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Vitamin D receptor polymorphisms as tool for early screening of severe bone loss in women patients with rheumatoid arthritis

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Abstract. – OBJECTIVE: Rheumatoid Arthritis (RA) is an autoimmune inflammatory disease that leads to local and systemic arthritis and bone loss.

Exploring genetic markers of candidate genes in osteoporosis and inflammatory cytokine genes could be a useful tool for the early identification of bone loss and fracture risk in RA patients. The target of this study is the evaluation and correlation between of Single Nucleotide Polymorphisms (SNPs) of Vitamin D Receptor (VDR) and possible effects on bone loss in RA.

PATIENTS AND METHODS: 40 Caucasian patients with RA (26 of them with a severe bone loss) and 40 healthy donors as control samples were genotyped for the VDR SNPs (called BsmI, ApaI, TaqI and FokI). The detection method is based on Restriction Fragment Length Polymorphism (RFLP).

RESULTS: Genotyping profile shown no difference between RA patients and controls. Only VDR-TaqI genotype (TT vs. tt) seem to influence the bone density in females, but not in males. The mean differences of Bone Mass Density (BMD) at the lumbar spine in RA women with the tt allele were 4.7% compared to 0.1% in women with the TT allele ($p < 0.05$).

CONCLUSIONS: The results of these studies support an association between specific VDR alleles and bone loss in RA. The TaqI t and BsmI B alleles were associated with an accelerated bone loss in RA, but not with a wfocal bone loss. These effects of VDR genotypes and vitamin D supplementation are not unexpected, given that the central pathological feature in RA is bone and joint destruction. The VDR SNPs genotyping should be a useful tool to screen early women RA patients with the bone loss.

Key Words:
Osteoporosis, VDR, Bone loss, Inflammatory cytokine genes, Genotyping, Vitamin D.

Introduction

Rheumatoid Arthritis (RA) is an autoimmune disease that exerts its greatest impact on the joints of the body that are lined with synovia due to inflammatory process. RA induces bone and cartilage damage; the bone destruction is associated with systemic osteoporosis and susceptibility to fragility fractures associated with inflammatory disease activity. Osteoporosis and related fragility fractures are one of the most common complications in patients with RA and markedly affect the quality of life in these patients. Erosive RA is associated with pain, disability and deformity, affecting all aspects of motor function from walking to fine movements of the hand, and early mortality; the degree of progressive damage is related to the duration and strength of the inflammation[1-2]

RA is an autoimmune disease with greater incidence among women. The pathologic immune response is not only affecting joints and bone but it is causing a systemic syndrome involving many other organs causing, either systemic disease (i.e., vasculitis, lung fibrosis) and/or increasing gene expression related to cancer development[3]. Heart conditions such as ischemic heart disease and cardiac failure have been shown to be more common in RA. Reduced mobility and inflammation are concurrent causes of osteoporosis. Unfortunately, the causes of this disease are still unknown.

The role of genetic variants in candidate genes for osteoporosis is low defined in RA related osteoporosis. In this regard, the role of vitamin D and its receptor (VDR) in bone loss in RA is high attractive.

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Vitamin D plays a major role in bone mineral homeostasis, for example by promoting the transport of calcium and phosphate, absorbed from food and supplements. In addition to bone remodeling, the calcium plays a role in the release of many hormones and enzymes. Vitamin D is found as vitamin D_2 (ergocalciferol) or vitamin D_3 (cholecalciferol), and after it enters the circulation it is bound to the vitamin D binding protein and transported to the liver where vitamin D undergoes a first hydroxylation; this form is then carried to the kidney for a second hydroxylation to generate the hormonal form $1,25[OH]_2D$. A deficit of vitamin D can lead to osteoporosis in the adult, causing fragility fractures, and rickets in the children. Several studies have evaluated whether the supplement of vitamin D prevents bone fractures, especially in postmenopausal women[4].

Vitamin D is also important for the nerve function for the movement of the muscles, and for the immune system action to fight bacteria and viruses. Vitamin D role in regulating the immune system had led scientists to explore if its deficiency contributes to the development of autoimmune diseases and if vitamin D supplements help boost the defenses against microbes. Epidemiologic evidence links vitamin D deficiency to autoimmune disease, musculoskeletal decline, cardiovascular disease, depression, dementia, infectious diseases, and cancer[5].

In the last years, it has been recognized to Vitamin D new anti-proliferative, pro-differentiation and immune-modulating shares; these actions are mediated by Vitamin D Receptor (VDR), a cytoplasmic/nuclear receptor belonging to the family of hormonal receptors. VDR is a phosphoprotein which binds the pseudo-hormone form $1,25\ (OH)_2D_3$ with high affinity, forming an heterodimer with retinoid X receptors (RXR), and regulates the expression of genes via zinc finger-mediated DNA binding and protein-protein interactions. VDR gene encodes a protein that regulates the transport and calcium homeostasis and has been proposed as the locus with greater genetic effect on Bone Mineral Density (BMD) in association studies. During the last years VDR have been found in a vast number of tissues and cell types, which also possess the enzymatic system to convert the vitamin D in the active form. The role of VDR has been well-known in a variety of metabolic disorders and in the regulation of inflammation, such as osteoblast function, macrophage/osteoclast differentiation, intestinal $Ca_3(PO4)_2$ absorption, renal vitamin D catabolism, parathyroid hormone synthesis and immune-modulation/cell differentiation. VDR is also one of the candidate genes of RA[6]. In addition, polymorphisms in the vitamin D receptor gene appear to influence the susceptibility to bone loss in RA. The allelic variations of gene VDR explain for 70% of the genetic effects on bone density.

There are several polymorphic sites in the 3' region of the VDR human gene identified by restriction endonuclease enzymes TaqI, BsmI, ApaI and another variant in the exon 2 recognized by FokI. The alleles are called respectively T-t, B-b, A-a and F-f depending on the presence or the absence of restriction site. These polymorphisms may affect the response to various dietary components with the possible risk of development of pathology, as widely ~~it~~ demonstrated by a functional involvement of the alleles of the VDR in calcium homeostasis and bone mineralization. Initial studies have allowed us to detect the interaction between the VDR gene, the absorption of calcium and the calcium levels in the diet [7].

TaqI is localized in the exon 9 of the VDR gene, on the codon 352, and it consists in a nucleotide variation T-C. The T nucleotide is also defined allele T, instead the C nucleotide allele t[8].

BmsI is localized in the intron 8 of the gene consisting in a nucleotide variation A-G, and it is associated with the variation of the transcript stability. The A nucleotide is also defined allele B, and the G nucleotide allele b[8].

FokI consists in a nucleotide substitution T-C on the start codon of the VDR gene translation (ATG ->ACG); this polymorphism determines the translation of 3 amino acids by the start site with consequent alteration of the protein, missing of 3 amino acids. The T nucleotide is also defined allele f, instead the C nucleotide is defined allele F[8].

The aim of this study was the evaluation of the impact of VDR genes polymorphisms on bone loss in patients with RA. In particular, a validated PCR-based method was optimized for the identification of the four polymorphisms of VDR gene, in the context of a possible correlation with bone loss in patients with RA. The interest of the study comes from the presence in the literature of conflicting data on the association between polymorphisms and bone loss, mainly due to the use of not comparable methods and because not always all four SNPs of VDR gene have been considered.

Patients and Methods

Patients and Clinical Laboratory Assessment

40 Caucasian patients (30 females and 10 males) with RA diagnosis undergoing follow-up at our institution (Department of Translational Medical

Table I. Characteristics of controls and RA patients in individual studies. Very high levels of CRP were found in RA patients with erosions ($^*p<0.01$).

	Controls	Bone loss	
		Yes	No
Number of patients	40	26	14
Mean age	40.3 ± 11.3	57.5 ± 12.5	58.2 ± 11.8
Disease duration (months)	/	105 ± 57	67 ± 52
CRP (g/L)	0.15 ± 0.23	*15.02 ± 2.80	*0.33 ± 0.41
Lumbar spine BMD (T-score)	0.36 ± 0.15	-1.64 ± 1.21	-1.54 ± 1.36
Femoral neck BMD (T-score)	0.58 ± 0.42	-1.58 ± 1.08	-1.18 ± 1.05

Sciences) were included in the study. In addition 40 healthy donors (30 females and 10 males) served as control. The mean age of RA patients was 57.5, while the mean age of controls was 40.3 years (Table I). The Rheumatoid Factor (RF), evaluated by a nephelometric assay (Siemens BN II, Healthineers Italia), was evaluated in all patients with RA; 32 (80%) of them were RF positive (Normal range: RF = 0-5 UI/ml). The C-reactive Protein (CRP) levels were also measured by a nephelometric assay (Siemens BN II, Healthineers, Italy) (Normal range: RP sensible = 0-0.30 mg/dl).

All RA patients satisfied the classification criteria of the American College of Rheumatology for RA. Informed consent was obtained from all patients and control subjects, the clinical trial complied with the Helsinki Declaration. All subjects were genotyped for VDR polymorphism.

Assessment of Bone Metabolism

At the time of the study, all RA patients underwent bone densitometry evaluation by a dual energy X-ray absorptiometry (DEXA) (Hologic, Inc., Bedford, MA, USA). We obtained the BMD (g/cm^2) of the second through fourth lumbar vertebrae and of the femoral neck, and T-score determinate (normal range: +1/-1). The variation coefficient in vivo tested before starting the study ranged 0.6-1.2% to the lumbar spine and 0.9-2.2% to the femoral neck. For the radiological evaluation of erosions it was used the Sharp score modified by Van der Heijde[9]. The healthy donors had normal mean BMD values (Table I). Radiographs of hands were available in all patients, and 26 (65%) of them had erosions (Table I). The healthy control group had no history of any rheumatologic disorder.

Determination of VDR Genes Polymorphisms

Genomic DNA was extracted by a new saline-method following the manufacturer's protocol of Ampli DNA extraction (Dia-Chem Srl, Molecular Biology, Naples, Italy). The BsmI, ApaI, TaqI and FokI polymorphisms have been studied on DNA from patients and controls RFLP method using by AMPLI set VDR Polymorphisms (Dia-Chem Srl, Molecular Biology, Naples, Italy) amplifying samples with specific primers and restriction analysis (Table II). This method is based on 4 consecutive steps.

First (extraction) step: extraction of genomic DNA from whole blood picked up in EDTA; optimal concentration of DNA is about 50 ng/µl, and stored at 4°C.

Second (DNA amplification) step: a PCR amplification reaction carried out using MIX PCR (ApaI-TaqI; BsmI; FokI) 20 µl, H_2O 27.5 µl, Taq-Polymerase 0.5 µl (5U/ µl), DNA 2 µl (100 ng). PCR conditions were: 35 PCR cycles performed with denaturation at 95°C for 30 seconds; annealing at 62°C for 30 seconds; elongation at 72°c for 1 minute. Positive controls (heterozygous and homozygous for major and minor alleles) and negative control (H_2O RNase/Dnase-free) also was added to each reaction.

Table II. Kit and storage

Name	Storage
Amplification	
Mix PCR ApaI-TaqI	-20°C
Mix PCR BsmI	- 20°C
Mix PCR FokI	- 20°C
H_2O sterile RNase/DNase Free	- 20°C
Taq Polymerase	- 20°C
Homozigote control (FokI)	- 20°C
Heterozigote control (ApaI-TaqI-BmsI)	- 20°C
Digestion	
Enzymes (ApaI, BmsI, TaqI, FokI)	- 20°C
Buffer	- 20°C
H_2O steril RNase/DNase Free	- 20°C

Table III. Restriction Fragments of polymorphic sites of VDR genes.

Restriction sites	PCR amplicon	Allelic variants	Fragments post-digestion (bp)
ApaI	745bp	A/A	745
		A/a	745+528+218
		a/a	528+218
TaqI	745bp	T/T	745
		T/t	745+293+251+201
		t/t	293+251+201
BmsI	822bp	B/B	822
		B/b	822+646+176
		b/b	646+176
FokI	267bp	f/f	267
		F/f	267+197+70
		F/F	197+70

Note: Wild type alleles are AA, TT, BB, ff. Minor polymorphic alleles are aa, tt, bb, FF.

Third (DNA enzyme restriction assay) step: the digestion procedure with enzyme BsmI, TaqI, FokI, ApaI were performed by incubation temperature of 65°C for TaqI and Bsml, 37°C for FokI and 30°C for ApaI; digested products were stored at -20°C until 4th step.

Fourth step: (electrophoresis of agarose gels) 10 µl of digested samples were run on 4% agarose Ethidium bromide-stained gels; a 100 bp molecular weight markers was used. The entire procedure was kept on ice.

The digested DNA-fragments of polymorphic sites of VDR gene were shown in Table III.

Statistical Analysis

Mean ± SD (standard deviation) values were calculated in all investigated parameters. Within the RA patients and healthy controls, frequencies of different polymorphisms investigated were calculated using the χ^2-test. Association between clinical laboratory markers and SNPs screened were determined by analysis of variance (ANOVA).

Results

Data by genotyping, CRP value and correlation with erosive disease were reported in Table IV. Furthermore, several results were retrieved:

a) There was no difference in the polymorphisms frequency between RA patients and controls.

b) Only TaqI VDR genotype (TT vs tt) seems to influence the bone density in females, but not in males. In fact, when compared to controls, the mean difference of BMD at the lumbar spine in RA women with the tt genotype was 4.7% lower than controls, as compared to a difference of 0.1% lower than controls in women with the TT genotype (p <0.05). Similarly, at the hip, RA women with the tt genotype had a bone density of 9.8% lower than controls while the TT group had a mean difference of 3.7% lower than controls (p <0.01). Interestingly, very high levels of CRP (15.02 ± 2.80) were found in RA patients with erosions (p <0.01) in respect to RA patients without erosion.

The TaqI t and BsmI B alleles are two polymorphisms in tight linkage Disequilibrium, so they were both associated with an accelerated systemic bone loss in RA, but surprisingly not with a focal bone loss.

The CRP seems to be a significant factor in determining bone loss (associated with immune-phlogosis) at the spine and hip in both men and women with RA. Patients with systemic bone loss have 15.02 g/L of CRP versus 0.33 g/L of patients without erosion.

Discussion

Although the multifactorial scenery of autoimmune diseases is well recognized, genetic factors are considered to be strong determinants of these conditions, and researchers have struggled to search for the genes responsible. Many genes have been studied in this context; the VDR gene being an example of those studied in RA patients[10]. Vitamin D plays a key role in calcium homeostasis and also contributes to regulation of the immune system[11]. Given the immunosuppressive effects of vitamin D and the potential

Table IV. Correspondences between clinical characteristics of patients with rheumatoid arthritis and VDR polymorphisms. The relation between TaqI tt allele and erosion is statistically significant (*$p<0.05$)

Vitamin D receptor allelic variants	Patient n. 40	High CRP* n. 24	Erosive disease (Yes) n. 26	(No) n. 14	Controls n. 40
FokI					
FF	24 (60%)	11 (46%)	11 (42%)	5 (36%)	18 (45%)
Ff	13 (32%)	11 (46%)	11 (42%)	5 (36%)	18 (45%)
ff	3 (8%)	2 (8%)	4 (16%)	4 (28%)	4 (10%)
TaqI					
TT	16 (40%)	10 (42%)	13 (50%)	9 (63%)	15 (38%)
Tt	18 (45 %)	11 (46%)	9 (35%)	3 (35%)	20 (50%)
tt	6 (15%)	3 (12%)	*4 (15%)	*2 (2%)	5 (12%)
BmsI					
BB	6 (15%)	3 (12%)	3 (12%)	2 (14%)	3 (7.5%)
Bb	16 (40%)	10 (42%)	13 (50%)	6 (43%)	20 (50%)
bb	18 (45%)	11 (46%)	10 (38%)	6 (43%)	17 (42.5%)
ApaI					
AA	36 (90%)	24 (100%)	25 (96%)	12 (86%)	34 (85%)
Aa	3 (7.5%)	0 (0%)	1 (4%)	1 (7%)	5 (12.5%)
aa	1 (2.5%)	0 (0%)	0 (0%)	1 (7%)	1 (2.5%)

*CRP C-Reactive Protein.

link between vitamin D deficiency and autoimmune diseases, VDR polymorphisms, which may influence VDR activity, have been studied as potential causes of autoimmune diseases, including RA[12].

The results of these studies support an association between specific VDR FokI, TaqI, BsmI, ApaI alleles and bone loss in RA[13]. The TaqI t and BsmI B alleles (in tight linkage) were associated with an accelerated systemic bone loss in RA, but surprisingly not with a focal bone loss. In addition, inflammation profile, as measured by CRP, was associated with bone loss and increased bone turnover. The FokI and TaqI polymorphisms were linked to early onset RA. These findings taken together support an immunoregulatory role for vitamin D mediated through VDR with effects on disease susceptibility and bone loss in RA. Further, lending credibility to this are findings from epidemiological studies in RA, which show that vitamin D supplementation may be associated with lower risk for the disease and lower disease activity. These effects of VDR genotypes and vitamin D supplementation are not surprising, given that the central pathological feature in RA is bone and joint destruction[14].

Furthermore, the VDR genotyping seem to be cost-effectiveness. Evermore, the cost of a genetic testing includes more than just the cost of the test and/or the kit itself selected from the available methods[15,16]. However, additional costs are genetic counseling; lab weres equipment and time-labor are potentially of greater magnitude and must be evaluated[17]. Furthermore, the major issues to consider for the clinical laboratories (who are responsible for providing VDR genotyping), are: (1) the availability of in vitro diagnostic (IVD)-cleared tests; (2) the current absence of public reimbursement; (3) the need for genotyping accuracy; (4) the need to find clinical expertise to interpret laboratory data results[18,19].

Based on these considerations, the clinician and the lab manager may join together to evaluate advantages and limitation, in terms of costs and availability, of the mainly appropriate methods to setting molecular diagnostics of VDR Genotyping[20].

Conclusions

The present findings further suggest that VDR polymorphisms seem to influence directly the bone loss in RA and prompt for additional studies on the possible involvement of vitamin D and VDR alleles in the development and progression of RA.

Conflict of Interest

Disclosures include relationships between VE with DIA-Chem whose products that are related to the subject matter of the manuscript. The other authors stated that there are no conflict of interest regarding the publication of this article.

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