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ORIGINAL ARTICLE

A TRAF6 genetic variant is associated with low bone mineral density in rheumatoid arthritis

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Abstract

Objectives This study was aimed to investigate the association of the single nucleotide polymorphism of tumor necrosis factor receptor associated factor 6 (TRAF6), rs540386, with low bone mineral density (BMD) among patients with rheumatoid arthritis (RA).

Methods TRAF6 rs540386 genotyping was performed by mutagenically separated PCR in a cohort of 188 (23 men, 165 women, median age, 56.2 years) adult RA patients and 224 age and gender-matched controls. BMD was measured using dual-energy X-ray absorptiometry (DXA) (Lunar Prodigy advance scans, GE Healthcare, USA).

Results Among the RA patients, 64 (55 women, 9 men) had low BMD comprising of 57 patients with osteoporosis and 7 with osteopenia. Whereas TRAF6 rs540386 was not associated with RA susceptibility, it was however found to be a risk factor for reduced lumbar spine *Z*-score in the recessive model (OR = 3.34, 95% CI = (1.01–11.00), $p = 0.038$). This association was confirmed further in the multivariate logistic regression analysis taking into account several potential confounding factors (OR = 3.34 (1.01–11.00), $p = 0.048$). In addition, mean total femur *Z*-score was found to be reduced in TT patients when compared to CC + CT patients (-1.30 ± 1.32 versus -0.60 ± 1.05, $p = 0.034$). No association between TRAF6 rs540386 and local bone damage was observed.

Conclusions This study for the first time ever demonstrated an association between a genetic variant of TRAF6 and low BMD among patients with RA. Further investigations are needed to elucidate the exact role of this variant.

Keywords Bone mineral density · Mutagenically separated polymerase chain reaction (MS-PCR) · Osteoporosis · Rheumatoid arthritis · rs540386 · Tumor necrosis factor receptor associated factor 6 (TRAF6)

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Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease targeting the synovial membranes afflicting severe structural damage to cartilages and bones with both local and systemic bone loss (BL). Osteoporosis (OP) is a potential complication of RA and affects approximately 15–36% of RA patients [1, 2]. Presence of RA poses a significantly higher risk of osteoporotic bone fractures compared to individuals without RA, particularly the vertebral fractures that positively correlate with RA regardless of the gender [3]. Although OP usually complicates long lasting and uncontrolled RA but it has also been shown to affect in 11% of patient with recent RA [4]. Long-term use of glucocorticoids in RA may contribute to the development of OP in RA patients; low bone mass density (BMD) however has been reported among RA patients never treated by glucocorticoids [5, 6]. Apart from the classical causes of OP such as menopause, diabetes, and the use of glucocorticoids, chronic inflammation has also been implicated in OP [7]. Moreover, a positive correlation between disease activity and biochemical markers of BL has also been reported in RA [8].

A number of studies have revealed a close interaction between the bone and the immune system, and both the systems share several regulatory pathways [9–11]. Bone destruction in RA is associated with aberrant activation of osteoclasts without equivalent activation of osteoblasts. Receptor Activator of Nuclear Factor κB (RANK)/RANK Ligand (RANKL) pathway has emerged as a crucial pathway mediating osteoclast related BL [12]. RANKL promotes osteoclast formation and augments osteoclast function and survival by interacting with its cognate receptor RANK. RANKL expression is mediated by a number of cytokines including IL-1, TNF-α, and IL-6 which play a pivotal role in the pathogenesis of RA [13]. Several studies support the hypothesis that there is a shift in bone homeostasis towards increased osteoclastic activity in RA. Patients with RA tend to have higher plasma levels of RANKL and lower plasma levels of osteoprotegerin (OPG), a decoy receptor of RANKL inhibiting osteo-resorption by preventing RANK/RANKL interaction [14].

RANK signaling is mediated by tumor necrosis factor (TNF) receptor associated factor 6 (TRAF6). TRAF6 is a unique molecule representing a focal point for different pathways involved in bone remodeling and inflammation. TRAF6 is a member of the TNF receptor associated factor (TRAF) family of proteins that mediate signaling from the TNFR family. Unlike the other members of the TRAF superfamily, TRAF6 also plays a pivotal role in the signal transduction of the interleukin-1 (IL-1) receptor/toll-like receptor (IL1R/TLR) superfamily [15, 16]. Activation of TRAF6 induces NF-kB activation resulting in transcription and secretion of a variety of inflammatory factors involved in causing synovial inflammation along with cartilage and bone destruction [17].

The intronic single nucleotide polymorphism (SNP) TRAF6 rs540386 has been previously studied in RA [18, 19] and systemic lupus erythematosus (SLE) [20] with conflicting results. Taking into account the role of TRAF6 both in inflammation and in bone remodeling, this study for the first time ever investigates the implications of TRAF6 rs540386 in RA and the associated BL in the disorder.

Material and method

Material

Patients

A total of 188 Tunisian adult patients with RA and 224 age and gender-matched controls of the same ethnicity were enrolled in the present study.

Diagnosis of RA was confirmed in accordance with the ACR/EULAR 2010 criteria [21]. Patients were recruited from the Department of Rheumatology of Farhat Hached Hospital between January 2013 and December 2015. Disease duration was defined as the duration of patient self-reported joint symptoms. Body mass index (BMI) was computed as weight in kilograms divided by height in meters squared (kg/m^2). Apart from the demographic and clinical details, data were also collected for 28-joint Disease Activity Score (DAS28), erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), anti-cyclic citrullinated peptide antibodies (anti-CCP) (Euroimmun®, Lubeck, Germany), and findings of postero-anterior radiographs of hands, wrists, and forefeet for the assessment of joint damage. Radiological damage was evaluated according to Sharp/van der Heijde. All RA patients were osteoporosis treatment-naïve.

This study was approved by Farhat Hached University Hospital ethics committee. Participants were enrolled in the study after obtaining their informed consent.

Methods

Genotyping

DNA was extracted from whole blood of patients and controls by the salt-out method. Primers were newly designed for TRAF6 rs540386 genotyping using mutagenically separated polymerase chain reaction (MS-PCR) (primers given upon request). PCR conditions were as follows: initial denaturation at 95 °C for 5 min, 34 cycles of denaturation at 94 °C for 30 s, annealing at 51 °C for 20 s, and extension at 72 °C for 30 s.

Bone-mineral density measurement

BMD was measured prior to any treatment for OP. BMD of lumbar spine 1–4 (L1–4), total proximal femur, and femoral neck were measured using dual-energy X-ray absorptiometry (DXA) (Lunar Prodigy advance scans, GE Healthcare, USA). BMD was automatically calculated from the bone area (cm^2) and bone mineral content (g) and expressed absolutely in g/cm^2. The results were expressed as *T*-score (standard deviation (SD) below the mean of young healthy adults) and as *Z*-score (SD below the age- and gender-matched mean reference value). After acquisition of the BMD *T*-score, osteopenia (*T*-score between − 1.0 and − 2.5) and osteoporosis (*T*-score below − 2.5) were defined according to the criteria of the World Health Organization [22]. To correct for age and gender heterogeneity, the measures were analyzed as *Z*-score. Low BMD was defined as *Z*-score < − 1.9.

Statistical analysis

Statistical analysis was performed using SPSS version 17.0 for Windows (SPSS Inc., Chicago, IL, USA). Chi-square test was used for testing Hardy-Weinberg equilibrium. Odds ratios (OR) and 95% confidence intervals (CI) were calculated to determine the association of TRAF6 rs540386 with the risk of RA and BMD. Chi-square test was used for categorical data. Student and Anova tests were used to compare means of variables with normal distribution. Mann-Whitney and Kruskal-Wallis tests were conducted for variables with non-normal distribution. To identify the variables associated with low BMD, a multivariate logistic regression analysis, where low BMD was the dependent variable, were conducted. Any variable with a $p < 0.2$ was integrated in this regression. A $p < 0.05$ was considered statistically significant.

In silico study

To test the possible effect of TRAF6 rs540386 on splicing, we applied the SNP function prediction tool, available at: http://www.cbs.dtu.dk/services/NetGene2/.

Results

Demographic, clinical, biological, and radiological characteristics

Among the 188 RA patients, 165 (87.7%) were females. Median age was 56.2 years. CRP was elevated in 113 out of 153 (73.8%) RA patients. Anti-CCP were present in 146 out of 186 (78.5%) RA patients. One hundred eighty-two (96.8%) RA patients were receiving glucocorticoid treatment.

One hundred sixty-four (87.2%) patients had radiological damage. Sharp/van der Heijde (SVHS) median score was 58 (0–242).

BMD

Low BMD was observed in 64 (34%) RA patients comprising of 57 (30.3%) patients with OP and 7 (3.7%) patients with osteopenia. Main characteristics were comparable between patients with normal and low BMD except for DAS28 (5.8 versus 6.2 respectively, $p = 0.01$). Patients with OP and patients with osteopenia were comparable (Table 1). Femoral neck, total femur and lumbar spine, and BMD results are detailed in Table 2.

No association between low BMD and the presence of local radiological damage was detected (data not shown).

TRAF6

TRAF6 and RA

TRAF6 rs540386 genotype frequencies among the patients and the controls were in Hardy-Weinberg equilibrium. TRAF6 rs540386 C allele was the most frequently observed among the patients and the controls. It was detected in 76.6% of patients and 81.5% of controls. Among all genotypes, CC was the most frequently observed genotype. It was present among 59.6% of patients with RA and 67.4% of controls. TRAF6 rs540386 was not associated with RA susceptibility in any model; codominant, dominant, or recessive (Table 3).

No association between TRAF6 rs540386 and DAS28, CRP, anti-CCP, or joint damage was detected even after stratification by age and gender.

TRAF6 and BMD

In the univariate analysis, TRAF6 rs540386 was found to be associated with a significantly reduced lumbar spine *Z*-score in the recessive model (TT patients versus CC + CT patients) (OR = 3.33, 95% CI = 1.01–11.00, $p = 0.038$).

TRAF6 rs540386 was not associated with reduced total femur *Z*-score or femoral neck *Z*-score. However, when mean *Z*-scores were compared, mean total femur *Z*-score was found to be reduced in TT patients when compared to CC + CT patients (− 1.30 ± 1.32 versus − 0.60 ± 1.05, $p = 0.034$) (Fig. 1). Median femoral neck *Z*-score and mean lumbar *Z*-score were also lower in TT patients. However, the difference was not statistically significant (− 0.85 +- 1.15 versus − 0.50 +-1.04, $p = 0.177$) and (− 1.90 ± 1.56 versus − 1.17 ± 1.4, $p = 0.056$) respectively.

Association of known risk factors of OP like smoking, diabetes, BMI, disease duration, DAS28, CRP, anti-CCP. and glucocorticoids was assessed. All variables with $p < 0.2$ were selected for the multivariate logistic regression with low

Table 1 Comparison of clinical features of patients with rheumatoid arthritis based on bone mineral density

	Low BMD (*n*=64)	Normal BMD (*n*=124)	*p*	Osteoporosis (*n*=57)	Osteopenia (*n*=7)	Normal BMD (*n*=124)	*p*
Mean age (years) ±SD	54.3±11.0	56.6±11.0	0.2	54.6±11.2	52.2±9.4	56.6±11.0	0.4
Median BMI (kg/m^2) ±SD	28.8±8.3	28.0±6.7	0.7	28.4±8.3	35.5±14.9	28.0±6.7	0.1
Median disease duration (months) ±SD	39.0±85.0	72.0±83.6	0.4	42.0±88.8	36.0±38.2	72.0±83.6	0.5
Median DAS28 ±SD	6.2±1.5	5.8±1.5	0.01*	6.2±1.5	6.1±1.9	5.8±1.5	0.2
Median ESR (mm/hr) ±SD	56.0±38.2	54.0±37.0	0.6	56.0±36.8	56.0±50.6	54.0±37.0	0.8
Median of elevated CRP (mg/L) ±SD	31.0±31.9	23.5±33.4	0.2	36.5±33.3	24.0±15.5	23.5±33.4	0.3
Median of positive anti-CCP antibodies (RU/mL) ±SD	125.0±76.6	110.0±76.3	0.7	130.0±78.4	120.0±79.6	110.0±76.3	0.9
Glucocorticoids use, n (%)	61.0 (33.5)	121.0 (66.5)	0.4	55.0 (30.2)	6.0 (3.3)	121.0 (66.5)	0.2

BMD bone mineral density, *BMI* body mass index, *DAS28* Disease activity score in 28 joints, *ESR* erythrocyte sedimentation rate, *CRP* C reactive protein, *CCP* cyclic citrullinated peptide

*Represents statistical significance

BMD as a dependent variable. Among those variables, only TRAF6 rs540386 was confirmed as an independent risk factor for low BMD ($p = 0.048$) (Table 4).

TRAF6 and local radiological damage

TRAF6 was not associated with local radiological damage in any model, and median SVHS were comparable in all groups (data not shown).

Table 2 Site specific assessment of bone mineral density among patients with rheumatoid arthritis

Bone mineral density	
Femoral neck (*n*=188)	
Mean BMD ±SD (g/cm^2)	0.826±0.114
Mean of Z-score ±SD	-0.46±1.05
Z-score < -1.9, n (%)	10 (5.3)
Total femur (*n*=188)	
Mean BMD ±SD (g/cm^2)	0.857±0.170
Mean of Z- score ±SD	-0.65±1.08
Z-score < -1.9, n (%)	18 (9.6)
Lumbar spine L1–L4 (*n*=188)	
Mean BMD ±SD (g/cm^2)	0.919±0.181
Mean of Z-score ±SD	-1.22±1.42
Z-score < -1.9, n (%)	59 (31.4)

BMD bone mineral density

In silico study

In silico study showed that TRAF6 rs540386 could not potentially affect splicing.

Discussion

In the present study, we examined for the first time ever the implication of a genetic variant of TRAF6 in low BMD in RA. In the multivariate logistic regression analysis taking into account several potential confounding factors, TRAF6 rs540386 was found to be an independent risk factor for low BMD. These findings highlight the potential impact of TRAF6 rs540386 on bone homeostasis in RA.

TRAF6 is among the molecules that have recently been identified as a family of molecules bridging the gap between immune function and bone homeostasis. It has been clearly demonstrated that TRAF6 governs osteoclastogenesis. Activation of NF-kB by RANK requires TRAF6 [23] and $TRAF6^{-/-}$ mice exhibit severe osteopetrosis and defective osteoclast formation. In vitro culture experiments have revealed that the precursors of osteoclasts derived from $TRAF6^{-/-}$ mice are unable to differentiate to functional osteoclasts in response to osteoclast differentiation factor (ODF) [24]. Moreover, it has been clearly demonstrated that precursors overexpressing TRAF6 differentiate into osteoclasts in the absence of

Table 3 TRAF6 rs540386 and risk for having rheumatoid arthritis

Alleles and genotypes	Patients (*n*=188)		Controls (*n*=224)		OR (95% CI)	*p*
	n	%	n	%		
C	288	76.6	365	81.5	1	0.08
T	88	23.4	83	18.5	0.74 (0.53-1.04)	
CC	112	59.6	151	67.4	1	0.24
CT	64	34.0	63	28.1	1.37 (0.89-2.09)	
TT	12	6.4	10	4.5	1.61 (0.67-3.87)	
CC	112	59.6	151	67.4	1	0.09
CT+TT	76	40.4	73	32.6	1.40 (0.93-2.10)	

additional signals from RANKL and that strength of TRAF6 signaling is crucial for osteoclastogenesis [25].

Evidence of genetic linkage to the chromosomal region harboring TRAF6 has previously been associated with severe forms of SLE [26]. A SNP-based genome-wide linkage scan for RA also incriminated this region [27]. A candidate gene study identified an association between RA and the intronic SNP rs540386 of TRAF6 in Caucasians [18]. In the present study, we did not find any association between TRAF6 rs540386 and RA. This could be a false-negative finding due to the limited number of patients that may limit our statistical power to detect small differences between groups. It is also possible that genetic susceptibility to RA involves different genetic polymorphisms in different ethnic groups. Indeed, a recent genome wide association study (GWAS) performed in Arabs did not confirm TRAF6 association with RA. Conversely, this study has reported two novel loci specific to Arab populations which have not been previously described among Caucasian and Asian populations [19]. These findings corroborate the implication of some population specific variants in RA.

TRAF6 has been found to correlate with the histological severity of synovitis and the number of infiltrated inflammatory cells [28]. Evidence suggests that TRAF6 plays a critical role in induction of pro-inflammatory effects and proliferation of RA fibroblast-like synoviocytes (RA-FLSs). Inhibition of TRAF6 in RA-FLSs has been shown to mitigate the mRNA levels and secretion of pro- inflammatory cytokines and matrix metalloproteinases. In addition, inhibition of TRAF6 decreases proliferation of RA-FLSs, blocks RA-FLSs in G0/G1-phase, and inhibits the cells to enter the S-phase and G2/M-phase [29].

Fracture is the most important clinical complication of osteoporosis. However, most genetic studies on osteoporosis have focused on BMD for being a highly heritable trait [30, 31] and a strong clinical predictor of osteoporotic fracture [32]. Several GWAS have been conducted and identified many genes implicated in low BMD and OP including gene encoding for OPG [33]. However, there is a lack of GWAS on OP complicating RA. In previous candidate gene studies, polymorphisms of genes involved in bone metabolism like the vitamin D receptor (VDR) gene has been incriminated in low BMD in Caucasians RA patients [34, 35]. OPG gene has been also associated with increased risk for hip fracture among Japanese patients with RA [36]; however, its association with low BMD was not confirmed in other populations [37].

Interestingly, our study showed that TRAF6 rs540386 was associated with low BMD but not with local bone damage as assessed by SVHS. These data suggest that local and systemic bone loss might be regulated by different mechanisms. In line with our results, increased synovial TRAF6 expression in RA was found to be associated with severity of synovitis but not with joint destruction [28].

TRAF6 maps to chromosome 11p12 and it covers approximately ~22 kb. It resides on the reverse strand of genomic DNA and encodes at least two reference transcripts. TRAF6 rs540386 (C/T) is located in the first intron of TRAF6 gene. Generally, the SNPs in introns may affect splicing, alternative splicing, and splicing efficiency. We assessed the possible effect of TRAF6 rs540386 on splicing using the SNP function prediction tool. Analysis indicated that TRAF6 rs540386 could not potentially affect splicing.

In addition to TRAF6, we have examined the role of other parameters potentially affecting BMD like smoking, disease

Table 4 Multivariate logistic regression analysis of factors associated with reduced lumbar spine Z-score

Variables	OR	95% CI	*P*
TRAF6 rs540386	3.33	1.01-11.00	0.048*
28-joint disease activity score (DAS28)	1.18	0.93-1.49	0.17
Diabetes	0.49	0.17-1.42	0.18

*Represents statistical significance

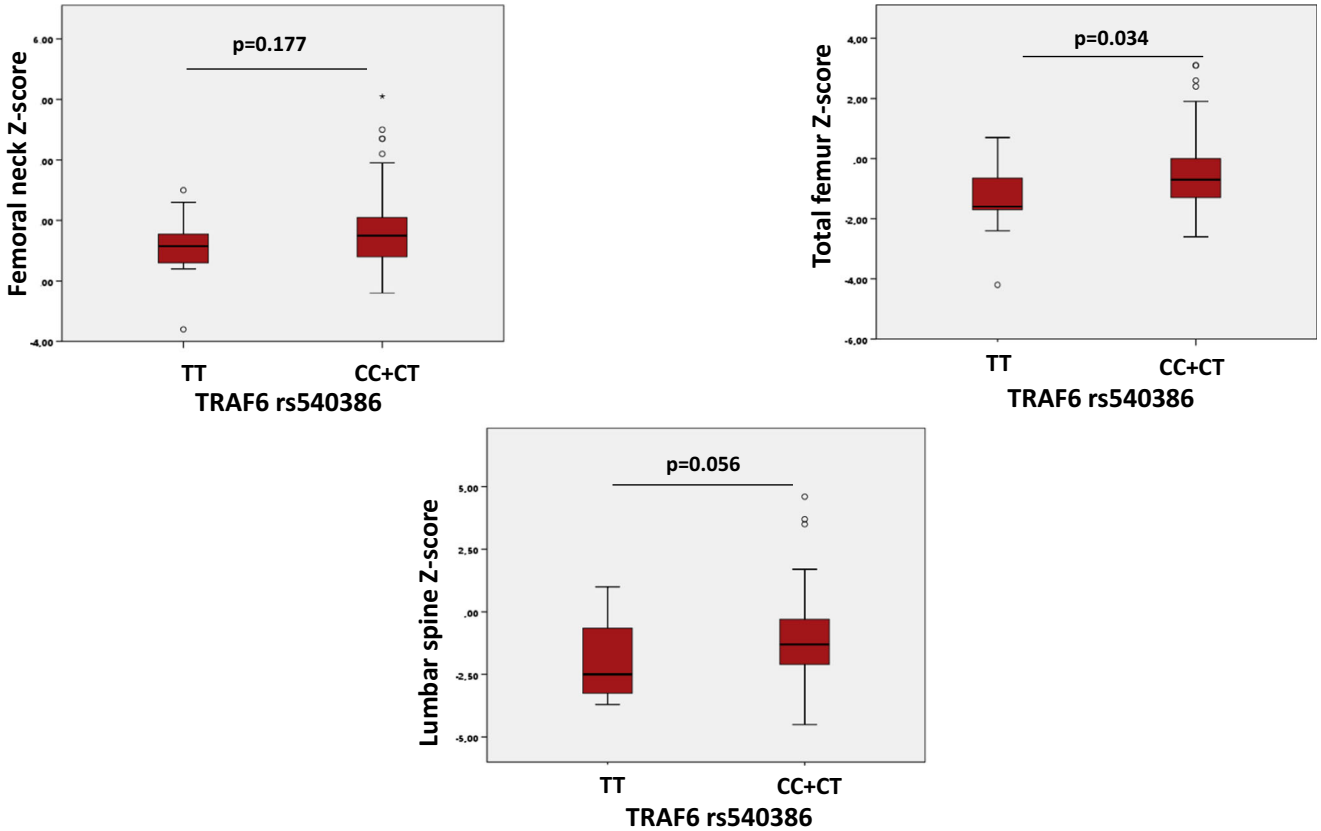

Fig. 1 Comparison of median femoral neck Z-score, mean total femur *Z*-score, and mean lumbar *Z*-score between TT and CC + CT patients. Mean total femur *Z*-score was reduced in TT patients when compared to CC + CT patients whereas median femoral neck *Z*-score and mean lumbar *Z*-score were statistically comparable

duration, BMI, DAS28, glucocorticoids, ESR, CRP, and anti-CCP. It was shown that human anti-citrullinated protein antibodies (ACPA) transferred to mice bind to osteoclasts and induce osteoclastogenesis [38]. In humans, early presence of bone erosions and the presence of osteopenia in RA patients with only a few weeks of disease duration and no exposure to glucocorticoids suggest that other factors than synovitis are involved. Kleyer et al. compared the microstructure of the metacarpophalangeal joints of ACPA positive and ACPA negative healthy individuals. They found that only ACPA positive individuals had alterations in cortical bone architecture [39]. In our cohort, we did not find any association between anti-CCP and BMD. Our results are in line with those reported by Nava-Valdivia et al. [37].

Our study has some limitations. Despite the inclusion of many potential confounders, we were unable to account for confounders such as alcohol, caffeine, physical activity, cumulative dose of corticoids, and parathyroid hormone/calcemia/vitamin D levels because of the insufficient information in patients' records.

In conclusion, a TRAF6 rs540386 variant was found to be associated with low BMD in RA. The mechanism by which this variant could affect TRAF6 function remains elusive. Our results are preliminary and need to be replicated in prospective multi-center trials with large numbers of patients. The exact role of this genetic variant needs to be further clarified by functional studies.

Taking into account the impact of this variant on BMD, eviction of OP risk factor like glucorticoids and supplementation with vitamin D would be advisable in patients harboring this genetic variant.

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Compliance with ethical standards

Disclosures None.

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