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ERAP1-ERAP2 haplotypes are associated with ankylosing spondylitis in Polish patients

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ABSTRACT

The objective of this case-control study was to evaluate the role of four single-nucleotide polymorphisms in the *ERAP1* (rs2287987, rs30187, rs27044) and *ERAP2* (rs2248374) genes and their haplotypes in predicting the risk for ankylosing spondylitis (AS) on a well-defined Polish population. Our study confirmed the strong association between the *HLA-B*27* allele and the disease. For all tested *ERAP1* SNPs we found significant differences in the minor allele and genotype distribution between patients and controls. The strongest association with AS was observed for rs30187. The minor T allele and homozygous TT genotype of this SNP significantly increased disease risk (OR = 1.56, 95%CI = 1.22–1.99, p = 0.0004 and OR = 2.52, 95%CI = 1.50–4.25, p = 0.001, respectively). In the case of rs2287987, minor C allele exerted a protective effect (OR = 0.64, 95%CI = 0.46–0.88, p = 0.008). In contrast to *ERAP1*, we observed no effect of rs2248374 in *ERAP2* on the disease. We also carried out *ERAP1-ERAP2* haplotype analysis to demonstrate a possible association of both genes with AS. Results showed that the haplotype H4, containing *ERAP1* SNPs associated with high enzymatic activity, together with the presence of ERAP2 expression, significantly increased the risk of AS (OR = 1.97, 95% CI = 1.21–3.21, p_{corr} = 0.048). By contrast, the haplotype H5 coding for low activity of ERAP1 and the lack of ERAP2 expression was strongly protective (OR = 0.41, 95% CI = 0.23–0.72, p_{corr} = 0.008).

1. Introduction

Ankylosing spondylitis is a common, highly heritable inflammatory arthritis, affecting up to 5 per 1000 western Europeans, and characterized by inflammation that predominantly affects the axial skeleton [1–3]. *HLA-B*27* allele, which is encoded in the MHC class I region, confers the greatest known risk for AS and is present in 85–95% of AS patients of European ancestry [2,4,5]. Nevertheless, only 2–5% of *HLA-B*27* positive individuals develop AS, suggesting that other genetic and environmental factors also contribute to the disease [5]. Several genome wide association studies (GWASs) [6–9] and case-control studies [10–15] have indicated that the gene for endoplasmic reticulum aminopeptidase 1 (*ERAP1*) is the second strongest locus associated with AS in Caucasians, just after *HLA-B*27* [16,17]. Interestingly, *ERAP1* has demonstrated association with AS only in those patients positive for *HLA-B*27* [8,18]. The primary known function of ERAP1 is trimming peptides in the endoplasmic reticulum (ER) to a suitable length for binding and presenting by MHC class I molecules, including HLA-B*27 [19]. Genetic interaction between *ERAP1* and *HLA-B*27* found in AS suggests that peptide trimming and presentation contribute to disease susceptibility [5]. Apart from ERAP1, there is a second member of the aminopeptidase family present in ER i.e. endoplasmic reticulum aminopeptidase 2 (ERAP2), which significantly influences the HLA-B*27:05-bound peptidome [20]. Polymorphism of the *ERAP2* gene was also found to be associated with AS in a limited number of studies [1,21]. However, there was one exception in a previous report where the crucial single nucleotide polymorphism (SNP), (rs2248374) that affects protein expression versus non-expression, showed no association with the disease [22]. Interestingly, unlike ERAP1, ERAP2 seems to play a role in both HLA-B27 positive and negative AS patients [21]. The aim of this study was to evaluate whether three frequently studied missense SNPs in *ERAP1* (rs30187 [R528K], rs27044 [E730Q] and rs2287987 [M349V]) and one intronic variant in *ERAP2* (rs2248374) contribute to disease susceptibility in Polish patients with AS. It has

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been shown that polymorphism rs30187 is the major determinant of ERAP1 activity and, indirectly, expression [23–26], whereas rs27044 exerts a direct influence on peptide length preference [27]. Furthermore, the reported effects of K528R and Q730E on the HLA-B*27 peptidome seem to be independent and additive [28]. The effect and biological role of rs2287987 (M349V) is not known, however it is localized in the active site of the enzyme [29] and may therefore affect peptide trimming. Of note this SNP is in very strong linkage disequilibrium (LD), (in nearly all tested populations), with two other *ERAP1* SNPs – rs10050860 (D575N) and rs17482078 (R725Q) [30], that were not included in this study. These two variants are also in complete LD with each other [30], and it was suggested that they can influence ERAP1 activity [31]. Nevertheless, the possible effect of D575N/R725Q is rather secondary in comparison to primary role of K528 [27,32].

We also carried out haplotype analysis including all the polymorphisms tested in this study to check possible influence of both gene polymorphisms on disease risk. According to our knowledge, there was only one similar study, carried out in multiplex AS families, that indicated an association of the *ERAP1-ERAP2* haplotype with familial ankylosing spondylitis in Canadians [33]. Considering that familial studies are frequently confounded by linkage disequilibrium effects, we were interested in analyzing *ERAP1-ERAP2* haplotype distribution in case-control study design.

2. Materials and methods

2.1. Study population

A total of 180 patients diagnosed with ankylosing spondylitis were enrolled in the study by the Silesian Hospital of Rheumatology and Rehabilitation in Ustroń (N = 131), and the Chair and Clinic of Rheumatology and Internal Diseases of Wrocław Medical University (N = 49). Diagnosis of AS was carried out according to the modified New York criteria [34]. For all patients, information about their gender and age at disease onset was available.

Healthy controls included 506 ethnically matched blood donors who had no history of AS or other spondyloarthrites. The male to female ratio was similar in patients and controls (0.176 in patients and 0.231 in controls). The study was approved by the bioethical committee of the Wrocław Medical University and by the Ethics Committee of Opole Voivodship (No. 192 from July 14, 2012). All subjects (patients and controls) provided their written, informed consent. Detailed characteristics of patients and controls are shown in Table 1.

2.2. DNA extraction

Genomic DNA was isolated using Invisorb Blood Midi kit (Stratec Molecular, Berlin, Germany) from 3 ml of frozen blood according to the manufacturer's instructions.

2.3. ERAP1 and ERAP2 typing

Three SNPs in *ERAP1* (rs27044, rs30187, rs2287987) and one in *ERAP2* (rs2248374) were genotyped using TaqMan SNP Genotyping Assay (Applied Biosystems, Foster City, USA) as described previously [35].

2.4. Determination of HLA-B*27 genotypes using tag SNP rs116488202

The *HLA-B*27* genotypes for all individuals were evaluated using the genotyping method applied previously by Lehr et al. [36]. In detail, genotyping of *HLA-B*27* tag SNP – rs116488202 (Assay ID number C_100629578_10) was performed using TaqMan SNP Genotyping Assay (Applied Biosystems, Foster City, USA) according to the manufacturer's instruction. The rs116488202T allele corresponds to *HLA-B*27* positivity. 7300 Real Time PCR System and SDS software ver. 1.4 (Applied Biosystems, Foster City, USA) were used to carry out PCR reactions and allelic discrimination, respectively.

2.5. Statistical analysis

All statistical tests including alleles, genotypes and haplotypes as well as Hardy-Weinberg Equilibrium (HWE) were calculated using PLINK software ver. 1.07 [37]. For HWE analysis, the significance threshold $p < 0.05$ was adjusted. HaploView program ver.4.2 was used for analysis and visualization of linkage disequilibrium (LD) between tested SNPs. The odds ratio (OR) and its 95% confidence interval (95% CI) were computed as a measure of effect size. P value < 0.05 was considered significant. P value for single haplotypes underwent Bonferroni correction for multiple comparisons. Correlation between tested markers and age of disease onset was also calculated using PLINK. Power calculations were performed using Quanto software ver. 1.2.4, with the following main settings: an unmatched case-control study design, hypothesis gene only, model of inheritance log-additive.

3. Results

All four tested polymorphisms in *ERAP1* and *ERAP2* genes were in Hardy-Weinberg equilibrium in AS patients and in controls. In contrast, there was a strong departure from HWE in patients (but not in controls) for rs116488202 that tagged *HLA-B*27* allele ($p = 1.837e-027$, Supplementary Table S1). Not unexpectedly, HLA-B*27 allele was significantly associated with disease (OR = 15.0, 95%CI = 10.70–20.8, $p < 0.0001$). Additionally, heterozygous and homozygous genotypes (including one and two copies of *HLA-B*27* allele) markedly enhanced the risk of AS (OR = 147.4 and 87.1, respectively, Table 2). For all tested *ERAP1* SNPs we found significant differences in minor allele and genotype distribution between patients and controls. However, the strongest association with AS was observed for rs30187. The minor T allele and homozygous TT genotype of this SNP significantly increased disease risk (OR = 1.56, 95%CI = 1.22–1.99, $p = 0.0004$ and OR = 2.52, 95%CI = 1.50–4.25, $p = 0.001$, respectively). Of note, the power of the test for rs30187T allele achieved a level of 92% (under the assumption of an odds ratio of 1.56, rs30187T allele frequency in controls 29.5%, AS prevalence in our population 0.5%, log-additive inheritance model, and 5% type I error rate (α)). Under the log-additive model, it would be necessary to test 120 patients and 337 controls to

Table 1
Characteristics of AS patients and controls.

	N (%)	Mean age (years) at the moment of blood sampling (Min-Max)	Mean age (years) at onset (Min-Max)	ESR (mm/h) mean ± SD	CRP (mg/L) mean ± SD
Patients	180	52.9 (19–80)	28.9 (15–52)	24.6 ± 19.4	18.9 ± 26.9
Females	27 (15.0%)	53.8 (19–78)	31.0 (18–52)	33.9 ± 22.8	20.9 ± 26.7
Males	153 (85.0%)	52.7 (21–80)	28.5 (15–50)	23.0 ± 18.4	18.6 ± 27.1
Controls	506	33.9 (14–63)	–	–	–
Females	95 (18.8%)	33.3 (14–63)			
Males	411 (81.2%)	34.11 (18–62)			

Table 2
Distribution of genotypes and minor alleles of studied *ERAP1/2* SNPs and *HLA-B*27* allele in AS patients (N = 180) and controls (N = 506).

GENOTYPE/MA	Cases N (%)	Controls N (%)	p	OR (95%CI)
ERAP1/rs27044				
CC	84 (46.7)	293 (57.9)	–	1*
CG	83 (46.1)	184 (36.4)	**0.01**	**1.57 (1.10–2.24)**
GG	13 (7.2)	29 (5.7)	0.24	1.56 (0.77–23.14)
G	109 (30.3)	242 (23.9)	**0.01**	**1.38 (1.06–1.81)**
ERAP1/rs30187				
CC	62 (34.4)	251 (49.6)	–	1*
CT	88 (48.9)	211 (41.7)	**0.02**	**1.54 (1.07–2.21)**
TT	30 (16.7)	44 (8.7)	**0.001**	**2.52 (1.50–4.25)**
T	148 (41.1)	299 (29.5)	**0.0004**	**1.56 (1.22–1.99)**
ERAP1/rs2287987				
TT	128 (71.1)	307 (60.7)	–	1*
TC	48 (26.7)	173 (34.2)	**0.04**	**0.66 (0.45–0.97)**
CC	4 (2.2)	26 (5.1)	0.06	0.37 (0.12–1.08)
C	56 (15.6)	225 (22.2)	**0.008**	**0.64 (0.46–0.88)**
ERAP2/rs2248374				
AA	53 (29.4)	132 (26.1)	–	1*
AG	87 (48.3)	247 (48.8)	0.53	0.87 (0.58–1.31)
GG	40 (22.3)	127 (25.1)	0.33	0.78 (0.48–1.26)
G	167 (46.4)	501 (49.5)	0.32	0.88 (0.69–1.12)
*HLA-B*27*†				
−/−	9 (5.1)	448 (88.5)	–	1*
−/+	160 (90.9)	54 (10.7)	< **0.0001**	**147.4 (71.1–305.6)**
+/+	7 (4.0)	4 (0.8)	< **0.0001**	**87.1 (21.5–351.4)**
+	174 (49.4)	62 (6.1)	< **0.0001**	**15.0 (10.7–20.8)**

* The reference group, MA – minor allele, OR – odds ratio, CI – confidence interval, p values < 0.05 are indicated in bold.
† Number of genotyped cases and controls for *HLA-B*27* = 176 and 506, respectively.

achieve 80% power.

In terms of rs2287987, minor C allele was associated with protection (OR = 0.64, 95%CI = 0.46–0.88, p = 0.008). The genotypes rs2287987TC and rs2287987CC were more frequent in controls than in cases, however the difference was significant (weakly) only for heterozygotes TC (OR = 0.66, 95%CI = 0.45–0.97, p = 0.04). The power of the test for rs2287987C allele achieved 80.1% (assuming an odds ratio of 0.64, rs2287987C allele frequency in controls 22.2%, AS prevalence in our population 0.5%, log-additive inheritance model, and 5% type I error rate (α)), whereas the minimum number of cases and controls required to achieve 80% statistical power amounted to 179 patients and 502 controls.

In contrast to *ERAP1,* the distribution of alleles and genotypes for *ERAP2* rs2248374 was similar in patients and controls, Table 2.

We also divided our patients and controls according to presence or absence of the *HLA-B*27* allele to find a possible interaction between tested *ERAP1*, *ERAP2* SNPs and *HLA-B*27*. However, because of the low number of *HLA-B*27* positive controls (N = 58, 11.5%) and an even lower number of *HLA-B*27* – negative patients (N = 9, 5.1%), we could not achieve a strong enough statistical power in our analysis, resulting in a lack of significant differences (Supplementary Tables 2 and 3).

We found eight *ERAP1/ERAP2* haplotypes with frequencies exceeding 1%. Haplotype distribution differed significantly between patients and controls (Omnibus p value = 0.0001), Table 3. Association with a risk for AS after Bonferroni correction was observed for the haplotype H4 – ERAP2(+), ERAP1 349M/528K/730E (OR = 1.97, 95% CI = 1.21–3.21, p_{corr} = 0.048). In contrast, the haplotype H5 – ERAP2(−), ERAP1 349 V/528R/730E was associated with protection (OR = 0.41, 95% CI = 0.23–0.72, p_{corr} = 0.008). The p value for the haplotypes H1 and H7 lost significance after correction.

We did not find any correlation between studied markers and age at disease onset (Supplementary Table S4).

LD analysis using D' parameter showed strong linkage between three SNPs of *ERAP1* both in patients and in controls. However, we observed only weak (controls) to moderate (patients) values of LD between SNPs of *ERAP1* and rs2248374 in *ERAP2* (Supplementary Fig. 1)

4. Discussion

In this research we examined the possible effect of three SNPs in *ERAP1* and one SNP in *ERAP2* on the prevalence of AS in a Polish cohort. In a single-locus analysis all three markers in *ERAP1* showed significant disease association and this result is entirely consistent with results obtained earlier for this gene in three studies including UK and Hungarian populations [6,10,14], whereas association of rs30187 and rs27044 but not rs2287987 was observed in Portuguese and Korean populations [12,38]. SNP rs30187 increased the risk of disease in three additional reports including Canadian and Iranian AS patients, respectively [11,33,39].

Notably, in our report the rs30187 SNP exerted the most significant effect. For this allele we achieved a very high 92% power to detect its association with the disease. Furthermore, the genotype rs30187TT (Lys528Lys) elevated the disease risk almost 2 times stronger than heterozygous rs30187CT genotype (Arg528Lys), which indicates a dose dependent nature of T risk allele.

In contrast, we observed no association for *ERAP2* rs2248374 with the disease, when considered separately. This result is in agreement with one previous study which also failed to detect *ERAP2* gene association in the Caucasian UK population. In the above mentioned report the frequency of minor rs2248374G allele in AS patients and controls was almost the same (47% in AS cases vs. 46% in controls) [22]. Conversely, Cortes et al. in their large International Genetics of AS Consortium Immunochip study found associations of *ERAP2* rs2248374 and rs2549782 with AS, however, only after controlling for the major *ERAP1* SNP (rs30187) and only in *HLA-B27* negative patients [1]. However, another study conducted by Robinson et al. [21] indicated an association of *ERAP2* SNPs with AS also in *HLA-B27* positive disease. The authors of these reports concluded that the influence of *ERAP2* on the disease is masked by the dominant effect of *ERAP1*. Unfortunately, we were unable to test such high numbers of patients and controls as did the cited authors. Likewise, the work of Harvey et al. [22] on *ERAP2*, was also based on smaller numbers of individuals. Additionally, in our study we were not able to detect *ERAP1 – HLA-B*27* epistasis either, because the numbers of *HLA-B*27*-negative patients and *HLA-B*27*-positive controls were far too small for such an analysis. Notably, we found only 9 out of 180 patients negative for *HLA-B*27* (5.1%).

We also carried out *ERAP1-ERAP2* haplotype analysis to demonstrate association of both genes with AS predisposition. Such analysis seems to be reasonable due to (i) close proximity of both genes, (ii) existence of substantial LD between them, as well as (iii) complementary function of gene products. According to our knowledge, this is the only one case-control study comparing the frequency of *ERAP1-ERAP2* haplotypes in respect of AS, except the work of Tsui et al. [33] which, however, analyzed *ERAP1-ERAP2* haplotypes in multiplex families with AS.

We demonstrated a strong difference in frequency for all haplotypes (Omnibus p value) between AS patients and controls. Detailed examination of single haplotypes revealed that the haplotype H4, containing *ERAP1* SNPs associated with high enzymatic activity (especially for the peptides ≤9–mers [27]) and together with the presence of ERAP2 expression, significantly increased the risk of AS. By contrast, haplotype H5 encoding for low activity of ERAP1 and lack of ERAP2 expression was strongly protective. Interestingly, a protective effect,

Table 3
Estimated ERAP1/2 haplotype frequencies for ankylosing spondilitis patients (N = 180) and healthy controls (N = 506).

	ERAP2	*ERAP1*							
Hap.ID	rs2248374	rs2287987M349V	rs30187R528K	rs27044E730Q	Cases %	Controls %	P	P_{corr}	OR (95%CI)
H1	G (−)	T (M)	T (K)	G (Q)	23.1	17.4	0.015	–	1.42 (1.06–1.91)
H2	A (+)	T (M)	T (K)	G (Q)	6.6	6.2	0.817	–	
H3	G (−)	T (M)	T (K)	C (E)	3.4	1.7	0.061	–	
H4	**A (+)**	**T (M)**	**T (K)**	**C (E)**	**8.0**	**4.0**	**0.006**	**0.048**	**1.97 (1.21–3.21)**
H5	**G (−)**	**C (V)**	**C (R)**	**C (E)**	**4.3**	**9.6**	**0.001**	**0.008**	**0.41 (0.23–0.72)**
H6	A (+)	C (V)	C (R)	C (E)	10.3	12.3	0.304	–	
H7	G (−)	T (M)	C (R)	C (E)	15.4	20.7	0.030	–	0.70 (0.51–0.97)
H8	A (+)	T (M)	C (R)	C (E)	28.7	27.8	0.723	–	

Omnibus p value for all haplotypes = 0.0001 (CHISQ = 29.04, df = 7); haplotypes with frequency < 1% in both cases and controls has been dropped; (+) – ERAP2 expression present, (−) – ERAP2 expression absent. P_{corr} – P Bonferroni corrected (x8), OR – odds ratio, CI – confidence interval, p values < 0.05 are indicated in bold.

although not significant after correction for multiple comparisons, was observed also for H7. This haplotype is very similar to H5 except for position 349, which effect on activity of ERAP1, according to available literature, is not known. Of note, the protective effect observed for rs2287987C allele (349 V) in single locus analysis observed in this and several previous reports [10,14,40], seems to be dictated by its presence on the common haplotype with the residues 528R and 730E, that actually determine the protective character of the H5 haplotype. However, we cannot also exclude that just 349 V in H5 haplotype is responsible for much stronger protective effect of this haplotype in comparison to effect observed for H7 including 349 M. Moreover, the effect of M349V may result from its almost absolute LD with rs10050860 (D575N) and rs17482078 (R725Q) [30] what is also observed in ERAP1 haplotypes described by Ombrello et al. [41] and Roberts [42].

Of note, results for haplotypes H4 as well as H5 and H7 are substantially concordant with earlier observations that highly active ERAP1 and presence of ERAP2 are associated with AS, whereas low activity of ERAP1 and lack of ERAP2 expression act protectively (earlier reports reviewed in refs.[43,44], and by López de Castro and Stratikos in this issue [45]. Somewhat surprisingly, we showed that the haplotype H1 encoding a highly active ERAP1 variant (especially for peptides ≥9–mers [27]), and lack of ERAP2 expression showed a trend (albeit non-significant after correction) towards association with AS. Interestingly, a similar predisposing result for our haplotype H1 equivalent – (ERAP1rs27044G/rs30187T/ERAP2rs2549782T) was described in the familial study of Canadians, mentioned earlier [33]. Although these authors typed for a different SNP in *ERAP2* (rs2549782), but this SNP is in almost absolute linkage disequilibrium in Caucasians [46,47], including our population ($R^2 = 0.994$) [35]. Hence, rs2549782 can be an excellent proxy for rs2248374, and therefore we could compare haplotypes in these two works. Although the significance level for H1 haplotype did not survive rigorous Bonferroni correction, the result is intriguing because of the only partial concordance with earlier observations and speculations. It encodes both highly active ERAP1 (risk factor for AS) and loss-of-expression variant of ERAP2 (protective factor) and yet increased the risk of AS in our population. The lack of expression of ERAP2 in this haplotype seems to be the key element because haplotype H2 (including identical substitutions for ERAP1 but rs2248374A allele in ERAP2 providing protein expression) exerted no effect.

Interestingly, a haplotype very similar to H1 (ERAP2-negative, ERAP1 highly active: 349M, 528K, 730Q) in our recently published report was also associated with an increased risk of psoriasis in *HLA-C*06:02*–positive patients [35]. Then, the result for H1 haplotype may not be accidental. However, further work needs to be done to examine the effects of this and other AS-associated *ERAP1-ERAP2* haplotypes described in this study.

The involvement of ERAP1 and ERAP2 aminopeptidases in shaping the HLA-B*27 peptidome is well established [27,44], however in the literature there is still too scarce data about how exactly both enzymes cooperate in this process. An intriguing issue is also what is the exact function of heterodimers ERAP1-ERAP2 when both enzymes are coordinately expressed in the ER, and whether reciprocal quantity of both enzymes (conditioned i.a. by rs2248374 genotype) exerts impact on MHC-I ligand editing. Interestingly, ERAP1-ERAP2 complexes can trim some peptides more powerfully than ERAP1 and ERAP2 separately [48,49]. In this context it would be also desirable to type in AS for rs75862629 located between *ERAP1* and *ERAP2* and recently described to inversely correlate with *ERAP1* and *ERAP2* transcription levels regardless of the variant rs2248374 in *ERAP2* [50].

In conclusion, we confirmed the association of tested *ERAP1* SNPs (rs30187, rs27044 and rs2287987) with susceptibility to AS. Unfortunately, we were unable to detect association of rs2248374 in *ERAP2* with the disease, probably due to the relatively low number of patients tested. However, we demonstrated associations with the disease for two haplotypes *ERAP1-ERAP2*. To our knowledge, this is the first study describing the effects of *ERAP1-ERAP2* haplotypes implicated in AS susceptibility in case-control study design. However, additional investigation in this field encompassing larger cohorts are needed to confirm our results.

Conflict of interest

The authors declare that no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.humimm.2019.02.004.

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