Association of the X-Chromosomal Genes *TIMP1* and *IL9R* with Rheumatoid Arthritis

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ABSTRACT. ***Objective.*** Rheumatoid arthritis (RA) is an inflammatory joint disease with features of an autoimmune disease with female predominance. Candidate genes located on the X-chromosome were selected for a family trio-based association study.

Methods. A total of 1452 individuals belonging to 3 different sample sets were genotyped for 16 single-nucleotide polymorphisms (SNP) in 7 genes. The first 2 sets consisted of 100 family trios, each of French Caucasian origin, and the third of 284 additional family trios of European Caucasian origin. Subgroups were analyzed according to sex of patient and presence of anti-cyclic citrullinated peptide (anti-CCP) autoantibodies.

Results. Four SNP were associated with RA in the first sample set and were genotyped in the second set. In combined analysis of sets 1 and 2, evidence remained for association of 3 SNP in the genes *UBA1*, *TIMP1*, and *IL9R*. These were again genotyped in the third sample set. Two SNP were associated with RA in the joint analysis of all samples: rs6520278 (*TIMP1*) was associated with RA in general ($p = 0.035$) and rs3093457 (*IL9R*) with anti-CCP-positive RA patients ($p = 0.037$) and male RA patients ($p = 0.010$). A comparison of the results with data from whole-genome association studies further supports an association of RA with *TIMP1*. The sex-specific association of rs3093457 (*IL9R*) was supported by the observation that men homozygous for rs3093457-CC are at a significantly higher risk to develop RA than women (risk ratio male/female = 2.98; $p = 0.048$).

Conclusion. We provide evidence for an association of at least 2 X-chromosomal genes with RA: *TIMP1* (rs6520278) and *IL9R* (rs3093457). (J Rheumatol First Release Sept 1 2009; doi:10.3899/jrheum.090059)

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Rheumatoid arthritis (RA) is an inflammatory joint disease with features of an autoimmune disease and a prevalence of about 1% in the European Caucasian population[1]. There is evidence for genetic influences on RA and heritability is estimated to be 60%[2]. Female sex is a well known risk factor for RA. The female to male ratio ranges between 3 and 4[3]. There may be a link between heritability and sex, as the female genome differs crucially from the male genome. The Y chromosome supplies males with several genes absent in the female[4], while incomplete X-inactivation or varying inactivation patterns may lead to gene-dosage skewing in females[5]. X-chromosomal abnormalities were observed in immunological diseases, e.g., a significantly higher rate of acquired X-monosomy[6] and significantly skewed X-inactivation[7]. Patients with Turner's syndrome are known to manifest common autoimmune features[8]. Whole-genome linkage studies for RA suggest among others the presence of loci of interest on chromosome X[9,10]. Thus, X-chromosomal genes are highly relevant candidate genes to test for association with RA.

For this study we selected 7 genes, *CD40LG*, *CD99*, *EIF2S3*, *IL9R*, *TIMP1*, *UBA1*, and *XIAP* (Table 1). Most of these genes are involved in pathways thought to be crucial for RA etiology, and evidence for their involvement in other immunological diseases exists as well.

CD99 and *IL9R* are situated within pseudoautosomal regions and have a functional homologue on the Y chromosome, whereas the other genes are restricted to the X chromosome. To our knowledge none of the genes we selected, with the exception of *TIMP1*, has been investigated for association with RA in candidate gene studies.

CD40LG is involved in the regulation of B cell functions and the production of autoantibodies[11]. CD99 is described to play a role in transport regulation of MHC class I molecules[12], lymphocyte adhesion[13], and induced T cell death[14]. EIF2S3 is the γ-subunit of the eukaryotic translation initiation factor (EIF2) and is only partially affected by X-inactivation[15]. EIF2 is involved in stress responses and apoptosis[16]. Insufficient apoptosis of inflammatory cells in synovial membrane as well as increased apoptosis, especially within the synovial lining, has been demonstrated in RA[17,18]. IL9R is a receptor for the cytokine interleukin 9 (IL-9) expressed on many hematopoietic cells including T cells[19], and it is also involved in early T cell development[20]. The gene product of TIMP protects extracellular matrix from degradation by inhibiting metalloproteinases (MMP)[21]. Secretion of MMP is required for the initial stage of angiogenesis[22], contributing to pannus formation in RA[23]. *TIMP1* (SNP rs5953060) was described to be associated with RA in a small Japanese cohort[24] and has also shown association with other immunity disorders like Crohn's disease[25] and systemic sclerosis[26]. UBA1 (also known as UBE1) catalyzes the first step in ubiquitin conjugation to mark cellular proteins for degradation[27]. Involvement of UBA1 in cell-cycle regulation and apoptosis can be demonstrated and provides a functional link to RA[28]. XIAP is a potent inhibitor of apoptosis and is involved in regulation of lymphocyte homeostasis[29].

Our aim was to investigate genetic variants of selected X-chromosomal genes in a candidate gene association study based on a family-trio approach in a European Caucasian population.

Table 1. Selected genes in order of chromosomal location (short arm p to long arm q). Data were acquired using Entrez Gene and Entrez Protein databases, as well as the UCSC Genome Browser Build March 2006. rs numbers according to dbSNP Build 127.

Gene	Name	Locus	SNP Investigated	Position/Type of Variation	Pseudo-autosomal	Inactivation Status[59]	Published Disease Associations
CD99	CD99 molecule	Xp22.32	rs311071 rs312258	Intronic Intronic	Yes	Not inactivated	—
EIF2S3	Eukaryotic translation initiation factor 2 subunit 3	X922.2–p22.1	rs16997659 rs12556742 rs12847067	Coding, nonsynonymous Intronic 3' downstream	No	Partial inactivation	—
TIMP1	Tissue inhibitor of metalloproteinase 1	Xp11.23	rs4898 rs6520278 rs5953060	Coding, synonymous Intronic Intronic	No	Partial inactivation	Rheumatoid arthritis[24] Asthma[57] Crohn's disease[25] Systemic sclerosis[26]
UBA1	Ubiquitin-like modifier activating enzyme 1	Xp11.23	rs4239963 rs2070169 rs4529579	Intronic Coding, nonsynonymous Intronic	No	Partial inactivation	—
XIAP	X-linked inhibitor of apoptosis	Xq25	rs7878958 rs7053190 rs9856	5' upstream Intronic Coding, 3' UTR	No	Unknown	—
CD40LG	CD40 ligand	Xq26	rs3092936	Intronic	No	Not inactivated	Systemic lupus erythematosus[11]
IL9R	Interleukin 9 receptor	Xq28	rs3093457 rs1973881	Intronic Intronic	Yes	Not activated	Asthma[58]

MATERIALS AND METHODS

Three sets of family trios, RA patient (i.e., the affected individual) and both parents, were genotyped. Detailed characteristics of the first 2 and parts of the third set have been described[30]. Briefly, the first 2 sets consisted of 100 family trios of French Caucasian origin. The third set consisted of 284 additional European Caucasian families, from France, Germany, Italy, Portugal, Spain, The Netherlands, and Belgium. All affected individuals fulfilled the American College of Rheumatology 1987 revised criteria for RA[31]. In addition the status of anti-cyclic citrullinated peptide autoantibodies (anti-CCP, also known as ACPA) was available for French and German RA patients (CCP-positive, n = 226; CCP-negative, n = 73). In our multistage approach all SNP were genotyped in the first sample set ("exploration set"). Markers with a significant association with RA (uncorrected $p < 0.05$) were then genotyped in the second sample set ("replication set"). When evidence increased in favor of an association, i.e., the p value of the association decreased in the combined analysis of both sets, markers were genotyped again in the third sample set (the multinational European replication set).

Genomic DNA was purified from fresh peripheral blood leukocytes or from Epstein-Barr virus-transfected cell lines using standard methods.

SNP were chosen based on their position in the gene, depending on gene length and validation status. Information from public databases (PupaSNP, UCSC Genome Browser, Ensembl) was used to aid in SNP selection. Selected SNP are listed in Table 1.

Genotyping was carried out using the genoSNIP technique (Bruker Daltonics, Billerica, MA, USA)[32]. Polymerase chain reaction primers were designed using MuPlex Vs 2.2. SBE-primer design was carried out using PrimExtend, an in-house software tool based on CalcDalton[33]. Primer sequences are shown in Table 2.

Samples of the third set were genotyped by applying a TaqMan 5' allelic discrimination assay (Applied Biosystems, Foster City, CA, USA) following the manufacturer's protocols.

For quality control purposes Mendelian laws of inheritance and Hardy-Weinberg equilibrium (HWE) in nontransmitted controls had to be fulfilled ($p > 0.01$). HWE analysis for nonpseudoautosomal genes was carried out in healthy female controls (mothers) only. All genotyping results fulfilled the quality control criteria. Genotype call-rate was more than 95%.

Statistical analysis. HWE was investigated using a chi-square test with 1 degree of freedom. Linkage and association analyses were performed using the transmission disequilibrium test (TDT)[34] and the genotype relative risk (GRR) test[35]. The TDT compares the transmission of SNP alleles from het-

Table 2. Polymerase chain reaction (PCR) primer pairs and genotyping primers used in this study.

Gene	SNP (rs number)	PCR Forward Primer	PCR Reverse Primer	SBE Primer
CD40LG	rs3092936	ACGTTGGATGCAGGCCTTATTATCTCCATT	ACGTTGGATGCAGTTCCCAGGGTAGAATC	bioTCCTTGGCTTTGACAGATATTG
CD99	rs311071	ACGTTGGATGATACAGAGACAGGAAGTGGG	ACGTTGGATGGTGCTTTGCAAATACTGTGT	bioACTGTGTGTTTTA(L)AAACGGAAGG
CD99	rs312258	ACGTTGGATGGCTGGGATTTGGAAGAAG	ACGTTGGATGTCATTAGCCAGTACATTGGT	bioTGTGTGTGG(L)GCCCTCGT
EIF2S3	rs16997659	ACGTTGGATGACACCTGACGAGTTTCCTAC	ACGTTGGATGCACCGTTTAACACCTCGA	bioAGCAAAAGAGAG(L)TCACCTGACTAAT
EIF2S3	rs12556742	ACGTTGGATGGTGCTAGGATTACAGGCG	ACGTTGGATGATTTCTACCTCATTGGAAATAC	bioTTACCTACAAATG(L)CAGTTGAATGAT
EIF2S3	rs12847067	ACGTTGGATGTGGATGGGATCAATGTACA	ACGTTGGATGGCCTTTCTGGATTAGATTTAAT	bioGGATGGGAT(L)AATGTACAGATGACC
IL9R	rs3093457	ACGTTGGATGCCACTGTGGCATTTGAGA	ACGTTGGATGCCTACAGGACTGGAAATTAGT	bioAAATTTTGCCATCTC(L)AAGAGATAC
IL9R	rs1973881	ACGTTGGATGCAACCGGCCTGTTACCA	ACGTTGGATGCTGGCTCCTGACACCTCTC	bioCTGTGAA(L)TTCCCGAGGGC
UBA1	rs4239963	ACGTTGGATGGTGCTATGGTTTCTATGGTTA	ACGTTGGATGGTCCTGGTTAAAGGAAGAGT	bioAGGAAGAGTGAGTCTC(L)GAAACAG
UBA1	rs2070169	ACGTTGGATGAGTGCCTCCAGGTATGTG	ACGTTGGATGCAGGTCTGAGCCAAACAC	bioTCCTCCCTTT(L)CATTCCTTAGC
UBA1	rs4529579	ACGTTGGATGGCTGGTTATTTATTTGTCATG	ACGTTGGATGGCATCTAGTGGGCAGAAGT	bioTTGTC(L)TGGGAGAGGGGATG
TIMP1	rs6520277	ACGTTGGATGATTCCTCACAGCCAACAG	ACGTTGGATGAGCTCAGCCAATCACAAG	bioTGCACATCAC(L)ACCTGCAGTTT
TIMP1	rs6520278	ACGTTGGATGGAAGTAGCAGGGGAAGGAT	ACGTTGGATGTGTTCTGGGCTCTGTGTC	bioCTACCCTGCAGGT(L)AGCCCTT
TIMP1	rs5953060	TTCAGTCTATCAGAAGGCC	CAAGAGTCCATCCTGCAGT	bioTGGCT(L)AGCTGCCAAGCTG
XIAP	rs7878958	ACGTTGGATGGTTCATATCTCCCAGTTGAC	ACGTTGGATGTCCTGCTAGAATATAAGCTCT	bioCACAAGGATCCT(L)GTTTTGTTCA
XIAP	rs7053190	ACGTTGGATGTACGCAGTGAGTGGCATT	ACGTTGGATGTGTCCAGAATAGGCAAGTC	bioAGACAGAAAGTAGA(L)TACTGGTTGCC
XIAP	rs9856	ACGTTGGATGCAAATTTAGTTGAGCTTTCTAAG	ACGTTGGATGGCTGAGGAAGAAATTCACA	bioCTGTATGAGTCAAACTGAAA(L)TGATTATT

bio: biotinylated group; (L): photo-cleavable base.

erozygous parents to affected offspring with a transmission ratio of 50% as expected by Mendel's law. The GRR test compares differences in genotype distribution between RA cases and "virtual controls" reconstructed from nontransmitted parental alleles. Haploview 4.1 software was used for gene-wide haplotype analysis[36]. Tests were also done in sample sets stratified for sex or anti-CCP status of RA patients. We used a 2-tailed test of interaction to assess significance of differences between subgroups[37].

For nonpseudoautosomal genes XTDT was applied as implemented in Haploview 4.1[38]. As proposed[39], males were treated like homozygous females for comparing allele frequencies by allele counting. Additionally, for GRR tests (Lathrop tests) only maternal reconstructed control genotypes and genotypes from corresponding affected female children were included.

RESULTS

In the first set, consisting of 100 French Caucasian family trios, 3 genes, *IL9R*, *TIMP1*, and *UBA1*, showed evidence for association. SNP with evidence for association were again genotyped in the second French Caucasian family trio set (100 additional trios). A combined analysis of set 1 and set 2 revealed a decreased p value for 3 markers. These SNP, rs4239963 (*UBA1*), rs6520278 (*TIMP1*), and rs3093457 (*IL9R*), were genotyped in the third European Caucasian sample set (284 additional trios). These data are summarized in Tables 3, 4, and 5. Details of family trio-based association analysis for all markers are shown in Tables 6, 7, and 8.

While the *UBA1* SNP rs4239963 showed significant association with RA in the first 2 sample sets, it was not found to be associated with RA in the combined analysis of all 3 sets, although the trend was the same as in sets 1 and 2, with the minor allele (C) being undertransmitted (Table 3).

Table 3. Results of family-trio TDT analysis for UBA1 (rs4239963, minor allele C): results in all samples in a given sample set; minor allele transmissions (no. of transmitted alleles: untransmitted alleles) are shown.

	Set 1	Set 1 & 2	Set 1 & 2 & 3
No. of informative families	31	69	192
Minor allele transmission	10:21	21:48	86:106
TDT p value	0.048	0.001	0.149

Table 4. Results of family-trio TDT analysis for *TIMP1* (rs6020277, minor allele C; rs6520278, minor allele T). Minor allele transmissions (no. of transmitted alleles: untransmitted alleles) are shown.

rs6520277	Set 1, male	Set 1 & 2, male	Set 1 & 2 & 3, male
No. of families	6	9	NI
Minor allele transmission	0:6	1:8	NI
TDT p value	0.014	0.020	NI
rs6520278	Set 1	Set 1 & 2	Set 1 & 2 & 3
No. of informative families	45	78	189
Minor allele transmission	15:30	29:49	80:109
TDT p value	0.025	0.024	0.035
rs6520278	Set 1, male	Set 1 & 2, male	Set 1 & 2 & 3, male
No. of informative families	7	9	21
Minor allele transmission	0:7	1:8	9:12
TDT p value	0.008	0.020	0.513

Male subgroup: family trios with male patients. NI: not investigated.

In contrast, SNP rs6520278 (*TIMP1*) was found to be significantly associated with RA in general, which is indicated by significant p values in the combined analysis of all 3 sets (p = 0.035; Table 4). The TDT showed the minor allele T was undertransmitted. Association of rs6520278 in families with male offspring could not be replicated in the European replication set. Additionally, the test of interaction revealed no significant difference between female and male subgroups for the SNP, as effect sizes (GRR minor vs major genotype) of the 2 subgroups did not differ significantly (p for interaction = 0.071). Another SNP, rs6520277 of *TIMP1*, also showed significant p value in families with male offspring in set 1, but this result could not be replicated in the second sample set.

SNP rs3093457 of *IL9R* was found to be significantly associated with RA in 2 subgroups in the combined analysis: families with anti-CCP-positive patients (p = 0.037) and families with male patients (p = 0.010), while an association of rs3093457 was only marginally significant in all family trios (p = 0.056; Table 5) and was not significant in families with female RA patients. In both subgroups the association was due to an increase of the homozygous minor genotype rs3093457-CC in RA cases. We also performed an interaction test to identify specific effects concerning anti-CCP status and/or sex. Comparing the GRR of rs3093457-CC for male and female subgroups revealed that the SNP affected males significantly more than females (p = 0.048). GRR in families with male offspring was about 3 times greater in the combined analysis of all sample sets (ratio of male/female GRR 2.98, 95% CI 1.01–8.79; Table 9). No significant difference between effect sizes was observed for anti-CCP-positive and negative subgroups.

DISCUSSION

We investigated SNP in 7 X-chromosomal genes for association with RA and were able to detect evidence for association for markers of 2 genes, *TIMP1* and *IL9R*. SNP rs6520278 of *TIMP1* showed a significant association in the combined analysis of all 3 sets (n = 484 family trios), with the minor T-allele being undertransmitted in RA patients (affected children), indicating a protective effect for this allele.

SNP rs6520278 was measured directly in at least 3 whole-genome association studies (WGAS) [the Spanish Upstream Regulatory Region study[40]; the British Wellcome Trust Case-Control Consortium (WTCCC) study[39]; the North American Rheumatoid Arthritis Consortium and Swedish Epidemiological Investigation of Rheumatoid Arthritis[41] studies], but p values were not significant. This might be due to disease heterogeneity or, if the analyzed variant is not a causative variant, to differences in the link-

Table 5. Results of family-based case-control analysis of *IL9R* (rs3093457, minor allele C).

	Set 1	Set 1 & 2	Set 1 & 2 & 3
No. of cases	89	180	437
Homozygous minor genotype vs others (Lathrop) p value	0.028	0.020	0.056
Minor allele GRR (95% CI)	2.34 (1.1–5)	1.96 (1.2–3.4)	1.46 (1–2.1)
	Set 1, male	Set 1 & 2, male	Set 1 & 2 & 3, male
No. of cases	12	20	55
Homozygous minor genotype vs others (Lathrop) p value	0.013	0.005	0.01
Minor allele GRR (95% CI)	15.49 (1.8–130.9)	11.23 (2.1–60)	3.75 (1.4–10.2)
	Set 1, a-CCP+	Set 1 & 2, a-CCP+	Set 1 & 2 & 3, a-CCP+
No. of cases	68	132	209
Homozygous minor genotype vs others (Lathrop) p value	0.019	0.008	0.037
Minor allele GRR (95% CI)	2.71 (1.2–6.2)	2.33 (1.2–4.3)	1.76 (1–3)

GGR: genetic relative risk; male subgroup: family trios with male patients; a-CCP+: subgroup positive for anti-cyclic citrullinated peptide antibodies, i.e., family trios with anti-CCP-positive patients.

age disequilibrium of the various sample groups. On the other hand, we found several markers in the WGAS in proximity (± 200 kb, as proposed[40]) to *TIMP1* associated with RA (Table 10) at the single-marker level.

UBA1 and *TIMP1* are both situated on the same chromosomal band (Xp11.23) and about 370 kb apart. We could not confirm an association of the *UBA1* gene with RA in the analysis of all 3 sample sets. However, in WGAS several SNP near the gene showed significant p values as well (Table 10). Given the proximity of *UBA1* and *TIMP1*, these data might indicate the presence of causative variants in this chromosomal region.

Linkage disequilibrium (correlation of alleles of 2 polymorphisms in a given population) was examined between SNP associated with RA in our study and SNP in proximity that are also associated with RA in WGAS. Because SNP data for rs4239963 (*UBA1*) were not available from HapMap (release 23) and the *IL9R* region was not covered by the cited WGAS, only *TIMP1* could be investigated. The SNP rs760580 correlated with rs6520278 of *TIMP1* as shown by high D' (0.545) and r^2 (0.222). Moreover, SNP rs760580 was associated with RA in the WTCCC study at the single-marker level (p = 0.044) and showed a protective effect of the minor allele, as did rs6520278.

TIMP1 SNP rs5953060 was described to be associated with RA in a small Japanese cohort (p = 0.02)[42]. While we could not replicate this association (p = 0.228; Table 6), we found linkage disequilibrium between rs5953060 and rs65020278 (D' = 1, r^2 = 0.607). Therefore it appears possible that rs5953060 in the Japanese study reflects association of the same unknown causative locus in the *TIMP1* region as did rs6520278 in our study due to different linkage disequilibrium among populations.

We did not find a significant sex-specific effect of rs6520278, although another *TIMP1* SNP investigated in our study, rs6520277, did hint at sex-specific effects of the gene. This SNP was significantly associated with RA in families with male children in the first set and in the combined analysis of the first and second sets. However, the small number of informative families of male RA patients did not allow for final conclusions. Further investigations are required to clarify possible sex-specific effects of *TIMP1*.

TIMP1 could influence the etiology of RA in several ways. It inhibits MMP[43,44] and subsequently prevents the degradation of cartilage[22]. The inhibition of MMP also may inhibit angiogenesis required for pannus formation[23,45]. A genetic association of *TIMP1* with RA therefore supports the hypothesis that modified angiogenesis might play an important role in the etiology of RA due to altered regulation of MMP via their interactions with TIMP1.

Synovial endothelial cells of patients with RA secrete decreased levels of TIMP1[46]. Levels of TIMP1 expression are affected by X-chromosomal inactivation[47,48], but *TIMP1* partially escapes X-chromosomal gene silencing[49]. *TIMP1* variants may also lead to differences in the level of expression, e.g., SNP might be involved in incomplete gene silencing or in other regulatory mechanisms. It remains to be seen whether allele-specific effects contribute to differences in TIMP1 expression.

Another SNP associated with RA in our study was rs3093457 in the *IL9R* gene. SNP near *IL9R* were not investigated in any of the WGAS, thus our findings are the only data available for this gene and this region. The homozygous minor genotype CC was marginally increased in all cases (p = 0.056) and was significantly increased in the anti-CCP-positive subgroup (p = 0.037) and in male RA patients (p = 0.01). The interaction test result further supports the sex-specificity of the association with males, who are 3 times more affected by this genotype than females. Sex-specific effects for *IL9R* have been described for bipolar disorder as well as childhood wheezing, an asthma characteristic, with associations limited to males[50,51]. The observed association of the X-chromosomal *IL9R* with RA would therefore provide further evidence for sex-specific disease mechanisms in RA.

There are several possibilities for *IL9R* involvement in the etiology of RA. Different *IL9R* splice variants affect the

Table 6. Results of TDT analysis for SNP in family trios of set 1.

SNP	Gene	TDT p value	Allele	Transmission Ratio
All family trios				
rs3092936	CD40LG	0.595	C	9:6
rs311071	CD99	0.569	T	41:36
rs312258	CD99	0.092	G	47:32
rs16997659	EIF2S3	0.819	C	10:9
rs12556742	EIF2S3	0.731	G	18:16
rs12847067	EIF2S3	0.739	G	19:17
rs3093457	IL9R	0.087	C	46:31
rs1973881	IL9R	0.887	G	25:24
rs4239963	UBA1	0.048	G	21:10
rs11558783	UBA1	0.513	G	12:9
rs4529579	UBA1	0.435	T	23:18
rs6520277	TIMP1	0.307	T	27:20
rs6520278	TIMP1	0.025	C	30:15
rs5953060	TIMP1	0.228	C	26:18
rs7878958	XIAP	0.758	C	22:20
rs7053190	XIAP	0.578	T	16:13
rs9856	XIAP	0.773	G	25:23
Family trios with male offspring				
rs3092936	CD40LG	1	—	0:0
rs311071	CD99	0.166	T	9:4
rs312258	CD99	0.206	G	7:3
rs16997659	EIF2S3	1	—	1:1
rs12556742	EIF2S3	0.564	G	2:1
rs12847067	EIF2S3	0.564	G	2:1
rs3093457	IL9R	0.011	C	9:1
rs1973881	IL9R	0.248	A	4:2
rs4239963	UBA1	0.564	G	2:1
rs11558783	UBA1	0.564	G	2:1
rs4529579	UBA1	0.655	C	3:2
rs6520277	TIMP1	0.014	T	6:0
rs6520278	TIMP1	0.008	C	7:0
rs5953060	TIMP1	0.103	C	5:1
rs7878958	XIAP	0.655	C	3:2
rs7053190	XIAP	0.655	T	3:2
rs9856	XIAP	1	—	2:2
Family trios with female offspring				
rs3092936	CD40LG	0.595	C	9:6
rs311071	CD99	1 > 0.1	—	32:32
rs312258	CD99	0.232	G	40:30
rs16997659	EIF2S3	0.808	C	9:8
rs12556742	EIF2S3	0.857	G	16:15
rs12847067	EIF2S3	0.862	G	17:16
rs3093457	IL9R	0.232	C	40:30
rs1973881	IL9R	0.647	G	23:20
rs4239963	UBA1	0.059	G	19:9
rs11558783	UBA1	0.637	G	10:8
rs4529579	UBA1	0.250	T	22:15
rs6520277	TIMP1	0.758	T	22:20
rs6520278	TIMP1	0.194	C	23:15
rs5953060	TIMP1	0.631	C	21:17
rs7878958	XIAP	0.746	C	20:18
rs7053190	XIAP	0.683	T	13:11
rs9856	XIAP	0.763	G	23:21

influence of IL-9, because they differ in IL-9-binding abilities[52]. Expression of IL-9 was shown to be correlated with inflammation events and infiltration of lymphocytes in allergic diseases[53]. The STAT pathway is the main signaling pathway of IL-9/IL-9R[54], and its role in RA is discussed[55]. *IL9R* is also involved in early T cell development[20], which is relevant for RA, as the balance between autoreactive T cells and regulatory T cells is essential for immune tolerance.

Table 7. Results of TDT analysis for selected SNP in family trios sets 1 and 2 combined.

SNP	Gene	TDT p value	Allele	Transmission Ratio
All family trios				
rs3093457	IL9R	0.191	C	83:67
rs4239963	UBA1	0.001	G	48:21
rs6520277	TIMP1	0.131	T	50:36
rs6520278	TIMP1	0.024	C	49:29
Family trios with male offspring				
rs3093457	IL9R	0.071	C	11:4
rs4239963	UBA1	0.157	G	6:2
rs6520277	TIMP1	0.020	T	8:1
rs6520278	TIMP1	0.020	C	8:1
Family trios with female offspring				
rs3093457	IL9R	0.391	C	73:63
rs4239963	UBA1	0.003	G	42:19
rs6520277	TIMP1	0.365	T	43:35
rs6520278	TIMP1	0.118	C	41:28

Table 8. Results of TDT analysis for selected SNP in family trios of sets 1, 2, and 3 combined.

SNP	Gene	TDT p value	Allele	Transmission Ratio
All family trios				
rs4239963	UBA1	0.149	G	106:86
rs6520278	TIMP1	0.035	C	109:80
Family trios with male offspring				
rs4239963	UBA1	0.853	C	15:14
rs6520278	TIMP1	0.513	T	12:9
Family trios with female offspring				
rs4239963	UBA1	0.056	G	91:67
rs6520278	TIMP1	0.023	C	96:67

Table 9. Results of interaction test for *IL9R* (rs3093457) in subgroups of the combined set 1 & 2 & 3.

	Male		Female
No. of cases	55		382
Minor allele GRR (95% CI)	3.75 (1.4–10.2)		1.26 (0.8–1.9)
Ratio of GRR (95% CI)		2.98 (1.01–8.79)	
p		0.048	
	a-CCP+		a-CCP–
No. of cases	209		72
Minor allele GRR (95% CI)	1.76 (1.0–3)		1.05 (0.3–3.1)
Ratio of GRR (95% CI)		0.6 (0.16–2.17)	
p		0.216	

GRR: genetic relative risk. Male and female subgroups: family trios with male or female patients; a-CCP+ and a-CCP– subgroups: anti-CCP-positive and negative subgroups, i.e., family trios with anti-CCP-positive or negative patients.

We provide evidence suggesting association of 2 X-chromosomal genes, *TIMP1* and *IL9R*, with RA. As in other studies of RA[39], the effects of the observed associations were modest. This might be a reason why only nominal significance was achieved. However, our multistage approach analyzing and combining multiple study cohorts allowed testing for such modest genetic effects[56]. It is necessary to verify the associations we observed in additional larger cohorts.

While our findings might not explain the female predominance in RA, they point out that different disease mechanisms might exist in females and males. To elucidate the genetic background of complex diseases such as RA it might be beneficial to consider sex-specific effects, e.g., using sex-stratified sample subsets for association studies.

Table 10. Comparison of SNP analyzed in our study with results of genome-wide studies. Minimum regional p value is the lowest significant p value of markers in a region ± 200 kb near a gene investigated in our study. Positions on chromosome X were according to dbSNP built 127. The following genome-wide data input was used: URR global data, NARAC/EIRA all available data, WTCCC RA cases versus CTL (58C, NBS = normal controls) for chromosomes 23 (= X) and 24 (pseudoautosomal X genes).

Gene	URR Minimum Regional p	NARAC/EIRA Minimum Regional p	WTCCC Minimum Regional p
CD40LG	0.095	0.035 (2)	NI
CD99	0.667	0.184	0.0004 (10)
EIF2S3	0.312	0.090	0.024 (2)
IL9R	NI	NI	NI
UBA1	0.044 (1*)	0.021 (2*)	0.111
TIMP1	0.0065 (3*)	0.058	0.0057 (8*)
XIAP	0.021 (3*)	0.160	0.0060 (3*)

* Number of regional SNP with significant p value; NI: no regional SNP investigated in whole-genome association studies; WTCCC: Wellcome Trust Case-Control Consortium; NARAC: North American Rheumatoid Arthritis Consortium; EIRA: Epidemiological Investigation of Rheumatoid Arthritis; URR: Upstream regulatory region.

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