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Research paper

# Effect of *MTHFR*, *TGFβ1*, and *TNFB* polymorphisms on osteoporosis in rheumatoid arthritis patients

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ABSTRACT

Diseases of the immune and the skeletal systems should be studied together for the deep interaction between them. Many studies consider osteoporosis (OP) as a risk factor for the prediction of disease progression in rheumatoid arthritis (RA). The aim of this research is to study the effect of four single nucleotide polymorphisms (SNPs) on RA patients with and without OP. The examined SNPs (*MTHFR* (C677T, and A1298C), *TGFβ1* (T869C), and *TNFB* (A252G)) were tested by genotyping 17 RA patients with OP and 72 RA patients without OP. Associations were tested using four models (multiplicative, dominant, recessive, and co-dominant). The studied SNPs were not significantly associated with the risk of OP in RA. *MTHFR*, *TGFβ1*, and *TNFB* polymorphisms don't appear to be clinically useful genetic markers for predicting RA severity in Egyptian women population.


## 1. Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by swollen and tender joints. RA is mainly distributed symmetrically on left and right joints (Saad et al., 2015). Osteoporosis (OP) is a common bone disease characterized by a reduction in bone mineral density (BMD) (Oishi et al., 2012; Kurt-Sirin et al., 2014). The association between RA and OP leads to erosive cartilage and bone destruction. Researchers believe that RA and OP have genetic causes for attacking the body joints and bones (Ranganathan, 2009; Saad et al., 2014).

SNPs are considered as the most common type of sequence variation in genomes. Most commonly, SNPs can serve as valuable genetic biomarkers; guiding biologists in detecting genes that are related to common diseases (Fareed and Afzal, 2013). The 1p36 chromosome region is associated with RA and OP (Karsak et al., 2005; Owen et al., 2013). The methylene tetrahydrofolate reductase (*MTHFR*) gene is located in the 1p36 region. The C677T and the A1298C are two common polymorphisms in the *MTHFR* gene (Lee and Song, 2010). There are controversial results of C677T for the association with OP in RA disease in different populations. The association between C677T and RA patients with OP was confirmed in the Mexican population (Brambila-Tapia et al., 2012). Other results did not show any association between bone fracture risk in RA patients and C677T in the Japanese population (Urano et al., 2009). The same studies did not show any significant association with the A1298C polymorphism (Urano et al., 2009; Brambila-Tapia et al., 2012).

TGFβ1, TGFβ2, and TGFβ3 are three isoforms of the TGFβ (transforming growth factor beta) protein (Pohlers et al., 2007). The *TGFβ1* gene is located in the 19q13 chromosome region (Jaakkola et al., 2004). TGFβ1 was found in the synovial fluid of RA patients (Menegatti et al., 2009). *TGFβ1* is associated with OP (Langdahl et al., 2008). T869C is a common polymorphism within the *TGFβ1* gene. By analyzing the possible influence of T869C on the association with OP in RA in the Egyptian population, the results showed significant associations (Hussein et al., 2014). The T869C polymorphism is highly suggested for the association with OP in RA in Korean, Italian and white UK populations although it was not statistically significant (Kim et al., 2004; Mattey et al., 2005; Ceccarelli et al., 2011).

Tumor necrosis factor beta (TNFB) is considered as a proinflammatory immunostimulatory cytokine. TNFB is also known as lymphotoxin alpha (LTA). The *TNFB* gene, which encodes the LTA protein, affects the degree of inflammation. *TNFB* polymorphisms can influence adhesion molecules and cytokines from different types of leukocytes. The *TNFB* gene is located

*Abbreviations:* ACR, American College of Rheumatology; BMD, bone mineral density; CI, confidence interval; LTA, lymphotoxin alpha; MHC, major histocompatibility complex; MTHFR, methylene tetrahydrofolate reductase; OR, odds ratio; OP, osteoporosis; $\chi^2$, Pearson chi square; PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism; RA, rheumatoid arthritis; SNP, single nucleotide polymorphism; TGFβ, transforming growth factor beta; TNFB, tumor necrosis factor beta.

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**Table 1**
2 * 2 contingency table for observed and expected values.

| | Case | Control | Total | Case | Control | Total |
|---|---|---|---|---|---|---|
| Risk | $O_{11}$ | $O_{12}$ | $O_{1+}$ | $E_{11}$ | $E_{12}$ | $E_{1+}$ |
| Wild | $O_{21}$ | $O_{22}$ | $O_{2+}$ | $E_{21}$ | $E_{22}$ | $E_{2+}$ |
| Total | $O_{+1}$ | $O_{+2}$ | M | $E_{+1}$ | $E_{+2}$ | M |

within the MHC (major histocompatibility complex) class III region on chromosome 6p21.3 (Kieszko et al., 2010; Li et al., 2014). The A252G polymorphism is located at position 1069 of intron 1 of the *TNFB* gene (Qian et al., 2011).

## 2. Materials & methods

### 2.1. Study population and data collection

In total, 89 subjects were enrolled in the case–control study: 17 RA patients with OP (cases) and 72 RA patients without OP (controls). All individuals in this study were Egyptian females. RA patients were diagnosed by physician investigators and followed the 1987 American College of Rheumatology (ACR) criteria (Arnett et al., 1988). All participants were available for genotyping. All patients were recruited from the Rheumatology Department and Outpatient Clinics of Cairo University Hospitals (Kasr El-Aini Hospital). The nature of the study was explained to all participants. The study was approved by the Ethical Committee of the Faculty of Medicine, Cairo University, and an oral and written consent was obtained from all participants. The ethical committee approved all the consent procedures. The data collection was composed of four polymorphisms included in three genes. The four SNPs were *MTHFR* C677T, *MTHFR* A1298C, *TGFβ1* T869C, and *TNFB* A252G.

### 2.2. Molecular genetic methods

DNA was extracted from peripheral blood using a QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol to be used for genotyping of the four SNPs *MTHFR* C677T, *MTHFR* A1298C, *TGFβ1* T869C, and *TNFB* A252G.

#### 2.2.1. MTHFR C677T genotyping

One set of forward 5′-CAT CCC TAT TGG CAG GTT AC-3′ and reverse 5′-GAC GGT GCG GTG AGA GTG-3′ primers were used for the amplification of a fragment of 265 bp, and then the amplified fragments were digested with the HinfI enzyme. The PCR profile was: initial denaturation at 95 °C for 5 min, denaturation at 94 °C for 30 s, annealing at 59 °C for 30 s, extension at 72 °C for 30 s for 35 cycles and followed at 72 °C for 10 min. At position 677 of the *MTHFR* gene, the C wild base, replaced by the T base, produces a cut site for the HinfI enzyme, which cuts the amplicons into two fragments of 171 and 94 bp. Then, the CC genotype would be reflected by a single band of 265 bp (uncut), the CT genotype by three bands of 265, 171 and 94 bp, and the TT genotypes by two bands of 171 and 94 bp.

#### 2.2.2. MTHFR A1298C genotyping

One set of forward 5′-CTT TGG GGA GCT GAA GGA CTA CTA C-3′ and reverse 5′-CAC TTT GTG ACC ATT CCG GTT TG-3′ primers was used for the amplification of a fragment of 241 bp and then the amplified fragment was digested with the MboII enzyme. The PCR profile was: initial denaturation at 95 °C for 5 min, denaturation at 94 °C for 30 s, annealing at 51 °C for 30 s, extension at 72 °C for 30 s for 35 cycles and followed at 72 °C for 10 min. At position 1298 of the *MTHFR* gene, the transversion of the wild A base, to C base produces a cut site for the MboII enzyme, which cuts the PCR product into two fragments of 211 and 30 bp. Then, the AA genotype results in a single band of 241 bp (uncut), the AC genotypes produce three bands of 241, 211 and 30 bp, and the CC genotype produces two bands of 211 and 30 bp. The digestion of 10 μl of PCR products was carried out with 1.5 U of the MboII restriction enzyme in 37 °C for 2 h.

#### 2.2.3. TGFβ1 T869C genotyping

DNA was genotyped by specific primers: 5′-TTCCCTCGAGGCCCTC CTA-3′ and 5′-GCCGCAGCTTGGACAGGATC-3′ to amplify a fragment of the *TGFβ1* gene, with denaturation at 96 °C for 10 min, followed by 35 cycles at 96 °C for 75 s, 62 °C for 75 s, 73 °C for 75 s, and a final extension at 73 °C for 5 min. MspA1I (New England Biolabs, Hitchin, UK) digestion of the 294 bp fragments at 37 °C for 3 h resulted in fragments of the T allele of 161, 67, 40, and 26 bp, and the C allele of 149, 67, 40, 26, and 12 bp. The samples were then analyzed by electrophoresis on 4% agarose gel stained with ethidium bromide and the genotypes were determined.

#### 2.2.4. TNFB A252G genotyping

Genotypes for *TNFB* were determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Specific oligonucleotide primers were used: 5′-CCGTGCTTCGTGCTTTGGACTA-3′ and 5′-AGAGGGGTGGATGCTTGGGTTC-3′, 782 bp fragments were amplified for the first intron of the TNFB gene. PCR products were digested with NcoI restriction enzyme and analyzed on 2% agarose gel. The *TNFB* digested product generated fragments of 586 and 196 bp or 782 bp for TNFB * 1 or TNFB * 2 homozygous individuals, respectively. For heterozygous individuals, three fragments (196, 586 and 782 bp) are detected.

### 2.3. Materials

The odds ratio (OR), its confidence interval (CI), and Pearson chi square ($\chi^2$) test were measured using *SNPAnalyzer 2.0* (Bioinformatics Unit, ISTECH Inc., Republic of Korea) (Yoo et al., 2008). OR is one of the most popular measures of the strength of association between a

**Table 2**
Case–control study — SNP analysis.

| | | *MTHFR* C677T | *MTHFR* A1298C | *TGFβ1* T869C | *TNFB* A252G |
|---|---|---|---|---|---|
| Multiplicative model | P | 0.537 | 0.748 | 0.814 | 0.225 |
| | $\chi^2$ | 0.38 | 0.103 | 0.056 | 1.473 |
| | OR (95% CI) | 0.781 (0.355–1.718) | 0.883 (0.412–1.89) | 1.094 (0.518–2.313) | 1.795 (0.691–4.66) |
| Dominant model | P | 0.728 | 0.728 | 0.696 | 0.374 |
| | $\chi^2$ | 0.121 | 0.121 | 0.153 | 0.791 |
| | OR (95% CI) | 0.827 (0.283–2.415) | 0.827 (0.283–2.415) | 0.552 (0.113–2.7) | 1.64 (0.548–4.914) |
| Recessive model | P | 0.703 | 0.836 | 0.385 | 0.487 |
| | $\chi^2$ | 0.145 | 0.043 | 0.756 | 0.483 |
| | OR (95% CI) | 0.441 (0.074–2.635) | 0.929 (0.266–3.244) | 3.525 (0.428–29.011) | 0 |
| Co-dominant model | P | 0.927 | 0.982 | 0.52 | 0.733 |
| | $\chi^2$ | 0.151 | 0.037 | 1.308 | 0.622 |


**Fig. 1.** Association analysis for examined SNPs with OP in RA disease. (a) Multiplicative model. (b) Dominant model. (c) Recessive model. (d) Co-dominant model. The horizontal line in each model represents the significance level of the p value (0.05). The figure was generated using the *SNPAnalyzer 2.0* program.

disease severity and a biomarker SNP. OR is the probability of disease severity presence compared with disease severity absence in exposed versus unexposed individuals. OR can be calculated using Eq. (1).

$$\mathrm{OR} = (a * d)/(b * c) \tag{1}$$

where, $a$ is the no. of exposed cases, $b$ is the no. of unexposed cases, $c$ is the no. of exposed controls, and $d$ is the no. of unexposed controls.

The CI is a formula that shows how to use a sample data to calculate an interval that estimates a point estimate (OR). A large CI marks a low level of precision of the OR, while a narrower CI indicates a reliable OR. Eq. (2) demonstrates the calculation of 95% CI (Szumilas, 2010).

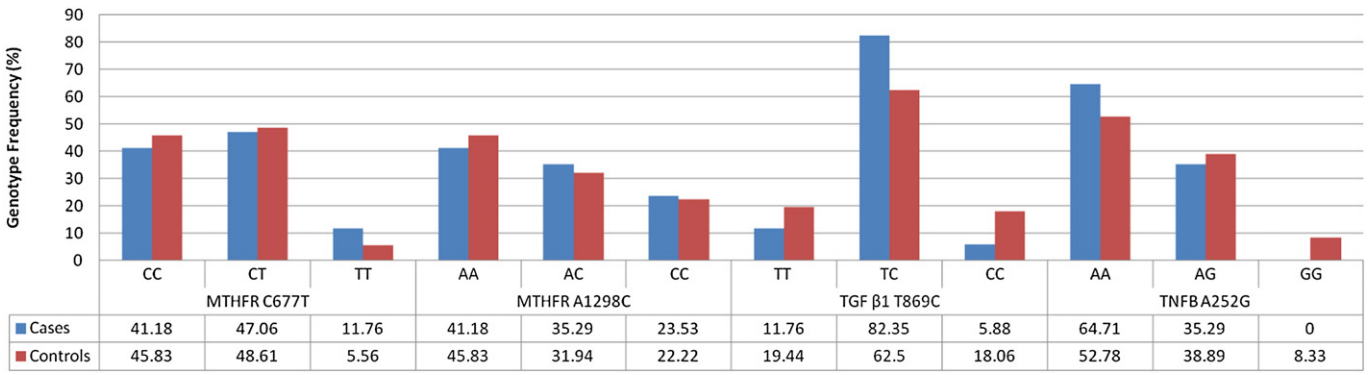
$$95\%\ \mathrm{CI} = e^{\ln \mathrm{OR} \pm 1.96\sqrt{1/a + 1/b + 1/c + 1/d}}. \tag{2}$$

### 2.4. Methods

The association between the four genetic polymorphisms and RA severity was assessed by the ORs with their corresponding 95% CI under four genetic models including the multiplicative model, the dominant model, the recessive model, and the co-dominant model. A two-sided p value less than 0.05 was considered statistically significant. The co-dominant model does not prescribe the link between the genotype and the phenotype. The dominant model compares risk in both the minor allele homozygote and the heterozygote genotypes combined to the unexposed major allele homozygote genotype. For the recessive model, the exposed group is the minor allele homozygote genotype and the unexposed group is the major allele homozygote and the heterozygote genotypes combined. For the multiplicative model, analysis should be done using alleles instead of genotypes. The exposed group is the minor allele, while the unexposed group is the major allele (Lewis, 2002).

The $\chi^2$ test is a formal statistical test used to analyze categorical data to verify the statistical significance of the results. The expected values can be calculated from Table 1 and Eqs. (3), (4), (5), and (6), where $O$ denotes the observed value in the cell, $E$ refers to the expected value, and $M$ is the total number of the studied samples. Eq. (7) shows the calculation of the $\chi^2$ result. Generally, the lower the $\chi^2$ value, the greater the likelihood that there is no significant difference between cases and controls (Clarke et al., 2011).

$$E_{11} = \frac{O_{1+}O_{+1}}{M} \tag{3}$$




| | CC | CT | TT | AA | AC | CC | TT | TC | CC | AA | AG | GG |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | MTHFR C677T | | | MTHFR A1298C | | | TGF β1 T869C | | | TNFB A252G | | |
| Cases | 41.18 | 47.06 | 11.76 | 41.18 | 35.29 | 23.53 | 11.76 | 82.35 | 5.88 | 64.71 | 35.29 | 0 |
| Controls | 45.83 | 48.61 | 5.56 | 45.83 | 31.94 | 22.22 | 19.44 | 62.5 | 18.06 | 52.78 | 38.89 | 8.33 |

**Fig. 2.** Genotype distributions in RA patients with and without OP.

$$E_{12} = \frac{O_{1+}O_{+2}}{M} \quad (4)$$

$$E_{21} = \frac{O_{2+}O_{+1}}{M} \quad (5)$$

$$E_{22} = \frac{O_{2+}O_{+2}}{M} \quad (6)$$

$$\chi^2 = \sum_{i=1}^{2}\sum_{j=1}^{2}\frac{(O_{ij}-E_{ij})^2}{E_{ij}}. \quad (7)$$

To be sure that the $\chi^2$ result gives a real statistical significant difference, the p-value should be looked up. A low p-value reflects a low expectation of finding these results by coincidence. A high p-value indicates a high probability of finding these results by chance. In case of a p-value of 1, it means that the two groups are not different at all (Chen et al., 2010).

## 3. Results

In this study, four SNPs were used to examine the association with OP linked to RA in the Egyptian population. The examined SNPs were *MTHFR* (C677T) (rs1801133), *MTHFR* (A1298C) (rs1801131), *TGFβ1* (T869C) (rs1982073), and *TNFB* (A252G) (rs909253). The average age of patients having OP was 46.16 ± 14.37 years. The average age of patients without OP was 41.72 ± 11.87 years. The average disease duration of patients having OP was 7.89 ± 5.86 years. The average disease duration of patients without OP was 6.33 ± 4.33 years.

The association between OP in RA and the studied polymorphisms has been examined in several studies. Contradictory results had arisen due to different populations, the age of the subjects, and the sample sizes of these studies. Table 2 represented the association between the examined SNPs and OP in RA patients. Four models were used to measure these associations which are multiplicative, dominant, recessive, and co-dominant models. The measured parameters were OR, its 95% CI, and $\chi^2$ with the corresponding p value. A graphical representation of the association results for the studied SNPs was shown in Fig. 1. From Table 2 and Fig. 1, all the studied SNPs didn't show any significant association with any of the used models.

Genotype frequencies for each SNP for RA patients with OP (cases) and without OP (controls) were presented in Fig. 2. From Fig. 2, the genotype frequencies of the studied SNPs didn't show any significant differences between cases and controls. A slightly higher frequency of the (TC) heterozygote genotype for *TGFβ1* (T869C) in cases (82.35%) with respect to controls (62.5%) was observed, which was not statistically significant.

## 4. Discussion

The genetic characteristics of the modern Egyptian population are a mixture of European, Middle Eastern, and African populations (Manni et al., 2002). This issue could explain the agreement/disagreement of our results with published data of other populations. Table 3 showed the influential genotype/allele in case of the presence of an association for the studied SNP with OP related to RA in the corresponding population. *TGFβ1* results differed in our study and the study of Hussein et al. (2014) for the Egyptian population. This variation may be due to the sample sizes (our study: cases 17, controls 72, Hussein et al.: cases 53, controls 107) or the age of patients (our study: 43.94 ± 13.12 years, Hussein et al.: 47.3 ± 9.3 years) or the disease duration (our study: 7.11 ± 5.095 years, Hussein et al.: 10.23 ± 7.5 years).

Our results were consistent with a Japanese population study that *MTHFR* (C677T) had no effect on OP in RA patients. This study added evidence to the hypothesis that *MTHFR* (A1298C) should not be used as a genetic biomarker for OP in RA. The result for *TGFβ1* (T869C) was in line with the findings in Korean, Italian, and white UK populations.

## 5. Conclusion

The results of this study suggested that *MTHFR* (C677T), *MTHFR* (A1298C), *TGFβ1* (T869C), and *TNFB* (A252G) didn't have major effects on OP in RA in the Egyptian population. In contrast to their role in RA

**Table 3**
Association status of our study and previous studies.

| | Population | Cs[a] | Ct[b] | G[c] | A[d] | Association |
|---|---|---|---|---|---|---|
| *MTHFR* C677T | Egyptian (Our study) | 17 | 72 | | | None |
| | Mexican Brambila-Tapia et al. (2012) | 41 | 30 | TT | T | Susceptible |
| | Japanese Urano et al. (2009) | 115 | 612 | | | None |
| *MTHFR* A1298C | Egyptian (Our study) | 17 | 72 | | | None |
| | Mexican Brambila-Tapia et al. (2012) | 41 | 30 | | | None |
| | Japanese Urano et al. (2009) | 115 | 614 | | | None |
| *TGFβ1* T869C | Egyptian (our study) | 17 | 72 | | | None |
| | Egyptian Hussein et al. (2014) | 53 | 107 | TT | T | Susceptible |
| | Italian Ceccarelli et al. (2011) | x[e] | x[e] | | | None |
| | Korean Kim et al. (2004) | x[e] | x[e] | | | None |
| | UK Mattey et al. (2005) | x[e] | x[e] | | | None |
| *TNFB* A252G | Egyptian (our study) | 17 | 72 | | | None |

[a] No. of cases.
[b] No. of controls.
[c] Genotype.
[d] Allele.
[e] Unknown.

susceptibility, the studied SNPs didn't show any association with disease progression in RA. There was no evidence explaining the effect of the examined SNPs in RA associating OP in some populations, while the association did not exist in the other populations. Further studies with extended samples are necessary to confirm our results.

## Declaration of interest

The authors have declared no conflicting interests.

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