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Association and Expression Study of *PRKCH* Gene in a French Caucasian Population with Rheumatoid Arthritis

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Abstract We study the association between three protein kinase C, eta gene polymorphisms (+8134C/T, rs912620, rs959728), and susceptibility to rheumatoid arthritis. One hundred French Caucasian rheumatoid arthritis trio families were genotyped. Relative quantification of protein kinase C, eta mRNA expression was performed from whole blood in 24 unrelated rheumatoid arthritis patients and in 16 healthy controls. Our results showed no significant association or linkage between the protein kinase C, eta polymorphisms, and rheumatoid arthritis. The protein kinase C, eta mRNA was expressed at lower level in rheumatoid arthritis unrelated patients than in healthy controls. This study shows that protein kinase C, eta gene is not a Rheumatoid Arthritis major susceptibility genetic factor in the French Caucasian population. Furthermore, the lower expression of this gene in rheumatoid arthritis patients comparing to healthy controls suggests that protein kinase C, eta could be associated with the patho-physiologic mechanism of rheumatoid arthritis.

Keywords *PRKCH* gene · rheumatoid arthritis · association study · gene expression · single nucleotide polymorphism

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Introduction

Rheumatoid Arthritis (RA) is a chronic inflammatory disease characterized by persistent joint synovial tissue inflammation associated with the destruction of affected joints [1]. During the RA development, autoreactive T cells are activated and recruited to joints mediating the synovial inflammation and finally causing tissue damage and cartilage and bone invasion [2]. Previous studies have shown that many autoreactive T cells, expressing high levels of CD45RO, are present in synovial fluid of RA patients [3] demonstrating that these autoreactive T cells are activated and have adopted an effector/memory phenotype [4]. Immunosuppressive drugs or anti-cell antibodies were used to eliminate or inhibit T cells causing an amelioration of the disease [5]. Hence, it is apparent that autoreactive T cells play important roles in synovial tissue inflammation in RA patients. The biologic mechanism of these autoreactive T cells in RA remains poorly studied.

Protein kinase C, eta (*PRKCH*) gene, which encodes the η isozyme of protein kinase C (PKCη), is a good functional candidate for susceptibility to RA because protein kinase C (PKC) plays an important role in signal transduction controlling T-cell activation. The PKC gene family consists of more than 11 members, including *PRKCH*/PKCη, and their individual products have been revealed to be involved in different cellular biological functions in various cell types [6]. Recent papers suggest that some isozymes of PKC are involved in critical functions of T cells [7, 8].

A previous study reported a significant association ($P< 0.05$) for a landmark SNP (rs767755), located in intron 2 of the *PRKCH* gene, with RA in a case-control Japanese population. Subsequent analysis of additional single nucleotide polymorphisms (SNPs) within this gene revealed multiple SNPs located in three distinct linkage disequilibrium blocks to be significantly associated with RA. In each linkage disequilibrium block the most significant associated SNP was reported (+8134C/T, rs912620, and rs959728). Furthermore, they have shown that *PRKCH* gene was expressed at high levels in resting T cells and this expression was downregulated by immune responses suggesting that PKCη is involved in signaling pathways to T cells [9].

In this study, we tested *PRKCH* +8134C/T, rs912620, and rs959728 SNPs for RA association and linkage in 100 French Caucasian trios. Moreover, the level of expression of *PRKCH* mRNA in 24 French Caucasian unrelated RA patients and in 16 French Caucasian healthy controls and the association between haplotypes of the SNPs tested and the level of expression in 24 unrelated RA patients were studied.

Methods

Patients and Healthy Controls

The study was approved by the Ethics Committees of Hôpital Bicêtre and Hôpital Saint Louis (Paris, France) and all subjects provided informed consent. One hundred RA trio families (one patient and both healthy parents) with the four grandparents of French Caucasian origin were recruited through a national media campaign. Characteristics of the RA trio families sample are reported in Table I. Among the 24 French Caucasian unrelated RA patients, 18 were women (mean±SD age at enrolment 53.6±12.1). Depending on the swollen/tender joint count at the time of the study, there were 17 severe RA patients (minimum five inflamed joints) and seven mild RA patients (less than five active joints). All RA patients have received disease-modifying anti-rheumatoid drugs (DMARDs) and corticosteroid therapy before the inclusion in the study. All RA patients satisfied the revised criteria of the American College of Rheumatology [10] according to the rheumatologist in charge of the patient. A rheumatologist university fellow reviewed all clinical data. Between the 16 French Caucasian healthy controls, 11 were women (mean±SD age at enrolment 47.8±7.5).

Molecular Genotyping Method

Genomic DNA of the 100 French Caucasian RA trio families and the 24 French Caucasian unrelated RA patients was isolated and purified from fresh peripheral blood leukocytes according to standard protocols. Genotyping of the *PRKCH* +8134C/T polymorphism was performed by the polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (PCR-RFLP) method [11]. The designed primers were: sense 5′ATGTGGTTTAT TATAGTATTCTACTTT3′ and anti-sense 5′ TACTCACC TGTTCCTACCTGCA 3′. Primers were tested using the BLAST algorithm to ensure that only the *PRKCH* gene was amplified. PCR amplification was performed on each sample in a 25-μl reaction volume consisting of 10× PCR buffer (Perkin-Elmer, Boston, MA, USA), 1.25 mM of each dNTP, 1.25 U of Taq Gold DNA polymerase (Perkin-Elmer), 3 mM MgCl2, 0.0125 nM of the two primers (Invitrogen, Cergy Pontoise, France) and 50 ng of genomic DNA, diluted to the final volume with H_2O on Eppendorf thermocycler. The PCR program was carried out using the following amplification protocol: 37 cycles of denaturation at 96°C for 30 s, with annealing temperature at 59°C for 30 s followed by an elongation step at 72°C for 1 min. One final cycle of the extension was performed at 72°C for 10 min. A 360-bp amplified fragment was digested with *NlaIII* (Ozyme, Montigny Le Bretonneux, France) generat-

Table I Characteristics of Rheumatoid Arthritis (RA) index cases from the investigated sample

	RA patients (n=100)
Females (%)	87
Mean age (± standard deviation, SD) at disease onset (years)	32 (±10)
Mean (± SD) disease duration (years)	18 (±7)
RA patients with bone erosions (%)	90
RA patients seropositive for rheumatoid factor (%)	81
RA patients seropositive for anti-cycle citrullinated peptides antibodies (%)	78
RA patients carrying at least one *HLA-DRB1*shared epitope allele (%)*	78

**DRB1*0101*, *DRB1*0102*, *DRB1*0401*, *DRB1*0404*, *DRB1*0405*, *DRB1*0408*, *DRB1*1001*.

ing two fragments (187 and 139 bp) when the restriction site located at the SNP locus was present (C allele). Two independent investigators assessed genotypes blindly. Genotyping of the *PRKCH* rs912620 and rs959728 SNPs was carried out with a Taqman 5′ allelic discrimination assay on an ABI 7500 real-time PCR machine (assays: C__7600119_10, C__7600090_10, respectively; Applied Biosystems, Foster City, CA, USA). Allele-specific probes were labeled with the fluorescent dyes VIC and FAM. PCR reaction was carried out in a total volume of 15 μl with the following amplification protocol: denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 92°C for 15 s, annealing and extension at 60°C for 1 min. Genotyping of each sample was automatically attributed using the sodium dodecyl sulfate (SDS) software for allelic discrimination. Ten percent of the samples chosen at random were genotyped again for quality control.

PRKCH mRNA Expression by Real-Time Quantitative Reverse Transcription-PCR

Total RNA of the 24 French Caucasian unrelated RA patients and 16 French Caucasian healthy controls from whole blood was extracted using a PAXgene Blood RNA kit (Qiagen, Hilden, Germany). The measure of the RNAs concentration was performed using the RNA RiboGreen dye (Invitrogen). The integrity of the RNAs was analyzed using the Agilent 2100 Bionalyzer (Agilent, Santa Clara, CA, USA). Reverse-transcription was performed in a total volume of 20 μl with SuperScript™ III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen) using 1 μg of each total RNA and random hexamers according to the manufacturer's protocol. The kit includes RNaseOUT™ recombinant ribonuclease inhibitor as an RNase protector. Real-time quantitative reverse transcriptase PCR (RT-PCR) analysis was executed on an ABI Prism 7500 machine, using TaqMan Gene Expression Assays probes (Applied Biosystems) for *PRKCH* (Hs00178933_m1) and TaqMan Endogenous Controls probes (Applied Biosystems) for *ACTB* (β-actin), *GAPDH* (Glyceraldehyde-3-phosphate dehydrogenase) and *B2M* (β-2-microglobulin). Each sample was tested in duplicate and a sample without template was included in each run as a negative control. The expression level of the *PRKCH* transcript was quantified using the threshold cycle (Ct) method and normalized to the amount of *ACTB*, *GAPDH*, and *B2M*. Samples showing Ct values>35 and duplicates with a Ct>0.3 were retested.

Haplotype Assignment

The *PRKCH* gene haplotypes obtained taking into account the unphased SNP genotype data of the 24 unrelated RA patients was performed using fastPHASE 1.2 software [12].

Statistical Analysis

After the hypothesis of an RA association profile of *PRKCH* in a French Caucasian population similar to that observed in Japanese RA patients, we used the reported allelic frequencies of the *PRKCH* +8134C/T SNP in Japanese RA patients (17%) and in controls (22.2%) [9]. Using the binomial distribution, we had 88.5% power to detect a trend in favor of an association: probability of having the frequency in patients superior to that in controls following the binomial distribution for n observations (0–100 in our trio index) [13].

The Hardy–Weinberg equilibrium was checked in the control group (constituted by the nontransmitted parental chromosomes from trio) before analysis.

The linkage and association analysis relied on the Transmission disequilibrium test (TDT) [14], which compares, for a given allele, its transmission from heterozygous parents to RA patients, with the transmission expected from Mendel's law (i.e., 50%). Secondly, we used the genotypes relative risk (GRR), which compares the affected offspring's genotype with the control genotype derived from nontransmitted parental chromosomes, using the method proposed by Lathrop [15]. Statistical significance was considered for $P<0.05$.

Results of relative mRNA expression are presented as the mean±standard deviation percentage. Statistical analysis of the relative expression of the *PRKCH* in RA patients and healthy controls was performed using the Mann–Whitney test and $P<0.05$ was considered significant.

Table II Transmission Disequilibrium Test (TDT) for *PRKCH* +8134C/T, rs912620, rs959728 SNPs, and RA (100 Trio families)

PRKCH SNPs	Allele	Transmitted	Nontransmitted	*T* (%)	*P* value
+8134C/*T*	*C*	40	40	50	1
rs912620 (*G*>*T*)	*G*	42	46	47.7	0.67
rs959728 (*C*>*T*)	*C*	17	22	43.6	0.42

T=Percentage of transmission from heterozygous parents

Table III Genotype Relative Risk (GRR) for *PRKCH* +8134C/T, rs912620, rs959728 SNPs, and RA (100 Trio families)

PRKCH SNPs	Genotypes	Lathrop *P* value (one genotype vs the others)	Global *P* value
+8134*C*/*T*	CC	0.63	1
	CT		
	TT	0.44	
rs912620 (*G*>*T*)	GG	0.46	0.90
	GT		
	TT	0.89	
rs959728 (*C*>*T*)	CC	0.28	0.61
	CT		
	TT	0.58	

The association between haplotypes of the SNPs tested and the level of expression in 24 unrelated RA patients was assessed by the Mann–Whitney test. Data are expressed as the mean±standard deviation and $P<0.05$ was considered significant.

Results

Hardy–Weinberg Equilibrium Check

The *PRKCH* +8134C/T, rs912620 and rs959728 SNPs were in Hardy–Weinberg equilibrium in the control sample investigated.

Test for Linkage and Association in the Trio RA Families

We test the *PRKCH* putative susceptibility alleles +8134C, rs912620-T and rs959728-T for which association with RA was the most significant in the case-control Japanese population [9].

We did not observe a linkage and association between the +8134C allele and RA: there was an equal transmission of +8134C allele and +8134T allele from heterozygous parents (40 transmitted *vs.* 40 nontransmitted, $P=1$) (Table II). There was no significant overtransmission of the rs912620-T allele from heterozygous parents (46 transmitted *vs.* 42 nontransmitted, $P=0.67$) (Table II). The same result is observed for the rs959728-C allele (22 transmitted *vs.* 17 nontransmitted, $P=0.42$) (Table II). The GRR analysis of the *PRKCH* +8134C/T, rs912620, and rs959728 SNPs showed no significant ($P>0.05$) association of the homozygous or heterozygous genotypes for susceptibility alleles with RA (Table III). The TDT analysis of *PRKCH* +8134C/T-rs912620-rs959728 haplotypes did not reveal any significant association for the seven haplotypes estimated (data not shown).

Expression Analysis

A relative quantification of *PRKCH* mRNA expression was performed in total RNA from whole blood in 24 unrelated RA patients and in 16 healthy controls. *PRKCH* was expressed in RA patients at lower level (−92%; $P<0.0001$) than in healthy controls (Fig. 1). Figure 2 reported the relative quantification of *PRKCH* mRNA in severe and

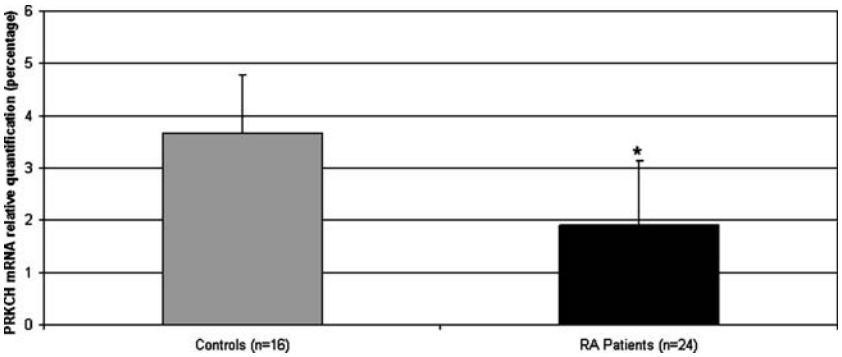

Fig. 1 Levels of *PRKCH* mRNA expression in peripheral blood from RA unrelated patients and healthy controls. *PRKCH* mRNA levels were determined by real-time quantitative RT-PCR and normalized to *ACTB*, *GAPHD*, and *B2M* levels. The expression level of the *PRKCH* transcript was quantified using the threshold cycle (Ct) method. Data are presented as the mean±SD percentage of the *PRKCH* mRNA expression. *$P<0.0001$ versus Controls, by Mann–Whitney test.

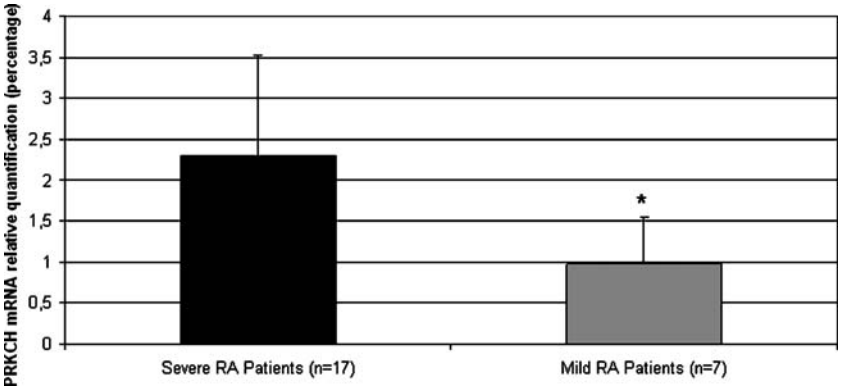

Fig. 2 Levels of *PRKCH* mRNA expression in peripheral blood from severe and mild RA patients. *PRKCH* mRNA levels were determined by real-time quantitative RT-PCR and normalized to *ACTB*, *GAPHD*, and *B2M* levels. The expression level of the *PRKCH* transcript was quantified using the threshold cycle (Ct) method. Data are presented as the mean±SD percentage of the *PRKCH* mRNA expression. **P*=0.008 versus severe RA patients, by Mann–Whitney test.

mild RA patients. The expression of *PRKCH* in severe RA patients was higher than in mild RA patients (*P*=0.008).

We selected three haplotype combinations: CTT/CTT (haplotype homozygous for the susceptible alleles), CTT/X (haplotype heterozygous for the susceptible alleles), and X/X (haplotype with no susceptible alleles). In the set of 24 unrelated RA patients, there were four RA patients with CTT/X haplotype, 20 with X/X and no one with CTT/CTT. Although the expression of *PRKCH* mRNA was lower in RA patients with CTT/X haplotype compared to X/X haplotype (Fig. 3), we did not observe a significant association (*P*=0.94).

Discussion

This study was designed to test the *PRKCH* +8134C/T, rs912620 and rs959728 SNPs for linkage to, and association with, RA in a French Caucasian familial population. In addition, we studied the level of expression of *PRKCH* mRNA in RA patients and in healthy controls and the association between haplotypes of the SNPs tested and the level of expression in RA patients.

Takata et al. [9] described the +8134C, rs912620-T, and rs959728-T alleles from SNPs located in three different linkage disequilibrium blocks susceptible for RA. We have confirmed that these susceptibility alleles were not in linkage disequilibrium in the French Caucasian population (data not shown). In the linkage disequilibrium block D described by Takata et al. [9], we have chosen to test the rs959728 SNP, which is more associated to RA than rs2230500 SNP (*P*=0.00011 vs *P*=0.0016). This functional SNP rs2230500 located in exon 9 (V3741I) was reported to increase the risk of cerebral infarction [16]. Our results showed a clear absence of RA linkage and association in the population investigated for the three SNPs tested. In good agreement with the TDT analysis, the GRR revealed a lack of association between the different genotypes and RA.

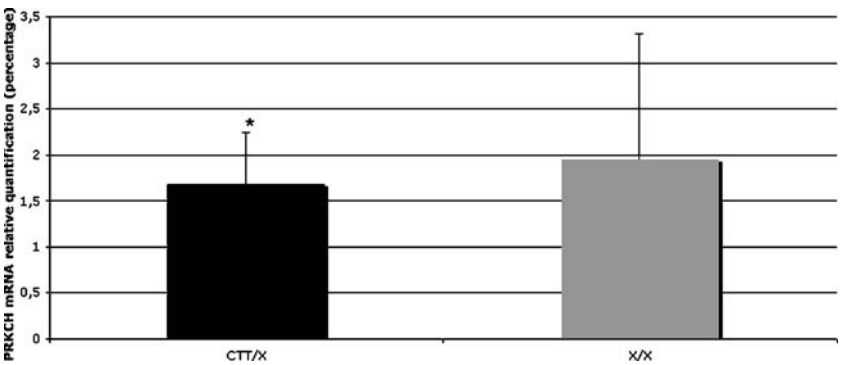

Fig. 3 Association between the level of *PRKCH* mRNA expression and *PRKCH* haplotype (+8134C/T-rs912620-rs95972824) in RA patients. *PRKCH* mRNA levels were determined by real-time quantitative RT-PCR and normalized to *ACTB*, *GAPHD*, and *B2M* levels. The expression level of the *PRKCH* transcript was quantified using the threshold cycle (Ct) method. Four samples in the CTT/X haplotypes and 20 in the X/X haplotypes were analyzed. Data are presented as the mean±SD percentage of the *PRKCH* mRNA expression. **P*=0.94 versus X/X haplotypes, by Mann–Whitney test.

The linkage analysis of *PRKCH* +8134C/T–rs912620–rs959728 haplotypes was not significant as none of the seven haplotypes described were overtransmitted (data not shown). The results were obtained with a particularly robust method, the Transmission Disequilibrium Test [14], which compares, for a given allele, its transmission from heterozygous parents to RA patients, with the transmission expected from Mendel's law (i.e., 50%). This family-based analysis avoids imperfect population match between patients and controls and permits the direct test of the Mendel's law. These results allowed us to exclude *PRKCH* as a major significant genetic factor in RA in a French Caucasian population. There were several genetic studies that reported differences in ethnic variations of polymorphisms associated with RA as *PADI4* and *SLC22A4* genes [17–19]. Moreover, *PRKCH* locus was not included among the suggested 19 non-HLA regions in the French Caucasian population [20]. The last genome-wide association study in a UK Caucasian population had found 9 SNPs that map to loci not associated previously to RA [21]. However, *PRKCH* locus was not present in these regions. Thus, other populations need to be studied to define extensively the involvement of this gene in the genetics of RA.

Even some PKC isozymes have been suggested to be involved in important functions of T cells [7, 8] the physiological function of PKCη in T cells has not yet been well documented and the pathway by which *PRKCH* SNPs may influence RA risk remains unknown.

This study is the first to show that *PRKCH* was expressed in RA patients at a lower level than in non-RA controls in peripheral blood cells. This result suggests that expression of the *PRKCH* gene is regulated through immune responses. This observation confirms the study of Takata et al. [9] who have shown that *PRKCH* gene is expressed in resting CD4+ cells (T helper/inducer cells) or CD8+ cells (T suppressor/cytotoxic cells) at higher levels than in resting CD19+ cells (B cells) or CD14+ cells (monocytes) and the expression in these cells were significantly downregulated by activation.

Our data also show the *PRKCH* mRNA highly expressed in severe RA patients comparing to mild RA patients, supporting the increase of expression of *PRKCH* during the progression of the disease from mild to severe RA. PKCη functions have been associated to the cytokine signaling cascade in monocytes and macrophages. Indeed, plasma levels of nitric oxide, a mediator of inflammation, were shown to be elevated in patients with severe RA, and a positive association between *PRKCH* and inducible nitric oxide synthase (*iNOS*) expression was observed in peripheral blood monocyte-derived macrophages from severe RA patients. Furthermore, this coexpression was not present in healthy controls [22].

Heale et al. [23] have confirmed these results reporting a PKCη-expression phenotype in monocytes for mild and severe RA patients and not for healthy controls. Moreover, there was a progressive and concordant expression of PKCη and iNOS phenotypes in monocytes from RA patients associated with the severity of the disease.

These studies provided evidence supporting the possible involvement of PKCη in immunologic activities of T cells, monocytes and macrophages, whereas our study highlighted an overall expression of *PRKCH* gene in whole blood from RA patients. The association of *PRKCH* differential expression and the patho-physiologic mechanism in RA should be then further investigated.

The relation between haplotypes and expression level of *PRKCH* gene was not identified, as the expression of *PRKCH* mRNA was lower in RA patients with CTT/X haplotype comparing to X/X haplotype but without significance. Nevertheless, the determination of haplotype effects should require an extensive examination of expression in different cell subsets and RA states.

Conclusions

We provided evidence against the involvement of the *PRKCH* gene in the genetics of RA in a French Caucasian familial population. However, replication studies in other independent Caucasian populations are required to confirm these results. Our study is the first to show that *PRKCH* was expressed in RA patients at lower level than in non-RA controls in peripheral blood cells. Further investigations in the regulation of *PRKCH* expression in RA are necessary to prove the involvement of PKCη molecular mechanisms in disease susceptibility, more specifically in signaling pathways of RA-specific immune response.

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