Research article

Association of common polymorphisms in known susceptibility genes with rheumatoid arthritis in a Slovak population using osteoarthritis patients as controls

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Abstract

Introduction Both genetic and environmental factors contribute to rheumatoid arthritis (RA), a common and complex autoimmune disease. As well as the major susceptibility gene HLA-DRB1, recent genome-wide and candidate-gene studies reported additional evidence for association of single nucleotide polymorphism (SNP) markers in the PTPN22, STAT4, OLIG3/TNFAIP3 and TRAF1/C5 loci with RA. This study was initiated to investigate the association between defined genetic markers and RA in a Slovak population. In contrast to recent studies, we included intensively-characterized osteoarthritis (OA) patients as controls.

Methods We used material of 520 RA and 303 OA samples in a case-control setting. Six SNPs were genotyped using TaqMan assays. HLA-DRB1 alleles were determined by employing site-specific polymerase chain reaction (PCR) amplification.

Results No statistically significant association of TRAF1/C5 SNPs rs3761847 and rs10818488 with RA was detected. However, we were able to replicate the association signals between RA and HLA-DRB1 alleles, STAT4 (rs7574865), PTPN22 (rs2476601) and OLIG3/TNFAIP3 (rs10499194 and rs6920220). The strongest signal was detected for HLA-DRB1*04 with an allelic P = 1.2*10^-13 (OR = 2.92, 95% confidence interval (CI) = 2.18 – 3.91). Additionally, SNPs rs7574865 STAT4 (P = 9.2*10^-6; OR = 1.71, 95% CI = 1.35 – 2.18) and rs2476601 PTPN22 (P = 9.5*10^-4; OR = 1.67, 95% CI = 1.23 – 2.26) were associated with susceptibility to RA, whereas after permutation testing OLIG3/TNFAIP3 SNPs rs10499194 and rs6920220 missed our criteria for significance (Pcorr = 0.114 and Pcorr = 0.180, respectively).

Conclusions In our Slovak population, HLA-DRB1 alleles as well as SNPs in STAT4 and PTPN22 genes showed a strong association with RA.

Introduction

Susceptibility to rheumatoid arthritis (RA) is influenced by both environmental and genetic determinants with a concordance rate in monozygotic twins between 12% and 30% and a λs ranging from three to seven [1]. One of the first known genetic loci responsible for susceptibility to RA was found within the major histocompatibility complex, namely immune response genes in the human leukocyte antigen (HLA) class II region [2]. Recent genome-wide association studies have confirmed known and identified new genetic determinants of RA [3]. The well studied associations with HLA-DRB1 and PTPN22 explain about 50% of the genetic contribution to RA disease susceptibility [4]. For other polymorphisms, strong associations with RA were demonstrated, namely for a single nucleotide polymorphism (SNP) in the STAT4 gene, for two independent alleles at chromosome 6q23 near OLIG3 and...
TNFAIP3 genes, and for SNPs near TRAF1 and C5 genes [5-9].

In contrast to recent studies, we performed a replication study of seven genetic polymorphisms in Slovak patients with chronic RA as cases and with chronic osteoarthritis (OA) as controls. RA and OA share some features of pathology, but in detail seem to be quite different entities [10-13]. For a functional variant in the GDF5 gene, it was recently shown that risk of both RA and OA is increased [14,15]. Therefore, more genetic markers might be involved in both diseases.

To the best of our knowledge, this is the first study aimed at examining a genetic association in a RA-OA case-control setting in a Slovak population.

Materials and methods

Study participants

A total of 520 Slovak individuals (87 males, 433 females) with the diagnosis of RA were recruited to this study. All RA cases fulfilled the diagnostic features based on the established American College of Rheumatology criteria [13]. Controls (60 males, 243 females) were unrelated individuals from Slovakia who did not have any indication of RA but were affected by OA and intensively characterized. Further phenotypic details are shown in Table 1. Our study population did not differ in gender between RA cases and RA-free OA controls. Controls with OA are significantly older but free of RA symptoms and are rheumatoid factor (RF) negative. Both serum anti-cyclic citrullinated peptide (CCP) and C-reactive protein levels are significantly lower in OA than in RA cases (Table 1).

Measurement of antibody against CCP was carried out using an anti-CCP-ELISA (Euroimmun, Lübeck, Germany) following the manufacturer’s instructions. From a total of 428 individuals (304 RA patients, 124 OA patients) anti-CCP antibodies were determined. Values less than 4.2 RU/ml were considered as anti-CCP negative. No value exceeded the proposed linear range of up to 196 RU/ml. The RF was determined by standard techniques in the Laboratories of the National Institute of Rheumatic Diseases, Piestany, Slovakia.

Written consent was obtained from the patients according to the current Declaration of Helsinki. The study was approved by the Ethical Committee of the National Institute of Rheumatic Diseases, Piestany, Slovakia.

Marker selection and genetic analyses

SNPs in or near the genes PTPN22, STAT4, OLIG3/TNFAIP3, and TRAF1/C5 were selected from recent genome-wide association studies with replication studies and candidate-gene approaches (Table 2) [4-9].

Genomic DNA was isolated from whole blood samples using the PureGene DNA Blood Kit (QIAGEN, Hilden, Germany). DNA samples were genotyped using 5’ exonuclease TaqMan® technology (Applied Biosystems, Foster City, CA, USA), as recently described [16]. In brief, for each genotyping experiment 10 ng DNA was used in a total volume of 5 μl containing 1 × TaqMan® Genotyping Master Mix (Applied Biosystems Foster City, CA, USA). PCR and post-PCR endpoint plate read was carried out according to the manufacturer’s instructions using the Applied Biosystems 7900 HT Real-Time PCR System (Foster City, CA, USA). Sequence Detection System software version 2.3 (Applied Biosystems, Foster City, CA, USA) was used to assign genotypes applying the allelic discrimination test. Case and control DNA was genotyped together on the same plates with duplicates of samples (15%).

Table 1

| Variable                          | RA cases (n = 520) | RA-free OA controls (n = 303) | P      |
|-----------------------------------|-------------------|-------------------------------|--------|
| Gender, % female (n)              | 83.3 (433)        | 80.2 (243)                    | ns     |
| Age at inclusion, years (range)   | 51.6 ± 11.2 (19 to 80) | 57.9 ± 13.5 (21 to 83)       | < 0.0001 |
| Age of onset, years               | 40.8 ± 12.7       | 50.7 ± 12.8                   | < 0.0001 |
| Duration of disease, years        | 10.8 ± 8.3        | 7.2 ± 6.8                     | < 0.0001 |
| RF, IU/ml                         | 149.8 ± 67.2      | -                             | -      |
| RF-positive, % (n)                | 53.8 (280)        | -                             | -      |
| anti-CCP antibody, RU/ml<sup>a</sup> | 67.5 ± 53.7   | 1.5 ± 6.4                     | < 0.0001 |
| anti-CCP positive, % (n)<sup>b</sup> | 78.6 (239)  | 3.2 (4)                       | < 0.0001 |
| CRP, μg/ml                        | 19.6 ± 23.7       | 5.1 ± 9.2                     | < 0.0001 |

Values denote means ± standard deviations unless indicated otherwise. CCP = cyclic citrullinated peptide; CRP = C-reactive protein; ns = not significant; RF = rheumatoid factor.

<sup>a</sup> anti-CCP antibody serum level was determined in 428 individuals.

<sup>b</sup> Values below 4.2 RU/ml were considered as anti-CCP negative.
**Table 2**

| SNP          | Positiona | Major allele (1) | Minor allele (2) | Gene/function               |
|--------------|-----------|------------------|------------------|-----------------------------|
| rs2476601    | chr 1: 114,179,091 | G               | A                | PTPN22/R620W                |
| rs7574865    | chr 2: 191,672,878  | G               | T                | STAT4/Intron                |
| rs10499194   | chr 6: 138,044,330  | C               | T                | intergenic between OLIG3 and TNFAIP3 |
| rs6920220    | chr 6: 138,048,197  | G               | A                | intergenic between OLIG3 and TNFAIP3 |
| rs3761847    | chr 9: 122,730,060  | A               | G                | intergenic between TRAF1 and C5 |
| rs10818488   | chr 9: 122,744,908  | G               | A                | intergenic between TRAF1 and C5 |

a according to NCBI build 36.3.
SNP = single nucleotide polymorphism.

To assess intraplate and interplate genotype quality. No genotyping discrepancies were detected. Assignment of genotypes was performed by a person with no knowledge of the proband’s affection status.

**HLA-DRB1** genotyping was carried out using PCR with **HLA-DRB1** low-resolution exon 2 sequence-specific primers as previously described [17]. Absence or presence of **HLA-DRB1** specific products was visualized by agarose gel electrophoresis, photographed, and documented.

**HLA-DRB1** alleles were classified according to the nomenclature proposed by the World Health Organization Nomenclature Committee for factors of the HLA system [18]. For shared epitope (SE) association with RA, the classification system from de Vries was employed [19]. Due to frequencies below 1% for protective **HLA-DRB1** allele *0402 and neutral alleles *0403, *0406, and *0407, we did not analyse the *04 group in high resolution and considered *04 in total as SE [20]. With only three alleles in our study population (one in OA controls and two in RA cases), **HLA-DRB1**10103 was not used as a separate genotype and therefore *01 was also considered as SE in total.

**Statistical analyses**

To determine whether the genotypes of cases and controls of all SNPs deviated from Hardy-Weinberg equilibrium, actual and predicted genotype counts of both groups were compared by an exact test [21]. Differences between dichotomous traits were calculated employing a chi-squared test. Genotypes were coded for both dominant and recessive effects (genotype 22 + 12 versus 11 and genotype 22 versus 11 + 12, respectively, with the minor allele coded as 2). The additive genetic model was calculated using Armitage’s trend test [22]. To test for epistatic interaction between SNP markers a logistic regression model based on allele dosage for each SNP was carried out. Differences in continuous variables between groups were calculated using a two-tailed t-test for normally distributed values or using the non-parametric Wilcoxon rank-sum test for variables failing normal distribution as determined by the Shapiro-Wilk test. Multiple logistic regression analysis was used to examine the association between SNPs and RA with **HLA-DRB1** genotypes as covariates. Prevalence odds ratios (OR) with their 95% confidence intervals (CI) were reported. Correction for multiple testing was carried out using the Bonferroni adjustment. For post-hoc power calculation Fisher’s exact test was used. A one-sided P ≤ 0.05 was considered statistically significant.

Association analyses were performed using JMP 7.0.2 (SAS Institute Inc, Cary, NC, USA) and PLINK v1.04 [23,24]. For analysis of linkage disequilibrium (LD) and for permutation testing HaploView v4.1 was employed [25,26]. Power analysis was carried out using G*Power 3.0.10 [27,28].

**Results**

**Genetic analyses – SNP marker association**

We analyzed six SNPs with prior evidence of association with RA in genome-wide association studies and candidate-gene approaches, namely in or near the genes **PTPN22**, **STAT4**, **OLIG3/TNFAIP3**, and **TRAF1/C5** (Tables 2 and 3) [4-9]. Additionally, **HLA-DRB1** alleles were determined in low resolution and classified in respect to the SE [see Table S1 in Additional data file 1].

For all six SNP markers analyzed, call rates were greater than 98.5% and no deviation from the Hardy-Weinberg equilibrium was observed both in RA cases and RA-free OA controls (Table 4). Between **TRAF1** and **C5** SNPs on chromosome 9 (rs3761847 and rs10818487, respectively) strong LD exist with an r² value of 0.99. Weak LD (r²= 0.08) was detected between the two SNPs on chromosome 6 (rs10499194 and rs6920220), whereas the other SNPs are unlinked (r² = 0) and lie on different chromosomes.

A strong association between two SNPs (rs7574865**STAT4** and rs2476601**PTPN22**) and RA was detected, whereas for **OLIG3/TNFAIP3** SNPs rs10499194 and rs6920220 nominal association was found. **TRAF1/C5** SNPs rs3761847 and rs10818488 did not reach statistical significance in our study.
population (Table 5). However, OR for all SNPs are shifted in the same direction as previously published (Table 3). After correction for multiple testing (six SNPs), allelic $P$-values were still significant for rs7574865 $STAT4$ and rs2476601 $PTPN22$ ($P_{corr} = 5.5 \times 10^{-5}$ and $P_{corr} = 5.7 \times 10^{-3}$, respectively), but not for the other four SNPs (Table 5). Different genetic models revealed no considerable stronger association than observed by comparison of allele frequencies [see Table S2 in Additional data file 1]. After 100,000 permutation testings, rs7574865 $STAT4$ still showed the strongest association signal ($P = 8.0 \times 10^{-5}$) with rs2476601 $PTPN22$ ($P = 5.9 \times 10^{-3}$). The other SNPs failed to reach a level of statistical significance (rs6920220 $OLIG3/TNFAIP3$, $P = 0.105$; rs10499194 $OLIG3/TNFAIP3$, $P = 0.152$; rs3761847 $TRAF1/C5$, $P = 0.966$; rs10818488 $TRAF1/C5$, $P = 0.996$).

Analysis of epistasis revealed no significant interaction between the six SNPs (best $P = 0.063$ for epistatic interaction between rs7574865 $STAT4$ and rs2476601 $PTPN22$, and between rs7574865 $STAT4$ and rs10499194 $OLIG3/TNFAIP3$ with $P = 0.073$). In particular, the two SNPs localized on chromosome some 6 between $OLIG3$ and $TNFAIP3$ genes (rs10499194 and rs6920220) showed no interaction ($P = 0.425$).

Gender-specific analyses showed no association between the six SNPs and RA in the male subgroup (87 cases, 60 controls) [see Table S3 in Additional data file 1]. However, in the female subgroup (433 cases, 243 controls) the SNPs rs7574865 $STAT4$, rs2476601 $PTPN22$, and rs10499194 $OLIG3/TNFAIP3$ were associated with susceptibility to RA [see Table S4 in Additional data file 1], even after correction for multiple testing ($P_{corr} = 2.8 \times 10^{-5}$, $P_{corr} = 9.0 \times 10^{-3}$ and $P_{corr} = 0.037$, respectively).

In a subset analysis of RA samples stratified to RF status, no association between SNPs and RF status were found by comparison of RF-positive and RF-negative RA cases [see Table S5 in Additional data file 1]. In contrast, RF-positive and RF-negative RA cases compared with OA controls showed effects for SNPs rs7574865 $STAT4$ and rs2476601 $PTPN22$ in the same order of magnitude (OR = 1.62 to 1.74) as the whole RA sample [see Tables S6 and S7 in Additional data file 1].

### Table 3

#### Power analysis of SNP markers

| SNP     | Published OR | Published MAF in controls | Ref | Current study's MAF in controls | Power b |
|---------|--------------|---------------------------|-----|-------------------------------|---------|
| rs2476601 | 1.98         | 0.10                      | [4] | 0.108                         | 94.5%   |
| rs7574865 | 1.27         | 0.22                      | [5] | 0.202                         | 35.9%   |
| rs10499194 | 0.75         | 0.21 to 0.31              | [6] | 0.315                         | 53.1%   |
| rs6920220 | 1.22         | 0.21 to 0.22              | [7] | 0.154                         | 23.4%   |
| rs3761847 | 1.32         | 0.37 to 0.45              | [8] | 0.387                         | 57.0%   |
| rs10818488 | 1.26         | 0.44                      | [9] | 0.390                         | 44.4%   |

OR = odds ratio; MAF = minor allele frequency; Ref = reference; SNP = single nucleotide polymorphism.

a combination of initial finding and replication (when available) in the cited study; effects from minor allele.

b Power was calculated for published OR and MAF in controls from the present study (Table 4) with 520 cases and 303 controls assuming a one-tailed $P = 0.05$.

### Table 4

#### SNP characteristics in RA-OA case-control sample

| SNP     | RA case genotypes | RA-free OA control genotypes |
|---------|-------------------|-------------------------------|
|         | 11    | 12    | 22    | MAF   | $P$ (HWE) | 11    | 12    | 22    | MAF   | $P$ (HWE) |
| rs2476601 | 356   | 144   | 14    | 0.167 | 1       | 239   | 61    | 2     | 0.108 | 0.551     |
| rs7574865 | 259   | 205   | 54    | 0.302 | 0.175   | 196   | 87    | 17    | 0.202 | 0.104     |
| rs10499194 | 281  | 200   | 37    | 0.264 | 0.910   | 149   | 116   | 37    | 0.315 | 0.062     |
| rs6920220 | 324   | 175   | 16    | 0.201 | 0.218   | 213   | 78    | 7     | 0.154 | 1         |
| rs3761847 | 186   | 243   | 87    | 0.404 | 0.648   | 117   | 133   | 49    | 0.387 | 0.275     |
| rs10818488 | 186  | 240   | 85    | 0.401 | 0.645   | 116   | 134   | 50    | 0.390 | 0.278     |

HWE = Hardy-Weinberg equilibrium; MAF = minor allele frequency; OA = osteoarthritis; RA = rheumatoid arthritis; SNP = single nucleotide polymorphism. Numbers of genotypes (11, 12, 22) according to alleles from Table 2.
To test for an influence of serum anti-CCP antibody on RA susceptibility, association analyses between SNPs and RA were carried out in stratified subgroups [see Tables S8 to S10 in Additional data file 1]. Only PTPN22 SNP rs2476601 reached statistical significance after correction for multiple testing when comparing anti-CCP-positive RA patients with OA controls ($P_{corr} = 2.5 \times 10^{-3}$).

Genetic analyses – HLA allele association

HLA-DRB1 alleles were determined in 795 individuals (96.6%). Borderline deviation from Hardy-Weinberg equilibrium was found for HLA-DRB1*01 in controls and for *07 in cases (Table 6).

Except for HLA-DRB1*01, all association results confirmed our assumption of HLA-DRB1 allele classification [see Table S1 in Additional data file 1] (Table 7). Highest signals for risk association to RA were observed for HLA-DRB1*04 and *10 (Table 7). HLA-DRB1*07, *12, *13, and *15 showed protective effects (Table 7). After correction for multiple testing (13 tests), alleles *04, *07, and *13 still remained significant ($P_{corr}$).

Table 5

| SNP        | Allelic P  | Allelic $P_{corr.}^a$ | Allelic OR (95% CI) | Locus |
|------------|------------|------------------------|---------------------|-------|
| rs2476601  | 9.5 × 10^{-4} | 5.7 × 10^{-3} | 1.67 (1.23 to 2.26) | PTPN22 |
| rs7574865  | 9.2 × 10^{-6} | 5.5 × 10^{-5} | 1.71 (1.35 to 2.18) | STAT4 |
| rs10499194 | 0.030       | 0.180                | 0.78 (0.63 to 0.98)  | OLIG3/TNFAIP3 |
| rs6920220  | 0.019       | 0.114                | 1.38 (1.05 to 1.80)  | OLIG3/TNFAIP3 |
| rs3761847  | 0.480       | 1                    | 1.08 (0.88 to 1.32)  | TRAF1/C5 |
| rs10818488 | 0.657       | 1                    | 1.05 (0.85 to 1.29)  | TRAF1/C5 |

$^a$ Bonferroni correction for six SNPs tested.

CI = confidence interval; OA = osteoarthritis; OR = odds ratio; RA = rheumatoid arthritis; SNP = single nucleotide polymorphism.

Table 6

| HLA-DRB1 allele | RA case genotypes $b$ | RA-free OA control genotypes $b$ |
|-----------------|------------------------|----------------------------------|
|                 | 0         | 1      | 2      | MAF | $P$ (HWE) | 0   | 1    | 2    | MAF | $P$ (HWE) |

$^a$ Allele numbering according to Table S1 in Additional data file 1.

$^b$ Numbers indicate counts of rare alleles.

HWE = Hardy-Weinberg equilibrium; MAF = minor allele frequency; OA = osteoarthritis; RA = rheumatoid arthritis.
In gender-specific analyses, we found associations to RA susceptibility in our male subgroup for HLA-DRB1*04 and protective effects for alleles *12 and *13 [see Table S11 in Additional data file 1]. However, after correction for multiple testing, only allele *13 achieved marginal statistical significance ($P_{\text{corr}} = 0.043$). The female subgroup showed almost the same pattern of association as the whole population, except for alleles *11 and *12 [see Table S12 in Additional data file 1], whereas after correction for multiple testing, alleles *04, *07, and *13 still met our criteria for significance ($P_{\text{corr}} = 6.9 \times 10^{-11}$, $P_{\text{corr}} = 2.1 \times 10^{-3}$, and $P_{\text{corr}} = 0.014$, respectively). In both genders, no inflation of association signals was caused by deviation from Hardy-Weinberg equilibrium [see Tables S11 and S12 in Additional data file 1].

Additionally, we carried out a subset analysis of RA samples stratified to RF status. Association between RA and HLA-DRB1 alleles *04, *07, and *11 was detected by comparison of RF-positive and RF-negative RA cases [see Table S13 in Additional data file 1], whereas after correction for multiple testing, alleles *04 and *07 still met our criteria for significance ($P_{\text{corr}} = 0.018$ for both alleles). Comparison of RF-positive cases with OA controls showed association signals for HLA-DRB1 alleles *04, *07, *10, *11, *12, and *13, after correction for multiple testing alleles *11 and *12 failed significance [see Table S14 in Additional data file 1]. Alleles *04, *13, and *15 were associated with RA when comparing RF-negative cases with OA controls [see Table S15 in Additional data file 1], but only risk allele *04 met significance criteria after correction for multiple testing ($P_{\text{corr}} = 5.3 \times 10^{-5}$).

Stratification for serum anti-CCP antibody showed risk effect of HLA-DRB1*04 and protective effect of allele *13 in RA patients [see Table S16 in Additional data file 1] even after correction for multiple testing ($P_{\text{corr}} = 0.025$ and $P_{\text{corr}} = 0.036$, respectively). Comparison of anti-CCP-positive RA cases with anti-CCP-negative OA controls revealed several association signals, whereas anti-CCP-negative RA cases did not [see Tables S17 and S18 in Additional data file 1].

Assuming a dominant genetic model for HLA-DRB1 alleles, we carried out a multiple logistic regression analysis to test for interactions between HLA-DRB1 alleles and the six SNPs. Taking into account all 13 HLA-DRB1 alleles, a significant association between RA and rs754865$_{\text{STAT4}}$ as well as rs2476601$_{\text{PTPN22}}$ remained ($P = 2.8 \times 10^{-4}$ and $P = 1.9 \times 10^{-3}$, respectively), whereas the other SNPs failed to reach the level of statistical significance (rs10499194$_{\text{OLIG3/TNFAIP3}}$: $P = 0.140$; rs6920220$_{\text{OLIG3/TNFAIP3}}$: $P = 0.079$; rs3761847$_{\text{TRAF1/ C5P}}$: $P = 0.771$; rs10818488$_{\text{TRAF1/C5P}}$: $P = 0.897$). After adjustment for only risk HLA-DRB1 alleles *04 and *10, for four SNPs significant association was detected (rs754865$_{\text{STAT4}}$: $P = 1.4 \times 10^{-5}$; rs2476601$_{\text{PTPN22}}$: $P = 1.2 \times 10^{-3}$;
rs6920220 <sub>OLIG3/TNFAIP3</sub> \( P = 4.6 \times 10^{-3} \); rs10499194 <sub>OLIG3/TNFAIP3</sub> \( P = 0.017 \) but not for rs3761847 <sub>TRAF1/C5</sub> and rs10818488 <sub>TRAF1/C5</sub> \( P = 0.790 \) and \( P = 0.943 \), respectively.

**Discussion**

This study investigated the relation between known susceptibility alleles and RA in a Slovak population. In contrast to recent studies, we compared RA cases with gender-matched OA controls. Therefore, this paper is the first to analyze the differences between RA and OA for known high-risk genetic polymorphisms.

Since the 1970s it has been known that variants in the *HLA* region on chromosome 6p21.3 are associated with RA [29]. In our study, the main effect to RA risk came from *HLA-DRB1*04 allele. Additionally, we found protective effects of *HLA-DRB1*07 and *13 in the whole study group. However, common SNP markers in genes *PTPN22* and *STAT4* also contributed to RA susceptibility, but no other SNPs analyzed. It is noteworthy, that, in contrast to other studies, *STAT4* SNP rs7574865 showed higher significance than *PTPN22* SNP rs2476601. One explanation may be our study design. By comparing RA with OA patients, genes with opposing effects will show higher OR.

For SNPs rs3761847 and rs10818488, localized between *TRAF1* and *C5* genes, we were not able to find a statistically significant association with RA. Recently, re-evaluation of RA susceptibility genes in the Wellcome Trust Case Control Consortium study revealed very moderate effect sizes for SNPs in the *TRAF1/C5* genomic region (OR = 1.08) [30]. The effect of *TRAF1/C5* alleles may have been over-estimated in the initial study (‘winner’s curse’). Therefore, in replication studies, the moderate effects have to be the basis for analysis.

The power to detect association in our study was only 12% (minor allele frequency = 39%, assumed OR = 1.08, one-tailed \( P = 0.05 \)). Hence, both missing power and ethnicity could explain the non-replication of these associations with RA in our Slovak population. For example, minor allele frequency for rs10818488 in controls is lower in our study (0.39) compared with published data in sample sets from the Netherlands, Sweden, and the USA (0.44) [9]. Another reason could be the pathophysiological identity in genetic susceptibility between RA and OA. Our study is designed to work out specific genetic differences to RA susceptibility in comparison to OA. As a consequence, common pathways would not be highlighted as association signals. It is important to note that in a recent study, an association was found with RA in the extended genomic segment including *TRAF1* but excluding the C5 coding region [31]. Therefore, more specific and potentially unlinked SNP markers may exist and should be taken into account.

We only found nominal significance for SNPs rs10499194 <sub>OLIG3/TNFAIP3</sub> and rs6920220 <sub>OLIG3/TNFAIP3</sub> identified by Plenge and colleagues as independent RA risk markers [6]. The two SNPs are located on chromosome 6q23 and are in weak LD. SNP rs10499194 <sub>OLIG3/TNFAIP3</sub> showed a pronounced effect on RA risk in a recessive model in our study sample (\( P = 0.014 \)), and, hence, might need larger populations to be detected with study-wide significance. Interestingly, minor allele frequency for rs10499194 <sub>OLIG3/TNFAIP3</sub> is on the upper end whereas that for rs6920220 <sub>OLIG3/TNFAIP3</sub> is below the frequencies from previously published studies [6,7]. Again, this may be caused by our study design or represent an ethnical characteristic. Perfect proxies of rs10499194 <sub>OLIG3/TNFAIP3</sub> are also associated with a risk of systemic lupus erythematosus [32]. Therefore, this genomic region might contribute to risk for autoimmune diseases and needs to be analyzed in further studies with higher power to detect an effect.

We were not able to show an association between the six SNPs and RA in the male subset of our population, which was likely to be due to a lack of power. However, gender-specific influence on association signal can not be excluded. Recently, in the Wellcome Trust Case Control Consortium genome-wide association study, a single SNP (rs11761231) generated a strong signal in the gender-differentiated analyses for RA, with an additive effect in females and no effect in males [4]. In contrast, a protective effect of the *HLA-DRB1*13 allele was obvious in our male subgroup with an OR of 0.32 (i.e. OR = 3.13 for susceptibility allele). One possible explanation is the moderate SNP OR between 1.38 and 1.67 in the whole sample and, therefore, a loss of power to detect this effect in the small male sample (87 cases, 60 controls).

Several limitations of our study have to be considered. The summarization of all *HLA-DRB1*01xx and *04xx alleles as SE alleles ignored the protective effects of *0103 and *0402 and the neutral effect of *0403, *0406, and *0407 subtypes. However, a recent report by Morgan and colleagues showed that the frequency of these alleles is very low [20]. Therefore, we may have underestimated the risk effect of *HLA-DRB1*01 and *04 alleles in this study but confirmed the association between *HLA-DRB1*04 SE and RA.

Our RA population is heterogeneous in relation to RF and anti-CCP. Another study showed that the *HLA-DRB1* SE alleles are only associated with anti-CCP-positive RA in a European population, where the combination of smoking history and SE alleles increased the risk for RA 21-fold [33]. Here, we found significant association to RA risk for *PTPN22* variant rs2476601 and *HLA-DRB1* alleles in anti-CCP-positive RA patients compared with OA controls. Analysis within our RA group divided into anti-CCP-positive and anti-CCP-negative subgroups revealed a pattern of association for *HLA-DRB1*-alleles similar to that found in the unstratified case-control set-
ting. It remains unclear whether we had too little power to
detect other effects or in fact found a significant causal inter-
action between serum anti-CCP antibody, HLA-DRB1 alleles,
and rs2476601_PTPN22 as previously described [33,34].

The ascertainment strategy used here was not aimed at col-
lecting special subgroups (e.g. only RF-positive RA cases with
detectable anti-CCP) and, therefore, is not presenting a partic-
ular form of pathology with a higher power to detect specific
genetic factors. However, this population reflects the clinical
reality and, hence, allows a better risk assessment for the gen-
eral patient with RA.

The predictive value of genetic markers for RA diagnosis is not
obvious when using a limited number of alleles [35]. However,
the knowledge of nearly all genetic variants contributing to
both RA and OA susceptibility in a given ethnicity may help to
prevent clinical mismanagement and avoid excessive costs.
Our population is the first aimed at identifying genetic differ-
ences between RA and OA and, therefore, allowing the dis-
section of genetic markers for diagnosis in the border area
between these two disease entities.

Conclusions
Our study demonstrates strong evidence that polymorphisms
in HLA-DRB1, PTPN22, and STAT4 genes contribute to RA
susceptibility in a comprehensively characterized Slovak case
population compared with a gender-matched OA control
group.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
KS carried out the SNP genotyping and statistical analysis and
drafted the manuscript. JR and SB collected the sample and
phenotyped the patients. HGW and SF carried out the HLA
typing. CH participated in study coordination and helped to
draft the manuscript. RS conceived of the study, and partici-
pated in its design and coordination and helped to draft the
manuscript. All authors read and approved the final manu-
script.

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Additional files
The following Additional files are available online:

Additional file 1
Word file containing 18 tables. Table S1 lists the HLA-
DRB1 allele classification. Table S2 lists the single
nucleotide polymorphism (SNP) association results from
different genetic models in rheumatoid arthritis (RA)-
osteoarthritis (OA) case-control sample. Table S3 lists
the SNP association analysis results in male RA case-
control sample. Table S4 lists the SNP association
analysis results in female RA-OA case-control sample.
Table S5 lists the SNP association analysis results in RA
patients with rheumatoid factor (RF) vs. RA patients
without RF. Table S6 lists the SNP association analysis
results in RA patients with RF vs. OA controls. Table S7
lists the SNP association analysis results in RA patients
without RF vs. OA controls. Table S8 lists the SNP
association analysis results in anti-cyclic citrullinated
peptide (CCP)-positive RA patients vs. anti-CCP-
negative RA patients. Table S9 lists the SNP association
analysis results in anti-CCP-positive RA patients vs. anti-
CCP-negative OA patients. Table S10 lists the SNP
association analysis results in anti-CCP-negative RA
patients vs. anti-CCP-negative OA patients. Table S11
lists the HLA-DRB1 association analysis results in male
RA case-control sample. Table S12 lists the HLA-DRB1
association analysis results in female RA case-control
sample. Table S13 lists the HLA-DRB1 association analysis
results in RA patients with RF vs. RA patients
without RF. Table S14 lists the HLA-DRB1 association
analysis results in RA patients with RF vs. OA controls. Table
S15 lists the HLA-DRB1 association analysis results in RA
patients without RF vs. OA controls. Table S16 lists the HLA-
DRB1 association analysis results in anti-CCP-positive RA patients vs. anti-CCP-negative RA
patients. Table S17 lists the HLA-DRB1 association analysis
results in anti-CCP-positive RA patients vs. anti-CCP-negative OA patients. Table S18 lists the HLA-
DRB1 association analysis results in anti-CCP-negative
RA patients vs. anti-CCP-negative OA patients.
See http://www.biomedcentral.com/content/
supplementary/ar2699-S1.doc
inated protein antibodies to very early rheumatoid arthritis diagnosis. Rheumatology (Oxford) 2008, 47:1208-1212.