Association of rheumatoid arthritis with \textit{Mdm2 SNP309} and genetic evidence for an allele-specific interaction between \textit{MDM2} and \textit{p53 P72R} variants: a case control study

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\textbf{Abstract}

\textbf{Objective}

This study examines two common, functional, single nucleotide polymorphisms (SNP) in the genes coding the human homolog of murine-double-minute-2 (\textit{MDM2}) and \textit{p53} in patients with rheumatoid arthritis (RA) based on the hypothesis that \textit{p53} may be an important negative regulator of the pro-inflammatory transcription factor nuclear factor \textit{kappa b} (NF\textit{\kappa}B).

\textbf{Methods}

Genomic DNA was obtained from 221 patients with RA who fulfilled at least 4 ACR criteria and from 521 healthy controls. \textit{Mdm2 SNP309} and \textit{p53 P72R} were genotyped by polymerase chain reaction and restriction enzyme analysis.

\textbf{Results}

In RA patients the frequencies of the \textit{mdm2 SNP309 G} allele and both G-containing genotypes were significantly reduced (G allele: OR: 0.75, 95\% CI: 0.59–0.95, \textit{p}=0.016; genotype TG: OR: 0.71, 95\% CI: 0.50–1.00; genotype GG: OR: 0.58, 95\% CI: 0.34–0.99; both: \textit{p}=0.049). Concerning \textit{p53 P72R}, no differences in allele or genotype frequencies were detected. A combined analysis of both polymorphisms revealed a significant interaction between them (\textit{p}=0.046). In individuals carrying \textit{≥1 p53 72R} allele, \textit{MDM2} had a protective effect, whereas in individuals homozygous for \textit{p53 72P}, \textit{MDM2} had the opposite effect.

\textbf{Conclusion}

The function of \textit{MDM2} depends on the \textit{p53 P72R} genotype, resulting in either an increased or reduced risk for RA. We suggest that in most cases \textit{MDM2} stabilizes the conformation of \textit{p53}, whereas in \textit{p53 PP-positive subjects MDM2 supports the degradation of p53}.

\textbf{Key words}

Mdm2 SNP309 and p53 R72P in RA / G. Assmann et al.

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The study was supported by grants from the Faculty of Medicine of the University of Saarland: HOMFOR 2007 (to Jan Voswinkel) and BMBF Kompetenznetz Rheuma (to Inga Melchers).

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Received on December 16, 2008; accepted in revised form on April 14, 2009.

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Introduction

Established rheumatoid arthritis (RA) is clinically characterized by chronic symmetrical inflammation of several joints associated with an increase in synovial tissue cell mass (pannus formation) (1, 2). Without treatment, inflammatory cells and synoviocytes will infiltrate the joint cartilage and subchondral bone, causing progressive articular damage. The skeletal complications of RA include focal bone erosions and juxtaarticular osteopenia at sites of active inflammation, leading to joint destruction, deformation and disability (3, 4). In addition to macrophages and lymphocytes, increased numbers of fibroblast- and macrophage-like synoviocytes are present in the rheumatoid synovium and play a central role in pathological processes (5, 6). Although rheumatoid synoviocytes are not malignant cells, their phenotype shows several features reminiscent of transformed cells, e.g. anchorage-independent growth or loss of contact inhibition in culture. Alterations in apoptosis have been suggested as one cause (6). The tumor suppressor protein p53 (53 kDa encoded on chromosome 17p13.1) and its most important regulator, MDM2 (90 kDa, chromosome 12q13–14), are positioned in the centre of a pathway that eliminates damaged cells through apoptosis (7). Moreover, recent in vitro and in vivo studies have indicated that p53 is one of the most important negative regulators of the pro-inflammatory transcription factor NFκB (8). TNF-alpha and interleukin 1-beta are known to be subject to regulation by NFκB, and increased expression of TNF-alpha and interleukin 1-beta causes activation of osteoclasts and collagenases and, subsequently, erosive bone lesions in many rheumatic diseases (9-11).

MDM2 is an ubiquitin ligase that binds p53 and blocks its function as a transcription factor. Ubiquitinilation marks p53 for degradation via the proteasome. On the other hand, p53 binds to the promoter of mdm2 and activates its expression. Among other factors, the balance between MDM2 and p53 regulates the expression of downstream genes, and depends on the protein level and functional activity of both molecules. Functional SNPs have been described in the two genes. Mdm2 SNP309 (T/G) is located in the promoter/enhancer region of the mdm2 gene and affects the binding of the transcription factor SP1. The G allele is believed to cause higher MDM2 levels and, consequently, less functional p53 in stressed cells (12). The SNP P53 P72R (codon 72; Pro72Arg; C/G) lies in the proline-rich domain of p53 and probably affects its structure and influences its functional activity; e.g. P53 72R has been reported to induce apoptosis more effectively than P53 72P (13). It is unclear at present whether these polymorphisms have an impact on NFκB and inflammation. Here we conducted a case-control study to evaluate whether p53 P72R and/or mdm2 SNP309 may be associated with RA.

Materials and methods

Study participants

We recruited 221 patients with RA from the outpatient rheumatology department at the hospital of the University of Saarland Medical School, Homburg/Saar, Germany, and from the Department of Rheumatology and Clinical Immunology, University Medical Center Freiburg, Germany. Blood donors from the Institute for Transfusion Medicine, University of Saarland Medical School, served as controls (n = 521, 36% female, mean age 34.8±11.3 years, range 18–65 years). Patients and controls were of central European Caucasian ethnicity. The ethics committees of the medical association of the Saarland, Germany approved the study and all participants gave their written informed consent.

All patients fulfilled ≥4 of the 1987ACR criteria for RA. Their mean age was 61.9±15.3 years (range 18–92 years) and their mean disease duration was 8.2±8.3 years (range 0–44 years). Most patients were female (73.3%) and rheumatoid factor (RF)-positive (75.6%). Anti-CCP antibodies were assayed in 175 patients and detected in 67.4%. Antibodies were tested by RF IgM ELISA (positive >20 RE/ml) and anti-CCP IgG ELISA (positive >5 RE/ml) (Euroimmun, Luebeck, Germany).

Competing interests: none declared.
Table I. Allele frequencies of p53 P72R and mdm2 SNP309 in patients with rheumatoid arthritis (RA) and in healthy controls.

<table>
<thead>
<tr>
<th></th>
<th>RA patients</th>
<th>Healthy controls</th>
<th>p-value</th>
<th>Odds ratio (95 % CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Npos/Nres (%)</td>
<td>Npos/Nres (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p53 P72R alleles</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R</td>
<td>331/430 (76.9)</td>
<td>766/1030 (74.4)</td>
<td></td>
<td>0.29</td>
</tr>
<tr>
<td>P</td>
<td>99/430 (23.0)</td>
<td>264/1030 (25.6)</td>
<td></td>
<td>0.87 (0.67-1.13)</td>
</tr>
<tr>
<td>mdm2 SNP309 alleles</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>286/420 (68.1)</td>
<td>636/1036 (61.4)</td>
<td></td>
<td>0.016</td>
</tr>
<tr>
<td>G</td>
<td>134/420 (31.9)</td>
<td>400/1036 (38.6)</td>
<td></td>
<td>0.75 (0.59-0.95)</td>
</tr>
</tbody>
</table>

1Data are given as Npos/Nres (number of positives/number of samples with result). Results are missing for 6 patients and 11 controls for p53 and for 11 patients and 3 controls for mdm2, respectively.

SNP genotyping

Genomic DNA was isolated from whole blood by standard procedures. DNA was diluted in water to a final concentration of 15 ng/μl and 5 μl (75 ng) per reaction was used. SNP analyses were performed as previously reported (14). In brief, SNPs were genotyped by PCR and subsequent differential enzymatic restriction. PCR products were cut with MspAI (Fermentas, St. Leon-Rot, Germany). An uncut fragment (251 bp) was observed in case of the C allele (72P), while the G allele (72R) resulted in two fragments of 107 and 144 bp, respectively. All fragments were separated on agarose gels and stained with ethidium bromide. Data are given as Npos/Nres (number of positives/number of samples with result). The results of Mdm2 SNP309 defining the promoter region were presented in the frequencies of T- and G-alleles or corresponding genotypes. However, the results of the p53 SNP 72 in the proline-rich domain were described in the frequencies of the corresponding protein formation to the respective polymorphism R (to allele G) and P (to allele C).

Statistical analysis

Data were analysed using SPSS and SAS statistical software. The differences between RA patients and controls were estimated using χ² tests for 2×3 tables and 2×2 tables, respectively. Differences in allele frequencies were quantified by odds ratios (OR) and 95% CI. The adjusted ORs (one polymorphism adjusting for the other one) were estimated by fitting a logistic model. A logistic model containing an interaction effect between p53 P72R and the mdm2 SNP309 was compared to the model without this interaction effect by applying the likelihood ratio χ² test, in order to determine whether the risk associated with one polymorphism depended on the genotype of the other polymorphism (15). The interaction effect was further described and illustrated by contrasting the mdm2 SNP309 TT genotype with carriers of the mdm2 SNP309 G allele. In order to avoid inflation of the type I error that may occur when testing various subgroups and groupings of genotypes, only the interaction test was considered to be confirmative.

Results

The genotype distributions for both of the SNPs tested were in Hardy-Weinberg-equilibrium. The allele and genotype frequencies are shown in Tables I and II. Analysis of both the allele and genotype frequencies of p53 P72R revealed no significant difference between RA patients and controls. In contrast, the mdm2 SNP309 allele frequencies of RA patients differed significantly from the allele frequencies in controls, with the minor G allele being less frequent.

Table II. Genotype frequencies of p53 P72R and mdm2 SNP309 in RA patients and healthy controls.

<table>
<thead>
<tr>
<th></th>
<th>RA patients</th>
<th>Healthy controls</th>
<th>p-value</th>
<th>Odds ratio (95 % CI)</th>
<th>Odds ratio (95 % CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Npos/Nres (%)</td>
<td>Npos/Nres (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p53 P72R genotypes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RR</td>
<td>131/215 (60.9)</td>
<td>285/515 (55.3)</td>
<td>1.0</td>
<td>1.04 (0.55 - 1.98)</td>
<td></td>
</tr>
<tr>
<td>PR</td>
<td>69/215 (32.1)</td>
<td>196/515 (38.1)</td>
<td>0.77</td>
<td>0.80 (0.41 - 1.55)</td>
<td></td>
</tr>
<tr>
<td>PP</td>
<td>15/215 (7.0)</td>
<td>54/515 (6.6)</td>
<td>0.31</td>
<td>0.96 (0.51-1.82)</td>
<td>1.0</td>
</tr>
<tr>
<td>mdm2 SNP309 genotypes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>98/210 (46.7)</td>
<td>193/518 (37.3)</td>
<td>1.0</td>
<td>1.73 (1.02 - 2.95)</td>
<td></td>
</tr>
<tr>
<td>TG</td>
<td>90/210 (42.9)</td>
<td>250/518 (48.3)</td>
<td>0.71</td>
<td>1.23 (0.72 - 2.09)</td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>22/210 (10.5)</td>
<td>75/518 (14.5)</td>
<td>0.049</td>
<td>0.58 (0.34-0.99)</td>
<td>1.0</td>
</tr>
</tbody>
</table>

1χ²-test with two degrees of freedom.
2Calculated with reference to p53 RR and mdm2 SNP309 TT.
3Calculated with reference to p53 PP and mdm2 SNP309 GG.
in RA patients (Table I). G-containing genotypes of \textit{mdm2} SNP309 (TG and GG) also showed significantly lower frequencies in RA patients (Table II). Furthermore, when adjusted for the p53 genotype, this effect was significant (chi-square test for trend, \( p = 0.046 \)).

For further analyses, G-positive genotypes of \textit{mdm2} SNP309 (TG and GG) were combined and compared with the G-negative TT genotype (Fig. 1). Once again RA patients were less frequently G-positive (53.3\%) than controls (62.7\%). Most controls (n=479/512; 93.6\%) and RA patients (n=191/205; 93.2\%) exhibited at least one p53 72R allele. The majority of the controls in this group were \textit{mdm2} SNP309 G-positive. RA patients were less frequently \textit{mdm2} SNP309G-positive than controls, in accordance with the overall observation, which indicates a protective effect of \textit{mdm2} SNP309G in p53 72R positive individuals (Fig. 1). A minority of both controls (n=33/512; 6.5\%) and RA patients (n=14/205; 6.8\%) were p53 72P homozygous. In this group, the majority of the controls were \textit{mdm2} SNP309G-negative (n=19/33; 57.6\%), whereas the majority of the RA patients were \textit{mdm2} SNP309G-positive (n=11/14; 78.6\%). Hence the \textit{mdm2} SNP309G-positive genotypes are not protective but detrimental in p53 72P homozygotes.

In order to formally assess the statistical significance of this observation, a model containing an interaction effect was fitted to the data (Table III). The likelihood ratio test for interaction was significant (\( p = 0.046 \), 4 degrees of freedom). The case control ratio exceeded the overall ratio (205/512 = 0.40) in the genotype combinations \textit{p53 RR/mdm2 SNP309 TT} (0.58), \textit{p53 PR/mdm2 SNP309 TT} (0.43), \textit{p53 PP/mdm2 SNP309 TG} (0.90) and \textit{p53 PP/mdm2 SNP309 GG} (0.50). Again, in subjects with p53 RR or p53 PR, the genotype \textit{mdm2 SNP309 TT} indicated an increased risk of RA, whereas in subjects with p53 PP the presence of the \textit{mdm2 SNP309 G}-containing genotypes (TG and GG) indicated an increased RA risk. Equivalently, for \textit{mdm2 SNP309 TT} carriers, p53 PP appeared to be protective, whereas for \textit{mdm2 SNP309 G} carriers p53 PP indicated an increased risk for RA.

**Discussion**

To our knowledge, this is the first study showing an association between the susceptibility for RA and \textit{mdm2} SNP309, either alone or in combination with the p53 72R genotype. We describe here a significant protective effect of the \textit{mdm2} SNP309G allele in most individuals, with the remarkable exception of p53 72P homozygous individuals where the \textit{mdm2} SNP309G allele may have detrimental effects – at least for RA.

There is evidence from p53 negative mice that the presence of p53 reduces the severity of collagen-induced arthritis and joint destruction, and the expression of pro-inflammatory cytokines like interleukin-6 and interleukin 1-beta and destructive enzymes, \textit{e.g.} collagenase-3 (16). Most probably these phenomena are due to the lack of apoptosis observed in the inflamed synovia of p53 negative mice. Apoptosis may also be deficient in RA synoviocytes, although p53 is expressed or even up-regulated in all stages of RA (17). P53 72P was shown to induce apoptosis less efficiently than p53 72R (13). However, in our study the susceptibility for RA was

![Fig. 1. Proportion of combined \textit{mdm2} SNP309 G-positive genotypes (\textit{mdm2} SNP309 GG/TG) in RA patients and healthy donors in relation to the p53 P72R genotype.](image)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>RR (%)</th>
<th>RP (%)</th>
<th>PP (%)</th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TT</td>
<td>60/103</td>
<td>30/70</td>
<td>3/19</td>
<td>93/192 (0.48)</td>
</tr>
<tr>
<td>TG</td>
<td>54/138</td>
<td>27/98</td>
<td>9/40</td>
<td>90/246 (0.37)</td>
</tr>
<tr>
<td>GG</td>
<td>11/42</td>
<td>9/28</td>
<td>2/4</td>
<td>22/74 (0.30)</td>
</tr>
<tr>
<td>Total</td>
<td>125/283</td>
<td>66/196</td>
<td>14/33</td>
<td>205/512 (0.40)</td>
</tr>
</tbody>
</table>

\(^1\text{Genotype combinations with a case/control ratio above the overall ratio of 205/512 = 0.40 indicate an increased risk of RA.}\)
not associated with p53 P72R, hence reinforcing the reports of earlier publications (18-20). Only 7.0% and 6.6% of RA patients and healthy controls, respectively, were homozygous for p53 72P. The genotypes within our healthy cohort were distributed as previously reported for the European population; differences in genotype distribution between ethnic groups have been described (23). A recent Italian study of 170 cases and 200 controls reported 9.8% (RA patients) and 9.5% (controls) p53 72P homozygotes, and the highest number of radiological erosions after 5 years of disease in the group of 14 p53 72P homozygous RA patients (20).

MDM2, which is regarded as the major negative regulator of p53, was detected in a small number of Australian samples of RA synovial tissue and in in vitro cultures of synoviocytes (21). Compared to samples from patients with osteoarthritis, MDM2 protein levels were higher in RA patients (21). Mdm2 SNP309G influences the efficiency with which the gene is expressed under stress, with the minor G allele leading to higher protein levels (12). Thus, one might expect to find a higher frequency of the mdm2 SNP309G allele and/or mdm2 SNP309G positive genotypes in RA patients. Our data show that the contrary is the case: the overall frequency of the mdm2 SNP309G allele was significantly lower in RA patients than in controls. Only in the small group of p53 72P homozygotes was the mdm2 SNP309G allele significantly increased in RA patients compared to healthy donors.

Our results indicate that Mdm2 modiﬁes the risk for RA in different ways depending on the protein structure of p53. In p53 72P homozygotes the presence of Mdm2 G-containing genotypes increases the risk for RA, probably by its well-known function as a negative regulator of p53, leading to ubiquitination and degradation of p53. In contrast, in p53 72R homozygotes we observed a gene dose effect, indicating that a higher amount of MDM2 protein may be protective in this setting (mdm2 SNP309 TT = reference: OR 1.0; TG: OR 0.58, CI 0.38 - 0.88, p=0.012; GG: OR 0.39, CI 0.19 - 0.80; p=0.010). Assuming deﬁcient apoptosis in RA, this effect might be due to the recently described chaperone-like activity of MDM2, which guides the binding of p53 to speciﬁc promoter sequences at physiological temperatures (22). It was suggested that MDM2 and p53, together with Hsp90, participate in a transient complex at the point in the pathway where p53 is either activated or degraded (22). MDM2, like many other regulatory molecules, may have two possibilities, one pointing towards degradation and the other towards stabilization of its client, p53. Further investigations in cell culture models of RA-derived synoviocytes with deﬁned mdm2 and p53 genotypes may contribute to elucidate the conditions of the interaction between MDM2 and p53.

We conclude that the function of MDM2 depends on the p53 P72R genotype, resulting in either an increased or a reduced risk for RA. We suggest that in most cases MDM2 stabilizes the conformation of p53, whereas in p53 PP-positive subjects MDM2 supports the degradation of p53.

Acknowledgements

We would like to thank the staff and patients of the University of Saarland Medical School and the University Medical Center, Freiburg, Germany for participating in this study.

References


