it was shown that plasma levels of Sestrin2 were elevated in patients with coronary artery disease (CAD) and were related to disease severity. High levels of Sestrin2 in patients with CAD may reflect a protective response against disease progression (15).

Our results also showed that the expression level of Sestrin2, an important gene involved in the regulation of inflammation, was increased in patients treated with etanercept. This finding may indicate that, in addition to inhibiting the TNF pathway, etanercept influences the expression of the Sestrin2 gene, thereby contributing to inflammation control through upregulation of this gene.

We also demonstrated that relative NRF2 mRNA expression was higher in the etanercept-treated group than in newly diagnosed patients. NRF2 is a basic leucine zipper protein responsible for regulating the expression of antioxidant proteins and protecting cells against oxidative damage caused by inflammation and tissue injury (16).

NFATc1 is the most strongly induced transcription factor following RANKL stimulation and is known to play a critical role in the efficient induction of mature osteoclasts (11). In this study, we found that the relative expression of NFATc1 was higher in the etanercept-treated group compared with the other two groups. In a study that investigated the expression levels of osteoclast-specific genes in cells from AS patients compared with healthy donors, the results showed that the expression of these genes, including NFATc1, was significantly decreased in cells collected from AS patients compared with healthy individuals. The authors concluded that the reduced expression of osteoclast-specific genes in circulating progenitors, as well as genes encoding bone-degrading proteins on the 21st day of culture, may indicate decreased osteoclastogenesis in AS, possibly due to a diminished response to osteoclastogenic stimuli (17).

In a study conducted on human apical periodontitis samples and mouse periapical tissues with apical periodontitis, the expression of NFATc1 was analyzed by RT-qPCR, and its expression was increased in both groups (18).

In another study, monocytes isolated from healthy individuals were irradiated with X-ray doses and subsequently differentiated into osteoclasts in vitro under conditions that mimicked the physiological bone microenvironment over a 21-day culture period. Although the frequency of apoptosis was low after irradiation, NFATc1 expression decreased until the end of the culture period (19).

Conclusion

Our results showed that the expression levels of these three genes, which play important roles in the regulation of inflammation, were increased under treatment with etanercept. These findings suggest that, in addition to its inhibitory effects on the TNF-α pathway, etanercept may also influence the expression of genes involved in the control of inflammatory processes.

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Ethical Statement

This study was approved by the Ethics Committee of Golestan University of Medical Sciences (IR.GOUMS.REC.1401.352). All procedures performed were in accordance with the principles of the Declaration of Helsinki and its later amendments.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Author Contributions

Z. H. and K. S. S. designed the experiments. M. A. and Z. F. collected the data. Z. F. performed the experiments. J. A. and M. G. analyzed the data. Z. F., J. A., and M. G. discussed the results and strategy. N. E. and Z. F. wrote and edited the manuscript; Z. H. and K. S. S. supervised and directed the study Z. H., Z. F., and K. S. S. approved the final version of the manuscript to be published. All authors reviewed and approved the final version of the manuscript.

Data aAvailability Statement

Not applicable

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