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Lack of linkage and association of adrenomedulin and its receptor genes in French Caucasian rheumatoid arthritis trio families

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ABSTRACT

Objective. *Rheumatoid arthritis (RA) is characterized by hyperplasia of fibroblast-like synoviocytes (FLSs), in part due to apoptosis resistance. Adrenomedullin, an anti-apoptotic peptide, is secreted more by RA than osteoarthritis FLSs. Adrenomedullin binds to a heterodimeric functional receptor, of calcitonin receptor-like receptor (CRLR) coupled with a receptor activity-modifying protein-2 (RAMP-2), which is also overexpressed by rheumatoid synoviocytes. Since adrenomedullin decreases RA FLS apoptosis, possibly contributing to the development of pannus, study of adrenomedullin and its receptor genes might reveal a linkage and association in French Caucasian RA trio families.*

Methods. *Within each of 100 families, one RA-affected patient and both parents underwent genotyping for polymorphisms of adrenomedullin, CRLR and RAMP-2, by PCR-restricted fragment-length polymorphism (RFLP) or Taqman 5' allelic discrimination assay. Statistical analysis relied on the transmission disequilibrium test, the affected family-based controls and the genotype relative risk. Haplotypes of CRLR were inferred, and linkage and association studies were performed.*

Results. *No significant transmission disequilibrium or association between the three genes and RA was observed. CRLR haplotypes revealed two major haplotypes, but no significant linkage with RA.*

Conclusion. *Our findings provided no significant linkage or association of adrenomedullin and CRLR-RAMP-2 genes with RA in the studied trio families. The two CRLR polymorphisms rs3771076 and rs3771084 should be investigated in larger samples.*

Introduction

The pathogenesis of rheumatoid arthritis (RA) is multifactorial, involving both genetic (HLA-DRB1 and PTPN22 genes) and environmental factors. Fibroblast-like synoviocyte (FLS) hyperplasia plays an important role in RA, and its activation is characterized by signalling cascade alterations and apoptosis pathway changes. Resistance to apoptosis may be due to a defective Fas-induced apoptosis pathway (1), effects of regulatory or antiapoptotic peptides and local synovial expression of the mutant p53 gene (2).

Adrenomedullin (ADM), an anti-apoptotic peptide, is expressed and secreted more by RA FLSs than osteoarthritic FLSs. ADM binds to a receptor, the calcitonin receptor-like receptor (CRLR) coupled with the receptor activity-modifying protein-2 (RAMP-2), which can activate the protein kinase A pathway (3). ADM and its receptor genes are good RA candidates because their anti-apoptotic effect is involved in RA pathogenesis. ADM is a survival factor of antigen-activated T cells and may act as a proinflammatory or anti-inflammatory factor. ADM's beneficial properties in biological functions suggest that it could be a potential therapeutic target (4). Subcutaneous administration of the entire ADM peptide to mice reduced the incidence and severity of collagen-induced arthritis by modulating T-cell functions ex vivo and regulating cytokine and chemokine production (5). However, ADM is also a potential pathogenic peptide because of its angiogenic and anti-apoptotic properties (6), which might be relevant in the pathogenesis of pannus in RA.

ADM is highly conserved and its 3' end is flanked by a microsatellite marker whose 19-repeats allele has been associated with essential hypertension (7). A Chinese study suggested that an A-to-G substitution at position –1984 in the promoter region of *ADM* likely increases transcription (8).

The CRLR gene, located at chromosome region 2q31-q32, has been suggested to be RA-linked in a French genome-wide scan (9), confirmed in a genome-wide linkage meta-analysis for RA including the French population (10). However this locus was not associated with RA in a recent case-control genome-wide association study in the British population (11). In a Japanese study, CRLR polymorphisms were found not to be associated with essential hypertension (12).

Because *ADM* and its receptor genes are implicated in the pathogenesis of RA, we aimed to perform a linkage and association study of ADM and CRLR-RAMP-2 in RA.

Patients and methods

Patients and their families

We included 100 French Caucasian RA "trio" families (the patient and parents). RA diagnosis fulfilled the 1987 American College of Rheumatology criteria (13). All individuals provided informed written consent. The study was approved by the ethics committee of the Hospital Bicêtre (Kremlin-Bicêtre, Assistance Publique-Hôpitaux de Paris).

Eighty-seven percent of the RA patients were females, mean age of RA onset was 32±10 years and mean disease duration was 18±7 years. Erosions were present in 90% of patients, and 81% were positive for IgM rheumatoid factor; 78% carried at least one shared-epitope HLA-DRB1 allele.

Genotyping

Blood samples were collected for DNA extraction and genotyping by standard methods. Because *ADM* and *RAMP-2* genes contain less than 5,000 base pairs (bp), one polymorphism per gene was studied. For *CRLR*, four polymorphisms spanning the whole gene were genotyped. Four polymorphisms were genotyped by PCR-restriction fragment length polymorphism (RFLP). PCR amplification involved use of the Eppendorf Mastercycler. Annealing temperatures were 60°C for CRLR-rs10194247 (primers: forward 5'-TGATTCACCAAATCCAAACTGA-3'/reverse 5'-TTGCACAGGCACTCTTTCTTT-3') and CRLR-rs3771076 (primers: forward 5'-TCCGTGTGAAACCAGAAAGA-3'/reverse 5'-CTGAGAAGGTCTGCCTGCAT-3') and 63°C for CRLR-rs3771084 (primers : forward 5'-TGCATGTGTGGGATTACAGA-3'/reverse 5'-GGTTAAGATATTT-CATCAGTCTCCT-3') and ADM-rs4399321 (primers: forward 5'-TCCACAGTGCTAGCTGAGAAA-3'/reverse 5'-AATGCTGCAGTGGGAGTTCT-3'). Restriction enzymes were respectively BclI, BanI, BfaI and HaeIII. Genotyping of CRLR-rs10931284 and RAMP-2-rs1078523 polymorphisms involved use of Taqman 5' allelic discrimination assay (assays C26197317_10 and C2160077_10, respectively) by real-time PCR on ABI 7500. CEPH controls were co-genotyped with all samples for genotyping quality control.

Statistical analysis

Hardy-Weinberg equilibrium was checked in controls (constituted by the untransmitted parental chromosomes), by use of chi-square test with one degree of freedom. The linkage analysis relied on the transmission disequilibrium test (TDT), which compared, for a given allele, the transmission of that allele from heterozygous parents to RA patients, with the transmission expected from Mendel's first law (*i.e.*, 50%), with a conformity chi-square test with one degree of freedom. For the association analyses, we used the affected family-based controls (AFBAC) to compare frequency of transmitted and untransmitted alleles, and the genotype relative risk (GRR), which compared the affected offspring's genotype with that of a control genotype derived from untransmitted parental chromosomes. The odds ratio (OR) and 95% confidence interval (CI) were estimated. CRLR haplotypes were inferred by use of the algorithm implemented in GeneHunter ('haplo' option). The TDT and haplotype relative risks were determined as described above. Linkage disequilibrium was tested for each pair of CRLR polymorphisms. The haplotype frequencies were estimated by use of GeneHunter and linkage disequilibrium by fbat software. A $p<0.05$ was considered significant.

Results

All controls showed Hardy-Weinberg equilibrium for all polymorphisms. TDT results remained not statistically significant, except for a trend of over-transmission (57%, $p=0.2$) for two CRLR polymorphisms (T allele of rs3771076 and rs3771084). AFBAC analysis showed a high frequency of transmitted T alleles for the same two polymorphisms (Table I).

The GRR analysis revealed a higher number of TT genotypes in patients than in controls (22 *vs.* 12, $p=0.2$) for CRLR-rs3771084, but it was probably not an effect of the T allele because this difference disappeared when the CT and TT genotypes were pooled. The four CRLR polymorphisms allowed us to generate 15 haplotypes, most with a frequency below 5%. None of the four polymorphisms showed a linkage disequilibrium with any other. We observed two major haplotypes in patients and controls, with a frequency of 49% for A-A-C-G and 21.2% for G-T-T-A (Table II).

The linkage analysis revealed an over-transmission of 60% of the T-T haplotype made up of the T alleles of CRLR-rs3771076 and CRLR-rs3771084 ($p=0.09$). Haplotypic TDT with the four polymorphisms and association analyses provided no significant results.

Discussion

We performed a linkage and association study of three candidate genes, ADM, CRLR and RAMP-2 with RA. Although the CRLR locus was suggested to be linked to RA (9) and was a good RA candidate gene by its function, we found no significant transmission disequilibrium or association. However, we did find a trend to

Table I. Results of the transmission disequilibrium test (TDT) and affected family-based controls (AFBAC).

Polymorphisms (allele)	TDT		AFBAC			
	T	P	Frequency of transmitted alleles	Frequency of untransmitted alleles	OR	95% CI
ADM-rs4399321 (A)	54%	0.4	0.73	0.69	1.2	0.8-1.8
CRLR-rs10194247 (G)	51%	0.8	0.28	0.26	0.9	0.6-1.4
CRLR-rs3771076 (T)	57%	0.2	0.42	0.36	0.8	0.5-1.2
CRLR-rs3771084 (T)	57%	0.2	0.39	0.33	0.8	0.5-1.2
CRLR-rs10931284 (G)	54%	0.5	0.38	0.35	1.2	0.8-1.7
RAMP-2-rs1078523 (G)	51%	0.8	0.47	0.45	1.1	0.7-1.6

T: percentage of transmission; OR: odds ratio; 95% CI: 95% confidence interval.

Table II. Frequencies of the haplotypes of *CRLR* constituted by the four studied polymorphisms.

Haplotype number	Details of the haplotype	Patients, n (%)	Controls, n (%)
1	A-T-C-A	4 (2.2)	3 (1.6)
2	A-T-T-A	12 (6.5)	9 (4.9)
3	A-A-C-A	90 (49.0)	94 (51.1)
4	G-T-T-G	39 (21.2)	32 (17.4)
5	A-A-T-G	3 (1.6)	1 (0.5)
6	A-T-T-G	10 (5.4)	10 (5.4)
7	A-T-C-G	2 (1.1)	6 (3.3)
8	G-T-T-A	3 (1.6)	2 (1.1)
9	G-T-C-G	5 (2.7)	4 (2.2)
10	A-A-C-G	9 (4.9)	6 (3.3)
11	G-T-C-A	1 (0.5)	1 (0.5)
12	A-A-T-A	4 (2.2)	7 (3.8)
13	G-A-C-A	1 (0.5)	4 (2.2)
14	G-A-C-G	0 (0.0)	4 (2.2)
15	G-A-T-G	1 (0.5)	1 (0.5)

over transmission for T alleles of two CRLR polymorphisms and also when combined in a haplotype. Analysis of CRLR haplotypes revealed two major haplotypes, but no significant linkage or association with RA.

To our knowledge, this is the first linkage or association study of the three genes with RA. A negative association for ADM-rs4399321 with essential hypertension was reported from Japan (14).

Here, we genotyped French Caucasian RA "trio" families, which gave information on linkage as well as association, without control population stratification bias. However, this approach was limited due to the low number of trio studied – 100. Indeed, our study might have lacked power, because of no information on the power calculation of this size of sample for the polymorphisms studied. We estimated *a posteriori* that for the CRLR-rs3771076 polymorphism which has a transmission disequilibrium of 57% with 45% of heterozygous parents, 740 trio families may be needed to observe a significant transmission disequilibrium with a power >80%.

Index cases have a young age at RA onset, since both parents are needed in this trio design, and a long disease duration, whereas RA is usually a later onset disorder. Thus, the index cases have some clinical characteristics that do not allow us to extrapolate our results to every RA patient. The polymorphisms were selected from a public database on the basis of their position and minor allele frequency to obtain data for enough heterozygous parents for TDT. As well, the polymorphisms have no known functional effect, although the analysis of the ADM promoter region revealed that at least two transcription factors participate in the regulation of ADM expression (15), and the 5' flanking region contains TATA, CAAT and GC boxes and a cAMP-regulated enhancer element.

The two CRLR polymorphisms, rs3771076 and rs3771084, should be investigated in larger samples.

Conclusion

In conclusion, our findings provide no evidence of linkage or association of the ADM-CRLR-RAMP-2 pathway with RA in this first sample.

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