ERAP1 association with ankylosing spondylitis is attributable to common genotypes rather than rare haplotype combinations

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We investigated the proposal that ankylosing spondylitis (AS) is associated with unusual *ERAP1* genotypes. *ERAP1* haplotypes were constructed for 213 AS cases and 46 rheumatoid arthritis controls using family data. Haplotypes were generated from five common *ERAP1* single nucleotide polymorphisms (SNPs)—*rs2287987* (M349V), *rs30187* (K528R), *rs10050860* (D575N), *rs17482078* (R725Q), and *rs27044* (Q730E). Haplotype frequencies were compared using Fisher's exact test. *ERAP1* haplotypes imputed from the International Genetics of AS Consortium (IGAS) Immunochip study were also studied. In the family study, we identified only four common *ERAP1* haplotypes ("VRNQE," "MKDRQ," "MRDRE," and "MKDRE") in both AS cases and controls apart from two rare (<0.5%) previously unreported haplotypes. There were no examples of the unusual *ERAP1* haplotype combination ("*001/*005") previously reported by others in 53% of AS cases. As expected, K528-bearing haplotypes were increased in the AS family study (AS 43% vs. control 35%), due particularly to an increase in the MKDRQ haplotype (AS 35% vs. control 25%, $P = 0.01$). This trend was replicated in the imputed Immunochip data for the two K528-bearing haplotypes MKDRQ (AS 33% vs. controls 27%, $P = 1.2 \times 10^{-24}$) and MKDRE (AS 8% vs. controls 7%, $P = 0.004$). The *ERAP1* association with AS is therefore predominantly attributable to common *ERAP1* haplotypes and haplotype combinations.

ERAP1 | ankylosing spondylitis | haplotypes

Ankylosing spondylitis (AS) is the archetype of a group of inflammatory conditions known as spondyloarthropathies because of their tendency to involve the spine. In the developed world, it is the most common form of inflammatory arthritis after rheumatoid arthritis (RA), affecting up to 1 in 200 Europeans. It is a polygenic disorder with more than 100 genetic influences reported to date (1–3). *ERAP1* (endoplasmic reticulum-associated aminopeptidase 1) was the second gene to be definitively associated with AS (4) after *HLA-B*27* (5). Both contribute to the Major Histocompatibility Complex Class 1 (MHC1) antigen processing pathway where ERAP1 plays a role in trimming peptides for optimal binding to MHC1 molecules, such as HLA-B27 (6, 7). The *ERAP1* association is restricted to *HLA-B*27*–positive cases or *HLA-B*27*–negative/*HLA-B*40*–positive cases (1, 8, 9). Several nonsynonymous *ERAP1* single nucleotide polymorphisms (SNPs) (encoding amino acids M349V, K528R, D575N, R725Q, and Q730E) are associated with AS, including the protective "loss of function" variant 528R (10, 11). Latterly, in a small but widely quoted study, it is suggested that the *ERAP1* association with AS is explicable by rare combinations of haplotypes [referred to as allotypes in Reeves et al. (12)] affecting ERAP1 function (13). In a series of in vitro studies, these putative rare haplotypes were synthesized and transfected into *ERAAP1*-deficient murine cell lines, to highlight differences in peptide trimming, the peptide repertoire generated, cell surface MHC1 expression, and T-cell proliferative responses (12, 13). For example, an MRDRQ haplotype [designated "*005" by Reeves et al. (12)] was reportedly not only overrepresented in AS but also exhibited impaired function compared with the MKDRQ haplotype (designated "*002") (12). The potentially profound mechanistic implications from this study prompted us to undertake a larger analysis of the *ERAP1* associations of AS using (*i*) family-based haplotypes and (*ii*) imputed haplotypes from 4,230 unrelated AS cases and 9,700 unrelated controls from the International Genetics of AS Consortium (IGAS) Immunochip study (1). Our results cast doubt on the existence of unusual/rare *ERAP1* haplotypes or haplotype combinations in AS with a significant pathogenic role. Instead, the association is largely attributable to common *ERAP1* genotypes.

Results

Manual Assignment of ERAP1 Haplotypes in the Family Study. We were able to assign unequivocal *ERAP1* haplotypes manually to all 213 AS family trios and the 46 RA controls. We identified only six haplotypes in total; these included four common haplotypes—VRNQE (I), MKDRQ (II), MRDRE (IV), and MKDRE (V)—and two rare (frequency 0.5%) haplotypes—VRNRQ (VI) and MRNRE (VII). *ERAP1* haplotype frequencies are shown in Table 1 and haplotype combination frequencies in Table 2. No examples of the putative *005, "*006," "*007," "*009," "*010," or "*012" *ERAP1* haplotypes were identified in our family cases or controls.

Significance

Ankylosing spondylitis (AS) is a common inflammatory arthritis of the spine. It is associated with two genes involved in antigen processing and presentation to the immune system, *HLA-B*27* and *ERAP1* (endoplasmic reticulum aminopeptidase 1), which act synergistically in AS. Previous reports have suggested that rare *ERAP1* variants associated with dramatically altered antigen processing function are responsible. In contrast, we show here conclusively that it is common variants of *ERAP1* that are mainly responsible for protection/susceptibility in AS rather than rare *ERAP1* variants and/or unusual combinations of *ERAP1* variants. This has important potential implications for future studies addressing the development of ERAP1 inhibitors as new treatments not only for AS but also in other diseases genetically associated with *ERAP1*.

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Table 1. ERAP1 haplotype frequencies in cases and controls

ERAP1 haplotypes	RA controls, *n* (%)	AS cases, *n* (%)	Immunochip controls, *n* (%)[†]	Immunochip AS cases, *n* (%)[†]	M349V *rs2287987*	K528R *rs30187*	D575N *rs10050860*	R725Q *rs17482078*	Q730E *rs27044*	OR	*P* value[‡]
Total	92	426	19,400	8,460							—
I (*001)	22 (24)	73 (17)	4,319 (22.3)	1,466 (17.3)	V	R	N	Q	E	0.7	3.8×10^{-21}
II (*002)	23 (25)	148 (34.7)[§]	5,152 (26.6)	2,760 (32.6)	M	K	D	R	Q	1.3	1.2×10^{-24}
III (*005)	0	0	14 (0.1)	23 (0.3)	M	R	D	R	Q	3.8	8.9×10^{-5}
IV (*008 or *011)	38 (41.3)	166 (39)	8,415 (43.4)	3,488 (41.2)	M	R	D	R	E	0.9	8.6×10^{-4}
V	9 (9.8)	35 (8.2)	1,319 (6.8)	658 (7.8)	M	K	D	R	E	1.1	0.004
VI	0	2 (0.5)	122 (0.6)	40 (0.5)	V	R	N	R	Q	0.7	0.1
VII	0	2 (0.5)	45 (0.2)	16 (0.2)	M	R	N	R	E	0.8	0.6

Roman numerals denote haplotypes defined in the current study. Corresponding alleles described by Reeves et al. (12) are shown in parentheses, where appropriate in the first column. OR, odds ratio.

[†]Using SHAPEIT for haplotype analysis excludes very rare haplotypes that exist in <0.05% of the population.

[‡]Fisher's exact test performed on Immunochip cases versus controls.

[§]Family cases versus controls, $P = 0.01$.

Sequencing revealed no examples of the putative polymorphisms at amino acid positions 82, 102, 115, 199, 581, 727, 737, 752, or 874 in any of the 48 AS cases in which full-length sequencing of *ERAP1* had previously been performed (10).

In the family study, only the MKDRQ haplotype frequencies differed significantly ($P = 0.01$) between the AS cases and RA controls, but our power to detect small differences reliably for the other haplotypes was weak. In AS, there was an increase in M349 (82% vs. 76%), K528 (43% vs. 35%), D575 (82% vs. 76%), R725 (83% vs. 76%), and Q730 (35% vs. 25%), consistent with the significant increase in the MKDRQ haplotype (AS 35% vs. RA controls 25%, $P = 0.01$). Other haplotypes were correspondingly less common in AS [VRNQE 17% vs. 24%; MKDRE 8% vs. 10%; MRDRE 39% vs. 41%; P = nonsignificant (NS)]. No significant differences in haplotype combination frequencies were observed between cases and controls; specifically, no examples of the *001/005 haplotype combination (VRNQE/MRDRQ), previously reported to be present in 53% of cases with AS, were seen in either cases or controls (Table 2). The frequency (54%) of R528/K528 heterozygotes in AS cases ($n = 116/213$) was almost identical to the frequency (52%) in RA ($n = 24/46$).

Estimation of ERAP1 Haplotypes Using Immunochip Data. Four common haplotypes—VRNQE (I), MKDRQ (II), MRDRE (IV), and MKDRE (V)—were imputed in 4,230 unrelated AS cases and 9,700 unrelated controls from the IGAS Immunochip study (Table 1). We also imputed two previously unreported rare haplotypes—VRNRQ (VI) and MRNRE (VII)—and only a very low frequency (0.3% in AS cases vs. 0.1% in controls) of haplotype III—MRDRQ (previously designated *005, which was reported to be present in 53% of AS cases) (12). No examples of the putative *006, *007, *009, *010, or *012 haplotypes were observed. In AS, there was an increase in M349 (82% vs. 77%), K528 (40% vs. 33%), D575 (82% vs. 77%), R725 (83% vs. 78%), and Q730 (33% vs. 27%), consistent with a significant increase in the major MKDRQ haplotype (AS 33% vs. controls 27%, $P = 1.2 \times 10^{-24}$).

Discussion

By studying parent-case trios to determine haplotypic phase with certainty, we confirmed only six *ERAP1* haplotypes in this AS family study; two of these were rare (frequency 0.5%), and only the same four common haplotypes were observed in the RA controls. We observed only four of the previously reported 13 putative *ERAP1* haplotypes (12, 13) despite investigating more than six times as many chromosomes in this family study and comprehensively sequencing the entire *ERAP1* gene (20 exons, intron–exon boundaries, and 5′UTR) in 48 AS cases previously (10). Specifically, we found no examples of eight previously postulated variants at V82I, I102L, F199S, L115P, L581S, L727A, V737A, R752G, or M874V (12, 13). This is particularly surprising

Table 2. *ERAP1* haplotype combination frequencies

Haplotype combination	RA controls, *n* (%)	AS cases, *n* (%)	*P* value[†]
	46	213	
II + IV (*002 + *011) MKDRQ + MRDRE	12 (26)	67 (31.5)	0.6
IV + IV MRDRE + MRDRE	9 (19.6)	28 (13.2)	0.3
I + II (*001 + *002) VRNQE + MKDRQ	7 (15.2)	31 (14.6)	1
I + IV (*001 + *008) VRNQE + MRDRE	5 (10.9)	28 (13.2)	0.8
I + I (*001 + *001) VRNQE + VRNQE	4 (8.7)	6 (2.8)	0.08
IV + V MR DRE + MKDRE	3 (6.5)	9 (4.2)	0.5
II + II (*002 + *002) MKDRQ + MKDRQ	2 (4.4)	18 (8.5)	0.5
II + V MKDRQ + MKDRE	2 (4.4)	12 (5.6)	1
I + VV RNQE + MKDRE	2 (4.4)	3 (1.4)	0.2
V + VI MKDRE + VRNRQ	0	6 (2.8)	0.6
IV + VII MRDRE + MRNRE	0	3 (1.4)	1
V + V MKDRE + MKDRE	0	1 (0.5)	1
IV + VI MRDRE + VRNRQ	0	1 (0.5)	1

Roman numerals denote haplotypes defined in the current study. Corresponding putative alleles described by Reeves et al. (12) are shown in parentheses in the first column. Amino acids are shown in the order: M349V (*rs2287987*), K528R (*rs30187*), D575N (*rs10050860*), R725Q (*rs17482078*), and Q730E (*rs27044*).

[†]Fisher's exact test performed on family AS cases versus RA controls.

given the reportedly high frequency (44.5% in controls) of the 82I polymorphism in the MKDRQ haplotype (previously designated *002) (12). The MRDRQ haplotype (III/*005) has also been reported to be common in cases (29%) and controls (11%) and to be an important component of the *001/*005 haplotype combination reportedly present in 53% of AS cases (12). In stark contrast, it was completely absent from our family study and inferred only at extremely low frequency (0.3%) when the large IGAS Immunochip dataset (1) was evaluated using SHAPEIT to estimate haplotype frequencies (Table 1). As expected, we observed a higher frequency of the K528 "susceptibility allele" in our cases (43%) than controls (35%). To our knowledge, the Reeves et al. study (12) is the only work ever to have reported a higher frequency of R528 (82%) than K528 (18%) in AS. This seems likely to represent either a sampling error due to small numbers (17 cases and 19 controls) or a technical error in genotyping. We also find no support for the contention that R528/K528 heterozygotes are either absent or rare in AS (0/17 in AS vs. 15/19 controls in their data) because the frequencies in our family cases (116/213) and controls (24/46) are very similar (54% vs. 52%). The frequencies of R528/K528 heterozygotes in the Immunochip European cases and controls are also very different from the data presented by Reeves et al. (12)—53% in cases (5,477/10,417) and 44% in controls (5,424/12,338) (1). Our family study also showed haplotype frequencies very close to those predicted by imputation from the large AS association study using the Immunochip custom genotyping platform (1). They are also consistent with previous family studies on *ERAP1*/*ERAP2* haplotypic associations with AS in Canada and recent results from a large study of *ERAP1* haplotypes in Behçet's syndrome (14, 15).

Overall our results confirm that *ERAP1* alleles with K528 predispose to AS and that 528R is protective. Haplotype I (VRNQE) appears to be the most strongly protective, whereas haplotype II (MKDRQ) is the most strongly associated with risk of AS. Comparisons between these two haplotypes would therefore appear to be the most relevant for future functional studies of *ERAP1* in AS. Researchers interested in the role of *ERAP1* in AS should concentrate on investigating the K528R variant. However, there may also be functional differences arising from other residues on the main AS risk haplotype II (MKDRQ) compared with other haplotypes associated with protection (haplotypes I and IV—VRNQE and MRDRE, respectively). Our results cast significant doubt on the hypothesis that rare hyperactive or hypoactive trimming *ERAP1* variants or unusual haplotype combinations are the key to understanding the *ERAP1* association with AS.

In conclusion, the association between AS and *ERAP1* is mainly attributable to common variants at this locus, although we have not formally excluded a minor role for rare alleles. The development of small-molecule ERAP1 inhibitors for the treatment of AS is an intriguing possibility that also has potential implications for other disorders linked with endoplasmic reticulum-associated endopeptidases, such as inflammatory bowel disease, psoriasis, and Behçet's syndrome.

Methods

Cases and Controls. We studied 213 AS case trios (AS-affected probands and their parents) fulfilling the modified New York criteria for AS (16). For comparison, we used 46 similar RA control trios (1987 American College of Rheumatology criteria for RA) (17), as RA is not associated with *ERAP1* (18). All samples were obtained from patients and controls following informed consent about the study according to the protocols approved by the National Research Ethics Service, Central Oxford Research Ethics Committee, and Oxford University Hospitals National Health Service Foundation Trust (98/5/023 for AS cases and 98/5/22 for RA controls).

Genotyping. Five common *ERAP1* SNPs were genotyped—*rs2287987* (M349V), *rs30187* (K528R), *rs10050860* (D575N), *rs17482078* (R725Q), and *rs27044* (Q730E)—using KASP technology (LGC Genomics) in all parent-case trio members and RA control trios. The accuracy of these *ERAP1* genotypes was confirmed by direct sequencing of genomic DNA in a subset of 12 of these individuals (see *ERAP1 Sequencing* below).

ERAP1 Sequencing. We designed primers around five SNPs (corresponding to M349V, K528R, D575N, R725Q, and Q730E) for sequencing genomic DNA in 12 *ERAP1* homozygous cases and controls (Source BioScience). We also sequenced genomic DNA corresponding to the previously reported polymorphisms at V82I, I102L, L115P, R127P, L581S, L727A, V737A, and R752G (12). The V82I polymorphism is particularly important because it distinguishes the two commonest putative haplotypes designated *001 (VRNQE) and *002 (MKDRQ) that have previously been reported in 44% of AS cases and 44.5% of controls, respectively (12). Primers were forward ATGGTGTTTCTGCCCCTCAA and reverse TCTCCGAAAGATTGCCAGCA (82, 102, 115, 127), forward GTTCTTCCAGTCTAGAGCTCCAT and reverse CCATAGTGACCAGGTTCCCAAA (M349V), forward GTGTTATTGCCAGCCCCAAA and reverse AGGAGCATTACCCAGTGTCC (K528R), forward GGGGATGTTTTGAGAGCTTGG and reverse GGCAACTACATCTCTGGCCAT (D575N, 581), and forward CTGTTTCCCTGTACAACGCC and reverse GCATGGCTGTCACCGTTTAA (R725Q, 727, Q730E, 737, 752). In this evaluation, we were also able to use historic sequencing data from our laboratory on 48 AS cases, which covered all exons, intron/exon boundaries, and 5′UTR of the *ERAP1* gene (10).

Assignment of Haplotypes and Statistical Analysis. Because each of the five SNPs used in this study had relatively high polymorphism information content, *ERAP1* haplotypes could be assigned in the family study to the index case and control samples by simple inspection of the trio pedigree data. Parental genotypes were used only to infer the index case and control *ERAP1* haplotypes. For completeness, we confirmed correct assignment of representative *ERAP1* haplotypes and genotypes with SHAPEIT (19) and in 12 *ERAP1* homozygous index cases and controls by direct sequencing. *ERAP1* haplotypes were assigned Roman numerals (I–VII) to distinguish them from the previously designated *ERAP1* haplotypes [termed allotypes by Reeves et al. (12)]: *001–*013.

ERAP1 haplotypes were also imputed in 4,230 unrelated AS cases and 9,700 unrelated, ethnically matched controls from the IGAS Immunochip study as previously described (1, 9). At the *ERAP1* locus (chr5:96104000–96190000; HG19 coordinates), 320 SNPs were used for inferring haplotypes with SHAPEIT (19) followed by imputation of untyped SNPs with IMPUTE version 2 using as a reference all samples from phase 3 of the 1000 Genomes Project (from IMPUTE2 website, released December 2013) (20). Haplotypes with imputed SNPs were saved with the -phase option in IMPUTE2. Post-imputation quality control used a threshold of 1.0 for "info" statistic produced by IMPUTE2. Of the genotyped SNPs listed above, only *rs2287987* required imputation in the IGAS dataset, as the other four SNPs had been typed on Immunochip and passed quality control as described (1). Fisher's exact test was applied to each haplotype, and the reported odds ratio assumes an additive effect.

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