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Identification of missense mutations in the *PCP4* and *CD109* genes to validate the effect of neutral genetic markers

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ABSTRACT: The marker-assisted selection exploits anonymous genetic markers that have been associated with measurable differences on complex traits. Because it is based on the linkage disequilibrium (LD) between the polymorphic markers and the polymorphisms which code for the trait, its success is limited to the population in which the association has been assessed. The identification of the gene with effect on the target trait and the detection of the functional mutations will allow selection in independent populations, while encouraging studies on gene expression. In a flock of sheep infected with *M. paratuberculosis*, a genome-wide scan, performed with the Illumina OvineSNP50 BeadChip, had identified two candidate genes, the *PCP4* and the *CD109*, located in proximity of the markers with significant allele substitution effect on the positivity level at paratuberculosis serological assessment. The coding region of the two genes was directly sequenced. Three missense mutations were detected: two in the *PCP4* gene and one in the second exon of the *CD109* gene, the latter showing a strong LD with the anonymous marker. Direct sequencing of the DNA of sheep of different populations showed that disequilibrium was maintained. Allele frequency at the hypothesized marker associated to immune response, calculated for other breeds of sheep, showed that the marker allele potentially associated to disease resistance is more frequent in the local breeds and in breeds that have not been submitted to selection programs.

Keywords: immune response; local sheep; genomics; genome-wide association

INTRODUCTION

The aim of marker-assisted selection (MAS) is to select specific DNA variations that have been associated with a measurable difference on complex traits. It is based on the linkage disequilibrium (LD) between the polymorphic markers and the polymorphisms which code for the trait. Therefore the marker must by necessity be close to the functional mutation for sufficient population-wide LD between the marker and the gene. Also for this reason, success of MAS has been limited in livestock and few studies reported substantial gains with real populations (Dekkers 2004). The possibility to detect the direct markers (i.e. polymorphisms that code for the functional mutations) opens the way to wider fields of applications, on one side because selection could be performed in independent populations, with no care of the LD extent; on the other side, the identification of the gene having major effect on the trait will allow a deeper knowledge of the function and the expression of the gene, as well as of the related gene network, and such knowlege might be of use also to other species and different target traits.

Paratuberculosis, or Johne's disease, is a chronic granulomatous enteritis caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), which af-

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fects ruminants. It causes substantially reduced production, higher susceptibility to acquire other diseases and infertility (Kennedy and Benedictus 2001). In a flock of MAP-infected sheep, Moioli et al. (2015) performed a Genome Wide Association Analysis (GWAS) with the OvineSNP50 BeadChip on 100 ewes representing the extreme divergent animals for the optical density (S/P) ratio obtained from MAP serological assay, assessed on about 700 sheep. The authors hypothesized the presence of 30 putative candidate genes of disease susceptibility, these genes being in close proximity of the polymorphic markers which showed a significant effect on the obtained serological value. While the majority of these genes did not appear to have a straightforward and recognizable effect on disease resistance, being responsible for basic cellular processes, two of them, the Purkinje Cell Protein 4 (*PCP4*), and the *CD109* genes appeared worth to be studied in more depth for their potential role in influencing the immune system. In fact, Jacobson et al. (2009), in the murine B cell co-receptor complex, demonstrated an increased expression of *PCP4* in wild type mice compared to complement-deficient animals, and correlated the increased expression of *PCP4* with B cell maturation into end stage phenotypes. On the other hand, the *CD109* belongs to the complement gene family and is a co-receptor of the Transforming growth factor beta (TGF-β), a type of cytokine which plays a role in immunity (Hashimoto et al. 2004).

The present study was conceived to search for the presence of further polymorphisms in the coding or regulatory region of *PCP4* and *CD109* genes that might explain and corroborate the effect of the two anonymous markers identified by Moioli et al. (2015) in influencing disease resistance.

MATERIAL AND METHODS

Genotyping results at the OvineSNP50 BeadChip for 100 sheep of the Sarda breed were available from the previous study (Moioli et al. 2015); the sheep represented the two tails of the distribution of the serological assessment for MAP diagnose, that had been performed on 759 sheep of the same flock using the ELISA test. DNA of 42 genotyped Sarda sheep for the present study was provided by the Animal Health Institute of Viterbo, Italy. Because the ELISA kit for MAP diagnosis attests positive results when S/P values > 70, the 42 Sarda sheep were chosen so to be equally distributed between positive and negative.

The genomic scaffolds encoding the *PCP4* and the *CD109* genes were obtained from the NCBI database (http://www.ncbi.nlm.nih.gov/genome/?term=ovis+aries). The size and the position of the coding regions of the *PCP4* and the *CD109* genes on the corresponding chromosomes – OAR1 and OAR8 – were identified by performing a standard nucleotide blast (Altschul et al. 1990) of the genomic scaffolds against the expressed sequence of the gene under investigation in the NCBI (www.ncbi.nlm.nih.gov/nuccore).

Because Moioli et al. (2015) had hypothesized that OAR1_278980576.1 and OAR8_270360, located in proximity of the *PCP4* and the *CD109* genes, were potential markers of MAP resistance, the genotyping results of the 100 Sarda sheep of the previous study were used to perform a χ^2 test of significance of differences of the allele frequencies between the 50 serologically positive and 50 negative sheep. The χ^2 test was performed on the genomic scaffolds encompassing the *PCP4* and the *CD109* genes, in order to restrain the genomic region to be submitted to direct DNA sequencing, because the average distance between markers of the OvineSNP50 BeadChip ranges from 60 to 200K. The χ^2 test values are deemed to provide solid indication of the region in which LD had been maintained (Lewis 2002).

Primer pairs to amplify the expressed regions of the genes were designed on the ovine sequence by using Primer3Plus software, Version 3.0, 2007 (www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi).

In a first step, DNA of 10 individuals (5 serologically positive and 5 negative) selected from the 42 Sarda sheep was amplified at each amplicon, under the assumption that this number was sufficient to detect the presence of further SNPs. Once the novel SNP had been detected, direct sequencing of the amplicon was performed on all 42 sheep. Direct sequencing of the coding regions of the putative candidate genes was performed on the 3500 Genetic Analyzer (Applied Biosystems, Foster City, USA). Allele frequencies and LD measures, for both the anonymous markers and the novel detected polymorphisms, were estimated using the Allele Procedure in SAS software.

Because all the sheep assessed for MAP diagnose belonged to the Sarda breed, to verify whether LD between the novel detected SNPs and the

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Table 1. The χ^2 test of differences between 50 serologically positive and 50 negative sheep at the markers falling in the region around the *PCP4* gene (genomic scaffold NW_004080164.1)

Marker	Gene	Position on OARv3.1	χ^2	P (χ^2)	Position in the gene
s49212.1		OAR1 258029257	1.2	ns	5' UTR
	PCP4 start	OAR1 258032168			
OAR1_278884883.1		OAR1 258068271	6.0	0.01	intron 1
OAR1_278927044.1		OAR1 258105475	0.7	ns	intron 2
	PCP4 stop	OAR1 258118984			
OAR1_278980576.1		OAR1 258148544	15.2	0.0001	3' UTR
	CD109 start	OAR8 273740			
OAR8_270360.1		OAR8 274469	17.5	0.00003	intron 2
OAR8_360354.1		OAR8 342710	13.0	0.0003	intron 8
OAR8_409032.1		OAR8 387749	3.6	ns	intron 26
OAR8_423752.1		OAR8 403376	0.6	ns	intron 31
	CD109 stop	OAR8 408954			

ns = non-significant

OvineSNP50 BeadChip anonymous markers were maintained also in different breeds, DNA of 33 more sheep of two Italian breeds – Altamurana and Comisana – was directly sequenced at the amplicons encompassing the coding regions of the putative candidate genes. These breeds and individuals were chosen because their DNA was available in the laboratory performing the present study, and because their genotyping results at the OvineSNP50 BeadChip were also available, thanks to a previous biodiversity project (Ciani et al. 2014).

Finally, under the hypothesis that local non-selected breeds are less susceptible to diseases, allele frequency at the hypothesized markers of MAP resistance was calculated in different Italian and related sheep breeds using the genotyping results obtained in the study by Ciant et al. (2014).

RESULTS

PCP4 gene. The genomic region encompassing the *PCP4* gene contained 4 markers of the OvineSNP50 BeadChip (Table 1); two of the markers, OAR1_278884883.1 and OAR1_278980576.1, showed significant difference in allele frequency, between serologically positive and negative sheep, after the χ^2 test results (Table 1). The nucleotide blast (Altschul et al. 1990) of the genomic scaffold NW_004080164.1 against the expressed sequence of *PCP4* (XM_004003899.1) showed that the gene is composed of 3 exons (Figure 1). The two markers, with significant difference in allele frequency, OAR1_278884883.1 and OAR1_278980576.1, were located, respectively, in the first intron and 29K downstream the stop codon of the gene. Therefore, hypothesizing that putative mutations influencing the target trait be located either in the first part, or in the 3'UTR of the gene, two amplicons were designed: the first, of 346 bp, encompassing exon 2 and part of the flanking regions; the second, of 580 bp, including exon 3 and the whole 3' UTR region (Table 2).

Direct sequencing of the amplicons described in Table 2 allowed the detection of one missense mutation in exon 2 (XM_004003899.1; g.97 G>C) producing the aa change from Glu to Gln (Table 3).

Moreover, five more mutations were detected in the amplicon encoding exon 3 and the 3' UTR region, of which one missense (XM_004003899.1; g.107 G>A) producing the aa change from Gly to Glu. Allele frequency was reported in Table 3.

The novel detected SNP in exon 2 and the marker in intron 1 (OAR1_278884883.1) showed no LD between each other (0.05) (Table 4).

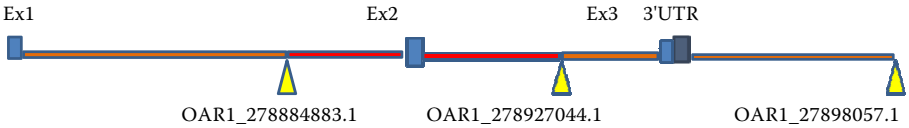

Figure 1. Structure of the *PCP4* gene in *Ovis aries* with indication of the position of the Illumina OvineSNP50 BeadChip markers

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Table 2. Amplicons encoding the *PCP4* and *CD109* genes and primers used for PCR

Gene	Genomic region	Amplicon size (bp)	Forward primer	Reverse primer
PCP4	OAR1 NW_004080164.1 exon 2	346	GGGAGGCAGTCTGTGACATT	GCCGATTTGGCTGAAAGTTA
PCP4	OAR1 NW_004080164.1 exon 3 + 3' UTR	580	TCCAGAGCCACTGTCTTCCT	TCTTGGAGGGAGAGGGAAAT
CD109	OAR8 NW_004080171.1 exon 1 + exon 2	952	TTCCTGGAGAGGGAAACAGA	GGCCACACCTTATCATTGCT
CD109	OAR8 NW_004080171.1 exon 2	499	ACTGCTGAGCCGGGAGTCT	GGCCACACCTTATCATTGCT

For the novel detected SNPs in the 3' UTR, LD between pair of SNPs was calculated for the markers with minimum allele frequency (MAF) higher than 0.10 and reported in Table 4; LD was week ($r < 0.45$) for all markers, and significant only in few cases.

CD109 gene. The genomic region encompassing the *CD109* gene contained 4 markers of the OvineSNP50 BeadChip (Table 1). The results of the χ^2 test of differences between 50 serologically positive and 50 negative sheep showed that only the two markers located in the upstream region of the gene have different frequencies in the two groups. The nucleotide blast (Altschul et al. 1990) of the *Ovis aries* breed Texel chromosome 8, Oar_v3.1, whole genome shotgun sequence (NCBI Reference Sequence: NC_019465.1) against the expressed sequence of *CD109* gene (XM_004011463.1) indicated that the gene encompasses position 20284 to 155500 of the scaffold NC_019465.1 and that the gene is composed of 34 exons (Figure 2). Only two

Table 3. Detected mutations in the *PCP4* gene (Accession No. XM_004003899.1) and frequency of the mutated allele

Position	Location	Frequency of the mutated allele	aa change
OAR1_278884883.1 C>A	intron 1	0.12	
g.97 G>A	exon 2	0.12	Glu > Gln
g.107 G>A	exon 3	0.07	Gly > Glu
g.235 G>A	3' UTR	0.03	
g.295 A>G	3' UTR	0.04	
g.297 A>G	3' UTR	0.36	
g.308 A>G	3' UTR	0.22	
g.491 A>G	3' UTR	0.09	
g.492 A>G	3' UTR	0.25	
OAR1_278980576.1 G>A	3' UTR	0.15	

Table 4. Linkage disequilibium (LD) measures between pair of SNPs in the 3' UTR of the *PCP4* gene (Accession No. XM_004003899.1)

SNP 1	SNP 2	LD	*P* (LD)
OAR1_278884883.1 C>A	g.97 G>A	0.05	ns
g.297 A>G	g.308 A>G	0.42	0.0001
g.297 A>G	g.491 A>G	0.17	ns
g.297 A>G	OAR1_278980576.1	0.31	ns
g.308 A>G	g.492 A>G	0.44	0.002
g.308 A>G	OAR1_278980576.1	0.24	0.02
g.492 A>G	OAR1_278980576.1	0.24	0.05

ns = non-significant

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Table 5. Detected mutations in the *CD109* gene and linkage disequilibium (LD) measures between the mutation and the marker

Breed	XM_004011463.1; g.128 A>G	OAR8_270360.1 (A>G)	Individuals	LD
	G allele frequency		*n*	
Sarda serologically positive	0.25	0.11	19	
Sarda serologically negative	0.54	0.52	23	
Sarda	0.41	0.34	42	0.74 ($P < 0.0001$)
Comisana	0.41	0.25	12	
Altamurana	0.83	0.79	21	
Other breeds				0.82 ($P < 0.0001$)
Total				0.79 ($P < 0.0001$)

markers, OAR8_270360.1 and OAR8_360354.1, showed significant difference between serologically positive and negative sheep (Table 1). Hypothesizing the presence of putative mutations in LD with the markers, in a first step it was decided to sequence the first exons of the gene. It was moreover noted that scaffold NC_019465.1 contained a gap of ~ 298 nt, possibly located in intron 1, according to the blast results against the expressed sequence (XM_004011463.1). One amplicon was therefore designed (Table 2) in order to identify the missing nucleotides; DNA of ten individuals was directly sequenced and the obtained novel sequence was deposited in the GenBank under the Accession No. KP242288. Within this sequence, a novel missense mutation in exon 2 (g.128 A>G; XM_004011463.1) was detected producing the aa change from Glu to Arg.

A second shorter amplicon was designed to amplify the DNA fragment encoding the mutation, with the forward primer on the novel identified sequence (Table 2), so to speed up the sequencing of more individuals. The novel detected mutation in exon 2 had a frequency of 0.25 in the positive and 0.54 in the negative sheep ($P < 0.01$) (Table 5). LD between the missense mutation and marker OAR8_270360.1 was significant (LD = 0.74; $P < 0.0001$).

Sequencing of individuals of different breeds at the CD109 gene to confirm LD. To verify whether LD between marker OAR8_270360.1 and the novel detected mutation were maintained also in different sheep populations, the amplicon encoding the Glu/Arg mutation was sequenced in 33 further sheep of Italian breeds (12 Comisana and 21 Altamurana), which had already been genotyped at the OvineSNP50 BeadChip in previous projects (Moioli et al. 2013; Ciani et al. 2014). The conservation of LD between the marker and the missense mutation in breeds differing from the Sarda would in fact substantiate the use of this marker, as a marker of natural immunity, for any individual which was genotyped for different purposes – genomic selection or biodiversity studies.

In Table 5, the measures of LD between marker and missense mutation in the Altamurana and Comisana breeds (0.82; $P < 0.0001$) were presented, with evidence that LD was maintained also in these breeds. Table 5 indicated also that Comisana and Altamurana had opposite allele frequencies at both the neutral marker (OAR8_270360.1) and the novel detected mutation.

The opposite allele frequency observed in the two specialized dairy breeds (Sarda and Comisana) with respect to a local hard non-selected breed (Altamurana) encouraged further analyses, thanks to the availability of the genotyping results obtained from a genetic diversity study of Italian sheep breeds (Ciani et al. 2014), in which genome-wide analyses had been performed on 24 non-related individuals for each breed. Frequency of the *G* allele at marker OAR8_270360.1, i.e. the hypothesized allele associated to the trait "disease resistance", was then calculated for each breed, and reported in Table 6.

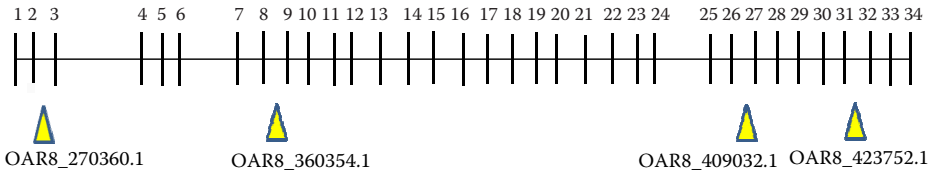

Figure 2. Structure of the *CD109* gene in *Ovis aries* with indication of the position of the Illumina OvineSNP50 BeadChip markers

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Table 6. Frequency of the OAR8_270360.1 *G* allele in some Italian sheep breeds

Breed	*G* allele frequency
Gentile	0.25
Delle Langhe	0.27
Comisana	0.31
Sambucana	0.31
Sarda	0.35
Pinzirita	0.38
Laticauda	0.38
Bergamasca	0.42
Biellese	0.43
Leccese	0.44
Bagnolese	0.52
Appenninica	0.50
Alpagota	0.50
Sopravissana	0.50
Valle del Belice	0.54
Massese	0.56
Fabrianese	0.60
Istrian	0.67

DISCUSSION

The present study had been formulated under the assumption that anonymous markers previously associated with disease resistance were in LD with missense mutations in the genes located in proximity of the markers, so to corroborate the influence of the gene on the target trait. Two putative genes were therefore scanned: *PCP4* and *CD109*.

PCP4 gene. The analysis of the *PCP4* gene allowed the detection of a missense mutation in exon 3 and four more SNPs, in the 3' UTR region; however, the low frequency of the missense mutation and the week LD between the other SNPs and the marker did not endorse a possible direct role of this segment of the gene in influencing the immune system. The discovery of another marker in intron 1 of the *PCP4* gene, with different allele frequency between seropositive and negative sheep, suggested to perform a further genomic scan in the first part of the gene, which allowed the detection of a further missense mutation in exon 2; this mutation, however, had a very low frequency in the analyzed population (0.12). Furthermore, no LD was registered between the mutation and the anonymous marker OAR1_278884883.1. In conclusion, for the *PCP4* gene, the suggestive association proposed by Moioli et al. (2015) might have been simply probabilistic, due to the limited size of the analyzed sample and the possible relationship between individuals of the same flock.

CD109 gene. The missense mutation in the *CD109* gene, on the other hand, was intriguing, because LD between marker OAR8_270360.1 and the mutation (0.79; $P < 0.0001$) was maintained also in breeds different from the Sarda, even in a breed where the mutation had opposite allele frequencies. For the Sarda breed, allele *G* was the minor allele at marker OAR8_270360.1, with the frequency of 0.34; but the serologically positive Sarda sheep showed a much lower frequency (0.11) while the negative sheep had a frequency of 0.52 (Table 5). Similar trend was detected for the mutation in LD with the marker. In the Comisana breed the *G* allele frequency was even lower than in the Sarda (0.25). Allele *G* was on the contrary the major allele in the Altamurana breed, this fact encouraging to perform further investigations. Tha Sarda and the Comisana breeds are two specialized Italian dairy breeds of sheep, which have been submitted to breeding programs for the improvement of milk yield, and have rapidly spread outside of their original geographical areas, so that they have largely replaced indigenous and low-productive breeds of sheep all over Italy, like the Altamurana. The latter is a dairy sheep, belonging to the subgroup of South European milk sheep (Pieragostini and Dario 1996), living in harsh environment, and producing no more than 50 l milk in a short lactation. Because the Altamurana breed, as well as several other Italian indigenous breeds, is maintained for conservation purposes, the discovery of markers in genes which play a role in the immune systems, with opposite allele frequency in selected and indigenous hardy breeds, would corroborate the usefulness of developing conservation programs for the endangered breeds.

Allele frequency of marker OAR8_270360.1 in other breeds. The exam of the genotyping results of further Italian breeds, obtained from previous genome-wide analyses of genetic diversity (Ciani et al. 2014), showed that the frequency of the hypothesized allele of disease resistance (*G* allele at marker OAR8_270360.1) in the majority of the breeds ranged between 0.40 and 0.50 (Table 6). The lowest frequency was obtained by the Sarda (0.35), Comisana (0.31), Delle Langhe (0.27), and

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Gentile (0.25); the first three breeds are the only Italian breeds under selection for the improvement of milk yield; the Gentile is the Italian merino, voluntarily improved during the past centuries with the use of foreign merino rams. On the other hand, the highest frequency of the *G* allele was obtained in the Massese (0.56), Fabrianese (0.61), and Istrian (0.67). It should be noted that Istrian was the only non-indigenous Italian breed and was included in the study because of close proximity to northern Italy sheep breeds (Ciani et al. 2014). Such proximity was confirmed by Salamon et al. (2015) who reported that Istrian population in Italy was restored, after near extinction, with stock from Slovenia. Moreover, although the study by Ciani et al. (2014) highlighted the genetic closeness of Istrian with other breeds of northern Italy (Bergamasca, Biellese, and Sambucana), the frequency of the *G* allele in the latter breeds was lower (0.31–0.43). Then, an obvious question to be raised was: Why the Istrian breed clearly differs from the above genetically similar breeds at that genomic fragment? Where does this allele come from? The Istrian sheep belongs to the Pramenka type, which includes several breeds and populations of sheep of the East Adriatic region. Genetic origin of these sheep was traced by Ferencakovic et al. (2013), who reported a surprisingly high number of mitochondrial DNA haplotypes, despite the overall maternal homogeneity, while Salamon et al. (2014), analyzing the genetic structure and variability of the Pramenka populations in order to give indications for conservation strategies, showed that the Istrian breed had maintained higher variability compared to other breeds (i.e. Kupres Pramenka), the distinctiveness being confirmed by the high number (10) of conserved private alleles (Salamon et al. 2014). Moreover, a previous study by Salamon et al. (2012) who analyzed the genetic diversity of the two Istrian populations, Croatian and Slovenian, separately, reported a higher inbreeding coefficient for the Slovenian; then the higher frequencies of peculiar alleles like the *G* allele are not surprising, the Istrian sheep of the present study having Slovenian origin. Adaptation to mountain enviroment of the Istrian breed was also inferred in the study by Salamon et al. (2014) in contrast with other Pramenka populations that had been submitted to hybridization programmes; while Kompan et al. (1999) wrote that Istrian sheep were bred mainly for their unusual characteristics – their distinct long-stepping walk and ability to graze in rocky terrain.

As regards to adaptation to harsh environment, also the Massese is an indigenous hardy dairy breed, grazing all the year in the hills and mounatins of Tuscany (Casoli et al. 1989), while the Fabrianese is a course wool breed kept for both meat and milk production, originating from local breeds of the Apennine and crossed to Bergamasca (Ciani et al. 2014). Also in this case, despite of the crossbreeding with the Bergamasca, the breed has maintained a higher frequency of the *G* allele. It could be argued that the reported frequencies of the *G* allele in different breeds are suggestive, because the study by Ciani et al. (2014) investigated a relatively small sample (24 individuals) for each breed; however, they offer further elements to consider the hypothesis of the *G* allele being associated with hardness, and substantiating the conviction of the *CD109* gene having a role in natural immunity.

Involvement of CD109 gene in immune response. The *CD109* is a protein-coding gene. Gene ontology annotations related to this gene include serine-type endopeptidase inhibitor activity. To our knowledge, no studies have examined the expression of this gene in livestock; on the other hand, in human medicine, *CD109* expression was correlated with the pathological grade and tumor stage of some carcinomas (Hagikura et al. 2010). Li et al. (2013) found that *CD109* marker was expressed in the hepatic progenitor cells (HPCs) and suggested that this marker could potentially be utilized to identify and isolate HPCs for further cytotherapy of liver diseases. Finally, Bizet et al. (2011) identified *CD109* as a TGF-β co-receptor and a negative regulator of TGF-β signalling. These authors demonstrated that *CD109* increases binding of TGF-β to its receptors promoting localization of the TGF-β receptors into the caveolar compartment and facilitates TGF-β-receptor degradation. Thus, *CD109* regulates TGF-β receptor endocytosis and degradation to inhibit TGF-β signalling.

The existing studies on immune response to infections, both in humans and in livestock, have compared the positive and the control groups using either the candidate gene approach, which is based on the analysis of pre-selected genes, or the GWAS, by exploiting anonymous marker panels of various density (Minozzi et al. 2010; Nalpas et al. 2013; Zare et al. 2014). However, so far, little

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overall consensus has emerged from these studies in terms of resistance loci, this being likely due, according to Riggio et al. (2014), to the apparent genetic complexity of the trait and the diversity of the studies, both for the considered breeds and the experimental approaches.

In the present study we proposed a novel application of the genotyping results obtained with panels of polymorphic markers; the new application consisting in uncovering the genes and the mutations that might play a direct role in the variation of the trait under investigation. Once the genes have been identified, specific q-RT PCR trials of gene expression for these genes could be designed, so to corroborate the effect of the markers. The recognition of genes that potentially play an important role in immune response in livestock will offer valuable reference in promoting sustainable animal farming.

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