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Major histocompatibility complex and other allergy-related candidate genes associated with insect bite hypersensitivity in Icelandic horses

Marie Klumplerova · Leona Vychodilova · Olga Bobrova · Michaela Cvanova · Jan Futas · Eva Janova · Mirko Vyskocil · Irena Vrtkova · Lenka Putnova · Ladislav Dusek · Eliane Marti · Petr Horin

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Abstract Insect bite hypersensitivity (IBH) is an allergic dermatitis of horses caused by bites of insects. IBH is a multifactorial disease with contribution of genetic and environmental factors. Candidate gene association analysis of IBH was performed in a group of 89 Icelandic horses all born in Iceland and imported to Europe. Horses were classified in IBH-affected and non-affected based on clinical signs and history of recurrent dermatitis, and on the results of an in vitro sulfidoleukotriene (sLT)-release assay with *Culicoides nubeculosus* and *Simulium vittatum* extract. Different genetic markers were tested for association with IBH by the Fisher's exact test. The effect of the major histocompatibility complex (*MHC*) gene region was studied by genotyping five microsatellites spanning the MHC region (*COR112*, *COR113*, *COR114*, *UM011* and *UMN-JH34-2*), and exon 2 polymorphisms of the class II *Eqca-DRA* gene. Associations with *Eqca-DRA* and *COR113* were identified ($p < 0.05$). In addition, a panel of 20 single nucleotide polymorphisms (SNPs) in 17 candidate allergy-related genes was tested. During the initial screen, no marker from the panel was significantly ($p < 0.05$) associated with IBH. Five SNPs associated with IBH at $p < 0.10$ were therefore used for analysis of combined genotypes. Out of them, SNPs located in the genes coding for the CD14 receptor (*CD14*), interleukin 23 receptor (*IL23R*), thymic stromal lymphopoietin (*TSLP*) and transforming growth factor beta 3 (*TGFB3*) molecules were associated with IBH as parts of complex genotypes. These results are supported by similar associations and by expression data from different horse populations and from human studies.

Keywords Horse · Insect bite hypersensitivity · Major histocompatibility complex · Association analysis

M. Klumplerova · L. Vychodilova · O. Bobrova · J. Futas · E. Janova · M. Vyskocil · P. Horin
Institute of Animal Genetics, Faculty of Veterinary Medicine, University of Veterinary and Pharmaceutical Sciences, Palackeho tr. 1/3, 61242 Brno, Czech Republic

M. Klumplerova · P. Horin (✉)
Ceitec VFU, University of Veterinary and Pharmaceutical Sciences, Palackeho tr. 1/3, 61242 Brno, Czech Republic
e-mail: horin@dior.ics.muni.cz

M. Cvanova · L. Dusek
Institute of Biostatistics and Analyses, Masaryk University, Brno, Czech Republic

I. Vrtkova · L. Putnova
Laboratory of Agrigenomics, Mendel University, Brno, Czech Republic

E. Marti
Department of Clinical Research-VPH, Vetsuisse Faculty, University of Berne, Länggass-strasse 124, 3001 Berne, Switzerland

Introduction

Insect bite hypersensitivity (IBH) is an important disease of the horse [1–3], caused by IgE-mediated reactions against salivary proteins from midges (genus *Culicoides*) and sometimes black flies (*Simulium* spp.). The mechanisms resulting in susceptibility or resistance to IBH still remain unclear. IBH is the result of a complex interplay of environmental and genetic factors, as illustrated by an interesting phenomenon. Due to the absence of *Culicoides* spp., IBH does not occur in Iceland. However, when Icelandic horses are imported to continental Europe and get exposed to *Culicoides*, they develop IBH with a much higher prevalence (50 %) than horses of this breed born in the same environment (3–10 %). IBH shares some similarities

with human atopic dermatitis [3]. In vitro degranulation of basophils stimulated with *Culicoides* allergens and determination of released sulfidoleukotrienes (sLT) can be used as an in vitro diagnostic test for IBH [4].

The fact that many horses exposed to *Culicoides* remain healthy under the same environmental conditions suggests that genetic factors can influence susceptibility to IBH. In recent studies, the heritability for IBH was estimated to range from 0.07 to 0.30 [5, 6]. Earlier studies have shown that horses from some families are more susceptible than others indicating that specific genes can contribute to disease susceptibility [7]. Major histocompatibility complex (*MHC*) class II genes were reported to be associated with IBH in different breeds [8, 9]. In humans, further chromosome regions and specific genes were found to be associated with allergies in general as well as with atopic dermatitis [10]. In a group of horses belonging to another breed and living in a different environment, we have identified genes coding for interferon gamma (IFNγ), transforming growth factor beta 1 (TGFβ1), involucrin (Ivl), Janus kinase 2 (JAK2), thymic stromal lymphopoietin (TSLP) and CD14 lymphocyte receptor (CD14) either associated with IBH or differentially expressed in the skin, or both [Vychodilova et al., Vet Immunol Immunopathol accepted].

Several approaches may be used for detecting genes contributing to resistance/susceptibility to diseases. Genome-wide association studies (GWAS) using large numbers of anonymous SNPs distributed over the genome are often used for this purpose [11]. As they do not always allow identification of specific genes, candidate gene studies can be a useful complementary tool. As compared to humans, domestic animal populations bred for a long time are more homogenous and more informative, especially for clinically oriented association studies, but with limited numbers of cases available. Icelandic horses exported to Europe represent an interesting model of IBH [2].

The aim of this work was to investigate associations of the *MHC* region and to identify genotypes in candidate gene SNPs associated with susceptibility to IBH in Icelandic horses imported from Iceland.

Materials and methods

The population and the disease

Eighty-nine horses of the Icelandic breed born in Iceland with no common parents and imported to Switzerland were used for the study. The horses had been living in Switzerland for at least 4 years to exclude that IBH susceptible horses that had not yet developed clinical signs of IBH would be included in the non-affected group. Most of the horses developed IBH within 2 years after import [12]. The non-affected horses were selected randomly from the same stables where IBH-affected horses were maintained. Clinical manifestation of IBH was assessed as described previously [4], based on clinical signs and history of recurrent dermatitis during summer. Furthermore, an in vitro sLT release test was used to confirm the diagnosis of IBH and to exclude horses that were sensitized to *Culicoides* allergens but had not (yet) shown clinical signs from the control group. All horses were sampled and tested during the summer season. The in vitro sLT release test was performed as described by Baselgia et al. [4]. Briefly: peripheral blood leukocytes were isolated and incubated with Concanavalin A as positive control, *Culicoides nubeculosus* and *Simulium vittatum* extracts and with buffer alone to determine the spontaneous sLT release. After 40 min incubation at 37 °C, supernatants were collected and released sLTs determined by ELISA in the cellular allergen stimulation test (CAST, Bühlmann laboratories, http://www.buhlmannlabs.ch/) following the manufacturers instructions. The cut-off described in Baselgia et al. [4] was used to classify the results as positive or negative test result. Only horses with a negative test result with *C. nubeculosus* ("CAST-*Cul*") were selected as control horses ($n = 43$) and all horses included in the IBH-affected group ($n = 46$) had a positive test result with this allergen. We know from previous studies that the CAST with *S. vittatum* has a lower sensitivity and specificity for diagnosis of IBH. These results ("CAST-*Sim*") were thus not used to confirm the diagnosis of IBH but were used as an additional phenotype. 42 out of the 46 IBH-affected horses gave a positive "CAST-*Sim*"result and seven out of the 43 non affected horses were positive with this extract. There were thus in total 49 horses with a positive and 40 horses with a negative "CAST-*Sim*" test results, respectively.

Major histocompatibility complex markers

One *ELA* class II gene and five *MHC*-linked microsatellite markers were used for analyzing associations with the *MHC* (Fig. 1). A PCR-RFLP genotyping system of *Eqca-DRA* exon 2 alleles was developed based on the GenBank sequences available. Amplification of the 307 bp long product was carried out with standard primers Be3 and Be4 [13]. Aliquots of PCR products were digested separately with restriction enzymes Cac8I, Hpy166II, BsaJI and produced specific fragments allowing identification of alleles *Eqca-DRA*0201* (M60100), *Eqca-DRA*0301* (L47172), *Eqca-DRA*0401* (AJ575295) and *Eqca-DRA*0501* (FJ716134). The remaining allele *Eqca-DRA*0101* (L47174) could be identified by manual editing, subtracting sequences of the other alleles previously identified by PCR-RFLP. The nomenclature suggested by us previously [14] was used for designating *Eqca* alleles.

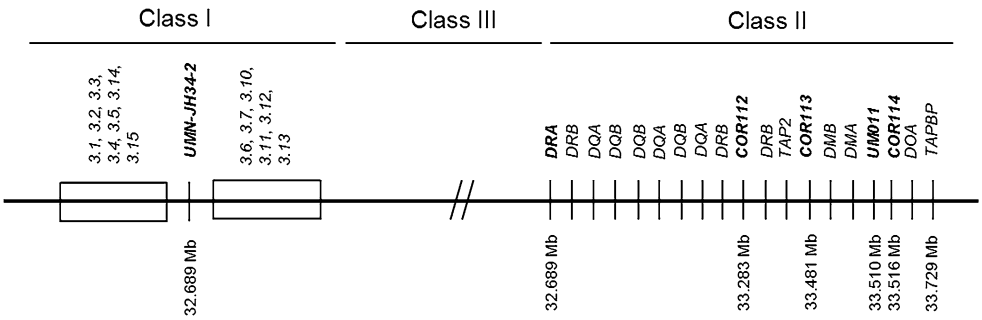

Fig. 1 MHC markers used for association analysis (in bold)

Length variations of five microsatellite markers located in the class I and class II regions of the horse *MHC* (*COR112*, *COR113*, *COR114*, *UM011* and *UMN-JH34-2*) were determined using an ABI PRISM 310 automated sequencer (Applied Biosystems, Foster City, CA, USA) based on primers and procedures published [15].

Single nucleotide polymorphism markers in allergy-related genes

SNPs in genes encoding the CD14 receptor (*CD14*), Fc fragment of the IgE receptor alpha chain (*FcER1A*), Interferon gamma (*IFNG*), Immunoglobulin heavy chain epsilon-like (*IGHE*), Interleukin 4 (*IL4*), Interleukin 4 receptor (*IL4R*), Interleukin 10 (*IL10*), Interleukin 13 (*IL13*), Interleukin 17 (*IL17A*), Interleukin 17 receptor (*IL17AR*), Interleukin 23 receptor (*IL23R*), Involucrin (*IVL*), Tyrosine-protein kinase JAK2-like (*JAK2*), Thymic stromal lymphopoietin (*TSLP*), Toll-like receptor 4 (*TLR4*), Transforming growth factor beta 1 (*TGFB1*), Transforming growth factor beta 3 (*TGFB3*) were studied (Table 1). Markers were identified by searching the Broad institute SNP database (http://www.broad.mit.edu/mammals/horse/) or newly developed by resequencing of pooled DNA samples from horses of the population under study (usually from 5 to 10 horses). Horse-specific primers were designed using Primer3 software (http://fokker.wi.mit.edu/primer3/) on annotated genes from the NCBI horse genome sequence assembly EcuCab2.0 (http://www.ncbi.nlm.nih.gov/mapview/map_search.cgi?taxid=9796) or genes predicted by BLAST search of horse genome for defined human/animal counterparts. Sequences with double or multiple peaks were aligned and analysed by the BioEdit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and putative restriction enzyme cleavage sites were assigned by NEBcutter V2.0 (http://tools.neb.com/NEBcutter2/index.php). SNP markers were then genotyped by PCR-RFLP by using restriction enzymes (New England Biolabs, Ipswich, MA, USA) corresponding to polymorphic sites. The fragments generated were run for 90 min at 300 V in 6 % PAGE and silver stained.

Data analysis

Association analysis was performed in IBM SPSS Advanced Statistics 19 for Windows (Release 19.0.1, © IBM Corporation 2010). Identification of host genotypes associated with the results of the CAST test was performed in two steps. After an initial screen allowing identification of individual markers potentially associated with IBH at $p_{raw} < 0.10$, a pairwise analysis of interactions of the markers identified was performed. All possible genotypes composed of individual marker variants were tested by binary logistic regression analysis to assess individual contributions of markers and of pairwise statistical interactions. The resulting odds ratios and confidence intervals were calculated based on a standard two-sided Fisher's exact test and Fisher's exact test based on Monte Carlo estimates (100,000 samples) of exact significance [16, 17]. Microsatellite alleles with frequencies <0.1 were pooled for this analysis. Bonferroni corrections for multiple comparisons were used based on [18], separately for different types of markers. *MHC*-linked multi-allelic microsatellite markers, the MHC *Eqca-DRA* expressed gene with multiple-SNP haplotypes, and independently segregating bi-allelic SNP markers are genetically different types of loci. Therefore, their associations with IBH were studied separately. Bonferroni corrections for multiple comparisons thus were made separately for five *MHC* microsatellites, three exon two alleles of *Eqca-DRA* and 20 candidate gene SNP markers. In specific genotypes, Bonferroni corrections were made for the numbers of genes involved in combined genotypes.

Results

MHC markers

Eqca-DRA was associated with CAST-*Cul* status ($p_{raw} = 0.012$, $p_{corr} = 0.036$). Genotypes with *Eqca-DRA*0501* were enriched in CAST-negative horses ($f = 0.390$) compared to cases ($f = 0.136$). For CAST-*Sim*, p_{raw} for

Table 1 Non-MHC candidate gene SNP markers used for analysis of associations with insect bite hypersensitivity

Candidate gene	Gene symbol	SNP genome position (*marker name used*)	SNP genome position (chromosome)	Type of marker	Primers 5′–3′ forward/ reverse
Fc fragment of IgE receptor, alpha chain	*FCER1A*	NW_001867419 G 1417759 A (*FcER1A*)	Eca5 38124382 G>A	Intronic	gtgccgtggctggaaggat/ gccaggaagaaattgctgttgc
Ig heavy epsilon chain	*IGHE*	NW_001876796 T 754996 C (*IGHE*)	Eca24 754996 T>C	Exonic	gtctccaagcaagccccatta/ tttaccagggtctttggacacctc
Interferon gamma	*IFNG*	NW_001867424 G 24587636 C (*IFNG/a*) G 24585624 C (*IFNG/b*)	Eca6 83346753 C>G 83344741 C>G	Intronic intronic	tactctggaactcagtcaattgctgaga/ gaaatggattctgactcctcttc
Interleukin 10	*IL10*	NW_001867416 A 2988098 G (*IL10*)	Eca5 2988098 A>G	Intronic	tctgccctgtgaaaataagagc/ tgtcaaactcactcatggcttt
Interleukin 13	*IL13*	NW_00186737 A 42920818 G (*IL13*)	Eca14 42920818 A>G	Intronic	cctgacccctctagagacctg/ acaggctgaggtccaagcta
Interleukin 17 receptor	*IL17RA*	NW_001867423.1 T 921254 C (*IL17RA*)	Eca6 27987884 T>C	3′UTR	gcaggcacacacctaaacct/ ggggacagaagatgaccaga
Interleukin 17A	*IL17A*	NW_001867389.1 T 49864780 C (*IL17A*)	Eca2049864780 T>C	Intronic	ctctctccttgcccattcag/ ggctgtcctgtgtcctatca
Interleukin 23 receptor	*IL23R*	NW_001867420.1 C 44757430 T (*IL23R*)	Eca5 93803835 C>T	Promotor	ttgaaaaggcagaacagaattt/ cctccatgacaccaactgaa
Interleukin 4	*IL4*	NW_00186737 G 42902615 A (*IL4*)	Eca14 42902615 G>A	Intronic	ccttgatcaaagaatgcctga/ tccaaaggccctgtgtaatc
Interleukin 4 receptor	*IL4R*	NW_001867375 G 2398183 A (*IL4R*)	Eca13 20794503 G>A	Exonic	cttcttccccctttaggaagtg/ gagttctgagggctgtgaggt
Involucrin	*IVL*	NW_001867419 C 44674251 T (*IVL/a*)	Eca5 44674251 C>T	Intronic	cagcacattctgccagtgac/ taatgctgctgctgctgttt
Janus kinase 2	*JAK2*	NW_001867420 T 26565472 C (*JAK2*)	Eca23 26565472 T>C	Intronic	ggggttaagaacaaggtgga/ tgtggaacccataaagctctg
LPS receptor molecule	*CD14*	NW_001867377 A 36268808 G (*CD14/a*) C 36269096 T (*CD14/b*)	Eca14 36268808 A>G 36269096 C>T	5‘UTR intronic	gagcctgagtcatcaggacattgc/ tggcttccaggctccacaca cgcagctctttccagagtcca/ cggaagttctcatcgtccacct
Thymic stromal lymphopoietin	*TSLP*	NW_001867377 C 66624113 G (*TSLP*)	Eca14 66624113 C>G	Intronic	gctggatgagaccgcagtccc/ gctgctcctcgtcagcatttgc
Toll-like receptor 4	*TLR4*	NW_001867396 A 21975271 G (*TLR4/a*) G 21975144 A (*TLR43/b*)	Eca25 21975271 A>G 21975144 G>A	Exonic exonic	ggcctcaaccatctctccacct/ ccacggtttaccatccagcaag
Transforming growth factor beta1	*TGFB1*	NW_001867363 C 11894258 A (*TGFB1*)	Eca10 11894258 C>A	Intronic	ttgactttcgcaaggatctg/ ggttgtgctggttgtacagg
Transforming growth factor beta3	*TGFB3*	NW_001867395 G 21504080 A (*TGFBR3*)	Eca24 21504080 G>A	Exonic	ggaaaaagtgtggctttcca/ tgatccaagattccccaaaa

*Eqca-DRA*0501* was 0.024, corresponding to p_{corr} 0.096. No microsatellite genotype was significantly associated with CAST-*Cul*/*Sim* after Bonferroni corrections and differences in microsatellite allelic frequencies ranged between 0.1 and 0.05. The *COR113* allele 257 was associated with negative results of the CAST test (Table 2). When only genotypes with selected resistance and/or susceptibility associated alleles were compared against each other, significant differences were observed for all loci. The strongest effect was observed for *UM011*. In 57 horses, the frequencies of genotypes with alleles 166 or 170 compared to genotypes carrying alleles 168 or 180 differed between cases and controls at $p = 0.0006$, while p values ranging from 0.045 to 0.006 were found for selected genotypes in *COR112*, *COR113*, *COR114* and *UMN-JH34-2*.

Interactions of different markers in composed genotypes could not be analyzed due to low numbers of animals with relevant combinations of alleles. Only the genotype *Eqca-DRA*0501/COR113 257* carrying two “resistant” alleles in both loci was informative. No carrier of this genotype was found among 46 CAST-*Sim* cases, while seven carriers were identified in the control group ($p_{raw} = 0.014$, corrected for two markers $p = 0.028$). No associations with heterozygosity in the *MHC*-linked markers were found.

Table 2 Associations between *MHC-ELA* markers and IBH in Icelandic horses at $p_{raw} < 0.100$ ($N = 85$)

Marker	Position	DA	Alleles tested	*Cul/Sim*	*f*(DAC) CAST positive	*f*(DAC) CAST negative	p_{raw}	p_{corr}	OR (CI)	*p* value (raw/corr) for differences in f(DA)
DRA	32690939	*DRA*0501*	3	Cul	0.136	0.390	0.012	**0.036**	0.247 (0.085–0.716)	0.017/0.051
DRA	32690939	*DRA*0501*	3	Sim	0.152	0.385	0.024	0.072	0.287 (0.102–0.805)	0.027/0.081
COR113	33480825	257	11	Cul	0.068	0.220	0.023	0.115	0.260 (0.065–1.040)	NS
COR113	33480825	257	11	Sim	0.043	0.256	0.020	0.100	0.152 (0.031–0.751)	0.004/**0.020**
COR113	33480825	253	11	Sim	0.283	0.103	0.039	0.195	3.447 (1.020–11.645)	0.021/0.105
UM011	33510120	170	10	Cul	0.023	0.195	0.013	0.065	0.960 (0.011–0.805)	0.015/0.075
UM011	33510120	170	10	Sim	0.022	0.205	0.013	0.065	0.074 (0.009–0.615)	NS

N number of horses, *DA* disease-associated allele, *Cul/Sim Culicoides/Simulium*, *f(DAC)* frequency of DA carriers, *OR(CI)* odds ratios (confidence interval), *f(DA)* frequency of DA, *NS* not significant before correction

Significant *p* values in bold

Allergy-related candidate gene SNPs

None of the SNP markers was significantly associated with CAST status at $p = 0.05$ (Fisher's exact test). Five statistically significant SNPs at $p = 0.1$ were then used for analysis of composed genotypes: *TSLP*, *IVL*, *CD14*, *IL23R* and *TGFB3* (Table 3). Out of all possible combinations tested, associations of genotypes composed

Table 3 Markers involved in composed genotypes ($p_{raw} < 0.100$)

Significant SNP	Genotypes/ alleles associated	*p* value for "*CAST-CUL*"	*p* value for "*CAST-SIM*"	Associated with
TSLP	GC	NT	0.057	Susceptibility
	CC			Resistance
IVL	TT	0.171 (allele T: 0.087)	–	Susceptibility
	CC+TC			Resistance
CD14/b	TC	0.086	0.073	Resistance
	CC			Susceptibility
IL23R	TT	0.191 (allele T: 0.099)	–	Resistance
	CC+CT			Susceptibility
TGFB3	AA	0.110 (allele: 0.053)	0.090	Resistance
	GG+AG			Susceptibility

Genotypes/alleles associations tested both for genotypes and/or alleles, *NT* not tested due to $p > 0.05$ for all individual markers

Table 4 Significant ($p_{corrected} < 0.05$) associations of composed genotypes with CAST status

Genotypes associated[a]	*p* values for CAST-*Cul* (p_{raw}/p_{corr})	*p* values for CAST-*Sim* (p_{raw}/p_{corr})	Odds ratios (CI 95 % OR)	Associated with
TSLP CC–*TGFB3*AA	NT	**0.018/0.036**	NC	Resistance
CD14/b CC–*IL23R* non-TT	**0.021/0.042**	NT	3.766 (1.218; 11.640)	Susceptibility
CD14/b CC–*TGFB3* non-AA	**0.021/0.042**	**0.009/0.018**	5.161 (1.339; 19.895)—*Cul* 6.143 (1.590; 23.736)—*Sim*	Susceptibility

CI 95 % OR confidence interval of odds ratios, *NT* not tested due to $p > 0.1$ for all individual markers, *NC* not calculable due to null values

[a] No composed genotype with *IVL* was significantly associated with IBH

Significant *p* values in bold

Table 5 Values of population linkage disequilibrium—LD (coefficient of correlation/*p*) among MHC-linked markers in Icelandic horses

UMN-JH34-2	xxxxx					
DRA	0.089/0.218	xxxxx				
COR112	0.098/**0.0001**	0.146/**0.0001**	xxxxx			
COR113	0.090/**0.001**	0.138/**0.0003**	0.134/**0.0001**	xxxxx		
UM011	0.094/**0.001**	0.131/**0.0002**	0.150/**0.0001**	0.182/**0.0001**	xxxxx	
COR114	0.108/**0.0001**	0.155/**0.0001**	0.162/**0.0001**	0.198/**0.0001**	0.208/**0.0001**	xxxxx
	UMN-JH34-2	*DRA*	*COR112*	*COR113*	*UM011*	*COR114*

Significant *p* values in bold

of *CD14/TGFB3, CD14/IL23R* and *TSLP/TGFB3* withstood corrections for multiple testing. No combined genotype containing *IVL* reached statistical significance (Table 4).

Discussion

Classification of the phenotypes observed is crucial for interpretation of the results of association analyses. We classified horses as resistant or susceptible to IBH based on the results of a cellular allergy test additionally to the clinical diagnosis. The sLT-release assay with *C. nubeculosus* extract had been shown previously to have a relatively good sensitivity (78 %) and very high specificity (97 %) for diagnosis of IBH [4]. This was important for the selection of the non-affected horses, as we wanted to exclude horses that were sensitized to *Culicoides* but had not (yet) developed clinical signs of IBH. On the other hand, the use of a positive sLT-release test results for selection of the IBH cases allowed including only horses with IBH caused by IgE-mediated reactions. In some cases, IBH may be caused by cell-mediated type IV hypersensitivity reactions [3].

Even when GWAS are available, studies of individual candidate loci can be useful for formulating more precise hypotheses on mechanisms of the disease studied [19, 20]. For equine IBH, this was confirmed by Andersson et al. [9]. Here, candidate genes were selected according to their role in immune responses and in allergic reactions as reported in horses and other species. The *MHC* region was shown to be associated with IBH in horses [8, 9]. The set of cytokines and of genes expressed in the skin (*IVL* and *TSLP*) was composed based on literary data and on results of our study on Old Kladruby horses where associations and/or differences in gene expression were found [Vychodilova et al., Vet Immunol Immunopathol accepted].

As compared to humans, domestic animal populations bred for a long time are more homogenous and more informative, especially for clinically oriented association studies with limited numbers of cases available. Icelandic ponies are an old breed, highly susceptible to IBH when imported to continental Europe [2, 12]. This is probably due to the fact that these horses are exposed to new allergens as adults [3]. However, the fact that about 50 % of these horses remain free of IBH even though subjected to the same environmental changes suggests that genetic factors also influence susceptibility to IBH in this group of horses.

Horses born in Iceland and imported to Switzerland were included in our study. Environmental influences could thus dilute the effect of genetic variation and consequently the power of the association analysis. However, it was our aim to investigate whether besides the environmental influences genetic factors contributing to susceptibility for IBH could be identified also in this group. Significant associations withstanding all over-conservative Bonferroni corrections confirmed this hypothesis. Since rather weak genetic effects could be anticipated, there is no reason to expect false positive results due to this approach. Similar associations for the *MHC-DRA* gene and for the *COR113* marker in Icelandic horses born and living in Sweden [9] support this conclusion.

However, false negative results must be considered. Only 89 horses living in a similar environment in terms of similar exposure to *Culicoides*, diagnosed with the same diagnostic method during the same season, belonging to the same breed but with no common parents, were available. This is a limitation of this study, like in many of clinical field studies on large domestic mammals. It is likely that the group analyzed did not allow detecting effects of further markers on IBH and negative results of this study cannot be interpreted as lack of association.

We confirmed effects of *ELA* class II markers observed previously in two distinct horse populations [9]. Due to within-breed variation, it is not surprising that the markers and alleles associated were not the same like in the populations analyzed. The LD values among markers (Table 5) are in agreement with their distances on *ECA20* (Fig. 1). Our data thus support the view that the *ELA* class II region is associated with clinical and cellular processes related to equine IBH across breeds and populations. It is not clear whether differences observed between CAST-*Cul* and

CAST-*Sim* reflect low numbers of cases analyzed or the specificity of allergic reactions.

Although Icelandic horses represent a model population for IBH investigations, no study analyzed non-MHC immunity-related candidate gene SNP markers associated in this context. Analysis of pairwise interactions in combined genotypes showed increased odds ratios and p values suggesting possible cumulative effects of selected genotypes/alleles. Only single markers associated at $p_{raw} < 0.05$ were used to increase the probability of finding p values significant after all corrections. Complex genotypes thus allowed identification of synergic interactions of genes with weak individual effects. This approach was used recently e.g. for analyzing interactions between markers underlying complex mechanisms of susceptibility to human Crohn's disease [21].

Support for biological plausibility of the results of the candidate gene study comes from two sides. First, the same *TSLP* marker (although through different alleles) was associated with clinical IBH in a genetically distinct population of Old Kladruber horses. [Vychodilova et al., Vet Immunol Immunopathol accepted]. As the SNP analyzed is an intronic *TSLP* marker, it is not supposed to have a direct effect on the phenotype. Second, in the same study, differences in gene expression in skin biopsies from affected and unaffected horses were observed for *CD14*. Statistical evidence for its association with IBH in Icelandic horses thus represents complementary information supporting the results obtained in Kladruber horses.

Data from human and animal studies also are in agreement with the assumption of biological plausibility of the associations observed. Immunity-related genes associated with IBH in this study were reported to be associated with atopic dermatitis in humans [22–24]. The TSLP-receptor gene was associated with atopic dermatitis in different dog breeds [25]. TSLP was shown to have a wide range of effects on cells of the immune system and on allergic inflammation [26]. Associations of atopic disease with *CD14* polymorphisms [24], supported by functional studies [27], were reported in humans. Besides immunity-related markers, genes related to skin structure and function were also associated with human AD [28]. Involucrin is a specialized cytoplasmatic protein of the keratinocytes, cells responsible for the structure of epidermis [29]. SNPs in the *IL23R* gene were associated with human psoriasis. It is known that genome regions associated with susceptibility to psoriasis, Crohn's disease and atopic dermatitis overlap in humans [30]. Futhermore, IL-23 signaling enhances Th2 polarisation [31] and Th2 cytokine production is increased in horses with IBH [32, 33]. Up-regulation of the *CD14* gene activity was observed in affected skin from human atopic dermatitis patients [27]. Transcription of *TGFB1* and *TGFB3* was enhanced in the regenerating epidermis and dermis in humans and pigs [34]. Expression of IL-10 and TGFβ1 molecules was found to be involved in IBH in horses [35].

Taken together, our results confirmed associations of *MHC* class II, *CD14* and *TSLP* markers with equine IBH observed in a different population. In addition, associations of IBH with two other immunity-related genes, *IL23R* and *TGFB3* were found. Genes associated with IBH correspond to those reported in human atopic dermatitis association studies. The results of statistical analyses supported by similar observations in genetically distinct populations and by gene expression data from another study suggest that the role of these genes in equine IBH merits to be further investigated.

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