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Amino acid signatures in the *Ovar*-DRB1 peptide-binding pockets are associated with Ovine Pulmonary Adenocarcinoma susceptibility/resistance

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ABSTRACT

Potential relationships between amino acid motifs of various alleles of the ovine major histocompatibility complex DR (*Ovar*-DR) molecule and occurrence of clinical OPA caused by JSRV were investigated in a case–control study. Latxa sheep ($n = 132$) screened for presence/absence of pulmonary OPA lesions were typed for their *Ovar*-DRB1 2nd exon alleles by PCR and sequence-based typing (PCR-SBT). The polymorphic amino acid residues derived from the obtained 34 DRB1 protein variants were subjected to a logistic regression-based association study. The amino acids at several positions showed significant associations with the presence/absence of pulmonary OPA lesions; some of the residues were located within the peptide binding cleft of the DRB molecule, including pockets P1, P4, P7 and P9.

1. Introduction

Ovine Pulmonary Adenocarcinoma (OPA) is a widespread chronic respiratory disease of retroviral origin which occurs in sheep and can reach high incidence in some flocks. OPA is a contagious tumour originating in the distal lung after infection by Jaagsiekte sheep retrovirus (JSRV) and is the main and most frequent tumoural process in sheep, causing major losses wherever a flock is affected (reviewed by Griffiths et al. [1]). Breed and family line differences in incidence and susceptibility to infection and disease progression suggest that host genetic factors influence the outcome of the disease [2–6].

MHC Class II molecules are cell-surface receptor glycoproteins that bind pathogen-derived peptides and present them to circulating T cells, thus causing the generation of adaptive immune responses against pathogens [7–9].

Class II molecules are formed by a non-covalent association of α and β chains encoded by distinct genes within the MHC. These domains form the peptide-binding site (PBS) for the MHC, part of which is encoded by the Class II DRB1 gene. This peptide-binding site contains several small cavities, or pockets that are highly variable and accommodate the side chains of the bound peptide. In particular, five pockets (numbered P1, P4, P6, P7 and P9) appear to play a critical role in peptide binding [9–12].

Several reports exist that link various amino acid motifs, particularly those located in P4, to resistance or susceptibility to infectious viral diseases in ruminants. In cattle, motifs in the homologous gene DRB3 have been associated with clinical mastitis [9,13], dermatophilosis [14]), Bovine Leukaemia Virus (BLV) [15] and Foot and Mouth Disease Virus (FMDV) [16].

In studies that analysed the role in diseases of the DRB1 gene in sheep (known as *Ovar*-DRB1), Nagaoka and co-workers [17] established that the SR (serine–arginine) motif at positions β70/71 was associated with susceptibility to the development of experimentally induced BLV-caused tumours, while the RK (arginine–lysine) motif at the same position was linked with resistance. In a subsequent study, Konnai et al. [18] selected sheep with the RK/RK or SR/SR genotype and investigated the immune responses to artificial BLV-infection in sheep. Different responses were detected, as it was found that the sheep carrying the resistant genotype RK/RK strongly expressed IFNγ, while the individuals with the susceptible genotype SR/SR expressed interleukin 2 (IL2).

In a previous association analysis performed in Latxa sheep at the nucleotide level, association was detected between the DRB1 gene and susceptibility/resistance to OPA [19]. Several alleles were linked with disease progression: alleles DRB1*0143 and DRB1*0323 were significantly associated with susceptibility ($p = 0.024$ and $p = 0.029$, respectively), and allele DRB1*0702 was significantly associated with resistance ($p = 0.012$). Interestingly,

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the resistant allele DRB*0702 encodes the motif SR at positions β70–71 whilst the susceptible allele DRB1*0143 encodes the motif RK. Therefore, these motifs seem to play a key role in resistance/susceptibility to the development of the tumours, but the mechanisms underlying resistance/susceptibility is different from that detected for BLV infection.

As pockets and motifs other than pocket 4 have also been shown to correlate with the immune response in both humans and cattle, we decided to extend the analysis to amino acid motifs located in other pockets to investigate the association between the DRB1 locus and the progression of Ovine Pulmonary Adenocarcinoma.

2. Materials and methods

This study was carried out based on the alleles described previously in a Latxa sheep sample [19] in which the 2nd *Ovar*-DRB1 gene was genotyped by PCR-SBT (sequence-based typing) aided by bioinformatic allele assignation [20], and subjected to a logistic regression association analysis at the nucleotide level. 37 DRB1 alleles were detected in 132 animals, of which 88 were controls and 44 had OPA lesions in the lungs.

The protein sequences coded by the 37 alleles identified for this locus were obtained from the GenBank database. The amino acid sequences were aligned and compared by ClustalW (http://www.ebi.ac.uk/clustalw/) and Bioedit software v.7.0.5.3 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) to identify polymorphic positions. Residues from positions β10–86 (77 amino acids in total) were scrutinised. Frequencies for each polymorphic residue and HWE were computed with Genepop v.4.0 [21]. The Wu–Kabat index was calculated to identify the most variable positions, where the variability is computed as the number of different amino acids at a specific position divided by the frequency of the most common amino acid at that position [22].

A logistic regression association analysis was performed, in which each polymorphic residue was analysed individually in addition to the β70/71 residues which were also analysed together. Logistic regression analyses in which flock effect was included were performed with the SAS software v.8.2 (SAS Institute, Cary, NC, USA). Odds ratios (OR), 95% confidence intervals and *p*-values were calculated in all analyses. OR values >1 were considered susceptible and values <1 resistant. A significance level of $p < 0.05$ was used for reporting statistical analysis results.

3. Results and discussion

The 37 *Ovar*-Mhc-DRB1 alleles present in the analysed Latxa population were translated to 34 protein variants, as alleles DRB1*0203 & *1101, *03011 & *0801 and *0308 & *0321 were identical at the amino acid level. In the analysed material, polymorphic residues were found to exist at positions 11–13, 16, 18, 26, 28, 31–32, 37–38, 42, 47, 51, 56–57, 59–60, 62, 66–67, 70–71, 73–74, 76 and 86, which means over 1/3 of the analysed amino acid positions are variable. Therefore, the analysed DRB1 protein fragment is remarkably polymorphic, and variability was almost as high as at the nucleic level. The alignment of the polymorphic residues for the 37 *Ovar*-DRB1 alleles present in the Latxa sheep population can be viewed in Table 1. The Wu–Kabat index highlighted those positions with an accentuated amino acid variation (Table 1, bottom row). The most polymorphic positions were 11 and 74, and both exhibited high index scores, 13.214 and 10.571 respectively. The next most variable positions were 67, 71 and 86, with values ranging from 7.400 to 8.706. All these amino acids showing high scores in the Wu–Kabat index are all located in pockets (P1, P4, P6 and P7). This is in agreement with previous literature stating that amino acids with high Wu–Kabat variability values are typically located in positions that are predicted to affect antigen presentation [23–25].

Frequencies of the most relevant polymorphic residues are shown in Table 2, calculated for the control and OPA groups.

In an attempt to understand the underlying biological mechanism of the association of *Ovar*-DRB1 and the presence of OPA lesions a residue-by-residue analysis was performed, in which all polymorphic residues were analysed by logistic regression analysis. Association was discerned at several residues (see Table 2). Most of the positions that resulted in significant *p*-values are key PBS residues located in the variable peptide-binding pockets.

Inside the pockets, significant associations were found for one or more residues in P1 (2), P4 (3), P7 (1) and P9 (2). Two different amino acidic residues yielded significant associations at position 74 (74A, resistant; 74T, susceptible), and two at position 86 (86F, resistant; 86I, susceptible). Moreover, amino acid residues at positions β37, 47, 60, 70 and the SR motif at positions β70/71 were significantly associated with resistance to OPA progression.

Concerning the amino acid residues located outside pockets, the presence of N (Asn) at position 42 was significantly associated with susceptibility to OPA, while variants at positions β31, 32, 51 and 42 were associated with susceptibility.

Those positions located outside the pockets are known to be located in the homodimerization patch (β51). MHC Class II homodimerization seems obligatory for CD4 binding, so a change of residue at that position may affect the process. As for 42N, it is coded by alleles DRB1*0143 and *0323 among others, alleles which were significantly associated with OPA progression at the nucleotide level. The area where this position is located (peptides β41–55) was shown to affect CD4–Class II interaction as seen in lymphocyte binding assays [26].

This work highlights the importance of P4 in sheep and OPA disease. Not only is this pocket highly polymorphic in the analysed sheep sample but also several residues located there had significant or near-significant association with disease progression. The critical importance of pocket 4 regarding infectious disease has been observed in a number of works [9,15–18,27,28], although it must also be pointed out that the amino acid positions located in P4 have been the most studied. This pocket is located in the centre of the PBC [16] and it has been proposed that the residues β13, 70, 71, 74 and 78 located in that pocket may exert a major and disproportionate influence on the outcome of T-cell recognition as compared with other polymorphic residues, as seen in assays of transfectants that expressed wild-type or mutant HLA-DR molecules with single amino acid [7,29]. Substitutions at these positions may cause, depending on the location of the residue, disruption of a critical TCR contact, prevention of peptide binding or a change in the conformation of the DRB molecule.

Moreover, significant associations were found for P1, P7 and P9 too, which may suggest an important role of these regions in the resistance or susceptibility to OPA development as well. Previous works have pointed out that several of the binding-pockets other than P4 have the potential to affect the peptide-binding affinity. P6, P7 and P9 have been found to be associated with mastitis and different immune responses to a Foot and Mouth Disease Virus (FMDV) peptide in cattle [16,30].

Allele DRB1*0702 (*1301 in the IPD database [31]) merits a mention, since it was significantly associated with resistance to OPA at the nucleotide level (OR = 0.181; $p = 0.012$) in our previous work (see [19]) and it stands out in this work, also regarding resistance to OPA progression. Several amino acid residues present in the protein sequence of this allele are significantly or near-significantly associated with resistance and intriguingly, exclusively coded by this single allele or sometimes by an additional low frequency allele. That was the case of position β31 and 32, position in which amino acid variants Y and T, respectively, were only

Table 1
Alignment of amino acid residues in variable positions of each *Ovar*-DRB1 allele detected in the analysed Latxa sheep sample. Wu–Kabat index values are shown in the bottom line. Alleles are identified following both the local and international (IPD) nomenclature.

Ovar-DRB1 allele		Position																										
Local nom.	IPD nom.	11	12	13	16	18	26	28	31	32	37	38	42	47	51	56	57	59	60	62	66	67	70	71	73	74	76	86
*0101	–	T	K	K	R	S	F	D	F	H	T	L	S	Y	A	P	D	K	Y	N	D	F	R	A	A	A	D	I
*0103	*0102	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	G
*0104	*0103	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	E	–	–	–	–	–	–	–	–	–	G
*0109	*1601	–	–	–	–	F	Y	–	–	Y	Y	V	N	–	–	–	–	–	–	–	E	L	–	K	–	N	–	G
*0114	*0303	–	–	–	–	–	–	–	–	Y	Y	A	–	–	–	–	–	–	–	–	E	I	–	R	T	E	–	G
*0115	*0308	–	–	–	–	–	–	–	–	Y	Y	A	–	–	–	R	S	E	–	–	E	I	–	K	–	–	–	G
*0116	*0304	–	–	–	–	–	–	–	–	Y	Y	A	–	–	–	R	S	E	–	–	E	I	–	R	T	E	–	–
*0117	–	–	–	–	–	–	–	–	–	–	Y	A	–	–	–	–	–	–	–	–	E	L	–	R	T	E	–	–
*0141	*0301	–	–	–	–	–	–	–	–	–	Y	A	–	–	–	–	–	–	–	–	E	I	–	R	T	E	–	–
*0142	–	–	–	–	–	–	–	–	–	Y	Y	V	–	–	–	R	S	E	–	–	E	I	–	R	T	E	–	–
*0143	–	–	–	–	–	F	–	–	–	Y	Y	V	N	–	–	–	–	–	–	–	E	L	–	K	–	T	–	G
*0201	*1001	S	T	S	H	F	–	–	–	Y	Y	V	–	–	–	–	–	–	–	–	E	I	–	R	T	E	–	–
*0203	*1101	S	T	S	H	F	–	–	–	Y	–	–	–	–	–	–	–	–	–	–	E	I	–	K	–	–	–	G
*03011	*0801	H	–	S	H	F	–	–	–	–	F	V	–	F	–	R	S	E	–	–	E	L	–	R	T	E	–	F
*03032	*1502	H	–	S	H	F	–	–	–	Y	Y	V	–	F	–	Q	S	E	H	–	E	L	–	R	–	E	–	–
*0308	*1605	H	–	S	–	–	Y	–	–	Y	Y	V	N	–	–	–	–	–	–	–	E	I	–	K	–	–	–	–
*0321	–	–	–	–	–	–	–	–	–	Y	Y	V	–	–	–	–	–	–	–	–	E	I	–	R	T	E	–	–
*0323	*0402	H	–	S	–	–	Y	–	–	Y	Y	V	N	–	–	R	S	E	–	–	–	–	Q	T	–	E	–	–
*0325	–	H	–	S	H	F	–	–	–	–	F	V	–	F	–	R	S	E	H	–	E	L	–	R	–	E	–	–
*0326	*0804	H	–	S	H	F	–	–	–	Y	Y	V	–	F	–	Q	S	E	–	–	E	L	–	R	T	E	–	F
*03411	*1201	H	–	S	H	F	–	–	–	Y	Y	V	–	F	–	–	E	–	–	–	E	I	–	K	–	–	–	–
*0353	–	R	–	S	–	–	–	–	–	Y	N	V	–	–	–	–	–	–	–	–	–	–	–	K	–	N	–	G
*0404	*2001	A	–	S	–	F	–	E	–	Y	Y	V	–	–	–	–	–	–	–	–	–	–	–	K	–	N	–	–
*0411	*0502	A	–	S	–	F	–	E	–	Y	–	–	–	–	–	–	–	–	–	–	E	L	–	K	–	N	–	D
*0412	*0501	A	–	S	–	F	–	E	–	Y	–	–	–	–	–	–	–	–	–	–	E	L	–	K	–	N	–	G
*0414	–	H	–	S	H	F	–	–	–	–	–	–	–	–	–	–	–	–	–	–	E	L	–	K	–	N	–	–
*0416	–	A	–	S	H	–	Y	–	–	Y	–	–	–	–	–	–	–	–	–	–	E	I	–	K	–	N	–	–
*0601	*1604	R	–	S	–	–	Y	–	–	Y	Y	V	–	–	–	–	–	–	–	–	E	L	–	K	–	N	–	–
*0605	*1801	R	–	S	–	–	Y	–	–	Y	Y	V	–	–	–	R	S	E	–	–	E	L	–	K	–	N	–	G
*0607	–	S	T	S	–	–	Y	–	–	Y	Y	V	–	–	–	–	–	–	–	–	E	L	–	K	–	N	–	–
*0608	–	H	–	S	–	–	–	–	–	Y	Y	A	–	–	–	R	S	E	–	T	E	L	–	R	T	E	–	G
*0702	*1301	S	–	S	H	F	–	–	Y	T	N	V	–	F	T	–	–	E	Q	–	–	–	S	R	T	–	–	F
*0801	*0901	Y	R	S	H	F	L	E	–	–	F	A	–	F	T	–	A	E	Q	–	N	I	Q	K	–	E	N	F
*1101	–	A	–	S	H	F	–	–	–	Y	Y	V	–	F	–	R	S	E	–	–	–	–	Q	T	–	E	–	G
*1301	*2101	S	T	S	H	–	L	–	–	–	F	V	–	F	–	–	–	E	–	–	–	–	S	R	T	–	–	–
Z92727	–	H	–	S	–	–	Y	–	–	Y	Y	V	N	–	–	–	–	E	–	–	–	–	Q	T	–	E	–	–
Z92731	–	H	–	S	H	F	–	–	–	Y	Y	V	–	F	–	–	–	E	–	–	E	L	–	R	T	E	–	F
	Wu–Kabat index	14.286	2.286	2.857	3.200	3.636	2.581	2.286	2.051	4.000	6.667	5.217	2.424	2.581	2.051	4.286	4.444	3.333	3.243	2.051	2.963	7.500	3.529	8.889	4.444	11.429	1.000	8.421

Position: number of residue position where different amino acids from the reference sequence (*0101) were found.
Amino acid abbreviations: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, Isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine; – same amino acid with reference sequence.

Table 2
Amino acid residue variant frequencies and association analysis results for polymorphic amino acid positions coded by the DRB1 gene.

	Position	Variants	Residue	Control	OPA	OR (95% CI)	*p*-Value
(a) Amino acid residues located within pockets							
Pocket 1	86	D, F, G, I	D[a]	0.023	0.011	0.366 (0.035–3.810)	0.401
			F	0.176	0.057	0.190 (0.057–0.629)	0.007**
			G	0.460	0.398	1.014 (0.430–2.393)	0.975
			I	0.341	0.534	2.636 (1.068–6.503)	0.035*
Pocket 4	13	K, S	K	0.341	0.455	2.321 (0.958–5.623)	0.062
			S	0.659	0.545	0.680 (0.213–2.168)	0.515
	26	F, L, Y	F	0.756	0.648	0.201 (0.036–1.110)	0.066
			L[a]	0.011	0.023	2.661 (0.319–22.18)	0.366
			Y	0.233	0.33	1.314 (0.586–2.947)	0.508
	70	Q, R, S	Q	0.068	0.148	2.582 (0.916–7.280)	0.073
			R	0.778	0.807	0.806 (0.163–3.983)	0.792
			S	0.153	0.045	0.237 (0.072–0.784)	0.018*
	71	A, K, R, T	A	0.136	0.091	0.764 (0.273–2.141)	0.609
			K	0.318	0.386	1.599 (0.712–3.593)	0.256
			R	0.483	0.386	0.589 (0.257–1.348)	0.210
			T	0.062	0.136	2.356 (0.809–6.867)	0.116
	74	A, E, N, T	A	0.330	0.193	0.416 (0.180–0.965)	0.041*
			E	0.398	0.489	1.378 (0.591–3.214)	0.457
			N	0.227	0.205	0.712 (0.307–1.652)	0.429
			T	0.045	0.114	4.321 (1.217–15.340)	0.024*
Pocket 6	11	A, H, R,	A	0.102	0.091	0.600 (0.210–1.714)	0.304
		S, T, Y	H	0.273	0.284	1.391 (0.616–3.140)	0.428
			R	0.097	0.068	0.774 (0.245–2.400)	0.663
			S	0.182	0.091	0.427 (0.162–1.124)	0.085
			T	0.341	0.455	2.321 (0.958–5.623)	0.062
			Y[a]	0.006	0.011	4.025 (0.191–85.022)	0.371
Pocket 7	28	D, E	D	0.949	0.966	–	–
			E[a]	0.050	0.034	0.568 (0.134–2.396)	0.441
	47	F, Y	F	0.244	0.114	0.331 (0.132–0.829)	0.018*
			Y	0.756	0.886	1.935 (0.196–19.10)	0.572
	67	F, I, L	F	0.364	0.273	0.530 (0.235–1.194)	0.126
			I	0.176	0.307	1.841 (0.776–4.368)	0.166
			L	0.460	0.42	0.739 (0.301–1.816)	0.510
Pocket 9	37	F, N, T, Y	F[a]	0.040	0.045	1.501 (0.349–6.450)	0.585
			N	0.153	0.034	0.169 (0.045–0.638)	0.009**
			T	0.227	0.216	0.827 (0.362–1.891)	0.653
			Y	0.580	0.705	1.082 (0.322–3.640)	0.898
	57	A,D, E, S	A[a]	0.006	0.011	4.025 (0.191–85.02)	0.371
			D	0.591	0.648	1.246 (0.384–4.051)	0.714
			E[a]	0.017	0.011	0.390 (0.036–4.205)	0.437
			S	0.386	0.33	0.844 (0.370–1.922)	0.686
	60	H, Q, Y	H[a]	0.040	0.034	1.955 (0.374–10.210)	0.427
			Q	0.153	0.045	0.169 (0.045–0.638)	0.009**
			Y	0.807	0.92	0.248 (0.012–5.247)	0.371
(b) Amino acid residues located out of pockets							
Additional residues	12	K, R, T	K	0.960	0.932	–	–
			R[a]	0.006	0.011	4.025 (0.191–85.02)	0.371
			T	0.034	0.057	2.252 (0.518–9.788)	0.279
	16	H, R	H	0.307	0.216	0.457 (0.200–1.046)	0.064
			R	0.693	0.784	1.231 (0.260–5.822)	0.793
	18	F, S	F	0.358	0.284	0.533 (0.235–1.207)	0.131
			S	0.642	0.716	0.991 (0.260–3.777)	0.990
	31	F, Y	F	0.852	0.966	1.526 (0.061–38022)	0.480
			Y	0.148	0.034	0.181 (0.048–0.689)	0.012*
	32	H, T, Y	H	0.244	0.25	1.011 (0.434–2.357)	0.979
			T	0.148	0.034	0.181 (0.048–0.689)	0.012*
			Y	0.608	0.716	0.872 (0.264–2.882)	0.823
	38	A, L, V	A	0.261	0.205	0.535 (0.223–1.283)	0.161
			L	0.227	0.216	0.827 (0.362–1.891)	0.653
			V	0.212	0.197	1.132 (0.421–3.042)	0.806
	42	N, S	N	0.119	0.284	4.056 (1.650–9.970)	0.002**
			S	0.881	0.716	0.416 (0.038–4.558)	0.473

Table 2 (*continued*)

	Position	Variants	Residue	Control	OPA	OR (95% CI)	*p*-Value
	51	A, T	A	0.847	0.955	0.248 (0.012–5.247)	0.371
			T	0.153	0.045	0.169 (0.045–0.638)	0.009**
	56	P, Q, R	P	0.614	0.67	1.109 (0.305–4.028)	0.875
			Q[a]	0.028	0.023	1.807 (0.275–11.87)	0.538
			R	0.358	0.307	0.700 (0.307–1.598)	0.397
	59	E, K	E	0.676	0.477	0.526 (0.205–1.345)	0.180
			K	0.324	0.523	2.017 (0.856–4.752)	0.109
	62	N, T	N	0.864	0.943	1.526 (0.061–38022)	0.480
			T	0.136	0.057	0.402 (0.122–1.322)	0.134
	66	D, E, N	D	0.364	0.273	0.530 (0.235–1.194)	0.126
			E	0.631	0.716	0.669 (0.207–2.168)	0.503
			N[a]	0.006	0.011	4.025 (0.191–85.02)	0.371
	73	A, T	A	0.557	0.443	1.659 (0.614–4.479)	0.318
			T	0.648	0.352	0.546 (0.239–1.248)	0.151
	76	D, N	D	0.994	0.989	–	–
			N[a]	0.006	0.011	4.025 (0.191–85.022)	0.371

Uncalculated due to sample distribution.
Position: antigen binding pocket number identified by Bondinas et al. [12].
[a] Amino acid variant with frequency ⩽0.05 in both controls and cases.
* $p < 0.05$.
** $p > 0.01$.

present in allele DRB1*702, and were associated with resistance to OPA (see Table 2). Similarly, variants 37N, 51T, 60Q and 70S were also associated with resistance and coded by allele *0702 plus an additional, low frequency allele. It may be possible that these amino acid variants somehow protect the host from the JSRV-induced tumour progression, although exact mechanisms of how this may happen will have to be elucidated. Interestingly, although allele DRB1*0702 was rare in their sample, related allele DRB1*07012 was associated with lower OPPV (Ovine Pulmonary Progressive Pneumonia Virus, also known as Visna/Maedi Virus) levels by Herrmann-Hoesing et al. [28]. Several residues coded by this allele and others were associated with a lower OPPV provirus level, most of which were also considered resistant in this work (Y31, T32, N37, T51 and Q60).

To our knowledge, this is the 1st study linking *Ovar*-DRB1 motifs and susceptibility or resistance to OPA. The analysis of polymorphic amino acid residues has proved to be useful as it offers functional perspective of the Class II DRB1 gene. It has shed light on the implication several amino acid variants may have in disease progression, and gives further evidence on the importance of the MHC Class II region in infectious disease. However, further studies involving neighbouring MHC loci and other susceptibility-related genes will be necessary to determine the extent of implication of the *Ovar*-DRB1 in the susceptibility/resistance to OPA.

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