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Immunology

Dog leukocyte antigen class II alleles and haplotypes associated with meningoencephalomyelitis of unknown origin in Chihuahuas

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ABSTRACT. Idiopathic non-infectious meningoencephalomyelitis (NIME), which is thought to be an immune-mediated disease, is a common inflammatory disease in dogs. Meningoencephalomyelitis of unknown origin (MUO), a subgroup of NIME, consists of necrotizing meningoencephalitis (NME), necrotizing leukoencephalitis, and granulomatous meningoencephalomyelitis. Recent studies have shown associations between disease development and dog leukocyte antigen (DLA) class II genes in NME in Pugs and in NIME in Greyhounds. This study focused on Chihuahuas, which have a high incidence of MUO and are one of the most common dog breeds in Japan. Because the development of MUO seems to be associated with DLA class II genes, we aimed to evaluate the association between DLA class II genes and MUO development in Chihuahuas. Blood samples were obtained from 22 Chihuahuas with MUO (MUO group) and 46 without neurological diseases (control). The allele sequences of three DLA class II loci were determined, and haplotypes were estimated from these data. In total, 23 haplotypes were detected. The frequency of one haplotype (DLA-DRB1*015:01--DQA1*006:01--DQB1*023:01) was significantly higher in the MUO group than in the control group (odds ratio, 7.11; 95% confidence interval, 1.37–36.81; *P*=0.0141). The results suggest that the development of MUO in Chihuahuas may be associated with DLA class II genes. Because the identified risk haplotypes differed from those of other breeds, the pathogenesis of NIME-related diseases may differ among dog breeds.

KEYWORDS: canine, dog leukocyte antigen class II, major histocompatibility complex, meningoencephalitis, meningoencephalomyelitis of unknown origin

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Meningoencephalomyelitis of unknown origin (MUO) in dogs is a subgroup of idiopathic non-infectious meningoencephalomyelitis (NIME); it is a common inflammatory disease generally consisting of necrotizing meningoencephalitis (NME), necrotizing leukoencephalitis, and granulomatous meningoencephalomyelitis [9, 17, 35]. Because histopathological examination is necessary for the definitive diagnosis of these diseases, in cases without histopathological diagnosis MUO is presumptively diagnosed based on clinical features such as dog breed, age at onset, neurological examination results, magnetic resonance imaging (MRI) findings, and cerebrospinal fluid (CSF) analysis [8, 17]. MUO predominantly affects young to middle-aged dogs. MUO can affect any dog breed, but is more common in toy and small breeds of dog (e.g., Pug, Maltese, Chihuahua, and Yorkshire Terrier) [7, 8, 10, 34]. The neurological signs of MUO in dogs vary depending on the brain region in which the lesion develops, and include seizure, circling,

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visual loss, and head tilt. The disease can be fatal if untreated [1, 8, 33]. Although the precise pathogenesis of MUO has not been clarified, an excessive immune response is considered the most important factor, since the neurological signs of MUO in dogs are ameliorated after administration of immunosuppressive therapies [10, 33, 39, 54].

The canine major histocompatibility complex, also described as dog leukocyte antigen (DLA), contains a family of genes that encode class I and class II molecules [48, 58]. The genes for DLA class II are encoded on chromosome 12 [13]. DLA class II molecules are essential components of antigen-presenting cells that present both exogenous antigens derived from phagocytosis and endogenous antigens derived from autophagy via DLA class II molecules on their surfaces. Polymorphisms of these molecules are important for various immune responses, such as antigen-specific immune responses and self-tolerance through priming CD4+ T cells by antigen presentation [23]. In addition, the polymorphisms of four DLA class II loci (i.e., DLA-DRA1, DLA-DRB1, DLA-DQA1, and DLA-DQB1) were defined using molecular methods [5, 12]. Of these, DLA-DRB1, DLA-DQA1, and DLA-DQB1 are highly polymorphic [48–50]. In recent years, many studies have reported that DLA class II genes are associated with the development of various diseases in dogs, including hypothyroidism (several breeds) [26, 32], hypoadrenocorticism (several breeds) [36], inflammatory bowel disease (German Shepherds) [45], chronic enteropathy (French Bulldogs) [40], NME (Pugs) [3, 18, 44], and NIME (Greyhounds) [52].

Chihuahuas are one of the most popular dog breeds in Japan [22] but they have a high incidence of MUO [11]. A genome-wide meta-analysis of single-nucleotide polymorphisms in DLA class II genes that had previously been associated with NME in Pugs identified single-nucleotide polymorphisms as risk factors for the development of NME in Chihuahuas [51]. This result suggests the presence of certain NME-associated DLA class II alleles and haplotypes in Chihuahuas, similar to Pugs, but this has not yet been confirmed. As mentioned, a definitive diagnosis of NME is difficult. The development of other types of MUO, such as necrotizing leukoencephalitis and granulomatous meningoencephalomyelitis, seems to be associated with DLA class II genes, as does NME. Therefore, we focused on MUO in Chihuahua. We aimed to establish the genetic factors associated with the development of MUO in this breed to help breeders evaluate the risk of MUO, as has been done for NME in Pugs [44]. We assessed the association between three DLA class II loci (DLA-DRB1, DLA-DQA1, and DLA-DQB1) and MUO development using DLA class II sequence data.

MATERIALS AND METHODS

Animals and blood samples

Blood samples were collected from Chihuahuas with MUO (MUO group) and without MUO or any other immune-mediated diseases (control group) at two institutions. In the MUO group, 22 dogs were recruited (15 at Nihon University, April 2015 to December 2020; seven at the Neurology and Neurosurgery Service of the Veterinary Specialists and Emergency Center, December 2019 to December 2020). During the same period, blood samples were collected from 46 control dogs (control group) at Nihon University. All blood samples were treated with ethylenediaminetetraacetic acid immediately after collection to suppress clotting pending further analysis. After obtaining the owner's consent for research use, blood was collected according to the guidelines for animal experiments and animal welfare at each collecting institution. The design of this study was examined and approved by the Ethics Committee of the Animal Medical Center of Nihon University.

Diagnosis of MUO and inclusion/exclusion criteria of controls

The diagnosis of MUO in Chihuahuas was made by board-certified veterinary neurologists (DI and SK) at each institution, based on age at onset, clinical history, MRI findings, and CSF analysis, if available. A CSF tap was not performed if MRI suggested elevated intracranial pressure due to the disease. In these cases, the diagnosis was based on the response to immunosuppressive therapies. The criteria for inclusion in this study were based on a previous report on MUO [17], as follows: (1) older than 6 months of age at the time of sample collection; (2) single, multiple, or diffuse intra-axial hyperintense lesions on T2-weighted images; and (3) for cases in which CSF analysis was performed, predominantly mononuclear cells in the CSF (the presence of pleocytosis in the CSF was not considered because 10–22% of MUO cases reportedly do not show this feature [17, 39]). The exclusion criteria were: (1) suspicion of infectious diseases commonly reported in Japan (canine distemper virus, Neospora, and Toxoplasma) by blood and CSF analysis and (2) having focal lesions in the spinal cord (myelitis) or optic nerves (optic neuritis).

The selection criteria for the control dogs were as follows: (1) Chihuahuas, (2) older than 7 years at sample collection (to avoid cases with a genetic predisposition to MUO being sampled prior to disease onset) [8], and (3) no history of neurological or immune-mediated diseases.

RNA extraction and complementary DNA (cDNA) synthesis

Total RNA was extracted from the peripheral leukocytes of 68 Chihuahuas using TRIzol LS Reagent (Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's protocol. The extracted total RNA was treated with DNase I (Thermo Fisher Scientific) and cDNA synthesis was performed using oligo-dT primers, followed by application of the Omniscript RT kit (Qiagen, Hilden, Germany).

Reverse-transcription polymerase chain reaction (RT-PCR)

The sequencing primer pairs for alleles of DLA-DRB1, DLA-DQA1, and DLA-DQB1 were as previously reported (Supplementary Table 1) [38]. Amplification using RT-PCR was performed on 20 µL of sample using a GeneAmp 9700 PCR System (Thermo Fisher Scientific). The sample consisted of 30 ng of cDNA template, 0.4 U of KOD FX polymerase (TOYOBO, Osaka, Japan), 1x PCR buffer, 2 mM of deoxyribonucleoside triphosphates, and 0.4 mM of each primer pair. The cycling parameters were as follows: thermal

denaturation at 94°C for 2 min, followed by 35 cycles of (1) 98°C for 10 sec; (2) 60°C for 30 sec (DLA-DRB1 and DLA-DQB1) or 57°C for 30 sec (DLA-DQA1); and (3) 68°C for 1 min.

Sanger sequencing and subcloning

The RT-PCR products were directly sequenced using an ABI3130 Genetic Analyzer (Thermo Fisher Scientific) in accordance with the BigDye Terminator protocol, Version 3.1 (Thermo Fisher Scientific). When novel and/or ambiguous alleles were obtained, the RT-PCR product was cloned into the pMD20 vector (Takara Bio, Kusatsu, Japan) using a TA cloning kit (TOYOBO), following the manufacturer's protocol, and then subcloning and Sanger sequencing were performed to confirm the allele sequences.

Determination of each DLA class II allele sequence and haplotype estimation

Each DLA class II allele sequence was obtained using Sequencher ver. 5.4.6 (Gene Codes Corp., Ann Arbor, MI, USA). The sequences were determined by comparison with known DLA-DRB1, DLA-DQA1, and DLA-DQB1 allele sequences published in the IPD-MHC canine databases [20] and GenBank [41]. Haplotypes containing all three loci were manually estimated as previously reported [31, 37, 40], using the previously identified haplotype data [14, 16, 18, 19, 25, 26, 30, 40, 44, 45, 52, 53, 55]. Haplotype IDs were determined in order of frequency over all samples.

Statistical analysis

2 × 2 contingency tables and the two-sided Fisher's exact test were used for computing odds ratios (ORs) with P values and 95% confidence intervals (CIs) for allele and haplotype frequencies and the numbers of dogs in the MUO and control groups. Following the recommendations of similar studies, Bonferroni correction was not applied [46, 53]. The relative predispositional effects (RPE) method was used to check for predispositional effects relative to each other in haplotype frequency in the MUO and control groups. Overall distribution of haplotypes at three loci was compared in the MUO and control groups using the Chi square test. The haplotype with the greatest predispositional effect was determined from the contribution of each haplotype to overall distribution. If a particular haplotype had the greatest predispositional effect, the remaining haplotypes were analyzed again after removing it; we repeated this procedure until there was no significant difference between the MUO and control groups [43]. GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA) was used for statistical analysis, and statistical significance was established at $P<0.05$.

RESULTS

The MUO group (22 dogs) had a median age of 41 months (range 7–146 months), with 10 males (seven castrated) and 12 females (11 spayed). The control group (46 dogs) had a median age of 133 months (range 85–204 months), with 28 males (18 castrated) and 18 females (12 spayed).

Analysis of all dogs (MUO and control) identified 15 DLA-DRB1 alleles (Table 1), seven DLA-DQA1 alleles (Table 2), and 17 DLA-DQB1 alleles (Table 3). The overall homozygosity of each locus was 22.1% for DLA-DRB1, 30.9% for DLA-DQA1, and 10.3% for DLA-DQB1. The most frequent alleles across all dogs were DLA-DRB1*015:01 (38.97%), DLA-DQA1*001:01 (36.76%), and

Table 1. Allele frequencies of the dog leukocyte antigen gene complex in 22 Chihuahuas with meningoencephalomyelitis of unknown origin and 46 controls: DRB1

DLA-DRB1	Total number of alleles		Number of alleles						Number of homozygous dogs			
			MUO		Control		*P* value	OR (95%CI)	MUO	Control	*P* value	OR (95%CI)
	2n=136	%	2n=44	%	2n=92	%			n=4	n=11		
001:01	4	2.94	3	6.82	1	1.09	NS	6.66 (0.67–65.99)	0	0		
002:01	2	1.47	0	0.00	2	2.17	NS	0.41 (0.02–8.66)	0	0		
006:01	8	5.88	4	9.09	4	4.35	NS	2.20 (0.52–9.25)	0	0		
009:01	33	24.26	8	18.18	25	27.17	NS	0.60 (0.24–1.46)	0	3	NS	0.27 (0.01–6.47)
012:01	11	8.09	5	11.36	6	6.52	NS	1.84 (0.53–6.39)	1	0	NS	9.86 (0.32–300.70)
013:01	2	1.47	0	0.00	2	2.17	NS	0.41 (0.02–8.66)	0	0		
015:01	53	38.97	17	38.64	36	39.13	NS	0.98 (0.47–2.05)	3	8	NS	1.13 (0.08–15.52)
015:02	1	0.74	1	2.27	0	0.00	NS	6.38 (0.25–159.90)	0	0		
017:02	4	2.94	1	2.27	3	3.26	NS	0.69 (0.07–6.83)	0	0		
018:01	4	2.94	2	4.55	2	2.17	NS	2.14 (0.29–15.75)	0	0		
020:01	7	5.15	1	2.27	6	6.52	NS	0.33 (0.04–2.86)	0	0		
035:01	1	0.74	1	2.27	0	0.00	NS	6.38 (0.25–159.90)	0	0		
046:01	1	0.74	0	0.00	1	1.09	NS	0.69 (0.03–17.18)	0	0		
073:01	2	1.47	0	0.00	2	2.17	NS	0.41 (0.02–8.66)	0	0		
113:01	3	2.21	1	2.27	2	2.17	NS	1.05 (0.09–11.87)	0	0		

NS, not significant; CI, confidence interval; OR, odds ratio.

Table 2. Allele frequencies of the dog leukocyte antigen gene complex in 22 Chihuahuas with meningoencephalomyelitis of unknown origin and 46 controls: DQA1

DLA-DQA1	Total number of alleles		Number of alleles						Number of homozygous dogs			
	2n=136	%	MUO 2n=44	%	Control 2n=92	%	*P* value	OR (95%CI)	MUO n=7	Control n=14	*P* value	OR (95%CI)
001:01	50	36.76	14	31.82	36	39.13	NS	0.73 (0.34–1.55)	2	7	NS	0.40 (0.06–2.80)
002:01	8	5.88	3	6.82	5	5.43	NS	1.27 (0.29–5.59)	0	0		
003:01	2	1.47	0	0.00	2	2.17	NS	0.41 (0.02–8.66)	0	0		
004:01	17	12.50	5	11.36	12	13.04	NS	0.85 (0.28–2.60)	1	0	NS	6.69 (0.24–187.40)
005:01:1	8	5.88	4	9.09	4	4.35	NS	2.20 (0.52–9.25)	0	0		
006:01	49	36.03	18	40.91	31	33.70	NS	1.36 (0.65–2.86)	4	7	NS	1.33 (0.21–8.29)
009:01	2	1.47	0	0.00	2	2.17	NS	0.41 (0.02–8.66)	0	0		

NS, not significant; CI, confidence interval; OR, odds ratio.

Table 3. Allele frequencies of the dog leukocyte antigen gene complex in 22 Chihuahuas with meningoencephalomyelitis of unknown origin and 46 controls: DQB1

DLA-DQB1	Total number of alleles		Number of alleles						Number of homozygous dogs			
	2n=136	%	MUO 2n=44	%	Control 2n=92	%	*P* value	OR (95%CI)	MUO n=2	Control n=5	*P* value	OR (95%CI)
001:01	2	1.47	0	0.00	2	2.17	NS	0.41 (0.02–8.66)	0	0		
002:01	7	5.15	4	9.09	3	3.26	NS	2.97 (0.63–13.88)	0	0		
005:01	2	1.47	0	0.00	2	2.17	NS	0.41 (0.02–8.66)	0	0		
007:01	5	3.68	3	6.82	2	2.17	NS	3.30 (0.53–20.47)	0	0		
008:01:1	32	23.53	8	18.18	24	26.09	NS	0.63 (0.26–1.54)	0	3	NS	0.14 (0.00–4.47)
008:02	10	7.35	2	4.55	8	8.70	NS	0.50 (0.10–2.46)	0	0		
013:02	1	0.74	1	2.27	0	0.00	NS	6.38 (0.25–159.90)	0	0		
013:03	10	7.35	2	4.55	8	8.70	NS	0.50 (0.10–2.46)	0	0		
013:03 + 017:01	9	6.62	4	9.09	5	5.43	NS	1.74 (0.44–6.83)	1	0	NS	11.00 (0.28–434.20)
013:04	4	2.94	1	2.27	3	3.26	NS	0.69 (0.07–6.83)	0	0		
020:01	6	4.41	1	2.27	5	5.43	NS	0.40 (0.05–3.57)	0	0		
020:02	9	6.62	4	9.09	5	5.43	NS	1.74 (0.44–6.83)	0	1	NS	0.60 (0.02–21.00)
022:01	17	12.50	2	4.55	15	16.30	NS	0.24 (0.05–1.12)	0	1	NS	0.60 (0.02–21.00)
023:01	**9**	**6.62**	**7**	**15.91**	**2**	**2.17**	**0.0053**	**8.51 (1.69–42.93)**	**1**	**0**	**NS**	**11.00 (0.28–434.20)**
026:01	7	5.15	3	6.82	4	4.35	NS	1.61 (0.34–7.53)	0	0		
028:01	4	2.94	1	2.27	3	3.26	NS	0.69 (0.07–6.83)	0	0		
057:01	2	1.47	1	2.27	1	1.09	NS	2.12 (0.13–34.67)	0	0		

Seventeen DLA-DQB1 alleles were found in the study population. DLA-DQB1*023:01 has a greater frequency in the MUO group than in the control group. DLA-DQB1*008:01:1 has an elevated frequency in both the MUO and control groups. Statistical significance was established at $P<0.05$. MUO, meningoencephalomyelitis of unknown origin; DLA, dog leukocyte antigen (i.e., canine major histocompatibility complex); NS, not significant; CI, confidence interval; OR, odds ratio. Bolded entries indicate risk-associated alleles.

DLA-DQB1*008:01:1 (23.53%). Twenty-three haplotypes at three loci were estimated from all dogs (MUO and control) (Table 4). Four haplotypes that were found in a single dog were classified as "Other single haplotypes", and two haplotypes that could not be estimated were classified as "Unidentified haplotypes". The haplotype frequencies varied from 0.74% to 23.53%.

In the MUO group, the allele frequency of DLA-DQB1*023:01 was significantly greater than that of the control group (P=0.0053), with an OR of 8.51 (95% CI, 1.69–42.93). The number of dogs with DLA-DQB1*023:01 in the MUO group was also significantly greater than that in the control group (Supplementary Table 4). Only two haplotypes contained DLA-DQB1*023:01, haplotype 5 (Hp.5: DLA-DRB1*015:01--DQA1*006:01--DQB1*023:01) and haplotype 20 (Hp.20: DLA-DRB1*015:02--DQA1*006:01--DQB1*023:01), which were classified as "Other single haplotypes". Differences in the constituent alleles between the two haplotypes were only found in the DLA-DRB1 alleles. DLA-DRB1*015:01 and DLA-DRB1*015:02 differed by one amino acid (T90A) outside the hypervariable region in exon 2 (Fig. 1). No other alleles were significantly different between the MUO and control groups (Tables 1–3, Supplementary Tables 2–4).

In the MUO group, the haplotype frequency of Hp.5 was significantly greater than that of the control group (P=0.0141), with an OR of 7.11 (95% CI, 1.37–36.81). The number of dogs with Hp.5 was also significantly greater than that of the control group (Supplementary Table 5). In the MUO group, six of the 22 (27.27%) dogs had Hp.5, whereas in the control group two of the 46

Table 4. Frequencies of dog leukocyte antigen-DRB1–DQA1–DQB1 haplotypes in 22 Chihuahuas with meningoencephalomyelitis of unknown origin and 46 controls

Haplotype ID	Haplotypes DLA-DRB1–DQA1–DQB1	Total number of haplotypes		Number of haplotypes						Number of homozygous dogs			
				MUO		Control		*P* value	OR (95%CI)	MUO	Control	*P* value	OR (95%CI)
		2n=136	%	2n=44	%	2n=92	%			n=1	n=5		
Hp.1	009:01–001:01–008:01:1	32	23.53	8	18.18	24	26.09	NS	0.63 (0.26–1.54)	0	3	NS	0.24 (0.01–8.62)
Hp.2	015:01–006:01–022:01	16	11.76	2	4.55	14	15.22	NS	0.27 (0.06–1.22)	0	1	NS	1.00 (0.02–40.31)
Hp.3	012:01–004:01–013:03 + 017:01	9	6.62	4	9.09	5	5.43	NS	1.74 (0.44–6.83)	1	0	NS	33.00 (0.44–2,473.00)
Hp.4	015:01–006:01–020:02	9	6.62	4	9.09	5	5.43	NS	1.74 (0.44–6.83)	0	1	NS	1.00 (0.02–40.31)
Hp.5	**015:01–006:01–023:01**	**8**	**5.88**	**6**	**13.64**	**2**	**2.17**	**0.0141**	**7.11 (1.37–36.81)**	**0**	**0**		
Hp.6	020:01–004:01–013:03	7	5.15	1	2.27	6	6.52	NS	0.33 (0.04–2.86)	0	0		
Hp.7	015:01–006:01–026:01	7	5.15	3	6.82	4	4.35	NS	1.61 (0.34–7.53)	0	0		
Hp.8	015:01–006:01–020:01	6	4.41	1	2.27	5	5.43	NS	0.40 (0.05–3.57)	0	0		
Hp.9	015:01–001:01–008:02	5	3.68	0	0.00	5	5.43	NS	0.18 (0.01–3.31)	0	0		
Hp.10	018:01–001:01–008:02	4	2.94	2	4.55	2	2.17	NS	2.14 (0.29–15.75)	0	0		
Hp.11	006:01–005:01:1–028:01	4	2.94	1	2.27	3	3.26	NS	0.69 (0.07–6.83)	0	0		
Hp.12	017:02–002:01–013:04	4	2.94	1	2.27	3	3.26	NS	0.69 (0.07–6.83)	0	0		
Hp.13	001:01–001:01–002:01	4	2.94	3	6.82	1	1.09	NS	6.66 (0.67–65.99)	0	0		
Hp.14	006:01–005:01:1–007:01	4	2.94	3	6.82	1	1.09	NS	6.66 (0.67–65.99)	0	0		
Hp.15	113:01–002:01–013:03	3	2.21	1	2.27	2	2.17	NS	1.05 (0.09–11.87)	0	0		
Hp.16	015:01–006:01–057:01	2	1.47	1	2.27	1	1.09	NS	2.12 (0.13–34.67)	0	0		
Hp.17	002:01–009:01–001:01	2	1.47	0	0.00	2	2.17	NS	0.41 (0.02–8.66)	0	0		
Hp.18	013:01–003:01–005:01	2	1.47	0	0.00	2	2.17	NS	0.41 (0.02–8.66)	0	0		
Hp.19	073:01–001:01–002:01	2	1.47	0	0.00	2	2.17	NS	0.41 (0.02–8.66)	0	0		
Other single haplotypes		4	2.94	3	6.82	1	1.09	NA	NA	0	0		
Unidentified haplotypes		2	1.47	0	0.00	2	2.17	NA	NA	0	0		

Twenty-three haplotypes were found in the study population. Hp.5 shows a greater frequency in the MUO group than in the control group. Hp.1 has an elevated frequency in both the MUO and control groups. Statistical significance was established at $P<0.05$. MUO, meningoencephalomyelitis of unknown origin; NS, not significant; CI, confidence interval; OR, odds ratio; NA, not applicable. Bolded entries indicate risk-associated haplotypes.

(4.35%) dogs had Hp.5. We found no dogs homozygous for Hp.5 in either group. No other haplotypes were significantly different between the MUO and control groups (Table 4, Supplementary Table 5).

DISCUSSION

In our study, Chihuahuas in the MUO group had higher allele and haplotype frequencies than the control group: DLA-DQB1*023:01 and Hp.5 (DLA-DRB1*015:01--DQA1*006:01--DQB1*023:01), respectively, and the results suggest that DLA-DQB1*023:01 and Hp.5 might be risk factors for MUO in Chihuahuas. Hp.20, which was the only haplotype other than Hp.5 that included DLA-DQB1*023:01, was detected in one Chihuahua in the MUO group. The difference between the two haplotypes was entirely due to a difference in the composition of the DLA-DRB1 alleles. DLA-DRB1*015:01 and DLA-DRB1*015:02 differed by only one base and one amino acid, and these were outside the immunologically important hypervariable region [47]. In humans, structural and functional changes that relate to the antigen presentation of histocompatibility complex molecules can reportedly be caused by slight changes

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               I--HVR1--I           I----HVR2----I               I-------HVR3--------I
                 10          20          30          40          50          60          70          80          90
DRB1*015:01 HFLEM VKFECHFTNG TERVRLLVRD IYNREEHVRF DSDVGEYRAV TELGRPDAEY WNGQKELLEQ RRAEVDTVCR HNYGVIESFT VQRR
DRB1*015:02 ----- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------A ----
```

Fig. 1. Amino acid alignment for dog leukocyte antigen-DRB1*015:01 and dog leukocyte antigen-DRB1*015:02 alleles. DLA-DRB1*015:01 and DLA-DRB1*015:02 differed by only one amino acid (T90A) outside the hypervariable regions of exon 2. DLA, dog leukocyte antigen (i.e., canine major histocompatibility complex); HVR, hypervariable region (gray boxes).

in the sequence of the hypervariable regions [15, 24]. It is possible that Hp.20 has an immunological effect similar to that of Hp.5 in Chihuahuas with MUO.

The haplotype identified in the present study, Hp.5, has been reported as a risk factor for hypoadrenocorticism in Cocker Spaniels, Springer Spaniels, and male Standard Poodles [36, 55], as well as for diabetes mellitus in several breeds [28]. It is presumed that Hp.5 may be a common genotype across breeds susceptible to immune diseases mediated by unknown mechanisms, and its presence in our results supports an immunogenetic basis for MUO in Chihuahuas.

Obvious differences were noted in the MUO-associated haplotypes between Pugs and Chihuahuas. In previous studies on Pugs, DLA class II polymorphism analysis indicated the presence of a risk haplotype (DLA-DRB1*010:01:1--DQA1*002:01--DQB1*015:01), a protective haplotype A (DLA-DRB1*015:02--DQA1*006:01--DQB1*023:01), and a protective haplotype B (DLA-DRB1*015:01--DQA1*006:01--DQB1*023:01) for the development of NME [3, 18, 44]. In our study, the risk haplotype for NME in Pugs was not detected. The protective haplotypes A and B matched with Hp.20 and Hp.5 of Chihuahuas in our study. A previous report on DLA class II polymorphisms across multiple breeds of dogs [30] indicated that the variations in DLA alleles and haplotypes are biased by breed, and the risk haplotype for NME in Pugs seems to be predominant in Pugs [30]. The observed difference in the risk haplotype suggests the possibility that the pathogenesis of MUO may differ between Chihuahuas and Pugs. In addition, intersections of risk and protective haplotypes have been reported previously: the risk haplotype (DLA-DRB1*015:01--DQA1*006:01--DQB1*003:01) for inflammatory bowel disease in German Shepherds [45] is protective against chronic enteropathy in French Bulldogs [40]. This may be due to linkage imbalances with other factors related to immunity near the DLA genes on chromosome 12 [4, 59]. For example, *IL17A*, which is located on chromosome 12 in dogs, specifies a cytokine produced by T helper 17 cells. It is reportedly associated with the development of autoimmune encephalomyelitis in mice [21]. Moreover, significant expression of *IL17R* mRNA has been observed in the brain tissues of dogs with granulomatous meningoencephalomyelitis [42]. Therefore, interleukin 17A might be another immune-related factor involved in the development of MUO. Because the RPE method was not used in the previous report ab out NME in Pugs [18], it is also possible that the protective haplotypes A and B appear to be protective due to the influence of a risk haplotype (DLA-DRB1*010:01:1--DQA1*002:01--DQB1*015:01), a strong risk factor. It is also possible that the potential protective effects of the protective haplotypes A and B in Pugs may be due to the restricted DLA diversity of this breed (six haplotypes), rather than to any functional significance [18, 30].

In Greyhounds, other haplotypes (haplotype C: DLA-DRB1*018:02--DQA1*001:01--DQB1*008:02; haplotype D: DLA-DRB1*015:01--DQA1*006:01--DQB1*022:01) have been detected as risk factors for NIME [52]. In the present study, the risk haplotype C was not detected. The risk haplotype D had a high frequency of detection here as haplotype 2, but was not significantly related to the development of MUO in Chihuahuas. A study on DLA class II polymorphisms across multiple breeds of dogs [30] reported that the risk haplotype C appeared to be predominant in Greyhounds, similar to the risk haplotype in Pugs with NME. Because of the difference in the risk haplotype, it is possible that the pathogenesis of NIME may also differ between Chihuahuas and Greyhounds.

In a previous report on Pugs with NME, homozygosity for the risk haplotype was associated with an increased risk for disease (OR 12.75) compared with heterozygosity (OR 1.13) [18]. Genetic analyses of NME associations in Pugs are commercially available, primarily to aid breeding programs [44], because it is possible to avoid forming the homozygous risk haplotype and thus reduce the likelihood of NME in Pugs. In the present study, Hp.5 was identified in 27.27% of the MUO group, and all cases were heterozygous. Therefore, it will be difficult to exclude the risk haplotype from Chihuahua breeding programs, because complete exclusion would result in a significant loss of genome-wide genetic diversity and possibly other negative consequences [18]. Therefore, to establish a genetic analysis method to aid the breeding of Chihuahuas without MUO, similar to Pugs and NME, it will be necessary to identify other genetic risk factors by referring to reports focusing on other breeds of dogs with MUO and on humans. In the medical field, major histocompatibility complex class I genes have been shown to be associated with neuro-Behçet's and neuro-Sweet's diseases, which are immune-mediated diseases that induce inflammation in the brain [56]. The same method of analysis is desirable for MUO in Chihuahuas.

Although several reports have analyzed DLA-DRB1, DLA-DQA1, and DLA-DQB1 alleles and haplotypes in various dog breeds [27, 29, 30], no coherent reports have focused on Chihuahua. This study is the first to reveal DLA-DRB1, DLA-DQA1, and DLA-DQB1 alleles and haplotypes in Chihuahuas. Notably, the haplotypes we identified in Chihuahuas were more varied (23 kinds) than those in other breeds reported so far (i.e., Labrador Retriever, 13; Beagle, 9; Newfoundland, 8; Doberman, 7; English Setter, 6; and Boxer, 5) [30]. Additionally, it is also notable that the overall homozygosity percentages for each locus in our Chihuahuas (22.1% for DLA-DRB1, 30.9% for DLA-DQA1, and 10.3% for DLA-DQB1) were lower than those reported in other breeds (i.e., American Akita, 29.7% for DLA-DRB1, 37.2% for DLA-DQA1, 26.6% for DLA-DQB1; German Shepherd, 46.7% for DLA-DRB1, 46.7% for DLA-DQA1, 26.7% for DLA-DQB1; Weimaraner, 39.3% for DLA-DRB1, 50.5% for DLA-DQA1, 21.4% for DLA-DQB1) [2]. The

reasons the Chihuahua breed has so many haplotypes and has low homozygosity for each locus are unknown. The low homozygosity of Chihuahuas may be due to their history of being transported from Mexico to the United States, where they may have been crossed with other small dogs [6, 57]. More detailed polymorphism analyses, including analysis of DLA class I genes in a large sample of Chihuahuas, are necessary to understand Chihuahua DLA diversity and immune response in the context of the major histocompatibility complex.

This study had several limitations. First, MUO sub-types were not diagnosed, as they must be distinguished histologically. Making classifications based on histopathological examination is a challenge that should be addressed in future studies. Second, the dog population included this study seemed somewhat small, despite the results indicating that Chihuahuas have more haplotypes than other breeds of dog. Nevertheless, intended our study to be a pilot study analyzing DLA alleles and haplotypes in Chihuahuas with and without MUO. Finally, we were not able to use an appropriate analysis software, e.g., SBTengine (Gendx, Utrecht, The Netherlands), due to research funding constraints. Therefore, our results may contain some small estimation errors.

CONFLICT OF INTEREST. We report no conflicts of interest related to this study.

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