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The role of A268V exon-7 polymorphism of PPARA in development of axial spondyloarthritis

Aksiyal spondiloartrit gelişiminde PPARA’nın A268V ekson-7 polimorfizminin rolü

Abstract

Objectives: Axial spondyloarthritis (axSpA) is a chronic inflammatory disease that mainly affects the axial skeleton. Peroxisome proliferator activated receptor alpha (PPARA) is an intracellular transcription factor, which play a role in inflammation and osteoblasting activity. This study is designed to investigate the relationship of NG_012204.2:p.Ala268Val polymorphism of PPARA with axSpA risk and its role in disease development.

Methods: This study was conducted with 168 patients and 181 controls. Genotyping was done with MALDITOF. Gene expression level was analyzed by quantitative real time PCR (RT-qPCR). The protein homology models of PPARA were created with ProMod3. Ligand binding dynamics were tested using the AutoDock4 docking program. Statistical evaluations were made with SPSS (ver24) and GeneGlobe.

Results: Our results showed that C>T polymorphism causing NG_012204.2:p.Ala268Val change was associated with disease risk (p=0.024) and T allele increased disease risk 1.7 times (95% CI=1.070–2.594). PPARA expression decreased (p<0.05) in individuals carrying the T allele. We determined that the ligand entry pocket was opened 1.1 Å in the polymorphic PPARA. Polymorphic change caused a decrease in the ligand binding affinity.

Conclusions: Our results provide an important contribution to elucidating the development of axSpA and demonstrate the potential of PPARA as a marker for the diagnosis of axSpA.

Keywords: axial spondyloarthritis; disease risk; inflammation; peroxisome proliferator activated receptor alpha; polymorphism.
arttırdığı (%95CI=1.070–2.594) gösterdi. T aleline taşıyan bireylerde PPARA ekspresyonu azaldı (p<0.05). Polimorfik PPARA’da ligand giriş cepinin 1.1 Å açılığına belirlendi. Polimorfik değişiklik, ligand bağlanma afinitelerinde bir azalmaya neden oldu.

Sonuç: Sonuçlarımız, axSpA’in gelişim mekanizması aydınlatmaya önemli bir katkı sağlar ve PPARA’nın axSpA tanısı için bir belirteç olarak potansiyelini göstermektedir.

Anahtar kelimeler: aksiyal spondiloartrit; hastalıktan enflamasyon; peroksizom çoaydırma; hipertrofik hücrelerin arası nükleus.

Introduction

Axial spondyloarthritis (axSpA) is a polygenic inflammatory disease [1]. Ankylosing spondylitis (AS) is a subtype of axSpA and presents radiographic manifestations [2]. It is known that approximately 6.5 million people worldwide suffer from AS [3]. The axial skeleton is the most severely affected by the disease [4]. New bone formation is important in axSpA. An increasing inflammation aggravate the disease, inducing new bone formation and ankylosis [5]. Inflammation can, over time, cause structural damage to the axial skeleton resulting in restriction of spinal mobility [6]. Other than by imaging, there is no direct test to diagnose axSpA. There is no serologic or blood marker specific for the disease [7]. The mean delay from the onset of symptoms to the diagnosis of axSpA can be as long as 14 years, with longer delays in females [8]. In recent years, studies have reported that the presence of axSpA increases the risk of cardiovascular disease and mortality [9]. Diagnosis of the disease in the early stages, before it causes structural damage and loss of the workforce among young people, is the priority of scientific research [10]. Therefore, there is an urgent need for biological markers to provide early diagnosis of the disease.

PPARA is an intracellular transcription factor, activated by fatty acids, which play a role in inflammation [11]. PPARα regulates in negatively control of pro-inflammatory proteins [12]. The activation of PPARα is related to the transcription of about 100 genes involved in fatty acid oxidation, lipid metabolism and inflammation [13]. PPARα plays multiple regulatory functions, including the control of macrophage activity and inflammation. PPARα ligands significantly reduce levels of pro-inflammatory cytokines (TNF, IL-6 and IL-1) [14, 15]. Studies show that many genetic factors may be associated with the risk of development and prognosis of axSpA [16, 17].

In this study, the association between PPARα, NG_012204.2:p.Ala268Val (rs1042311) polymorphism and disease risk was investigated in patients with axSpA.

Patients and Methods

Study protocol

In the study design, the association between NG_012204.2:p.Ala268Val change and disease risk was shown among case (n=168) and control (n=181) groups. Afterwards, expression levels of PPARα gene from blood tissue of 14 patients (TT) with risk allele and 14 healthy control (CC) were examined. The change induced by NG_012204.2:p.Ala268Val polymorphism in protein structure was modeled. The effect of polymorphic change on PPARα ligand binding affinity was investigated. The study was recruited at Turgut Özal Medical Center of İnönü University, Malatya, Turkey, from 2016 to 2018.

Study groups

This study comprised 168 axSpA patients and 181 controls recruited from the department of Rheumatology. A complete clinical evaluation was performed for all patients. Healthy individuals were selected for control group. Data collection form included information such as gender, age, disease duration. BASDAI (Bath Ankylosing Spondylitis Disease Activity Index) was used to calculate disease activity scores.

SNP selection and genotyping

We retrieved the reference SNPs (rs) identified for PPARα from the NCBI database and identified potential functional SNPs based on the following criteria: (i) located in exon regions; (ii) axSpA has not been associated with disease risk in published genome-wide association studies; (iii) potential functional SNPs identified using the SNPinfo software; (iv) causing a missense change; (v) SNPs located in and around the PPARα ligand binding site. As a result, the rs1042311 polymorphic region was examined in this study.

DNA isolation from peripheral blood samples was performed using Invisorb Spin Blood Mini Kit™ (Catalog Number: 1031100300). To ensure successful multiplex PCR, it was ensured that DNA concentrations were at least 20 ng/µl for each sample. Target polymorphic region (rs1042311- NG_012204.2:p.Ala268Val) were amplified by multiplex PCR using designed primer pairs and probe. The forward and reverse primers sequence were AGGTGGAGTGGTGATAGCCGAGACG and AGGTGGAGTGGCCAGCAGT GAGAGATGGC, respectively. The sequence of single base extension probe was GGGTGCATCCA-GAACCAAGGAG. The reaction was performed as 45 cycles with pre-denaturation at 95 °C for 3 min, denaturation at 95 °C for 30 s, binding at 56 °C for 30 s, elongation at 72 °C for 1 min and final elongation at 72 °C for 5 min. The total volume of reaction was 5 µL with the 2.5 mM of MgCl2, 2 ng of sample DNA, 1 enzyme unit, 0.1 µM of primer and 500 µM of dNTP as the final concentration in PCR. Next, 0.5 µL of Shrimp alkaline phosphatase was added to the samples for dNTP neutralisation after multiplex PCR, and the samples were then incubated...
at 37 °C for 40 min. The samples were further incubated at 85 °C for 5 min for enzyme inactivation. Next, 0.041 µL 1X enzyme, 0.940 µL elongation primer and 0.2 µL termination mixture were added to the samples for the identification of the polymorphic base, and PCR was performed as 42 cycles with pre-denaturation at 95 °C for 30 s, denaturation at 95 °C for 5 s, binding at 52 °C for 5 s, elongation at 80 °C for 5 s and final elongation at 75 °C for 2 min. The genotyping analysis was performed by MALDI TOF.

**Quantitative gene expression analyzes**

The gene expression changes of wild type and polymorphic of PPARA were analyzed by qPCR. Total RNA was isolated from peripheral blood using an RNA extraction kit according to the manufacturer’s instructions (RNaseasy Plus Mini Kit, QIAGEN). RNA was reverse transcribed using a cDNA conversion kit (QIAGEN, RT² HT First Strand Kit, Cat No: 330411). The cDNA in combination with RT2 SYBR® Green qPCR Mastermix (Cat. No. 330529) was used with RT2 qPCR assays. qPCR procedure was performed in a total reaction volume of 25 µL. The Ct cut-off was set to 35. The forward and reverse primers sequence for PPARA were TATCGTCCGGGTGGTT, respectively. The forward and reverse primers sequence for GAPDH were TGGGTGTGACATGAGAA and GCTAAGCAGTTGGTGGTGC, respectively.

For each sample, data of the PPARA were normalized with the GAPDH. In the study, the Ct values belonging to 3 repetitions of the samples were averaged. Ct averages of the GAPDH gene were subtracted from the Ct averages of the target gene and the data were normalized. The 2^ΔΔCT formula was applied to the data of the normalized samples and the obtained raw data were made suitable for the statistical study to be used. In order to determine the difference between gene expression levels between case and control groups, statistical study to be used. In order to determine the difference between gene expression levels between case and control groups, statistical study to be used. In order to determine the difference between gene expression levels between case and control groups, statistical study to be used. In order to determine the difference between gene expression levels between case and control groups, statistical study to be used. In order to determine the difference between gene expression levels between case and control groups, statistical study to be used. In order to determine the difference between gene expression levels between case and control groups, statistical study to be used.

The correlation of NG_012204.2:p.Ala268Val replacement with disease risk was analyzed by logistic regression test (SPSS 24.0, Chicago). Power analysis was perform by using PS Power and Sample Size Program 3.1.2. Hardy-Weinberg equilibrium was evaluated by χ2 analysis. Demographic data were given as mean ± standard deviation and min/max values. “p” value smaller than 0.05 was regarded as statistically significant. The statistical evaluation of the difference between PPARA expression levels between the case and control groups was done with t-test by GeneGlobe application.

**Modeling of polymorphic PPARA**

The homology model of polymorphic PPARA was built based on the target-template alignment using ProMod3 (ver3.1.1) [18]. IK7L (protein data bank) was selected as template. Protein sequence data was edited with MegaX [19]. ProSA and Molprobity were used for structural validation and model of wild type and polymorphic PPARA [20, 21]. Topological differences of wild type and polymorphic PPARA were calculated with the i-Tasser TM-Score [22]. The modeling results were visualized with PyMOL™ (ver2.4.1).

**Protein stability analyzes**

The changes in protein stability was performed using mCSM stability [23], DUET [24], DynaMut2 [25] and SDM [26].

**Docking**

The modeled structures of both wild type and polymorphic PPARA were used as targets and 2-(1-methyl-3-oxo-3-phenyl-propylylamo)3-{4-[2-(5-methyl-2-phenyl-oxazol-4-yl)-ethoxy]-phenyl}-propionic acid (CID 446642) was used as ligands for molecular docking using AutoDock 4.2 [27]. Kollman charges were added to the PPARA wild type and polymorphic homology models. Gasteiger partial charges were applied to the ligands. Docking were performed with a grid dimension of 74 × 92 × 88 (–17.189, –11.029, –5.596) with a grid spacing of 0.33 Å around the binding pocket. Docking simulations were performed Lamarckian genetic algorithm (LGA) [28]. The main selected LGA parameters were 100 runs, 2.7 × 10^7 generations and population size of 300. A maximum of 2.5 × 10^7 energy evaluations was applied for each experiment. The results were clustered according to binding energy scores using a tolerance of 2.0 Å RMSD. Docking results were visualized with Discovery SV (ver20.1, DDS Biovia).

**Statistical analysis**

The study group clinical and demographic data showed that the incidence of the disease in men was found to be 2.8 times higher than in women (p<0.05). The mean age of the patient group was 37.93 ± 11.09 and the mean age of the control group was 39.75 ± 10.42. Data obtained by genotyping in rs1042311 (NG_012204.2:p.Ala268Val) polymorphic region are given in Table 1. It was found that C>T polymorphism causing NG_012204.2:p.Ala268Val change was associated with disease risk (p=0.024) and T allele increased disease risk 1.7 times (95% CI=1.070–2.594). The BASDAI score of the patients was 6.78 ± 1.32.

The data of the qPCR study results with patient individuals carrying the T allele are given in Table 2. It was determined that PPARA expression decreased (p<0.05) in individuals carrying the T allele (Figure 1).

| A268V         | Control n=181 | Case n=168 | MAF* | p (p<0.05) | OR (95%CI) |
|---------------|---------------|------------|------|------------|------------|
| CC            | 87            | 63         | 0.36 | 0.024      | 1.67(1.070–2.594) |
| CT            | 83            | 0.29       | 89   | 2.594      |
| TT            | 11            | 16         |      |            |

*MAF, Minor allele frequency.
done with ProSA and molprobity. The quality scores of the variant model were found within NMR quality limits (Z score \(-8.64\)) (Figure 2). Structural and conformational differences between the polymorphic model and the model of wild type were analyzed with the i-Tasser’s TM-score tool. TM-score was 0.99. RMSD value was 1.397. The results indicate high similarity between wild type and polymorphic PPARA. Protein stability analyzes revealed that the polymorphic change destabilising the protein. DynaMut2, mCSM, DUET and SDM ΔΔG scores were \(-0.690\), \(-0.478\), \(-0.450\) and \(-1.46\) kcal.mol\(^{-1}\), respectively.

In this study, it was determined that NG_012204.2:p.Ala268Val change cause a decrease in ligand binding affinity. The ligand (2-(1-methyl-3-oxo-3-phenyl-propylamino)-3-\{4-\[2-(5-methyl-2-phenyl-oxazol-4-yl)-ethoxy\]-phenyl\}-propionic acid) was docked to the ligand binding site of wild type PPARA, the binding energy was between \(-11.58\) and \(-6.82\) kcal mol\(^{-1}\) and was found 94 number of distinct conformational binding clusters. The binding energy of polymorphic type was between \(-10.92\) and \(-6.26\) kcal mol\(^{-1}\) and was found 100 number of distinct conformational binding clusters (The top 10 conformation with the lowest binding energy for wild type and polymorphic PPARA are given in Table 3). The considering the lowest binding energies, the wild type with ligand interacted at 22 points (Figure 3), while the polymorphic type interacted at 19 points (Figure 4). The hydrogen bond interaction was observed to be 5 (Thr279:OG1, Ala333:N, Phe273:CA, Ser280:OG, Cys276:SG with ligand) in wild type PPARA and 3 (Lys358:HZ1, Lys358:HZ2, His440:CE1 with ligand) in polymorphic.

We determined that the polymorphic change causes an alteration in the protein three-dimensional structure in the ligand entry pocket (Figure 5). We determined that the distance between the residues 268:CA and 244:CA in the ligand entry pocket is 7.7 Å in the wild type PPARA, while this distance is 8.8 Å in the polymorphic PPARA. Also, the distance between positions 268:CB and 244:SD was 3.7 Å in wild type and 6.3 Å in polymorphic PPARA.

**Discussion**

axSpA is the typical form of a family of diseases known as spondyloarthritis characterized by inflammatory processes and new bone formation [29]. Irreversible joint and organ involvement, which significantly affects the quality of life of the patients due to the late diagnosis, is one of the most important problems [30]. The first study revealed that NG_012204.2:p.Ala268Val polymorphism of PPARA is associated with the risk of axSpA disease. It has been
shown that the change of alanine to valine at the 268th position in the protein structure causes a change in the ligand entry pocket, that may affect functional properties and gene expression. It was confirmed by qPCR analysis that polymorphic change caused a decrease in PPARA expression levels. The data to be obtained from the study will contribute to the identification of genetic markers that will contribute to the diagnosis of the disease in the early period, before spinal deformation progresses, and to elucidate the complex pathogenesis of the disease.

Inflammatory diseases trigger local cellular or environmental stress in certain tissue regions, leading to the activation of innate immune responses, the production of inflammatory mediators, and tissue damage [31]. PPARA has an important role in reducing inflammatory eicosanoid, is an endogenous PPARA ligand. Like other PPARA ligands, it induces the transcription of the genes of β- and ω-oxidation pathways that neutralize and reduce LTB4, to regulate inflammatory response. PPARA regulates the duration of the inflammatory response by limiting cytokine expression and inducing genes that metabolize LTB4 [32, 33].

New bone formation in axSpA plays an important role in the pathogenesis of the disease. Osteoblasting activity and ankylosis emerge as an important phenomenon that reduces patient comfort and quality of life by causing deformation in

![Figure 2](image.png)

**Figure 2:** Quality (Z) score of homology model of polymorphic PPARA.

![Figure 1](image.png)

**Figure 1:** PPARA expression fold change.

| Wild type PPARA-ligand | Binding Energy | LigandEff | ICons | Vdw-hb DE | Elect. | Internal |
|------------------------|----------------|----------|-------|-----------|--------|----------|
| −11.58                 | −0.3           | 3.23 nM  | −15.02| 0.16      | −1.58  |
| −11.49                 | −0.3           | 3.70 nM  | −14.91| 0.14      | −1.1   |
| −11.27                 | −0.3           | 5.66 nM  | −14.73| 0.18      | −1.84  |
| −11.23                 | −0.3           | 5.83 nM  | −14.6 | 0.09      | −1.39  |
| −11.13                 | −0.29          | 6.9 nM   | −14.52| 0.1       | −1.64  |
| −11.12                 | −0.29          | 7.06 nM  | −14.53| 0.13      | −1.31  |
| −11.09                 | −0.29          | 7.39 nM  | −14.39| 0.01      | −2.14  |
| −11.08                 | −0.29          | 7.53 nM  | −14.47| 0.1       | −1.56  |
| −11.06                 | −0.29          | 7.8 nM   | −14.4 | 0.06      | −1.76  |
| −11.03                 | −0.29          | 8.26 nM  | −14.4 | 0.09      | −1.96  |

| Polymorphic PPARA-ligand | Binding Energy | LigandEff | ICons | Vdw-hb DE | Elect. | Internal |
|--------------------------|----------------|----------|-------|-----------|--------|----------|
| −10.92                   | −0.29          | 9.89 nM  | −12.93| −1.27     | −1.39  |
| −10.79                   | −0.28          | 12.42 nM | −13.23| −0.84     | −1.51  |
| −10.78                   | −0.28          | 12.54 nM | −12.74| −1.32     | −1.19  |
| −10.66                   | −0.28          | 15.32 nM | −13.18| −0.76     | −1.39  |
| −10.38                   | −0.27          | 24.63    | −13.5 | −0.16     | −1.13  |
| −10.32                   | −0.27          | 27.3     | −12.46| −1.14     | −2.37  |
| −10.3                    | −0.27          | 28.39    | −13.18| −0.39     | −1.8   |
| −10.27                   | −0.27          | 29.62    | −12.44| −1.11     | −1.8   |
| −10.22                   | −0.27          | 32.23    | −12.41| −1.09     | −2.11  |
| −10.1                    | −0.27          | 39.59    | −13.03| −0.35     | −1.62  |

LigandEff, Ligand efficiency; ICons, inhibition concentration; Vdw-hb DE, vander-walls – hydrogen bonds dissolve energy; Elect, elektrostatic energy; Internal, internal energy.
vertebral bones. Excessive bone and ankylosis formation in axSpA is thought to be caused by the immune response and accompanying inflammation [34].

Previous studies conducted by our study group showed that the polymorphic changes in 5′ untranslated region of showed that PPARA is associated with disease risk PPARA (p<0.001, OR=2.262, 95% CI=1.462–3.499 for rs1800204; p=0.040, OR=1.561, 95% CI=1.020–2.391 for rs4253657; p=0.005, OR=1.851, 95% CI=1.200–2.855 for rs139090922) and this association may result in alteration in the expression level of the gene [35].

It is thought that PPARA, which we evaluated in our study, also inhibits osteoclasts and causes an increase in bone mineral density, and may play a role in cortical bone development, which plays a role in the pathogenesis of the disease [36].

TNF (Tumor Necrozis Factor) down-regulates bone formation and induces bone resorption by inducing proteins such as Dkk-1 and Sclerostin. Although it is a downstream regulator of bone formation, TNF does not have a key trigger role in the cortical bone formation process. For this reason, the roles of cytokines and mediators, whose activities change with the disease, in the formation of new bone should be examined. In our study, it was observed that the expression of PPARA positively associated with osteoblasting activity decreased. Despite the indirect effect of TNF on osteoclasting activity, the decrease in the expression of PPARA, which is thought to have a positive role in ossification and ankylosis, which is thought to occur due to inflammation and inflammatory response, and is found to be associated with disease development [35], was found to be compatible with the disease activity index scores (BASDAI).

It is known that amino acid changes seen in the protein primary sequence will cause changes in the 3-dimensional structure and functional properties of the protein [37, 38]. PPARA is a ligand-activated transcription factor. Activation of PPARA by agonist ligands triggers conformational changes, including stabilization of C-terminal helix-12 and enhancement of heterodimerization of PPARA with the retinoid-X receptor [39]. These conformational changes result in the activation of nuclear receptor coactivators and ultimately gene transcription [40].

PPARA consists of four functional sites: modulator region, DNA binding domain, hinge and ligand binding domain [41]. The PPARA ligand binding domain contains 2 different binding sites that interact with each other [42]. In this study, the ligand binding domain between H2′ and H3, called the omega loop, was examined. This region promotes local stabilization of the ligand binding site and activation function-2 (AF-2) in the ligand-PPARA interaction. In addition to ligand recognition and binding, the ligand binding site has additional functions such as regulating interactions with cofactors involved in signal transduction during transcription and binding to homodimerization or heterodimerization partners [43]. It is thought that positional changes due to polymorphic changes in the PPARA omega loop region may affect

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**Figure 3:** Illustration of wild type PPARA interaction with ligand at lowest binding energy. (A) Surface illustration of ligand receptor interaction, (B) reverse slide illustration, (C) diagram illustration of ligand receptor interaction.

**Figure 4:** Illustration of polymorphic PPARA interaction with ligand at lowest binding energy. (A) Surface illustration of ligand receptor interaction, (B) reverse slide illustration, (C) diagram illustration of ligand receptor interaction.
functional properties of PPARA. We found that the NG_012204.2:p.Ala268Val polymorphism caused 1.1 Å positional opening in the ligand entry pocket of PPARA (Figure 5), which resulted in a decrease in ligand affinity (Table 3) and a decrease in expression of PPARA. Sapone et al. showed that the NG_012204.2:p.Leu162Val mutation in DNA binding domain of PPARA consisting of two zinc finger motifs increased PPARA expression up to 5-fold in the presence of a potent PPARA activator [44].

Conclusion

This is the second study in which PPARA polymorphism is associated with axSpA disease risk. In the first study carried out by our study group, it was shown that some polymorphic changes in 5′UTR were associated with disease risk [35]. PPARA polymorphisms have been shown to be associated with cancer, cardiovascular diseases and metabolic disorder risk [45]. This study showed that PPARA polymorphism could be an important marker for the diagnosis of axSpA. This study, in which changes in genetic and protein structure are revealed, provides important data for elucidating the molecular mechanism of the disease. In order to elucidate the pathogenesis of the disease, it is necessary to enlighten the changes that occur in the relationship of PPARA with the genes it interacts after polymorphic change.

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Ethical approval: Ethical rules were followed in this study and written consent of volunteers was obtained. The study protocol was approved by the ethics committee of Malatya Clinical Investigations (2016/44). The study followed the ethical standards of the Helsinki Declaration.

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