Vitamin D receptor genotypes are not associated with rheumatoid arthritis or biochemical parameters of bone turnover in German RA patients

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Abstract

Objective

Vitamin D is known to exert immunomodulatory effects. An overrepresentation of the b allele of the vitamin D receptor (VDR) has been detected in autoimmune diseases as type-1-diabetes and multiple sclerosis. VDR polymorphisms have been shown to influence bone metabolism and bone density. The aim of the present study was to examine the distribution of VDR alleles in German rheumatoid arthritis (RA) patients and their relation to bone turnover parameters.

Methods

62 German RA patients were included and compared to 40 controls. Three VDR alleles were examined (Bsm I, Taq I and Fok I). In addition, serum intact osteocalcin (OC), parathyroid hormone, bone specific alkaline phosphatase (B-ALP), the carboxyterminal extension peptide of type I procollagen, 25-OH-vitamin D and urinary deoxypyridinoline (DPD) excretion were measured. Furthermore, C-reactive protein, erythrocyte sedimentation rate and rheumatoid factor were measured.

Results

We found a slightly higher frequency of the bB and tT-genotype in RA patients compared to controls, which was not statistically significant. OC and B-ALP were found to be significantly higher in RA patients with positive correlations between bone formation and resorption parameters indicating higher bone turnover in RA patients with maintained coupling. CRP in RA patients correlated with DPD and inversely with PTH. VDR genotype showed no association with bone turnover, family history or the presence of rheumatoid factor.

Conclusions

Our results suggest that VDR polymorphisms do not play a major role in RA predisposition in Germans.

Key words

Rheumatoid arthritis, genetics, vitamin D receptor, bone metabolism.
VDR genotypes in rheumatoid arthritis/ B. Goertz et al.

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Introduction
Rheumatoid arthritis (RA) is a systemic inflammatory autoimmune disease manifesting with inflammation of joints and resulting in progressive joint destruction (1). The aetiology of RA is not completely understood yet. The HLA-DRB1 shared epitope and certain HLA-DRB1-alleles (2-4) have been shown to be associated with RA. Epstein-Barr virus infection and an estrogen receptor polymorphism have also been examined concerning an association with RA risk with contradicting results (5-8).

Apart from corticosteroids, nonsteroidal antiinflammatory drugs (NSAIDs) and disease-modifying antirheumatic drugs (DMARDs) such as methotrexate or azathioprin, and high dose administration of 1-alpha(OH)D3 (alphacalcidiol) have been shown to reduce disease activity (9).

Vitamin D exerts immunomodulatory effects including suppression of T-cell proliferation affecting mainly the CD45RO+ subpopulation (10), inhibition of the production of the T-cell derived cytokines interleukin-2 and interferon-gamma (11-13), and the inhibition of interleukin-12 production by activated macrophages (14). The metabolic effects of vitamin D are mediated by the vitamin D receptor (VDR).

The VDR gene consists of 11 exons and contains 4 polymorphic regions, three of which are located at the 3’-end of the gene and can be detected by restriction enzymes Bsm I, Apa I and Taq I; one is located in the start codon and can be detected by restriction enzyme Fok I.

Although the functional consequences of VDR polymorphisms are not fully understood, certain VDR alleles appear to be associated with autoimmune diseases like multiple sclerosis (15) or type-1 diabetes mellitus (16, 17). Furthermore, association between vitamin D receptor polymorphisms and bone turnover or bone mineral density have been reported in some populations (18, 19), but seem to be modified by age and calcium intake (20, 21).

The aim of this study was to assess the distribution of VDR polymorphisms Bsm I, Taq I and Fok I in RA patients compared to controls, assess differences in bone metabolism between RA patients and controls, and search for associations between bone metabolism and genotype or therapy.

Materials and methods
Subjects
Sixty-two German RA patients were recruited from the rheumatologic outpatients clinic of the IIId Medical Department of the University of Giessen. Diagnosis of RA was made according to the 1987 revised ACR criteria (22). Using the ACR 1991 revised criteria for the classification of global functional status (23), the disease stages were: stage I in 2, stage II in 31, stage III in 27 and stage IV in 2 patients. The family history for RA was available in 59 patients and was positive in 8 patients (14%) and negative in 51 (86%) patients. All patients received NSAIDs or Cox-2-inhibitors in addition to various combinations of DMARDs with or without additional corticosteroids. At the time of blood sampling 40/62 (65%) patients were receiving corticosteroids, the mean duration of steroid treatment was 60.8 ± 82.7 months. 31/62 (50%) patients received vitamin D metabolites for the prevention of corticosteroid induced osteoporosis, 59/62 (95%) received DMARDs (methotrexate, leflunomide, azathioprin, chloroquine, gold). Eight patients suffered from corticosteroid-induced osteoporosis and were treated with bisphosphonates, calcium and vitamin D metabolites.

Control samples were drawn from 40 German persons of the hospital staff and outpatients without autoimmune or bone-related diseases and not taking drugs influencing bone metabolism. In women menopausal status was documented. Informed consent was obtained from all study subjects. Clinical and laboratory data of the patients and controls are listed in Table II.

Bone turnover
As parameters of bone turnover we measured serum bone specific alkaline phosphatase (B-ALP) (Alkphase-B, Metra Biosystems, Osnabrueck, Germany) (24), serum intact osteocalcin

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### Table I. Primer sequences, length of PCR product and restriction fragments and digestion temperature.

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Direction</th>
<th>Primer sequence</th>
<th>Length (PCR product)</th>
<th>Length (digestion fragments)</th>
<th>Digestion temp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>BsmI</td>
<td>Sense</td>
<td>5’-cag age atc cac tga cag cgg cag tag tga-3'</td>
<td>800 bp*</td>
<td>BB: 800 bp*&lt;br&gt;bb: 650/150 bp*</td>
<td>65°C</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>5’-cag age atc cac tga cag cgg cag tgg tgg-3'</td>
<td>740 bp*</td>
<td>TT: 495/245 bp*&lt;br&gt;tt: 255/290/205 bp*</td>
<td>70°C</td>
</tr>
<tr>
<td>TaqI</td>
<td>Sense</td>
<td>5’-cag age atc cac tga cag cgg cag tag tga-3'</td>
<td>265 bp*</td>
<td>FF: 265 bp*&lt;br&gt;ff: 196/69 bp*</td>
<td>37°C</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>5’-cag age atc cac tga cag cgg cag tgg tgg-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*bp = base pairs

### Table II. Clinical data and laboratory data of patients and controls.

<table>
<thead>
<tr>
<th></th>
<th>RA (n = 62)</th>
<th>Controls (n = 40)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Men (number/age (years))</td>
<td>13/58 ± 12.4</td>
<td>9/52.8 ± 15.5</td>
</tr>
<tr>
<td>Women (number/age (years))</td>
<td>49/57.4 ± 14.8</td>
<td>31/53.2 ± 15.2</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.6 ± 4.33</td>
<td>27.67 ± 5.32</td>
</tr>
<tr>
<td>Premenopausal</td>
<td>14 (29%)</td>
<td>12 (39%)</td>
</tr>
<tr>
<td>Postmenopausal</td>
<td>35 (71%)</td>
<td>19 (61%)</td>
</tr>
<tr>
<td>Age at onset (years)</td>
<td>47 ± 15.7</td>
<td></td>
</tr>
<tr>
<td>Disease duration (months)</td>
<td>124.9 ± 103.2</td>
<td></td>
</tr>
<tr>
<td>Rheumatoid factor +/- (n = 58)</td>
<td>39/67 (19/33%)</td>
<td></td>
</tr>
<tr>
<td>B-ALP (U/l)</td>
<td>24.6 ± 8.4</td>
<td>21.3 ± 5.8</td>
</tr>
<tr>
<td>OC (ng/ml)</td>
<td>22.0 ± 11.9</td>
<td>17.2 ± 9.8</td>
</tr>
<tr>
<td>PICP (ng/ml)</td>
<td>90.4 ± 29.4</td>
<td>90.5 ± 29.1</td>
</tr>
<tr>
<td>PTH (pg/ml)</td>
<td>28.1 ± 16.0</td>
<td>29.4 ± 13.1</td>
</tr>
<tr>
<td>DPD (nmol/mmol creatinine)</td>
<td>8.8 ± 3.3</td>
<td>7.9 ± 2.0</td>
</tr>
<tr>
<td>25-OH-D₃ (ng/ml)</td>
<td>23.6 ± 17.1</td>
<td>24.6 ± 14.1</td>
</tr>
</tbody>
</table>

BMI: body mass index; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; B-ALP: serum bone specific alkaline phosphatase; OC: serum osteocalcin; PICP: serum type I C-terminal extension peptide of procollagen; PTH: serum parathyroid hormone; DPD: urinary deoxypyridinoline; 25-OH-D₃: serum 25-OH-Vitamin D.

(Nichols Institute Diagnostics, San Juan Capistrano, CA, USA) (25), serum type I C-terminal extension propeptide of procollagen (PICP) (Prolagen-C, Metra Biosystems, Palo Alto, CA, USA) (26), 25-OH-Vitamin D (25-OH-D₃) serum levels (27) (Immunodiagnostik, Ben- heim, Germany), serum intact parathyroid hormone (PTH) (Nichols Institute, San Juan Capistrano, CA, USA) (28), and urinary excretion of free deoxypyridinoline (DPD) (Immulite, Diagnostic Products Corporation, CA, USA) (29), which was corrected for urinary creatinine concentrations (Jaffè Method, Boehringer Mannheim systems, Mannheim, Germany).

#### Markers of disease activity

RA activity was measured by C-reactive protein (CRP) (Boehringer Mannheim Systems, Mannheim, Germany) and erythrocyte sedimentation rate (ESR). CRP and ESR correlated highly significantly (p < 0.001, r = 0.58). Hence, in the following only correlations with CRP are described.

Furthermore, rheumatoid factor was measured (RHEFTM, Beckman Instruments Inc, Fullerton, California).

#### Genotyping

Genomic DNA was extracted from peripheral blood leukocytes as previously described (30). In brief, erythrocytes were lysed by mixing 3 ml of EDTA blood samples with 9 ml of lysis-buffer (0.32 M saccharose, 0.01 M Tris, 5 mM MgCl₂, 1% Triton X) followed by 10' incubation at 4°C, centrifugation, resuspension of the leukocyte pellet in 1.5 ml Sodium-EDTA-buffer (75 mM NaCl, 25 mM EDTA, pH 8.0) and 200 ml 10% SDS and overnight digestion at 37°C with Proteinase K (30 µg/ml). After protein precipitation with 500 µl 6 M NaCl solution, DNA was precipitated by adding 2 volumes of Ethanol (-20°C) followed by a washing step with 70% Ethanol and suspension of the DNA in Tris-EDTA-buffer (10 mM Tris-EDTA, 0.1 mM EDTA, pH 8.0). Polymerase chain reaction (PCR) amplification of the gene segments flanking the enzyme restriction sites was performed using a thermocycler (Hybaid PCR Sprint, Hybaid-AGS, Heidelberg, Germany) and primers described previously (31-33). PCR consisted of activation of Taq-polymerase for 5' at 94°C followed by 45 cycles of 94°C for 1', 58°C (BsmI) or 60°C (Taq I, Fok I) for 1' and 72°C for 1'30''. Afterwards extension of PCR products was performed at 72°C for 10'. Primer sequences, restriction fragment lengths and digestion temperatures are listed in Table I. PCR products were then digested by Bsm I (Hybaid-AGS, Heidelberg, Germany), Taq I (Hybaid-AGS, Heidelberg, Germany) and Fok I (New England Biolabs, Frankfurt/Main, Germany) according to the manufacturer's conditions and genotypes were determined by agarose gel electrophoresis. Genomic polymorphisms were described as FF, BB and TT (absence of restriction site in both alleles), ff, bb,
tt (presence of restriction site in both alleles) and Ff, Bb and Tt (heterozygous) according to the standard nomenclature (Fig. 1a-c).

Statistical analysis
Explorative data analysis was done using the following statistical tests. Mann-Whitney’s U-test was used for comparison of bone turnover and age between patients and controls. It was also used to compare bone turnover between osteoporotic and non-osteo- porotic patients and between patients with and without DMARD or steroid medication. Kruskal-Wallis test was performed for assessment of associations between VDR genotypes and bone turnover parameters and between VDR genotypes and age of onset. Correlations within biochemical parameters of bone turnover and between bone turnover and CRP or age in RA patients were assessed by Spearman’s rank correlation test. Chi² (χ²)-test was used to compare the distribution of vitamin D receptor genotypes in patients and controls, between patients with positive and negative family history and between seropositive and seronegative patients. All statistics were done using StatView™ II software (Abacus concepts, Inc.).

Results
No significant difference in the distribution of age (Man-Whitney-test) and sex (χ²-test) was found between patients and controls. Serum calcium and creatinine were in the normal range in all individuals and did not differ significantly between the patient and control groups.

Frequency of VDR alleles in RA patients and controls
Distribution of Taq I and Bsm I VDR genotypes was different in patients and controls when comparing single polymorphisms, with a lower frequency of BB and tt genotypes and higher frequency of heterozygotes in RA patients (p=0.026 for Bsm I, p=0.017 for Taq I, Table III). Because of the explorative approach no correction for multiple testing was made. Assuming a level of significance of p=0.015 according to the three polymorphisms tested, the difference in distribution of Bsm I and Taq I polymorphisms were no longer significant, although the difference in Taq I genotypes was close to statistical significance.

No difference in genotype distribution was observed between seropositive and seronegative patients. A significant difference in genotype distribution between patients with positive and negative family history was found only for the Fok I polymorphism (p = 0.0012, χ² = 13.37, df = 2). Of the patients with positive family history none had the fF genotype (3/8 ff, 5/8 FF) whereas 23/54 (43%) patients with negative family history had the fF genotype (2/54 ff, 29/54 FF). No association between genotype and age at onset was found. Furthermore we detected linkage between b and T as well as between B and t alleles (p = 0.0001, χ² = 83.81, df = 4). In patients and controls taken together, 93% of individuals homozygous for the t genotype also were homozygous for the B allele. We detected no difference concerning linkage between the patient and control groups and no linkage between Fok I genotypes and Bsm I or Taq I genotypes.
Biochemical parameters of bone turnover

B-ALP and OC were significantly higher (both p < 0.05) in RA patients compared to controls, indicating higher bone turnover (Table II).

CRP in RA patients correlated with DPD (p < 0.01, r = 0.4) and negatively with PTH (p < 0.05, r = -0.30) and a trend for an association with PICP (p = 0.08, r = 0.23) was observed. Furthermore, in RA patients we found positive correlations between the bone formation parameters B-ALP and OC (p < 0.01, r = 0.35), B-ALP and PICP (p < 0.001, r = 0.44) and OC and PICP (p < 0.001, r = 0.51). DPD in RA patients correlated with B-ALP (p < 0.01, r = 0.52) and PICP (p < 0.01, r = 0.35), indicating maintained coupling of bone formation and resorption processes. In patients B-ALP and PICP correlated significantly with age (p < 0.01, r = 0.42 for B-ALP; p < 0.01, r = 0.40 for PICP). No association between VDR genotypes and biochemical parameters of bone turnover was detected in either in RA patients (Table IV) or in controls (data not shown).

No association between bone metabolism and steroid medication or duration of steroid treatment was detectable. Nor was any difference observed between patients receiving DMARDs and patients not receiving DMARDs.

No difference concerning VDR genotypes, bone turnover or disease activity was found between patients suffering from secondary osteoporosis and non-osteoporotic patients.

Discussion

For autoimmune diabetes and autoimmune encephalomyelitis an association with VDR genotype has been shown (15-17). Thus, an association between VDR genotype and RA predisposition is conceivable.

Synovial inflammatory cells, chondrocytes and synovial stromal cells express the VDR (34). Vitamin D is able to suppress activity of autoimmune diseases such as autoimmune diabetes or experimental autoimmune encephalomyelitis (35, 36) and has also been shown to reduce disease activity in RA patients (9). The functional consequences of the VDR polymorphisms are not fully understood yet. Originally, differences in mRNA stability between the alleles were assumed, but this was not confirmed in later studies (37). A recent study revealed differences in the production of osteocalcin by osteoblast-like cells of different genotypes, resulting in higher osteocalcin levels after stimulation of cells with genotypes including homozygosity for the T- and the b-allele, and suggested that the polymorphisms affect individual responsiveness to vitamin D (38). In the present study we found a slightly – not statistically significant – higher frequency of the tt and bB genotypes in German RA patients compared to controls. The distribution of VDR alleles in controls was similar to that previously reported for healthy Caucasians (39-44). Thus, at least in our study an association between VDR genotype and RA risk cannot be established. Although this is in accordance with the results of recently published studies in Korean and Spanish RA patients (45, 46), our results should be confirmed in a larger German population as the sample size in our study was smaller than that of the aforementioned studies.

In contrast to the study of Garcia et al. (46), we did not detect any association between VDR genotype and age at onset. We did find a difference in distribution of the Fok I polymorphism between patients with a positive or a negative family history, but the number of

Table III. Distribution of VDR genotypes in RA patients (n = 62) and controls (n = 40).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>RA no. (%)</th>
<th>Controls no. (%)</th>
<th>p-value (χ² test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>bb</td>
<td>10 (16)</td>
<td>11 (27)</td>
<td></td>
</tr>
<tr>
<td>bB</td>
<td>43 (69)</td>
<td>17 (43)</td>
<td>0.026</td>
</tr>
<tr>
<td>BB</td>
<td>9 (15)</td>
<td>12 (30)</td>
<td></td>
</tr>
<tr>
<td>tt</td>
<td>4 (6)</td>
<td>10 (25)</td>
<td></td>
</tr>
<tr>
<td>tT</td>
<td>34 (55)</td>
<td>14 (35)</td>
<td>0.017</td>
</tr>
<tr>
<td>TT</td>
<td>24 (39)</td>
<td>16 (40)</td>
<td></td>
</tr>
<tr>
<td>ff</td>
<td>5 (8)</td>
<td>3 (7)</td>
<td></td>
</tr>
<tr>
<td>fF</td>
<td>23 (37)</td>
<td>23 (58)</td>
<td>0.117</td>
</tr>
<tr>
<td>FF</td>
<td>34 (55)</td>
<td>14 (35)</td>
<td></td>
</tr>
</tbody>
</table>

Biochemical parameters of bone turnover

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In patients B-ALP and PICP correlated significantly with age (p < 0.01, r = 0.42 for B-ALP; p < 0.01, r = 0.40 for PICP). No association between VDR genotypes and biochemical parameters of bone turnover was detected in either in RA patients (Table IV) or in controls (data not shown).

No association between bone metabolism and steroid medication or duration of steroid treatment was detectable. Nor was any difference observed between patients receiving DMARDs and patients not receiving DMARDs.

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Table IV. Comparison of bone turnover by patients genotypes.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>B-ALP (μL/L)</th>
<th>OC (ng/ml)</th>
<th>PICP (ng/ml)</th>
<th>PTH (pg/ml)</th>
<th>DPD (nmol/mmol Krea)</th>
<th>25-OH-D3 (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>bb (n=10)</td>
<td>24.64±6.58</td>
<td>26.81±13.82</td>
<td>91.49±28.27</td>
<td>23.59±10.19</td>
<td>8.36±3.4</td>
<td>29.5±21.88</td>
</tr>
<tr>
<td>BB (n=9)</td>
<td>26.5±5.89</td>
<td>21.72±14.31</td>
<td>85.36±22.71</td>
<td>40.26±16.72</td>
<td>10.23±3.75</td>
<td>21.22±29.89</td>
</tr>
<tr>
<td>tt (n=4)</td>
<td>23.03±3.45</td>
<td>32.88±12.28</td>
<td>95±16.75</td>
<td>45.47±21.26</td>
<td>10.28±3.71</td>
<td>16.75±4.11</td>
</tr>
<tr>
<td>tT (n=34)</td>
<td>25.57±9.62</td>
<td>20.31±11.97</td>
<td>94.79±31.69</td>
<td>29.16±17.24</td>
<td>9.08±3.06</td>
<td>23.03±18.48</td>
</tr>
<tr>
<td>TT (n=24)</td>
<td>23.69±7.19</td>
<td>23.24±11.19</td>
<td>83.12±27.02</td>
<td>23.97±11.10</td>
<td>8.36±3.46</td>
<td>25.67±14.20</td>
</tr>
<tr>
<td>ff (n=5)</td>
<td>27.5±9.48</td>
<td>18.6±1.44</td>
<td>101.63±37.2</td>
<td>37.68±34.23</td>
<td>7.23±1.23</td>
<td>22.6±16.79</td>
</tr>
<tr>
<td>fF (n=23)</td>
<td>25.54±8.62</td>
<td>21.02±9.91</td>
<td>92.07±33.96</td>
<td>23.83±10.73</td>
<td>9.25±2.72</td>
<td>20.04±12.85</td>
</tr>
<tr>
<td>FF (n=34)</td>
<td>23.81±8.37</td>
<td>23.49±13.74</td>
<td>87.97±25.65</td>
<td>30.17±16.08</td>
<td>8.90±3.72</td>
<td>16.24±12.53</td>
</tr>
</tbody>
</table>

B-ALP: serum bone specific alkaline phosphatase; OC: serum osteocalcin; PICP: serum type I C-terminal extension propeptide of procollagen; PTH: serum parathyroid hormone; DPD: urinary deoxypyridinoline; 25-OH-D3: serum 25-OH-Vitamin D.
patients with a positive family history was relatively small and no difference in Fok I genotypes between RA patients and controls was found. This result might thus not reflect a true association. Gough et al. reported of a higher rate of bone loss in British women with RA and the tt genotype (47). Unfortunately bone density data were not available in our study. Bone turnover was higher in RA patients than in controls without any association to the VDR genotype. Therefore a predisposition to more rapid bone loss in a certain genotype cannot be derived from our data. The lack of an association between VDR genotype and bone turnover is in keeping with the conflicting results reported in the literature concerning the influence of VDR genotypes on bone turnover (18, 19, 48).

Surprisingly, no association between bone turnover and steroid medication, duration of steroid treatment or DMARD therapy was detectable. It has to be noted, however, that due to the advanced state of disease in many of our patients, therapy consisted of various combinations of DMARDs with or without steroids. Hence, well-defined therapy groups could not be formed for statistical comparison and the overlapping effects of the simultaneously administered methotrexate and NSAIDs may be the reason that the well documented effects of steroid medication on bone turnover were not found in our study (49-51). Increased periarticular bone turnover has been shown before by histomorphometry (52). There was no significant difference in age, BMI, sex distribution and percentage of postmenopausal women between patients and controls. Therefore the higher bone turnover in RA patients detected in our study was most probably caused by inflammation-induced increased bone turnover in the vicinity of the affected joints. It has to be noted, however, that lifestyle factors influencing bone were not considered. The correlation between CRP and DPD and the negative correlation between CRP and PTH found in our study has been described before by Oelzner et al. (53), who attributed these correlations to an increase in calcium release due to inflammatory bone resorption with the consecutive inhibition of PTH secretion.

In summary, our study is the first to examine the prevalence of VDR polymorphisms in German RA patients. As in Korean and Spanish RA patients, no statistically significant association between VDR polymorphisms and RA susceptibility has been found. In contrast to other autoimmune diseases, VDR polymorphisms do not play a crucial role in RA predisposition.

Acknowledgement
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