CHAPTER 4

PADI4 polymorphism predisposes male smokers to rheumatoid arthritis

Y. Kochi
M.M. Thabet
A. Suzuki
Y. Okada
N.A. Daha
R.E.M. Toes
T.W.J. Huizinga
K. Myouzen
M. Kubo
R. Yamada
Y. Nakamura
K. Yamamoto

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ABSTRACT

Objective: To elucidate the differential role of peptidyl arginine deiminase 4 (PADI4) polymorphism in rheumatoid arthritis (RA) between Asian and European populations, possible gene-environmental interactions among the PADI4 polymorphism, sex and smoking status were analysed.

Methods: Three independent sets of case–control samples were genotyped for single nucleotide polymorphisms in PADI4; Japanese samples (first set, 1019 RA patients, 907 controls; second set, 999 RA patients, 1128 controls) using TaqMan assays and Dutch samples (635 RA patients, 391 controls) using Sequenom MassARRAY platform. The association of PADI4 with RA susceptibility was evaluated by smoking status and sex in contingency tables and logistic regression models.

Results: In the first set of Japanese samples, PADI4 polymorphism (rs1748033) showed a greater risk in men (OR_{allele} 1.39; 95% CI 1.10 to 1.76; p_{trend}=0.0054) than in women and in ever-smokers (OR_{allele} 1.25; 95% CI 1.02 to 1.53; p_{trend}=0.032) than in never-smokers. Moreover, the highest risk was seen in male ever-smokers (OR_{allele} 1.46; 95% CI 1.12 to 1.90; p_{trend}=0.0047). Similar trends were observed in the second set of Japanese samples as well as in Dutch samples.

Conclusion: PADI4 polymorphism highly predisposes male smokers to RA, and the genetic heterogeneity observed between Asian and European populations may be partly explained by differences in smoking prevalence among men.

INTRODUCTION

Rheumatoid arthritis (RA) is a multigenic disease caused by interactions between genetic predispositions and environmental factors that result in abnormal immune response and joint destruction. The HLA-DRB1 region is considered to be the major genetic determinant of RA susceptibility, but recent genetic studies have revealed multiple non-human leucocyte antigen susceptibility genes for RA [1]. Among these, the peptidyl arginine deiminase 4 (PADI4) gene, which encodes a post-translational modification enzyme that converts arginine to citrulline residues in proteins, is thought to have significant relevance in RA pathogenesis as anti-citrullinated protein antibodies (ACPA) are specifically observed in the sera of patients [2, 3].

The association of the PADI4 polymorphism with RA susceptibility was first reported in a Japanese population [2] and has been replicated in several Asian populations [4, 5]. Conversely, inconsistent results have been observed in populations of European ancestry [6-8]. A meta-analysis confirmed the association in Asian populations, but not in European populations [6-7]. The genetic heterogeneity observed between different populations could be partly explained by the difference of disease severity between the study populations, as the PADI4 polymorphism was reported to influence erosive joint status [9]. However, it could also be explained by unknown gene–gene or gene–environmental interactions with PADI4, and the higher magnitude of risk with PADI4 in Asian populations suggests the presence of these interacting factors.

Smoking is one of the well-established environmental factors in RA [10], and several studies have described associations with the appearance of ACPA in RA patients [3]. Klareskog *et al* [11] first reported that citrullinated proteins were detected in bronchoalveolar lavage cells from smokers but not in those from non-smokers. A later study by Makrygiannakis *et al* [12] showed that a significantly increased PADI2 expression and a higher trend of PADI4 expression were observed in bronchoalveolar lavage cells from smokers compared with non-smokers. These lines of evidence suggest that the up-regulated expression of PADI enzymes provoked by smoking may promote the citrullination of proteins in the lung, leading to citrulline autoimmunity in RA [3].

The present study examined possible interactions between PADI4 polymorphism, sex and smoking status, and discusses the resulting influence on the genetic heterogeneity in PADI4 observed between Asian and European populations.

METHODS

Subjects

Japanese RA patients (first set n=1019, second set n=999) were provided by the Leading Project for Personalized Medicine in the Ministry of Education, Culture, Sports, Science and Technology, Japan (BioBank Japan) [13]. Unrelated Japanese controls (first set n=907, second set n=1128) were recruited through Midousuji Rotary club and several medical institutes in Japan. These Japanese case–control sets were independent from that used in the previous study [2]. Dutch cohorts and RA patients were previously described [14]. RA patients (n=635) were part of the Leiden Early Arthritis Clinic, which comprises an inception cohort of patients with recent-onset arthritis (duration of symptoms <2 years). Those patients were diagnosed with RA within the first year after their initial visit. All individuals with RA met the 1987 revised criteria of the American College of Rheumatology for RA [10]. The characteristics of the cohorts are described in detail in supplementary **table 1** (available online only). All subjects entered into this study provided informed consent prior to participation in the study, and all study protocols were preapproved by the ethics committees of each institute.

Smoking status

Smoking status was determined for each individual on the basis of self-reported information. An ever-smoker was defined as a person who had smoked tobacco, cigarettes or pipes at any stage in their life, whereas a never-smoker was defined as someone who had never smoked any of these. Smoking status was available for all the samples in the first Japanese case–control set, cases in the second Japanese set and a part of the Dutch RA patients (52.9%), but not for the control subjects in the second Japanese and Dutch sets.

SNP genotyping

The four exonic single-nucleotide polymorphisms (SNP) comprising two major transcripts of PADI4 (rs11203366=padi4_89, rs11203367=padi4_90, rs874881=padi4_92 and

Table 1 Association of the PADI4polymorphism and RA stratified with sex and smoking status in a Japanese population*

		Sum		MAF			p Value for
	Set	Case	Contol	Case	Contol	Per allele OR	trend test
rs11203367							
All	1st	1019	907	0.43	0.40	1.14 (1.00 to 1.29)	0.045
	2nd	996	1124	0.42	0.40	1.09 (0.96 to 1.23)	0.16
Men	1st	190	672	0.48	0.39	1.44 (1.14 to 1.81)	0.0022
	2nd	185	448	0.44	0.40	1.18 (0.92 to 1.51)	0.19
Women	1st	829	235	0.42	0.41	1.02 (0.82 to 1.25)	0.84
	2nd	811	676	0.41	0.39	1.07 (0.92 to 1.24)	0.31
Ever-smoker	1st	337	488	0.47	0.39	1.35 (1.11 to 1.65)	0.0024
	2nd	302	1124	0.43	0.40	1.15 (0.95 to 1.38)	0.12
Never-smoker	1st	682	418	0.41	0.40	1.03 (0.86 to 1.23)	0.71
	2nd	694	1124	0.41	0.40	1.06 (0.92 to 1.21)	0.36
Male ever-smoker	1st	155	451	0.50	0.39	1.61 (1.24 to 2.09)	0.00031
	2nd	145	448	0.46	0.40	1.25 (0.96 to 1.63)	0.10
Male never-smoker	1st	35	221	0.39	0.40	0.92 (0.54 to 1.54)	0.77
	2nd	40	448	0.39	0.40	0.95 (0.59 to 1.52)	0.84
Female ever-smoker	1st	182	37	0.44	0.47	0.86 (0.52 to 1.42)	0.56
	2nd	157	676	0.41	0.39	1.05 (0.82 to 1.35)	0.64
Female never-smoker	1st	647	197	0.41	0.40	1.04 (0.83 to 1.32)	0.68
	2nd	654	676	0.41	0.39	1.08 (0.92 to 1.26)	0.31
rs1748033							
All	1st	1018	904	0.37	0.35	1.12 (0.98 to 1.27)	0.089
	2nd	996	1125	0.36	0.34	1.08 (0.95 to 1.22)	0.20
Men	1st	190	669	0.42	0.34	1.39 (1.10 to 1.76)	0.0054
	2nd	185	448	0.40	0.34	1.25 (0.97 to 1.60)	0.08
Women	1st	828	235	0.36	0.36	1.00 (0.81 to 1.24)	0.96
	2nd	811	677	0.35	0.34	1.05 (0.90 to 1.22)	0.50
Ever-smoker	1st	336	485	0.40	0.35	1.25 (1.02 to 1.53)	0.032
	2nd	302	1125	0.38	0.34	1.19 (0.99 to 1.43)	0.055
Never-smoker	1st	682	418	0.36	0.34	1.07 (0.89 to 1.28)	0.47
	2nd	694	1125	0.35	0.34	1.03 (0.90 to 1.19)	0.59
Male ever-smoker	1st	155	448	0.44	0.34	1.46 (1.12 to 1.90)	0.0047
	2nd	145	448	0.41	0.34	1.34 (1.02 to 1.75)	0.039
Male never-smoker	1st	35	221	0.36	0.34	1.09 (0.64 to 1.85)	0.75
	2nd	40	448	0.34	0.34	0.96 (0.59 to 1.56)	0.90
Female ever-smoker	1st	181	37	0.37	0.41	0.87 (0.52 to 1.45)	0.60
	2nd	157	677	0.36	0.34	1.06 (0.82 to 1.37)	0.60
Female never-smoker	1st	647	197	0.36	0.35	1.03 (0.81 to 1.30)	0.79
	2nd	654	677	0.35	0.34	1.04 (0.89 to 1.22)	0.55

* rs112033673 (T/C, T is the minor allele) and rs174803 (T/C, T is the minor allele) were genotyped for the test. Both case and control subjects were stratified with smoking status in the first set, whereas only case subjects were stratified with smoking status in the second set.
MAF, minor allele frequency; PADI4, peptidyl arginine deiminase 4; RA, rheumatoid arthritis.

rs1748033=padi4_104) were genotyped [2]. Two of these SNP (rs11203367 and rs1748033) tag the three haplotypes (two common haplotypes and one rare haplotype, see supplementary **table 2**, available online only) and provide full information for PADI4. These were also tested in the Dutch population. In the Japanese population SNP were genotyped using predesigned TaqMan SNP genotyping assays (Applied Biosystems, Carlsbad, California, USA).

Fluorescence was detected using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). In the Dutch population SNP were genotyped using time-of-flight mass spectrometry-based Sequenom MassARRAY Platform (Sequenom, San Diego, California, USA). Genotyping assessment was made for over 95% of samples, for all of the polymorphisms genotyped. All SNP were in Hardy–Weinberg equilibrium in control subjects according to $\chi 2$ statistics ($p>0.01$).

Statistical analysis

The case–control association of each SNP was tested with the Cochran Armitage trend test and the $\chi 2$ test. Genotype and allele frequencies for patients and controls were used to calculate the OR and the 95% CI using the method of Woolf [15]. Gene–environmental interactions were assessed by both 'case-only' analysis and logistic regression analysis [16]. All statistical analyses were performed using Plink software [17].

RESULTS AND DISCUSSION

A significant association between the PADI4 polymorphism and RA susceptibility was observed in the whole set of case–control subjects in the first Japanese set (rs11203367; per allele OR (OR_{allele}) 1.14; 95% CI 1.00 to 1.29; p value for a trend test (P_{trend})=0.045; **table 1**). In a stratified analysis with sex, the PADI4 polymorphism was significantly associated only in men (OR_{allele} 1.44; 95% CI 1.14 to 1.81; P_{trend}=0.0022), but not in women (OR_{allele} 1.02; 95% CI 0.82 to 1.25; P_{trend}=0.84). Similarly, when subjects in both cases and controls were stratified for smoking status, the PADI4 polymorphism had a greater effect in ever-smokers (OR_{allele} 1.35; 95% CI 1.11 to 1.65; P_{trend}=0.0024) compared with never-smokers (OR_{allele} 1.03; 95% CI 0.86 to 1.23; P_{trend}=0.71). Further stratification analysis with sex and smoking status revealed that the PADI4 polymorphism had the highest risk in the subpopulation of male ever-smokers (OR_{allele} 1.61; 95% CI 1.24 to 2.09; P_{trend}=0.00031). Similar findings were also observed, when only ACPA-positive patients were analysed (supplementary **table 3**, available online only).

To support these observations, we also analysed other case–control sets in the Japanese population and Dutch population (unstratified controls for smoking status were used in both

sets as no information was available). In the second Japanese set, the highest risk in the subpopulation of male ever-smokers was replicated in rs1748033 (OR_{allele} 1.34; 95% CI 1.02 to 1.75; P_{trend}=0.039; **table 1**). In the Dutch set, the association of the PADI4 polymorphism (rs1748033) was statistically significant in a dominant model (OR_{dom} 1.32; 95% CI 1.02 to 1.72; P_{dom}=0.03; **table 2**), but not in a trend test, when evaluated in total (P_{trend} =0.14). When patients were stratified by sex or/and smoking status and compared with control subjects, OR in the dominant model was higher for men (OR 1.36; 95% CI 0.90 to 2.06; p=0.13) than for women and was higher for ever-smokers (OR 1.56; 95% CI 1.06 to 2.31; p=0.02) than for never-smokers. Furthermore, it was highest in male ever-smokers (OR 1.79; 95% CI 0.98 to 3.27; p=0.043).

Table 2 Association of PADI4 polymorphism and RA stratified with sex and smoking status in a Dutch population*

	Sum		MAF		Genotype frequency test (dominant model)	
	Case	Contol	Case	Contol	OR	p Value
rs11203367						
All	646	385	0.44	0.42	1.19 (0.90 to 1.56)	0.2
Men	218	180	0.47	0.40	1.49 (0.96 to 2.33)	0.063
Women	398	188	0.42	0.43	1.06 (0.73 to 1.56)	0.7
Ever-smoker	174	385	0.43	0.42	1.14 (0.76 to 1.70)	0.5
Never-smoker	178	385	0.45	0.42	1.06 (0.72 to 1.57)	0.7
Male ever-smoker	76	180	0.45	0.40	1.46 (0.78 to 2.71)	0.2
Male never-smoker	40	180	0.53	0.40	1.38 (0.62 to 3.10)	0.4
Female ever-smoker	98	188	0.41	0.43	0.99 (0.58 to 1.72)	0.9
Female never-smoker	138	188	0.43	0.43	0.99 (0.61 to 1.61)	0.9
rs1748033						
All	635	391	0.34	0.30	1.32 (1.02 to 1.72)	0.03
Men	215	183	0.35	0.28	1.36 (0.90 to 2.06)	0.13
Women	389	191	0.32	0.31	1.33 (0.93 to 1.91)	0.11
Ever-smoker	158	391	0.36	0.30	1.56 (1.06 to 2.31)	0.02
Never-smoker	178	391	0.31	0.30	1.06 (0.73 to 1.53)	0.7
Male ever-smoker	70	183	0.38	0.28	1.79 (0.98 to 3.27)	0.043
Male never-smoker	41	183	0.35	0.28	1.35 (0.65 to 2.82)	0.4
Female ever-smoker	88	191	0.34	0.31	1.48 (0.86 to 2.55)	0.13
Female never-smoker	137	191	0.30	0.31	1.03 (0.65 to 1.64)	0.9

* rs112033673 (T/C, T is the minor allele) and rs174803 (T/C, T is the minor allele) were genotyped for the test. Only case subjects were stratified with smoking status.
MAF, minor allele frequency; PADI4, peptidyl arginine deiminase 4; RA, rheumatoid arthritis.

These stratified analyses suggested gene–environmental inter-actions between PADI4 and sex, and/or between PADI4 and smoking status. We performed case-only analysis to test these interactions statistically, by comparing the allele frequency of the PADI4 polymorphism in the stratified subpopulation of patients (the first and second Japanese sets were combined). Allele frequency was significantly higher in men than in women (rs11203367; 0.48 vs 0.42; P_{trend}=0.0016) and in ever-smokers than in never-smokers (rs11203367; 0.47 vs 0.41; P_{trend}=0.00077), suggesting the presence of gene–environmental interactions for PADI4. Similar results were obtained for rs1748033. In addition to stratified analyses using the contingency tables, we analysed these gene–environmental interactions using logistic regression models. The first Japanese set was used for analysis because of the availability of smoking status. The PADI4 polymorphism was associated with RA susceptibility in an additive model, adjusted by sex and smoking status (rs11203367; OR_{add} 1.18; 95% CI 1.01 to 1.38; P_{add}=0.035). When an interaction term between SNP genotype and sex (a product term of genotype×sex) was introduced into the regression model, the logistic coefficient for the term was significant (p=0.029). Similarly, when an interaction term between SNP genotype and smoking status (a product term of genotype×smoking status) was introduced into the model, the coefficient for the term was again significant (p=0.034). We also added the age of subjects into the model, because it could be a confounding factor considering that smoking prevalence has been decreasing in recent decades, especially in Japanese men (OECD Health Data, 2009) [18]. The interaction term for SNP and smoking remained significant (p=0.038), whereas the significance level of the interaction term for SNP and sex became marginal (p=0.075).

Finally, we examined the association between the PADI4 polymorphism and ACPA status in the patients of Japanese sets. The allele frequency of PADI4 showed a higher trend in ACPA-positive patients compared with ACPA-negative patients (rs11203367; 0.43 vs 0.41; P_{trend}=0.54). When the genotype frequency was compared in a recessive model, the PADI4 polymorphism was significantly associated with the ACPA status in ever-smokers (rs11203367; OR_{rec} 2.33; 95% CI 1.23 to 4.39; P_{rec}=0.0072; **table 3**), suggesting that the PADI4 polymorphism may be involved in the appearance of ACPA in smokers.

Table 3 Association of PADI4 polymorphism and ACPA status in a Japanese population*

	Sum		MAF		Genotype frequency test (recessive model)	
	ACPA+	ACPA−	ACPA+	ACPA−	OR	p Value
rs11203367						
All	1614	401	0.43	0.41	1.25 (0.93 to 1.68)	0.14
Men	295	80	0.46	0.45	1.22 (0.65 to 2.28)	0.52
Women	1319	321	0.42	0.40	1.27 (0.90 to 1.78)	0.17
Ever-smoker	523	116	0.46	0.41	2.33 (1.23 to 4.39)	0.0072
Never-smoker	1091	285	0.41	0.41	0.99 (0.70 to 1.39)	0.96
Male ever-smoker	245	55	0.49	0.44	1.90 (0.85 to 4.25)	0.11
Male never-smoker	50	25	0.34	0.48	0.28 (0.08 to 1.01)	0.045
Female ever-smoker	278	61	0.43	0.39	3.20 (1.11 to 9.22)	0.024
Female never-smoker	1041	260	0.41	0.41	1.09 (0.75 to 1.56)	1
rs1748033						
All	1614	400	0.37	0.37	1.39 (0.98 to 1.98)	0.063
Men	295	80	0.41	0.42	1.40 (0.69 to 2.83)	0.34
Women	1319	320	0.36	0.36	1.41 (0.93 to 2.12)	0.10
Ever-smoker	523	115	0.40	0.38	2.15 (1.08 to 4.28)	0.026
Never-smoker	1091	285	0.35	0.37	1.13 (0.75 to 1.72)	0.54
Male ever-smoker	245	55	0.43	0.41	2.09 (0.84 to 5.16)	0.10
Male never-smoker	50	25	0.30	0.44	0.34 (0.08 to 1.43)	0.13
Female ever-smoker	278	60	0.37	0.36	2.28 (0.78 to 6.65)	0.12
Female never-smoker	1041	260	0.35	0.36	1.27 (0.81 to 1.98)	0.29

Anti-citrullinated protein antibody (ACPA)+ and ACPA−, ACPA-positive and ACPA-negative rheumatoid arthritis (RA) patients, respectively.
*rs112033673 (T/C, T is the minor allele) and rs174803 (T/C, T is the minor allele) were genotyped for the test. Case subjects of Japanese sets (first and second) were combined for analysis.
MAF, minor allele frequency; PADI4, peptidyl arginine deiminase 4.

Gene–environmental interactions in RA susceptibility have been well described between polymorphisms in HLA-DRB1 and PTPN22 genes and smoking habit in populations of European descent [19, 20]. Our observations here indicate that the PADI4 polymorphism is another genetic risk that would interact with smoking in RA susceptibility, although why this interaction is prominent in men remains to be solved. The status of sex hormones may influence the role of PADI4, as it is profoundly involved in the onset of RA [21]. Another possible explanation could be gender differences in smoking behaviour, which has also been argued in other smoking-related diseases [22]. Quantitative analysis of smoking history, such as pack-years smoked, may be needed to investigate further for the gender difference.

Smoking prevalence rates differed highly among the populations, and the attribution of smoking to the onset of RA may thus differ among populations. A recent epidemiological survey has shown that smoking prevalences are generally higher in men from Asian countries than in western European countries: Japan, 45.8%; Korea, 46.6%; UK, 25.0%; The

Netherlands, 35.0%; Sweden, 13.9%; and USA, 19.1% in 2005 [18]. Considering our observation that the PADI4 polymorphism has the highest risk in male ever-smokers, the attribution of the PADI4 polymorphism may be relatively high in populations with high smoking prevalences among men, such as Japan and Korea, corresponding to the positive results in association studies for PADI4 polymorphisms in these countries [2, 4, 5].

In conclusion, the PADI4 polymorphism highly predisposes male smokers to RA, and the genetic heterogeneity observed in the PADI4 polymorphism between populations of Asian and European countries may be partly explained by differences in smoking prevalences among men.

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