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

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Received 3 December 2013; accepted with revision 17 February 2015
Available online 27 February 2015

KEYWORDS
Rheumatoid arthritis; Gene–gene interaction; MAP2K4; Alternative splicing; RNA expression

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.

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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...
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 Hild, 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 AACACACAGCATGGTC-3′ and Reverse 5′-CACAAAGAG CAGGATGAGGTC-3′. The specific primers for splicing isoform MAP2K4_v1 with skipped exon 5 were created to span the
juncture of exons 4 and 6 (Forward 5′-CAGAGAGACTGTGA AAGCACTAAA-3′; Reverse 5′-CAGACATCGAGGCGACATC-3′). Each primer system was used in a separate experiment with various cDNA templates. The enzyme used for PCR reaction was AmpliTaq Gold, together with GeneAmp dNTP Mix (Applied Biosystems, Branchburg, NJ, USA). Evaluation of PCR products was performed by PAGE on NOVEX TBE pre-cast gradient 4–12% gels (Invitrogen, Life Technologies Europe), stained with 3/10,000 GelRed (Biotium Inc.). Digitalization was done with Gel Doc XR+ Imaging System (Bio-Rad).

2.5. Quantitative PCR

Pre-made, real-time gene expression assay Hs00387426_m1 (Applied Biosystems, Life Technologies, Europe), with probe spanning exons 3–4 of the original sequence was used for general expression readout from the MAP2K4 locus.

Two custom TaqMan assays from Applied Biosystems, with similar amplicon sizes, were designed to probe the canonical form of MAP2K4_v1 (Forward 5′-AGTGATGCCCCATACCTGTC A3GT-3′; Reverse 5′-CAGAGTAGCATGACTCTCCAG-3′; Reporter 5′-FAM*-CCACAGCTCCCTCGT-NFQ**-3′) and splicing isoform MAP2K4_v2 with skipped exon 5 (Forward 5′-AGTGATG TGCCCATACCTGTCAGT-3′; Reverse 5′-TGGAAGGGTTGATGA TGCTCCTCCTC-3′). For primer design, the on-line Custom TaqMan Assay Design Tool from Applied Biosystems was used. The experiments were done at least in triplicate. Three non-template controls were included in each experiment. Expression measurements were made using the delta–delta relative quantification method (ΔΔCt Cycle threshold) with an ABI 7900HT Sequence Detector and Sequence Detection System Software 2.4. The ZNF592 gene was used as an endogenous control, as previously described [13]. The mean ΔΔCt values for controls were used as a calibrator.

2.6. Transfection and stimulations

Transfection plasmids E5 and noE5 were based on cDNA sequences of the canonical MAP2K4 and the putative variant (lacking exon 5) respectively – inserted into a PrecisionShuttle mammalian vector with N-terminal His-DDK tag (PS100018, OriGene Technologies Inc., MD, USA). Both constructs were manufactured by, and purchased from, Blue Heron Biotech LLC, WA, USA.

For transfection, HEK293 cells (2 × 10^5 cells/well) were seeded in a 6-well tissue culture plate. The day after, cells were transfected with 2 μg of E5, noE5 or pcDNA empty vector using GeneJuice transfection reagent (Merck Millipore, Sweden), according to the manufacturer's instruction. The cells were collected 24 h after transfection by scraping from the culture plates and lysed for Western blotting.

All the cell lines were purchased from ATCC and kept in Dulbecco’s modified medium or RPMI-1640 medium – supplemented with 10% fetal bovine serum (Gibco, Life Technologies, Sweden), L-glutamine, and penicillin/streptomycin – in a 37 °C incubator with 5% CO2. The authenticity of the cell lines was verified using genotyping.

TNF was purchased from Peprotech, Scandinavia.

2.7. Western blotting

To identify MAP2K4 isoforms we used the commercial antibodies for a canonical MAP2K4 protein (Santa Cruz, USA). By comparison of antibody specificity using protein-BLAST, we selected a monoclonal antibody against the C-terminus of the protein, which was predicted as a common domain for known and new isoforms.

Cell lysates were prepared in 50 mM Tris–HCl, pH 7.4, 1% NP40, 150 mM NaCl, 1 mM EDTA, 1% glycerol, and protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). 20 μg of total protein was separated by 4%–12% PAGE (XCell SureLock™ Mini-Cell Electrophoresis System, Invitrogen, Sweden) and transferred to PVDF-membranes using the iBlot® dry transfer system (Invitrogen by Life Technologies, Sweden). The membranes were blocked in 5% milk in PBS-Tween20 (1 × PBS plus 0.1% Tween-20). Blocking was followed by incubation with: primary antibody at +4 °C overnight (mouse monoclonal anti-MAP2K4: Santa Cruz, sc-166168), or 1 h at room temperature (GAPDH: #AB9485, Abcam); and secondary antibody (HRP-conjugated anti-mouse from Rockland, #20789) for 1 h at room temperature. The PVDF membranes were developed using Western Lightning™ ECL from PerkinElmer, Sweden AB. Images were obtained with a LAS-1000 from Fujifilm. Molecular weight standards were: Biotinylated Protein Ladder (Cell Signaling Technology®, In Vitro AB, Sweden) and Spectra TM Multicolor Broad Range Protein Ladder (Fermentas, ThermoFisher Scientific, Sweden).

2.8. Statistics

The interaction was tested for 195 SNPs from the 12 loci for MAPKK, MAPKKKs and JNK1 against HLA-DRB1 SE alleles (DRB1*01, *04 and *10). The calculation of interaction was performed with an algorithm, based on R programming language [14]. To assess genetic interaction, relative excess-risk (departure from additivity) was calculated for the group of individuals, carrying both prospective risk markers, and represented as the attributable proportion of risk due to interaction (AP) with 95% CI. The cohort was additionally stratified for ACPA status. Gender was treated as a potential confounder. Bonferroni correction of p-values for AP was used with study-wide threshold for the significance of 2.6 × 10^{-4}. All SNPs were tested for interaction with SE alleles using the binary dominant/recessive model.

Quantitative expression data was analyzed by the non-parametric Mann–Whitney U (2 groups) or Kruskal–Wallis (3 groups) tests. The threshold for significance was p = 0.05.

3. Results

3.1. SNP rs10468473 from MAP2K4 is in statistical interaction with HLA-DRB1 in ACPA-positive RA

We performed interaction analysis on pairwise combinations of HLA-DRB1 SE alleles and 195 SNPs from 12 genetic loci encoding known MAP2K, MAP3Ks and MAP4Ks upstream of JNK1 and p38 (including JNK1), in 1921 RA cases and 1079 healthy controls (EIRA cohort). Our data indicate that a single SNP, rs10468473, is in a relatively strong interaction with HLA-DRB1
SE alleles in the development of ACPA-positive RA (AP = 0.79, p = 4.4 × 10⁻⁶, 95% CI [0.51–1.0]) (Supplementary Fig. S1, Supplementary Datasheet SD1). We found little evidence for the interaction of HLA-DRB1 SE alleles with other genetic variations in MAP2K and MAP3K related loci, after correction for multiple comparisons had been performed. The SNP rs10468473 A/G is located 21 kb downstream of the MAP2K4 gene, with minor allelic frequency of 0.097 in our healthy control population (HapMap-CEU MAF 0.093).

To improve the representation of the individuals, positive exclusively for rs10468473 risk allele in our interaction analysis, we performed additional genotyping for this SNP in 861 RA patients and 1200 controls from EIRA study (not previously included in GWAS). In the extended cohort, rs10468473 from the MAP2K4 locus remained in significant interaction with SE (AP = 0.434, p = 0.041, 95% CI [0.018–0.85]) (Fig. 1). These results imply that the MAP2K4 polymorphism and SE alleles are associated with a non-linear increase in risk for ACPA-positive RA. Additionally, we performed haplotype analysis for all 22 GWAS SNPs from the MAP2K4 locus in our cohort of 1167 Swedish Caucasians, but could not observe a strong linkage of rs10468473 to other MAP2K4 SNPs (Supplementary Fig. S2).

### 3.2. Association of rs10468473 genotype with MAP2K4 expression

To find possible mechanisms, explaining the risk association of genetic variation in the MAP2K4 locus, we investigated the allelic expression of the MAP2K4 gene in mRNA from peripheral blood mononuclear cells of 44 RA patients and 44 matched controls with known rs10468473 genotype. Our data showed significantly elevated MAP2K4 expression in RA patients heterozygous for rs10468473, compared to individuals homozygous for A allele (Fig. 2A). This difference in MAP2K4 expression between different rs10468473-genotypes could not be observed in healthy controls (Fig. 2B). Due to the low frequency of the minor allele, individuals with the homozygotic state of the G were not present in our expression panel.

#### 3.3. MAP2K4 gene is expressed as alternative putatively protein-coding transcripts

We performed an exploratory study by using generic PCR to identify a spectrum of MAP2K4 mRNA products, detectable in PBMC. We identified a series of transcripts with altered exonic composition, and a prevalence of “cassette-exon”-type splicing events in the coding part of the Serine/Threonine protein kinase catalytic domain (Fig. 3A) Specifically, exon 5 skipping had been reported previously for various transcripts of human (ENST00000538465; ENST00000538465) and mouse (ENSMUST00000125598) origin, and was featured in our material (Fig. 3B). In a screening in mRNA from the PBMC, we could observe and sequence transcripts with exon 5 skipping in mRNA from 19/20 RA patients and 20/20 controls (Supplementary Fig. S3).

Importantly, the absence of exon 5 alone does not result in a shift of open reading frame. To assess the possibility of translation for MAP2K4 transcript with skipped exon 5 (assigned as V2), we transfected HEK293 cells with V1- and V2-containing plasmids. Both V1 and V2 transfection products could be detected by using commercially available mouse monoclonal anti-MAP2K4 in lysates of transfected HEK293 cells. Proteins with similar molecular weight were also detectable in non-transfected T-lymphocyte cell lines by Western Blot, using commercially available mouse monoclonal anti-MAP2K4 against the C-terminal part of MAP2K4 (Fig. 3C).

#### 3.4. Differential expression of MAP2K4 transcripts reflects genetic and phenotypic features of RA

We investigated the production of MAP2K4 transcript with skipped exon 5 (assigned as MAP2K4_V2) in relation to the expression of canonic full isoform (assigned as MAP2K4_V1), in PBMC from 38 RA cases and 43 controls, using probe-based RT-qPCR with probes spanning exonic junctions 4/5 and 4/6, respectively. When taken as a ratio (V2/V1), the expression

![Figure 1](image1.png)

**Figure 1**  SNP rs10468473 from the MAP2K4 locus, when combined with SE, provides a significant, non-additive increase in risk for ACPA-positive RA. Representative data shown for extended EIRA cohort. The statistical interaction is represented by attributable proportion (AP). The analysis was based on 2010 ACPA-positive cases and 2280 healthy controls. Data presented as relative risk (RR) for carriers of different risk genotypes: SE — individuals with shared epitope risk genotype; rs10468473 — individuals with MAP2K4 rs10468473_A risk genotype; rs10468473_A + SE — individuals with both MAP2K4 rs10468473 and SE risk genotypes; reference line (dashed) — unaffected individuals; the area of the squares represents the log-size of the genotype group; whiskers show RR confidence intervals.
of the transcripts was significantly different for RA patient and healthy control groups (Fig. 4A). The transcript ratio is primarily influenced by the increased expression of MAP2K4_V1 in RA patients (Supplementary Fig. S4A–B). We replicated these findings in an independent material of whole blood mRNA from 80 RA patients and 80 healthy individuals (data not shown).

In RA patients, we identified a significant correlation and a dosage effect between the number of carried SE alleles and the MAP2K4_V2/V1 expression ratio (Fig. 4B). Within the same group, we could also observe a correlation between the MAP2K4 variant expression ratio and RA-related serological factors, i.e. the presence of ACPA (measured as anti-CCP-antibodies) and presence of rheumatoid factor (RF) (Fig. 4C, D). These differences in V2/V1 expression ratio were consistently correlated with the increased expression of MAP2K4_V2 isoform in groups of individuals positive for SE-alleles and serological markers (Supplementary Fig. S5A–B and C–D, respectively). There was no significant connection between smoking habits and the expression ratio of MAP2K4 variants within the group of RA patients in our study (data not shown).

3.5. MAP2K4 isoforms are differentially expressed in lymphatic cell-lines under TNF stimulation

We studied the effect of TNF stimulation on the transcript expression ratio of MAP2K4 in lymphatic and myeloid cell-lines. The expression of MAP2K4 V1/V2 was measured at four different time points in Jurkat, Raji, and U937 cell lines treated with TNF. The transcripts of MAP2K4 were differentially expressed in T-cell-like Jurkat cell-line at 24 h of stimulation (Fig. 5A). There was no significant difference in transcript ratio for Raji and U937 (Fig. 5B, C).

4. Discussion

Our data suggest that the MAP2K4 gene is an important candidate gene for RA that was overlooked in GWAS studies, due to the fact that it only elevates risk for disease in combination with HLA-DRB1 SE alleles. The differential expression of mRNA possibly points to the important contribution of MAP2K4 splicing isoforms for disease development, which may be specific for the function of this kinase in T-cells. Therefore, the interaction between HLA-DRB1 SE alleles and MAP2K4 alleles, observed in our data, could arise due to mechanisms downstream of antigen presentation.

Recently, studies of gene–gene interaction effects have proven to be instrumental in understanding gene function [15]. Although straightforward functional interpretation of interaction between genes can be challenging, incorporating prior data on functional pathways and molecular biology could be an effective strategy of improving and expanding the applicability of gene–gene interaction studies [16].

The results of our study, with analysis of genetic variations in MAPKK and MAPKKK related loci, imply an interaction between a single member of the MAP signaling pathway, MAP2K4, and HLA-DRB1 SE loci, that may contribute to the risk of autoantibody-positive rheumatoid arthritis. Significant interaction between MAP2K4 and SE in ACPA-positive RA was
initially observed in the EIRA cohort that was later substantially extended with additional rs10468473 genotyping data. These results constitute an interaction profile that is connected to increased risk of ACPA-positive rheumatoid arthritis and can be helpful for clinical prediction in the future. Based on the interaction of MAP2K4 \textit{SNP} with the major risk factor for RA, it is possible that \textit{MAP2K4} plays a role in RA pathogenesis only in a specific subgroup of individuals with HLA-DRB1 \textit{SE} alleles. Notably, rs10468473 MAF is higher in Asian populations than in Caucasians. It would be interesting to investigate found effects in relation to RA in these populations.

Importantly, we observed association of the rs10468473 genotype with \textit{MAP2K4} mRNA expression, in our material of PBMC from RA patients. This allows us to assume that rs10468473, or other SNPs in high linkage disequilibrium (LD) with it, is potentially related to the regulation of expression in the \textit{MAP2K4} locus, and could be involved in inflammatory responses in RA.

To achieve the required variety of functions, almost all immunologically relevant genes undergo alternative splicing [17]. It has been suggested that the balance in production of alternatively-spliced gene transcripts could be relevant for disease progression and, hence, for the development of potential therapies [18]. Additionally, the isoforms of proteins with a function in immunity, such as CD44, TNFR2, IL-32, and IL-6R, were also shown to influence antigen processing and have a clinical relevance for autoimmunity [19–22]. Hytönen A.-M. et al. have demonstrated that a higher level of soluble IL-4R isoforms in asthma patients, compared with controls, has biological consequences [23]. It was also demonstrated...
that the ratio of PTPN22 splice forms is shifted in RA patients, compared to healthy controls, which can have an influence on immune response [13].

In this study we characterize splicing events in MAP2K4, resulting in partial lack of catalytic domain coding sequence. According to the range of discovered splice variants, spliced-out exon 5 could be characteristic for several MAP2K4 transcripts. Specifically, MAP2K4_V2 could be consistently identified with a specific primer-set in mRNA from PBMC of RA patients and controls. The skipping of exon 5 does not introduce premature translation termination codons within an open reading frame and could be effectively translated into a protein product, as confirmed by our transfection experiments in HEK293. However, it has to be noted that HEK293 cells are

Figure 4  Expression of V1 and V2 mRNA isoforms in PBMC of 38 RA patients and 43 controls. A. An expression ratio for full and exon-5-deficient MAP2K4 transcripts (V1 and V2 respectively) is significantly different in PBMC samples from healthy controls and RA patients. Data shown as relative quantification ratio (RQ MAP2K4 V2/V1) for 38 RA patients and 43 controls, analyzed with the Mann–Whitney U test (p < 0.005). B. Within the group of RA patients, MAP2K4 transcripts V1 and V2 were differentially expressed in SE-negative (NO SE), SE-positive (SE), and double-SE-positive (DOUBLE SE) individuals. Data shown as relative quantification ratio (RQ MAP2K4 V2/V1) for 38 RA patients, analyzed by Kruskal–Wallis ANOVA (p < 0.001). C. The expression ratio of MAP2K4 transcripts V2/V1 was different in RF-negative (NO RF) and RF-positive (RF) RA patients. Data shown as relative quantification ratio (RQ MAP2K4 V2/V1) for 38 RA patients, analyzed with the Mann–Whitney U test (p < 0.05). D. In RA patients, anti-CCP-negative and anti-CCP-positive profiles correlated with MAP2K4 transcript expression ratio V2/V1. Data shown as relative quantification ratio (RQ MAP2K4 V2/V1) for 38 RA patients, analyzed with the Mann–Whitney U test (p < 0.001).
likely not to have an identical MAP2K4 splicing profile to that of hematopoietic cells.

The possibility of translated, exon-5 deficient MAP2K4 isoforms is further supported by the existence of a splicing variant with an in-frame alternative transcription start site in exon 4, combined with exon 5 skipping, reported in expression data from 11 normal human tissues in the EASANA Genosplice micro-array database (Tissues; GSHG0012237) and a protein-coding transcript of the same structure, confirmed in mouse (ENSMUST00000125598). This type of splicing event could have a distinctive effect on the function of a putative protein product of MAP2K4, since exon 5 constitutes a part of the catalytic center of MAP2K4. Currently, further proteomic analysis is necessary to elucidate the origin and structure of these protein isoforms, detected in our study with MAP2K4 antibodies against the C-terminal part of the protein.

Our results show differential expression of the canonical and alternatively spliced MAP2K4 mRNAs in RA patients, compared to healthy controls. In our data, the MAP2K4 V2/V1 expression ratio also reflects other genetic and phenotypic features of the disease in the RA group, namely ACPA status, RF positivity, and number of SE alleles. The decreased V2/V1 ratio in RA patients could be chiefly attributed to the increased expression of canonical MAP2K4 transcript, while the correlations with SE alleles and serological markers within the RA group are driven by V2 (NoE5).

It has been reported that SNPs located in untranslated areas may contribute to the regulation of gene splicing [24]. However, we could not see a clear association between the rs10468473 or 21 other common MAP2K4 SNP genotypes with the expression of individual splicing variants (canonical and lacking exon 5) of the MAP2K4 gene. Further work is needed.

Figure 5  Expression of MAP2K4 isoforms in stimulated Jurkat, U937 and Raji cells. Cells were left untreated (control) or were treated with 50 ng/ml TNF for the presented time periods. Experiment was repeated in biological triplicates. Data analyzed by TaqMan quantitative RT-PCR and presented as a relative quantification ratio (RQ. MAP2K4 V2/V1). The error bars indicate the standard deviation.
to clarify whether downstream genomic variation directly influences MAP2K4 expression and transcript abundance, or represents LD with other unidentified SNPs within the locus.

TNF signaling leads to the activation of JNK, p38 and ERK MAPKs [25]. The observed effect of TNF stimulation on the transcript ratio in our experiments in the Jurkat cell-line, but not in Raji or U937, may indicate a cell-type specific splicing profile of MAP2K4 in pro-inflammatory conditions — and a topic for further analyses of mechanisms of involvement in RA and development of treatment strategy. This is in line with the established effect of anti-TNF treatment in RA and suggests how T-cell specific expression of MAP2K4 isoforms could be functionally involved in RA pathogenesis through the regulation of proliferation in the T-cell population.

Alternative mRNA transcripts can have a regulatory function by competing with the canonical transcript and shifting the amount of the main variant in the total gene output [26]. This knowledge can support the functional significance of splicing in MAP2K4 transcripts, even when the translation efficiency for non-canonical products is lower. It also seems reasonable to consider the possibility of NMD-subjected transcripts potentially influencing a mRNA-level regulatory capacity in disease pathogenesis [27]. It remains to be determined whether, and if so, how the balance of MAP2K4 splice variants may functionally affect the activation of downstream JNK and p38 in the context of autoimmunity. The quantity of an alternative MAP2K4 isoform could potentially interfere with complex formation and activation of downstream p38 and JNK MAPKs. Both JNK and p38 regulate interferon-γ-mediated gene expression, with p38 activating innate immune genes, while JNK acts on genes connected to antigen presentation [5]. Moreover, MAPKs are the main regulators of pro-inflammatory cytokine production, which is considered to be one of the major driving factors of RA [6]. If the gene expression of MAP2K4 is constitutively effected by a specific genotype it could potentially contribute to the development of autoimmunity over time.

5. Conclusion

In conclusion, we find that rs10468473 affects the expression of the MAP2K4 gene and is in statistical interaction with HLA-DRB1 SE alleles in the context of rheumatoid arthritis. The cassette exon 5 in MAP2K4 transcripts may represent a functionally and clinically meaningful splicing event that could potentially have a major impact on protein isoforms of MAP2K4. This may be a useful finding for risk prediction and development of potential therapies.

Abbreviations

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<tr>
<td>RA</td>
<td>rheumatoid arthritis</td>
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<td>HLA</td>
<td>human leukocyte antigen</td>
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<td>SE</td>
<td>shared epitope</td>
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<td>MAPK</td>
<td>mitogen activated protein kinase</td>
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<td>EIRA</td>
<td>Epidemiological Investigation in Rheumatoid Arthritis</td>
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<td>minor allele frequency</td>
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Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

This work was supported by the Swedish Research Council (Vetenskapsrådet), Vinnova (COMBINE project), Autocure and BeTheCure consortia. Christina Ottersson was instrumental in the collection of samples for this study. We would like to thank Peter Gregersen for providing access to the data collected as part of the NARAC project. We are also grateful to Bo Ding and Henrik Källberg from the Institute of Environmental Medicine, Karolinska Institutet for their support with handling and analysis of GWAS data. We thank Iryna Kolosenko from the Department of Oncology–Pathology, Karolinska Institutet for providing her expertise and assistance in protein identification and cell-line transfection experiments.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.clim.2015.02.011.

References


