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Genetic and Expression Analysis of CASP7 Gene in a European Caucasian Population with Rheumatoid Arthritis

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ABSTRACT.
Objective. To study the possible role of the caspase 7 (CASP7) gene in susceptibility to rheumatoid arthritis (RA) in a European Caucasian population.
Methods. CASP7 rs2227309 single nucleotide polymorphism (SNP) was genotyped in 197 French RA trio families and in 252 European RA families available for replication using Taqman allelic discrimination assay. Relative quantification of caspase 7 isoforms α and β mRNA expression was performed from whole blood in 25 unrelated patients with RA and in 15 healthy controls by real–time quantitative reverse transcription–polymerase chain reaction. The genetic analyses for association and linkage were performed using the comparison of allelic frequencies, the transmission disequilibrium test, and the genotype relative risk.
Results. We observed, in the first sample, a significant association of rs2227309-AA genotype with RA [p = 0.03, odds ratio (OR) 2.11 (95% CI 1.0–4.6)]. The second sample did not show any significant association of the AA genotype with RA [p = 0.6, OR 0.87 (95% CI 0.4–1.8)]. When the 2 samples were combined, no significant association of the AA genotype [p = 0.3, OR 1.32 (95% CI 0.8–2.2)] was observed. CASP7 isoforms α and β mRNA were expressed in patients with RA at lower level than in healthy controls (−89%, p = 0.003 and −47%, p = 0.01; respectively). Conclusion. CASP7 rs2227309 SNP was not associated with RA in a European Caucasian population. Nevertheless, CASP7 isoforms α and β could have an involvement in the apoptosis process in RA.

Key Indexing Terms: RHEUMATOID ARTHRITIS, GENE EXPRESSION, CASP7 GENE, APOPTOSIS, ASSOCIATION STUDY, SINGLE NUCLEOTIDE, POLYMORPHISM

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Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease characterized by persistent synovial tissue inflammation associated with the destruction of affected joints1. An imbalance between cell proliferation and insufficient apoptosis of synovial macrophages, fibroblasts, and lymphocytes is one mechanism that might contribute to persistence of RA2. The increase of osteoblast apoptotic cell death may contribute to periarticular bone loss in patients with RA, while impaired apoptosis of rheumatoid synovial cells appears to cause hyperplasia of the synovial tissues3. Studies in animal arthritis models suggest that an increase of the induction of synovial cell apoptosis ameliorates synovial tissue hyperplasia4,5. Additionally, the dysregulation of apoptosis is involved in a large variety of human diseases including cancer, autoimmune diseases, and neurodegenerative disorders6.
caspases involved in apoptotic responses are classified into 2 groups according to their structure and function. The first group is composed of initiator caspases (caspase-2, 8, 9, 10), which contain N-terminal adapter domains that allow for their autocleavage and the activation of downstream caspases. The second group is composed of effector or executioner caspases (caspase-3, 6, 7), which lack N-terminal adapter domains and are cleaved and activated by initiator caspases.

CASP7 gene is located in the chromosomal area 10q25.1-10q25.2. Caspase 7 exists as an inactive proenzyme, which undergoes proteolytic processing at conserved aspartic residues to produce 2 subunits, a large and small one, that dimerize to form the active enzyme. The precursor of this caspase is cleaved by caspases 3 and 10. Alternative splicing results in 4 transcript variants, encoding 3 distinct isoforms (α, β, and Δ), the majority of which retain catalytic activity.

In the pro-caspase 7 isoforms α and β, upon activation in vivo, a short N-terminal sequence is removed and an “IQADSG” site is cleaved, generating the active caspase 7 isoforms with catalytic activity. Pro-caspase β uses the same start codon as the variant α but has a distinct C-terminus compared with the other variants. Thus, the caspase 7 isoform β lacks the active site of the enzyme and may act as a dominant inhibitor for active pro-caspase 7 isoforms.

CASP7 gene mutations are present in human malignancies, and the inactivating mutations of CASP7 gene might lead to the loss of its apoptotic function and contribute to the pathogenesis of human solid cancers. Additionally, CASP7 has been proposed as a positional candidate for susceptibility to insulin-dependent diabetes mellitus (IDDM). In the field of genetic factors analysis in RA, a previous study reported a significant association for an rs2227309 (A/G) single nucleotide polymorphism (SNP) [allele G, p = 0.001, odds ratio (OR) 1.32 (95% CI 1.11–1.56); genotype GG, p = 0.0005, OR 1.47 (95% CI 1.18–1.83)], located in exon 7 of the CASP7 gene, with RA in a case-control Spanish Caucasian population. Further, there was a statistically significant deviation in the relative expression of the mRNA-encoding CASP7 isoform β versus functional isoforms in healthy individuals stratified according to their rs2227309 genotypes.

Our aim was to confirm the role of the CASP7 gene in susceptibility to RA in a European Caucasian population. We used RA familial material to test CASP7 rs2227309 SNP for RA associations and linkages. Moreover, the expression level of CASP7 isoform α and β mRNA in unrelated French Caucasian patients with RA and in French Caucasian healthy controls and the relation between genotypes of the SNP tested and the level of expression in these 2 groups were studied.

MATERIALS AND METHODS

Study populations. The study was approved by the Ethics Committees of Hôpital Bicêtre and Hôpital Saint-Louis (Paris, France) and all subjects provided informed consent. RA families were recruited through a national media campaign. All patients with RA satisfied the revised criteria of the American College of Rheumatology according to the rheumatologist in charge of the patient. A rheumatologist university fellow reviewed all clinical data. Sample 1 and 2, used for association and linkage studies, consisted of 197 French Caucasian unrelated trio families (1 RA patient and both parents) with the 4 grandparents of French Caucasian origin and of 252 European Caucasian unrelated trio families (Caucasian families from France, Italy, Portugal, Spain, Belgium, and The Netherlands). Characteristics of the RA French Caucasian families (sample 1) and European Caucasian families (sample 2) are reported in Table 1. Among the 25 French Caucasian unrelated patients with RA used for the expression study, 19 were women [mean ± standard deviation (SD) age at enrolment 53.4 ± 11.1 yrs]. Among the 15 French Caucasian healthy controls used for the expression study, 11 were women [mean ± SD age at enrolment 46.9 ± 6.6 yrs].

Molecular genotyping method. Genomic DNA of the 449 European Caucasian RA trio families and the 25 French Caucasian unrelated patients with RA was isolated and purified from fresh peripheral blood leukocytes according to standard protocols. Genotyping of the CASP7 rs2227309 SNP was performed with a Taqman 5' allelic discrimination assay on an ABI-7500 real-time polymerase chain reaction (PCR) machine (assay: C_500778_10, Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s protocol. Genotyping of each sample was automatically attributed using specific software for allelic discrimination. Ten percent of the samples chosen at random were genotyped again for quality control.

CASP7 isoform α/β mRNA expression. Total RNA from whole blood was extracted from 25 French Caucasian unrelated patients with RA and 15 French Caucasian healthy controls using a PAXgene Blood RNA kit (Qiagen, Hilden, Germany). The measure of the RNA concentration was performed using the RNA RiboGreen dye (Invitrogen). RNA integrity was analyzed with the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). Reverse transcription (RT) was performed with SuperScript™ III First-Strand Synthesis SuperMix for quantitative RT–PCR (Invitrogen) according to the manufacturer’s protocol. Real-time quantitative RT-PCR analysis was executed on an ABI-Prism 7500 machine, using TaqMan gene expression assay probes (Applied Biosystems) for CASP7 isoform β (Hs01032058_m1) and CASP7 isoform α (Hs01029847_m1) and TaqMan endogenous controls probes (Applied Biosystems) for β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and β₂-microglobulin. Each sample was tested in duplicate and a sample without template was included in each run as a negative control. Expression of the CASP7 isoform α and β mRNA was quantified using the threshold cycle (Ct) method and normalized to the amount of β-actin, GAPDH, and β₂-microglobulin. Samples showing Ct values > 35 and duplicates with a Ct > 0.3 were retested.

Statistical analysis. Using the previously reported frequencies of GG genotypes for CASP7 rs2227309 in Spanish patients with RA (56%) and in con-
controls (46.4%)\textsuperscript{13}, power to detect an association was calculated using the method described by Garnier, et al.\textsuperscript{15}

Hardy-Weinberg equilibrium was checked in the control group (constituted by the nontransmitted parental chromosomes from the trio subjects) prior to analysis.

The association analysis relied first on the Affected Family Based-Controls (AFBAC), which compares the allelic frequencies between chromosomes transmitted to the RA cases and nontransmitted parental chromosomes\textsuperscript{16}. The linkage analysis relied on the transmission disequilibrium test (TDT), which compares, for a given allele, its transmission from heterozygous parents to patients with RA, with the transmission expected from Mendel’s law (i.e., 50 %)\textsuperscript{17}. Second, we used the genotype’s relative risk (GRR), which compares the affected offspring’s genotype with the control genotype derived from nontransmitted parental chromosomes, using the method proposed by Lathrop\textsuperscript{18}. The OR and 95% confidence interval (CI) were estimated using the method of Woolf\textsuperscript{19}, as modified by Haldane\textsuperscript{20}. The significance of the p value was assessed at 5%, leading to replication tests in sample 2.

Results of relative mRNA expression are presented as the mean ± SD percentage. Statistical analysis of the relative quantification of CASP7 isoform α and β mRNA expression in patients with RA and healthy controls was performed using the Mann-Whitney test, and p < 0.05 was considered significant. The association between genotypes of the SNP tested and the expression in 25 unrelated patients with RA and in 15 healthy controls was assessed by the Mann-Whitney test. Data are expressed as the mean ± SD, and p < 0.05 was considered significant.

RESULTS

Power calculation. Using the reported frequencies of GG genotypes for CASP7 rs2227309 in Spanish patients with RA (56%) and in controls (46.4%)\textsuperscript{13}, association analysis of 449 trio families (RA samples 1 + 2) provides 100% power to reach statistical significance (p < 0.05).

Hardy-Weinberg equilibrium. The CASP7 rs2227309 SNP in the 2 samples investigated was in Hardy-Weinberg equilibrium in the control group.

Test for linkage and association in RA families. Sample 1: A total of 591 French Caucasian individuals from 197 trio families (1 RA case and both parents) were analyzed. The rs2227309-A allele frequency was slightly higher in RA index cases than in virtual controls (p = 0.5; Table 2A). There was a nonsignificant overtransmission of the rs2227309-A allele from heterozygous parents (p = 0.5; Table 3A). The GRR analysis of the CASP7 rs2227309 showed a significant association of the homozygous AA genotype with RA (p = 0.03; Table 4A). The same tests were performed in subgroups stratified for rheumatoid factor positivity, anti-cyclic citrulinated peptide positivity, and presence of the HLA-DRB1 shared epitope, and no significant associations/linkages were detected (data not shown).

Sample 2: The significant association observed for CASP7 rs2227309-AA genotype in Sample 1 led to a replication test in Sample 2 with the hypothesis of an AA genotype association with RA. A total of 756 European Caucasian individuals from 252 trio families were analyzed. One hundred thirteen families were of French origin and 139 were from continental Western European countries. In this sample, we did not observe a trend for association and linkage (AFBAC, p = 0.6; TDT, p = 0.6; Table 2B and 3B) of the allele A with RA. The GRR did not show a significant association of the AA genotype with RA (p = 0.6) (Table 4B). The same tests were performed in the 36 Spanish trio families from Sample 2 and no significant associations/linkages were detected (data not shown).

Samples 1 + 2: The combination of the 2 samples, indicated by the absence of any significant clinical difference between them, showed no significant association nor linkage of the allele A with RA (Table 2C and 3C). The GRR test did not show a significant association of the CASP7 rs2227309-AA genotype with RA (p = 0.3; Table 4C).

Expression analysis. A relative quantification of CASP7 isoform α and β mRNA expression was performed in total RNA isolated from whole blood of 25 unrelated patients with RA and 15 healthy controls. CASP7 isoform α and β mRNA were expressed in patients with RA at lower level than in healthy controls (−89%, p = 0.003 and −47%, p = 0.01, respectively; Figure 1). The expression level of isoform α mRNA in each group was higher than the expression level of isoform β mRNA (Figure 1), and the mRNA expression ratio (isoform α vs isoform β) in healthy controls was higher compared to patients with RA (+32%).

A nonstatistically significant deviation was observed when comparing the mRNA expression ratio (isoform α vs isoform β) in patients with RA (Figure 2A) and in healthy control samples (Figure 2B) stratified according to their rs2227309 genotypes (AA vs AG, AA vs GG, AG vs GG; p > 0.05).

DISCUSSION

Caspase 7 is crucial for apoptosis, and contributes to mitochondrial events such as the loss of mitochondrial membrane potential and the release of proapoptotic factors such as cytochrome c and apoptosis-inducing factor\textsuperscript{21}. Caspase 7 is an executioner caspase that requires cleavage to turn into the active isoforms α or Δ\textsuperscript{7}. The caspase 7 isoform β, which is not cleaved, may act as a dominant inhibitor of the active forms\textsuperscript{10}. Deregulation of apoptosis synovial cells was one of the mechanisms suspected to be involved in RA physiopathology\textsuperscript{2}.  

Table 2. Affected family-based controls (AFBAC) analysis in rheumatoid arthritis trio families.

<table>
<thead>
<tr>
<th>CASP7 rs2227309 (A/G) SNP</th>
<th>Allele</th>
<th>RA Cases</th>
<th>Controls</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Sample 1, n = 197</td>
<td>A</td>
<td>0.26</td>
<td>0.24</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>0.74</td>
<td>0.76</td>
<td></td>
</tr>
<tr>
<td>B. Sample 2, n = 252</td>
<td>A</td>
<td>0.26</td>
<td>0.27</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>0.74</td>
<td>0.73</td>
<td></td>
</tr>
<tr>
<td>C. Sample 1+ 2, n = 449</td>
<td>A</td>
<td>0.26</td>
<td>0.26</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>0.74</td>
<td>0.74</td>
<td></td>
</tr>
</tbody>
</table>

SNP: single nucleotide polymorphism.
Table 3. Transmission disequilibrium test in RA trio families.

<table>
<thead>
<tr>
<th>CASP7 rs2227309 (A/G) SNP</th>
<th>Allele</th>
<th>Transmitted</th>
<th>Non-transmitted</th>
<th>Transmission from Heterozygous Parents, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A</td>
<td>G</td>
<td></td>
</tr>
<tr>
<td>A. Sample 1, n = 197</td>
<td></td>
<td>76</td>
<td>68</td>
<td>52.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>68</td>
<td>76</td>
<td>47.2</td>
</tr>
<tr>
<td>B. Sample 2, n = 252</td>
<td></td>
<td>96</td>
<td>104</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td></td>
<td>104</td>
<td>96</td>
<td>52</td>
</tr>
<tr>
<td>C. Sample 1 + 2, n = 449</td>
<td></td>
<td>172</td>
<td>172</td>
<td>50</td>
</tr>
</tbody>
</table>

SNP: Single nucleotide polymorphism.

Table 4. Genotype relative risk analysis in RA trio families.

<table>
<thead>
<tr>
<th>CASP7 rs2227309 (A/G) SNP</th>
<th>Genotypes</th>
<th>RA Cases</th>
<th>Controls</th>
<th>Odds Ratio (95% CI)</th>
<th>Lathrop p value (one genotype vs the others)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. Sample 1, n = 197</td>
<td>AA</td>
<td>20</td>
<td>10</td>
<td>2.11 (1.0–4.6)</td>
<td>0.03 (AA vs AG + GG)</td>
</tr>
<tr>
<td></td>
<td>AG</td>
<td>62</td>
<td>74</td>
<td>0.76</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>115</td>
<td>113</td>
<td>1.04 (0.7–1.6)</td>
<td>0.94 (GG vs AA + AG)</td>
</tr>
<tr>
<td>B. Sample 2, n = 252</td>
<td>AA</td>
<td>15</td>
<td>17</td>
<td>0.87 (0.4–1.8)</td>
<td>0.5 (AA vs AG + GG)</td>
</tr>
<tr>
<td></td>
<td>AG</td>
<td>99</td>
<td>103</td>
<td>0.94</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>138</td>
<td>132</td>
<td>1.1 (0.8–1.6)</td>
<td>0.7 (GG vs AA + AG)</td>
</tr>
<tr>
<td>C. Sample 1 + 2, n = 449</td>
<td>AA</td>
<td>35</td>
<td>27</td>
<td>1.32 (0.8–2.2)</td>
<td>0.4 (AA vs AG + GG)</td>
</tr>
<tr>
<td></td>
<td>AG</td>
<td>161</td>
<td>177</td>
<td>0.86</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>253</td>
<td>245</td>
<td>1.07 (0.8–1.4)</td>
<td>0.7 (GG vs AA + AG)</td>
</tr>
</tbody>
</table>

SNP: Single nucleotide polymorphism.

Figure 1. Levels of CASP7 isoform α and β mRNA expression in peripheral blood from unrelated RA patients (RA) and healthy controls (HC). CASP7 isoform α and β mRNA levels were determined by real-time quantitative RT-PCR and normalized to β-actin, GAPDH, and β₂-microglobulin levels. Expression of CASP7 isoform α and β transcripts was quantified using the threshold cycle (Ct) method. Data are presented as mean ± SD percentage of the CASP7 isoform α and β mRNA expression. *p < 0.0001 vs controls; **p = 0.01 vs controls; Mann-Whitney test.
A previous study reported a significant association (p < 0.05) for rs2227309-GG genotype of the CASP7 gene with RA in a case-control Spanish Caucasian population study\textsuperscript{13}. This SNP produces a K249R change in the isoform β and a synonymous change in the isoform α. Further, this polymorphism is not located in the active site of the caspase 7 protein. Our results showed an overtransmission of the rs2227309-A allele from heterozygous parents and a significant association of the homozygous rs2227309-AA genotype with RA in Sample 1. This significant association led to a replication test in Sample 2 with the hypothesis of an AA genotype association with RA. In this sample, we observed neither a trend for association and linkage of the A allele nor a significant association of the AA genotype with RA.

Figure 2. Levels of CASP7 isoform α vs isoform β mRNA expression in RA patients (A) and in healthy controls (B) stratified according to their rs2227309 genotypes. CASP7 isoform α and β mRNA levels were determined by real-time quantitative RT-PCR and normalized to β-actin, GAPDH, and β\textsubscript{2}-microglobulin levels. Expression of CASP7 isoform α and β transcripts was quantified using the threshold cycle (Ct) method. Data are presented as mean ± SD percentage of the CASP7 isoform α vs isoform β mRNA expression in patients with RA and in healthy controls (AA vs AG, AA vs GG, AG vs GG: p > 0.05 by Mann-Whitney test).
Moreover, the combination of the 2 samples did not show any significant association of the CASP7 rs2227309-AA genotype with RA. This family-based analysis avoids an imperfect population match between patients and controls, permitting the direct test of Mendel’s law. The allele frequencies that we described in patients with RA (A = 26% and G = 74%) were the same as those observed in Spanish patients with RA13. However, the allele frequency inferred from parental nontransmitted chromosomes (A = 26% and G = 74%) was different from those observed in Spanish controls (A = 31.6% and G = 68.4%)13. Altogether, our results allowed us to exclude CASP7 rs2227309 SNP as a major significant genetic factor in RA in a European Caucasian population. To exclude CASP7 gene association with RA, other tagSNP should be tested. CASP7 locus was not included among the 19 suggested non-HLA regions in the French Caucasian population linked to RA22. Further, the last genome-wide association study in a UK Caucasian population found 9 SNP that map to loci not associated previously to RA23, and the CASP7 locus was not present in these regions.

Our study is the first to show a statistically significant difference in the expression of CASP7 isoform α and β mRNA between patients with RA and healthy controls in peripheral blood cells. When we compared the expression of these 2 isoforms in healthy controls or in patients with RA, we observed higher mRNA expression of the α isoform compared to the β isoform. Further, the decrease of mRNA CASP7 isoform β expression in patients with RA compared to healthy controls was more significant than observed for CASP7 isoform α mRNA expression. Thus, in patients with RA, peripheral blood cells could have a lower apoptotic activity based on the lower relative quantity of the functional isoform α compared to healthy controls.

As the decrease of isoform β mRNA expression in patients with RA was less significant than the decrease observed for isoform α, isoform β may act as a dominant inhibitor of active forms. Thus, peripheral blood cells from patients with RA may have lower apoptotic activity based on the higher proportion of the nonfunctional isoform (β isoform) compared to the active one (α isoform).

Reports have demonstrated that impaired apoptosis is a characteristic of several autoimmune diseases, and administration of factors that stimulate apoptosis decreases inflammation3,5,5,24,25. Further, in mice, the knockout of caspase 7 decreases the apoptotic process21. Nevertheless, to complete our observed data, further investigations should be done in specific cells such as T and B lymphocytes, monocytes, macrophages, and synoviocytes.

We cannot exclude the effects of methotrexate (MTX) in the apoptotic process of patients with RA because in our study, 92% of patients with RA have received MTX treatment. However, the mechanism of MTX action in autoimmune diseases remains unclear. An influence of MTX on apoptosis in peripheral blood mononuclear cells (PBMC) has been demonstrated only under nonphysiological conditions, for example after stimulation with mitogens26,27. Employing stimulation with conventional antigens, other studies have failed to demonstrate an apoptotic effect of MTX on PBMC, and instead attributed the effect of MTX on T cells to suppression of T cell activation and reduced expression of T cell adhesion molecules27. Finally, a recent study reported that MTX inhibits proliferation in Candida albicans (CA)- and tetanus toxoid (TT)-stimulated CD4+ T cells, and induces apoptosis in a subset of stimulated CD4+ T cells. These authors suggested that stimulation of T cells with the conventional antigens CA and TT more probably resembles stimulation with self-antigens occurring in autoimmune diseases, including RA, and the antiproliferative and proapoptotic effects of MTX reflect the actions of this drug in the treatment of rheumatic diseases28. Further investigations should be done in RA patients without MTX treatment to clarify this point and to identify the exact mechanism of MTX in RA.

Our findings provide evidence against the involvement of the CASP7 rs2227309 SNP in the genetics of RA in a European Caucasian family population. Our study is the first to show that CASP7 isoform α (functional isoform) and isoform β (nonfunctional isoform) mRNA were expressed in patients with RA at lower levels than in healthy controls in peripheral blood cells. Further investigations of the regulation of CASP7 expression in RA are required to prove the involvement of CASP7 in disease susceptibility, more specifically in the signalling pathways of RA apoptosis.

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