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Gene–gene interaction and RNA splicing profiles of MAP2K4 gene in rheumatoid arthritis

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Abstract
We performed gene–gene interaction analysis, with HLA-DRB1 shared epitope (SE) alleles for 195 SNPs within immunologically important MAP2K, MAP3K and MAP4K gene families, in 2010 rheumatoid arthritis (RA) patients and 2280 healthy controls. We found a significant statistical interaction for rs10468473 with SE alleles in autoantibody-positive RA. Individuals heterozygous for rs10468473 demonstrated higher expression of total MAP2K4 mRNA in blood, compared to A-allele homozygous. We discovered a novel, putatively translated, "cassette exon" RNA splice form of MAP2K4, differentially expressed in peripheral blood mononuclear cells from 88 RA cases and controls. Within the group of RA patients, we observed a correlation of MAP2K4 isoform expression with carried SE alleles, autoantibody, and rheumatoid factor profiles. TNF-dependent modulation of isoform expression pattern was detected in the Jurkat cell line. Our data suggest a genetic interaction between MAP2K4 and HLA-DRB1, and the importance of rs10468473 and MAP2K4 splice variants in the development of autoantibody-positive RA.

1. Introduction

Rheumatoid arthritis (RA) is a common autoimmune disease, characterized by chronic inflammation and severe impairment of the joints, that affects about 1% of the world population. *HLA-DRB1* shared epitope (SE) alleles are the major genetic risk factors for this condition [1]. RA patients positive for anti-citrullinated protein antibodies (ACPAs) constitute two thirds of all RA cases and represent a group with higher disease severity. Along with rheumatoid factor (RF) and the number of

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carried SE alleles, ACPA-positivity is one of the major factors determining poor prognosis for RA development [2,3].

TNF blockade is an efficient treatment of RA and a significant fraction of the patient population demonstrates good or very good response to this medicine. However, not all patients achieve full remission and some side effects are observed. One of the recent developments in biological therapy for RA is targeting mitogen-activated protein kinases (MAPKs), in particular p38 and JNK. MAPKs are considered to be among the key inflammatory mediators for autoimmune diseases due to their involvement in the regulation of immune response to stress stimuli [4]. Both JNK and p38 regulate interferon-γ-mediated gene expression, however, p38 activates genes related to innate immunity, while JNK acts on genes connected to antigen presentation [5,6]. MAPKs mediate TNF-receptor signaling and are important regulators of pro-inflammatory cytokine production, which is considered to be a major pathogenic factor in RA [6].

However, clinical trials with direct inhibitors of MAPKs were characterized by limited efficacy, partially due to side effects. It has been suggested that focusing on targets upstream of the p38 and JNK pathway may provide fine regulation of downstream events, resulting in more advantageous clinical strategies [4].

Genetic associations with complex diseases, e.g. RA, exhibit a moderate to low effect on incidence risk. Recently, we have demonstrated that interaction between genes may explain risk more successfully than the independent influence from separate variations [7,8]. Analysis of gene–gene and gene–environment interactions, combined with existing biological knowledge, has been described as a useful tool in discerning disease-associated genetic loci [9].

In this work, we assess the relevance of candidate genetic loci involved in MAPK signaling to rheumatoid arthritis, by investigating gene–gene interaction between loci that encode known MAP2Ks, MAP3Ks and MAP4Ks with *HLA-DRB1* SE alleles in two independent cohorts. Based on our findings, we proceeded to look at the association of transcript-specific expression of *MAP2K4* with the distinctive phenotype of ACPA-positive RA in mRNA from the peripheral blood of RA patients and healthy individuals. Finally, to address the potential role of *MAP2K4* transcripts in RA-related inflammatory processes, we investigated the response of *MAP2K4* transcript expression to TNF stimulation in lymphatic cell-lines.

2. Materials and methods

2.1. Patients and controls

The interaction study was based on the Swedish Epidemiological Investigation of Rheumatoid Arthritis (EIRA) cohort (1921 RA cases and 1079 healthy controls of Caucasian ethnicity), and was performed on data available from our genome-wide association analysis [11]. Initially, the number of ACPA-positive patients in the RA group constituted 1149 individuals. To reach higher numbers in groups with underrepresented genotypes, the number of ACPA-positive RA patients was increased to 2010 and the number of healthy controls was increased to 2280. The expansion of the EIRA cohort was done in full adherence to initial selection criteria.

The RNA expression experiments utilized PBMC-samples from 44 RA patients and 44 controls, and an independent source of whole blood samples from 80 RA patients and 80 controls from the Swedish population; controls were selected with consideration to gender, age and ethnicity for the patient group. RA patients were selected at the Rheumatology Clinic at Karolinska University Hospital on two occasions, and all correspond to ACR 1987 criteria for rheumatoid arthritis [10]. Subjects with any significant health problem, other than rheumatoid arthritis, and non-RA related laboratory abnormalities were not included. Information about anti-citrullinated protein antibody (ACPA) status, smoking habits and medication (for RA patients) was obtained from medical records. Informed consent was obtained from all participants in all cohorts, in compliance with the latest version of the Helsinki Declaration. Regional ethical committees at all sites have approved the study.

2.2. Genotyping

Genotyping for the MAP loci was performed by Illumina 300K chip in our previous GWAS study [11]. Additionally, we performed genotyping of rs10468473 using TaqMan allelic discrimination assay (Life Technologies, Europe) according to the manufacturer's instructions. HLA-DRB1 data was obtained and reported for this cohort previously [12]. The validation of genotyping data for rs10468473 demonstrated 99.8% consistency with GWAS data.

The North-American Rheumatoid Arthritis Cohort (NARAC) dataset was genotyped on the Illumina Infinium HumanHap550 platform for 545,080 SNPs [11].

2.3. RNA extraction and cDNA synthesis

The RNA extraction was performed either from whole blood or from PBMC samples in two independent materials. For whole blood samples (80 RA patients and 80 healthy controls), blood was collected into PAXgene Blood RNA Tubes and total RNA was extracted with the PAXgene Blood RNA kit (PreAnalytiX, Feldbachstrasse, Switzerland) according to the manufacturer's protocol. For PBMC (44 RA patients and 44 healthy controls), blood was collected in sodium citrate vacuum tubes and PBMC were isolated by Ficoll-Paque (GE Healthcare Life Sciences, Uppsala, Sweden) separation.

For PBMC, after incubation, 5×10^5 cells of each sample were harvested and lysed with RLT buffer from the RNA Miniprep kit (QIAGEN Hilden, Germany). Subsequently, RNA was extracted, with RNeasy Mini (QIAGEN, Hilden, Germany). An additional step of DNase digestion was performed with RNase-Free DNase Set (QIAGEN). Samples were stored at −80 °C prior to cDNA synthesis, which was performed with an iScript cDNA synthesis kit following the manufacturer's protocol (Bio-Rad, Hercules, CA, USA).

2.4. Detection of splicing variants by generic PCR

To cover the canonical sequence of the catalytic domain of *MAP2K4*, the following primers were used: Forward 5′-CTGAG AACACACAGCATTGAGTC-3′ and Reverse 5′-CACAAAAGAG CAGGATGAGGTC-3′. The specific primers for splicing isoform *MAP2K4*_v1 with skipped exon 5 were created to span the