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ORIGINAL ARTICLE - GENES AND DISEASE

Determination of *IL1R2*, *ANTXR2*, *CARD9*, and *SNAPC4* single nucleotide polymorphisms in Iranian patients with ankylosing spondylitis

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Abstract Ankylosing spondylitis (AS) is a chronic inflammatory disease of unknown origin, while both genetic and environmental factors have been demonstrated to be etiologically involved. Recent genome-wide association and replication studies have suggested that anthrax toxin receptor 2 (*ANTXR2*), interleukin-1 receptor 2 (*IL1R2*), caspase recruitment domain-containing protein 9 (*CARD9*), and small nuclear RNA-activating complex polypeptide 4 (*SNAPC4*) seem to be associated with AS pathogenesis. This case–control study was performed on 349 unrelated AS patients and 469 age- and gender-matched healthy controls, to investigate whether these non-MHC genes (*IL1R2* rs2310173, *ANTXR2* rs4333130, *CARD9* rs4077515, and *SNAPC4* rs3812571) influence the AS risk in Iranian population. *ANTXR2* rs4333130 allele C ($p = 0.0328$; OR 0.744, 95 % CI 0.598–0.927) and genotype CC ($p = 0.0108$; OR 0.273, 95 % CI 0.123–0.605) were found to be significantly protective against AS. No other associations were found between AS and studied genes. The association between *ANTXR2* rs4333130 and AS was independent of HLA-B27 status. Moreover, we found clinical disease severity scores (BASDAI and BASFI) and pain score were higher in *ANTXR2* rs4333130 CT genotype. However, we observed that *CARD9* allele C ($p = 0.012$) and genotype CC ($p = 0.012$) were significant protective factors against AS only in HLA-B27-negative patients, and *IL1R2* rs2310173 genotype GT was mildly protective against AS only in HLA-B27-negative status. These findings support the role of non-MHC pathogenic pathways in susceptibility to AS and warrants more comprehensive studies focusing on these non-MHC pathways for developing novel therapeutic strategies.

Keywords Ankylosing spondylitis · *IL1R2* · *ANTXR2* · *CARD9* · *SNAPC4*

Introduction

Ankylosing spondylitis (AS) belongs to the family of spondyloarthropathies and is a chronic inflammatory disease that primarily affects the sacroiliac joints and the axial skeleton [1]. AS patients have pain, stiffness, initial bone and joint erosion, and ultimately ankylosis and fibrosis [2, 3]. Inflammation may also involve extra-articular sites such

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as the uveal tract, tendon insertions, proximal aorta, and, rarely, lungs and kidneys [3].

The importance of genetic factors in AS susceptibility became clear after discovery of HLA-B27 as one major contributor to AS hereditability [4]. More exactly, genes within the MHC region influence nearly 25 % of AS hereditability, so that approximately 90 % of AS patients express the HLA-B27 antigen [4–6], but only about 73.5 % of Iranians patients [7] have HLA-B27. Moreover, minor contributions from genes outside of the MHC locus have been recognized to be associated in predisposing individuals to AS, which contribute up to 25.39 % of the overall heritability of AS [4]. To date, more than 14 susceptibility genes or genetic loci are identified to be associated with AS susceptibility based on genome-wide association studies regarding of individuals from the USA, UK, and Australia (the Australo–Anglo–American Spondylitis Consortium or TASC, in collaboration with the Wellcome Trust Case Control Consortium) [3, 8, 9].

Endoplasmic reticulum aminopeptidase 1 (*ERAP1*) is determined as the strongest non-MHC gene associated with AS [4, 10]. The other genetic loci beyond MHC that contribute in AS susceptibility include *IL17–IL23* axis genes, *IL1* region genes including *IL1R2*, *IL12B*, and *TNF* pathway-associated genes, Anthrax toxin receptor 2 (*ANTXR2*), and caspase recruitment domain-containing protein 9 (*CARD9*) [4]. Also *SNAPC4* (9q34.3) which is in linkage disequilibrium (LD) with *CARD9* is shown to be associated with AS in Han Chinese population [11]. To the best of our knowledge, lots of these beyond MHC genes, such as *ERAP1*, are highly correlated with HLA-B27 [4].

Some major questions regarding heritability of AS remained to be answered, for example, whether or not these beyond MHC genes are correlated with AS. Thus, we performed this study to investigate whether these non-MHC genes (*IL1R2*, *ANTXR2*, *CARD9*, and *SNAPC4*) affect the AS risk in Iranian population.

Patients and materials

Study population

The study group consists of 349 unrelated AS patients and 469 age- and gender-matched healthy controls. Diagnosis of AS patients was based on 1984 modified New York criteria. All patients were enrolled from Rheumatology Research Center of Tehran University of Medical Sciences, Shariati Hospital, and Iranian Association of AS. Ethics committee of Tehran University of Medical Sciences approved this study (according to the Declaration of Helsinki), and written informed consent was obtained from all individuals.

AS patients were examined for disease severity and functional capacities by a protocol based on the Assessment of Spondyloarthritis International Society (ASAS) core set [12], which include disease activity by Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) [13]; function by Bath Ankylosing Spondylitis functional Index (BASFI) [14]; damage or deformity of the spine by Bath Ankylosing Spondylitis Metrology Index (BASMI) [15]; Bath Ankylosing Spondylitis Global Index (BASG); and pain score. Validated Persian versions of BASDAI and BASFI questionnaires have been provided in our previous studies [16]. The Ankylosing Spondylitis Quality of Life (ASQoL) questionnaire was employed for the assessment of quality of life.

Genotyping

Genomic DNA was extracted from peripheral blood samples with standard phenol–chloroform–proteinase-K method [17]. Samples were genotyped for 4 selected SNPs including *IL1R2* (rs2310173), *ANTXR2* (rs4333130), *CARD9* (rs4077515), and *SNAPC4* (rs3812571). Real-Time PCR allelic discrimination TaqMan genotyping assays (Applied Biosystems, Foster city, USA) were applied to genotyping each study participant, using ABI StepOne Plus Real-Time PCR system. Reactions were processed following standard protocols of Applied Biosystems. The allelic call was performed by the analysis of allelic discrimination plots, using ABI SDS version 1.4 software.

Statistical analysis

Data analysis was performed using IBM SPSS Software (version 20). Quantitative data with normal distribution are expressed as Mean ± SD, and non-normally distributed characteristics are shown as Median (interquartile range; IQR). The odds ratio (with 95 % CI) and p values were calculated for each allele and genotype. The genotype distributions of all selected SNPs were tested for deviation from the Hardy–Weinberg equilibrium in control group using Chi-square test. p value of less than 0.001 was considered statistically significant for HWE [18]. For investigating the associations between genetic markers (alleles, genotypes) and clinical status (AS vs. healthy controls), Chi-square test and logistic regression were performed. p values less than 0.05 were considered statistically significant after adjusting for false discovery rate (FDR) by Benjamini–Hochberg method [19]. In addition, Mantel–Haenszel test was performed to discover the possible epistasis/interactive effects of HLA-B27 with studied genes.

Results

Demographic information

The patients group included 285 men and 64 women, with median (IQR) age of 37 (14) years and disease duration of 15 (12) years. Most of AS patients (75.1 %) were HLA-B27-positive. The healthy controls included 387 men and 82 women, with the median (IQR) age of 36 (16) years. No significant differences were observed between AS patients and healthy controls regarding age ($p = 0.082$) and sex ($p = 0.752$). Control subjects and their family did not report to have any autoimmune and rheumatic diseases. Only 4.5 % of healthy controls were HLA-B27 -positive.

Association of AS with *IL1R2*, *ANTXR2*, *CARD9*, and *SNAPC4*

The allelic and genotype frequencies of studied SNPs are demonstrated in Table 1. We did not observe any significant deviation from Hardy–Weinberg equilibrium in healthy controls. *ANTXR2* (rs4333130) allele C ($p = 0.0328$; OR 0.744, 95 % CI 0.598–0.927) and genotype CC ($p = 0.0108$; OR 0.273, 95 % CI 0.123–0.605) were found to be significantly protective against AS. More exactly 31.3 % of healthy controls had allele C compared to 25.4 % of AS patients, and 7.2 % of healthy controls had genotype CC as compared to 2.3 % of AS patients. No significant associations were observed for other studied SNPs (Table 1).

Table 1 Allele and genotype frequencies of single nucleotide polymorphisms of *IL1R2* (rs2310173), *SNAPC4* (rs3812571), *CARD9* (rs4077515), *ANTXR2* (rs4333130) are provided for AS and healthy controls

SNP	Alleles/genotypes	AS ($n = 349$) *N* (%)	Control ($n = 469$) *N* (%)	*p* value[†]	Power (%)[‡]	OR (95 % CI)
IL1R2 rs2310173	G	359 (51.4 %)	464 (49.5 %)	0.43	62.59	1.082 (0.889–1.316)
	T	339 (48.6 %)	474 (50.5 %)			Referent
	GG	96 (27.5 %)	104 (22.2 %)	0.16	99.25	1.17 (0.787–1.738)
	GT	167 (47.9 %)	256 (54.6 %)			0.827 (0.587–1.166)
	TT	86 (24.6 %)	109 (23.2 %)			Referent
	HWE	0.431	0.047			
SNAPC4 rs3812571	C	194 (27.8 %)	239 (25.5 %)	0.392	74.96	1.126 (0.902–1.405)
	G	504 (72.2 %)	699 (74.5 %)			Referent
	CC	33 (09.5 %)	33 (7.0 %)	0.442	90.44	1.399 (0.834–2.347)
	CG	128 (36.7 %)	173 (36.9 %)			1.035 (0.77–1.391)
	GG	188 (53.9 %)	263 (56.1 %)			Referent
	HWE	0.107	0.535			
CARD9 rs4077515	C	490 (70.2 %)	698 (74.4 %)	0.118	96.67	Referent
	T	208 (29.8 %)	240 (25.6 %)			1.235 (0.992–1.536)
	CC	158 (45.3 %)	251 (53.5 %)	0.122	99.81	Referent
	CT	174 (49.9 %)	196 (41.8 %)			1.410 (1.06–1.876)
	TT	17 (4.9 %)	22 (4.7 %)			1.228 (0.632–2.383)
	HWE	0.0003	0.035			
ANTXR2 rs4333130	C	177 (25.4 %)	294 (31.3 %)	**0.0328**	99.78	**0.744 (0.598–0.927)**
	T	521 (74.6 %)	644 (68.7 %)			Referent
	CC	8 (2.3 %)	34 (7.2 %)	**0.0108**	100	**0.273 (0.123–0.605)**
	CT	161 (46.1 %)	226 (48.2 %)			0.827 (0.623–1.099)
	TT	180 (51.6 %)	209 (44.6 %)			Referent
	HWE	<0.0001	0.0095			

OR odds ratio, *CI* confidence interval, *HWE* Hardy–Weinberg equilibrium *p* value

[†] FDR-adjusted *p* value for multiple testing using the Benjamini–Hochberg method

[‡] We used the formula power $= 1 - \chi'^2_{df,\lambda}$ where $\chi'^2_{df,\lambda}$ is a left-tail non-central Chi-square distribution with degree of freedom (df) and non-centrality parameter $\lambda = Nw^2 = N \times \left(\sum_{i=1}^{m} \left((p_{0i} - p_{1i})^2 / p_{0i}\right)\right)$; performed by IBM™ SPSS version 20 *compute variable* formula [1-NCDF. CHISQ(quant, df, nc)]

Significant associations are shown in bold

Table 2 Mantel–Haenszel test is provided to determine the epistasis between HLA-B27 and alleles of single nucleotide polymorphisms of *IL1R2* (rs2310173), *SNAPC4* (rs3812571), *CARD9* (rs4077515), *ANTXR2* (rs4333130)

SNPs	Alleles	HLA-B27	Status		Mantel–Haenszel	OR (95 % CI)	χ^2
			AS ($n = 349$)	Control ($n = 469$)	p value		p value
			N (%)	N (%)			
IL1R2 rs2310173	G	Negative	82 (22.8 %)	261 (96.3 %)	0.786	0.864 (0.612–1.22)	0.405
		Positive	277 (77.2 %)	10 (3.7 %)		1.57 (0.685–3.597)	0.283
	T	Negative	92 (27.1 %)	253 (94.8 %)		1.157 (0.82–1.633)	0.405
		Positive	247 (72.9 %)	14 (5.2 %)		0.637 (0.278–1.46)	0.283
SNAPC4 rs3812571	C	Negative	51 (26.3 %)	125 (96.2 %)	0.168	1.29 (0.88–1.894)	0.192
		Positive	143 (73.7 %)	5 (3.8 %)		1.427 (0.523–3.891)	0.486
	G	Negative	123 (24.4 %)	389 (95.3 %)		0.775 (0.528–1.137)	0.192
		Positive	381 (75.6 %)	19 (4.7 %)		0.701 (0.257–1.913)	0.486
CARD9 rs4077515	C	Negative	112 (22.9 %)	382 (95.3 %)	**0.012**	**0.624 (0.432–0.902)**	**0.012**
		Positive	378 (77.1 %)	19 (4.7 %)		0.681 (0.25–1.859)	0.451
	T	Negative	62 (29.8 %)	132 (96.4 %)		**1.602 (1.109–2.315)**	**0.012**
		Positive	146 (70.2 %)	5 (3.6 %)		1.468 (0.538–4.004)	0.451
ANTXR2 rs4333130	C	Negative	43 (24.3 %)	151 (95.0 %)	0.185	0.789 (0.532–1.17)	0.237
		Positive	134 (75.7 %)	8 (5.0 %)		0.687 (0.288–1.642)	0.396
	T	Negative	131 (25.1 %)	363 (95.8 %)		1.267 (0.855–1.878)	0.237
		Positive	390 (74.9 %)	16 (4.2 %)		1.455 (0.609–3.477)	0.396

OR odds ratio, *CI* confidence interval

Significant associations are shown in bold

Associations between HLA-B27 and *IL1R2*, *ANTXR2*, *CARD9*, and *SNAPC4*

Interestingly, Mantel–Haenszel test ($p = 0.012$, Tables 2 and 3) showed that *CARD9* allele C ($p = 0.012$; OR 0.624, 95 % CI 0.432–0.902) and genotype CC ($p = 0.005$; OR 0.477, 95 % CI 0.289–0.789) are significant protective factors against AS in HLA-B27-negative patients, while it is not meaningful in HLA-B27-positive ones. *IL1R2* genotype GT showed to be mildly protective against AS in HLA-B27-negative patients (Table 3).

Clinical severity scores

In the whole patients' population, numeric rate scale of pain score ($p = 0.006$), ASQOL ($p = 0.032$), BASFI ($p = 0.036$), and BASDAI ($p = 0.017$) were significantly different between *ANTXR2* rs4333130 genotypes. Interestingly, ANOVA followed by Tukey's post hoc analysis revealed that *ANTXR2* rs4333130 CT genotype has higher disease severity scores as compared to TT genotype. These findings were the same in AS patients (data not shown). Clinical characteristics of AS patients are compared between HLA-B27-positive and HLA-B27-negative patients (supplementary Table 1). We observed a slightly significant higher BASMI score and axial immobility in HLA-B27-positive individuals. Moreover, we observed that AS patients with positive HLA-B27 were significantly younger at the initiation of disease symptoms and at the time of diagnosis, as compared to HLA-B27-negative patients ($p < 0.001$; supplementary Table 1).

Discussion

Recently many genes outside MHC region have been investigated for AS susceptibility [4, 9]. Lots of minor contributions to the AS heritability have been discovered such as *IL1R2*, *ANTXR2*, *CARD9*, and *SNAPC4*; however, the data have been mostly limited to Caucasian and East Asian ethnicities, with no replications in Iranian population.

IL1R2 gene is located at chromosome 2q12, which its product, IL1 receptor type 2, inhibits IL1 activity by acting as a decoy target for IL1 [4]. In the total of population, *IL1R2* SNP rs2310173 was not associated with AS, which is in line with the findings of Han Chinese population [20]. However, we found that *IL1R2* genotype GT was mildly protective in HLA-B27 status.

We observed that *ANTXR2* (rs4333130) was significantly associated with AS risk in Iranian patients, which is in line with patients from the USA and UK [3]. However, it is different from the findings of Han Chinese population [3,

Table 3 Mantel–Haenszel test is provided to determine the epistasis between HLA-B27 and genotypes of single nucleotide polymorphisms of *IL1R2* (rs2310173), *SNAPC4* (rs3812571), *CARD9* (rs4077515), *ANTXR2* (rs4333130)

SNPs	Alleles	HLA-B27	Status		Mantel–Haenszel	OR (95 % CI)	χ^2
			AS ($n = 349$)	Control ($n = 469$)	*p* value		*p* value
			N (%)	*N* (%)			
IL1R2 rs2310173	GG	Negative	24 (25.0 %)	61 (96.8 %)	0.393	1.224 (0.705–2.124)	0.472
		Positive	72 (75.0 %)	2 (3.2 %)		1.895 (0.405–8.858)	0.523
	GT	Negative	34 (20.4 %)	139 (95.9 %)	**0.036**	**0.545 (0.332–0.894)**	**0.016**
		Positive	133 (79.6 %)	6 (4.1 %)		0.556 (0.162–1.913)	0.959
	TT	Negative	29 (33.7 %)	57 (93.4 %)	0.150	**1.754 (1.028–2.993)**	**0.038**
		Positive	57 (66.3 %)	4 (6.6 %)		0.556 (0.162–1.913)	0.346
SNAPC4 rs3812571	CC	Negative	7 (21.2 %)	20 (100.0 %)	0.734	1.037 (0.423–2.543)	0.937
		Positive	26 (78.8 %)	0 (0.0 %)		–	0.612
	CG	Negative	37 (28.9 %)	85 (94.4 %)	0.247	1.497 (0.91–2.464)	0.111
		Positive	91 (71.1 %)	5 (5.6 %)		0.745 (0.23–2.414)	0.758
	GG	Negative	43 (22.9 %)	152 (95.6 %)	0.156	0.675 (0.414–1.1)	0.114
		Positive	145 (77.1 %)	7 (4.4 %)		0.885 (0.274–2.861)	0.839
CARD9 rs4077515	CC	Negative	31 (19.6 %)	138 (95.2 %)	**0.005**	**0.477 (0.289–0.789)**	**0.004**
		Positive	127 (80.4 %)	7 (4.8 %)		0.672 (0.208–2.171)	0.504
	CT	Negative	50 (28.7 %)	106 (95.5 %)	**0.014**	**1.925 (1.177–3.149)**	**0.009**
		Positive	124 (71.3 %)	5 (4.5 %)		1.258 (0.389–4.065)	0.701
	TT	Negative	6 (35.3 %)	13 (100.0 %)	0.549	1.39 (0.512–3.777)	0.587
		Positive	11 (64.7 %)	0 (0.0 %)		–	0.999
ANTXR2 rs4333130	CC	Negative	1 (12.5 %)	13 (86.7 %)	**0.033**	0.218 (0.028–1.693)	0.204
		Positive	7 (87.5 %)	2 (13.3 %)		**0.137 (0.025–0.747)**	0.053
	CT	Negative	41 (25.5 %)	125 (96.9 %)	0.991	0.941 (0.578–1.532)	0.807
		Positive	120 (74.5 %)	4 (3.1 %)		1.690 (0.497–5.751)	0.396
	TT	Negative	45 (25.0 %)	119 (95.2 %)	0.465	1.242 (0.764–2.022)	0.382
		Positive	135 (75.0 %)	6 (4.8 %)		1.063 (0.334–3.382)	0.918

OR odds ratio, *CI* confidence interval

Significant associations are shown in bold

20]. *ANTXR2* is located at chromosome 4q21 and encodes a receptor for anthrax toxin, and its protein binds to type IV collagen and laminin, which suggests the role of *ANTXR2* in extracellular matrix adhesion. While the expression of *ANTXR2* has been found to be non-different between AS patients and healthy controls [9], we observed that genotype CT of *ANTXR2* rs4333130 exhibit significantly higher pain and disease activity scores. These findings have to be investigated in other molecular dimensions to provide sufficient evidence for the pathogenic role of *ANTXR2*.

We discovered that *CARD9* is associated with AS only in HLA-B27-negative Iranian AS patients. These findings guide us toward the hypothesis that different immunologic pathways are operative in pathogenesis of AS in HLA-B27-negative patients in comparison with HLA-B27-positive ones. The product of *CARD9* is an adaptor molecule that incorporates with other molecules and transduces signaling to the canonical NFκB pathway [4]. Actually *CARD9* plays a key role in the IL17–IL23 pathway, signaling through NFκB to induce production of TNF, IL6 and IL23 (but not IL12), and differentiation of T cells secreting IL17 and IL23 [21]. *CARD9* gene is located on chromosome 9q34, which its association with AS susceptibility was discovered in GWAS studies [3, 9] and was confirmed in replication study of UK population [22].

The mechanisms underlying relatively low HLA-B27 positivity of Iranian population is not exactly found. The differences in HLA-B27 frequencies in various ethnicities have been previously observed. For example, HLA-B27 does not exist in African blacks of unmixed ancestry and is present only in 8 % of white and 2–4 % of the American black population [23]. HLA-B27 is positive in healthy Iranian population to the extent 3.3 % (refer to Iran Royan Cord Blood Bank of 15,600 healthy blood donors, http://www.allelefrequencies.net/pop6001c.asp?pop_id=2813). Moreover, another study showed that only 3.95 % of

normal Iranian individuals in Isfahan (a large city in central Iran) are positive for HLA-B27 [24]. Also less than 60 % of American black patients are positive for HLA-B27 [23]. It should be noted that variations in HLA-B27 subtypes and existence of race-specific HLA-B27 subtypes might be the other cause of these differences. Therefore, it is highly suggested for future studies to investigate the presence of specific HLA-B27 subtypes in Iranian population. Altogether, the fact that a higher percent of Iranian AS patients are HLA-B27 negative (≈25 %) [6, 25–27] as compared to other populations (≈10 %) warrants studying genes outside MHC as potentially attributed markers to AS pathogenesis. Our result regarding the association of *CARD9* rs4077515 with HLA-B27-negative AS might intrigue the initiative molecular studies to better elucidate the enigmatic pathogenesis of AS.

Also we found no associations between *SNAPC4* and AS, which is not in line with the findings of Han Chinese population [11]. Actually the associations of *SNAPC4* and AS rests upon the hypothesis that *CARD9* (associated with AS) is in LD with *SNAPC4*. However, because a different *SNAPC4* SNP (rs11145835) is studied in Han Chinese AS patients, we cannot conclude these data are paradoxical.

Conclusion

In summary, we found significant associations between *ANTXR2* (rs4333130) and AS, regardless of HLA-B27 status. Moreover, clinical disease severity scores (BASDAI and BASFI) and pain score were higher in *ANTXR2* rs4333130 CT genotype. We observed that *CARD9* and *IL1R2* SNPs are only associated with HLA-B27-negative AS. These findings support the role of non-MHC pathogenic pathways in susceptibility to AS and warrants more comprehensive molecular studies focusing on these non-MHC pathways for developing novel therapeutic strategies.

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Compliance with ethical standard

Conflict of interest There is no conflict of interest to declare.

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