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Identification of immuno-inhibitory molecules in Mongolian native cattle and yak

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Abstract

The immuno-inhibitory molecules PD-1, PD-L1, TIM-3, GAL-9, LAG-3, and CTLA-4 from blood samples of Mongolian native cattle and yak were characterized through cloning and sequencing. As these molecules are involved in cell-mediated immune responses, identifying the differences in their reactions against the pathogens found in bovine species may be beneficial. The amino acid sequences of these molecules were predicted for the purpose of characterizing their functional domains, such as the signal peptide, extracellular domain, transmembrane region, and intracellular domain. Amino acid alignment showed that the sequences of these immuno-inhibitory molecules from Mongolian native cattle and yak were highly homologous to those from other bovine species. As a preliminary application of the genetic information, we conducted expression analysis of PD-L1 in bovine viral diarrhea virus (BVDV)-infected yak by using real-time polymerase chain reaction, and PD-L1 mRNA expression in peripheral blood mononuclear cells derived from BVDV-infected yak was significantly upregulated compared to that of uninfected-yak. Further studies are necessary to assess whether these molecules play roles in disease progression during chronic infection of Mongolian native cattle and yak.

Key Words: *Immuno-inhibitory molecule, Mongolian native cattle, Mongolian yak.*

Introduction

The maintenance of immune homeostasis is important for host survival, and over-reaction of immune responses to pathogens can result in inflammatory tissue damage[10)]. Therefore, the balance of immune responses is regulated by co-stimulatory and inhibitory signals[11)]. The inhibitory signals are mediated by immuno-inhibitory molecules such as programmed cell death 1 (PD-1), programmed cell death-ligand 1 (PD-L1), T-cell immunoglobulin and mucin domain 3 (TIM-3), galectin 9 (GAL-9), lymphocyte activation gene 3 (LAG-3), and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4)[30)]. These immuno-inhibitory molecules are expressed on various

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immune cells and negatively regulate immune function during chronic infection or cancer[8,15,16].

PD-1 is a member of the immunoglobulin (Ig) superfamily. This protein has been identified as a cell-surface receptor that interacts with the PD-L1 ligand and can inhibit the function of antigen-specific T cells[6]. PD-1 is mostly expressed on the surface of T cells, but it is also expressed on B cells, natural killer (NK) T cells, activated monocytes, and dendritic cells. In contrast, PD-L1 is constitutively expressed on a variety of cells including T and B cells, dendritic cells, macrophages, mesenchymal stem cells, and bone marrow–derived mast cells[24]. The PD-1/PD-L1 pathway is involved in the immune dysfunction associated with the development of several tumor types and chronic infections through the transmission of an inhibitory signal that reduces cytokine production and suppresses T cell proliferation[2,42].

TIM-3 is a member of the TIM family and has been identified as a transmembrane protein composed of Ig and mucin-like domains[1]. TIM-3 is mostly expressed in dysfunctional T cells and negatively regulates the immune responses occurring during chronic infection and the development of cancer[12]. Therefore, it contains an intracellular tyrosine-kinase phosphorylation motif, which is a critical mediator of intracellular signaling and of intracellular responses to extracellular signaling[28,34].

GAL-9, a member of the galectin family (also called S-type lectins), is secreted by several cells and has been identified as the ligand for TIM-3[51]. GAL-9 consists of N- and C-terminal carbohydrate-binding domains connected by a link peptide and binds to TIM-3 *via* a carbohydrate chain[47,51]. GAL-9-induced intracellular calcium flux, aggregation, inhibition of cell proliferation and cytokine production, and T cell death are known to be TIM-3-dependent[18,41]. Therefore, the TIM-3/GAL-9 pathway is closely associated with immune exhaustion and disease progression in organisms experiencing chronic infections and tumors[4,44].

CTLA-4 and LAG-3 are members of the Ig superfamily and have been identified as membrane proteins[14,48]. CTLA-4 is expressed on activated effector cells and regulatory T cells that bind to CD80 (B7-1)/CD86 (B7-2) molecules of antigen-presenting cells, while LAG-3 is expressed on various immune cells, such as T lymphocytes, NK cells, eosinophils, monocytes, and dendritic cells[49,50]. LAG-3 has 4 extracellular Ig-like domains, with conserved structural similarities between domains 1 and 3, as well as between domains 2 and 4[21]. CTLA-4 and LAG-3 play a role in the down-regulation of immune responses during the progression of chronic diseases and in facilitating immune evasion by several pathogens that cause chronic infections and tumors[16,23,26].

Mongolians have practiced animal husbandry for a long time. Mongolian livestock graze in free-range pastures, which is associated with a high risk of exposure to infectious agents transmitted by wildlife. In our previous field surveys, several pathogens were detected in Mongolian livestock, but none of the animals showed visible clinical signs of infection by the pathogens detected, which included bovine viral diarrhea virus (BVDV), bovine leukemia virus, *Mycobacterium avium* subsp. *paratuberculosis*, bovine gammaherpesvirus-2, and *Anaplasmaovis*[35–39]. However, there is no detailed information on the role of immuno-inhibitory molecules during disease progression for Mongolian native animals. Thus, molecular identification of PD-1, PD-L1, TIM-3, GAL-9, LAG-3, and CTLA-4 from Mongolian native cattle and yak was performed through cloning and sequencing analysis in this study.

Materials and Methods

Sample collection and RNA extraction: A total of 108 blood samples, including 68 samples from yak (in Tsenkhersoum of Arkhangai province) and 40 samples from Mongolian native cattle (in Lunsoum of Tuv province), were collected in 2014 for RNA extraction. The RNA was isolated from whole blood by using TRIzol® reagent (Thermo

Table 1. Primer sets for detection and characterization of immunoinhibitory molecules in Mongolian native cattle and yak

Gene	Product size bp		Primers (5'-3')	Reference
PD-1	894	513	Forward: ATGGGGACCCCGCGGGCGCT Reverse: GATGACCAGGCTCTGCATCT	Mingala *et al.*, (2011)
		504	Forward: AATGACAGCGGCGTCTACTT Reverse: TCAGAGGGGCCAGGAGCAGT	
PD-L1	870		Forward: CGGCAGGTCATTCCAGAAA Reverse: CCAAACCACAGGCTGAGAA	This study
CTLA-4	666		Forward: ATGGCTTGCTCTGGATTCCA Reverse: TCAATTGATGGGAATAAAATAAGGC	Mingala *et al.*, (2011)
GAL-9	972		Forward: GGGAGAAGTGGCAGTGGCTACAGA Reverse: ATCCAGATAGCAGCACAGGGCAG	Okagawa *et al.*, (2012)
LAG-3	1551	1031	Forward: CCTGATCTGTCTGGCTTTCC Reverse: GAGAAGACTGGATCCCCACA	This study
		773	Forward: TGTGGGGATCCAGTCTTCTC Reverse: AGCTGAGGAGATGAGGATGG	
TIM-3	843		Forward: AAACGGCACCTAAACAGAGC Reverse: GACAACACCAAGCCCCTAGA	Okagawa *et al.*, (2012)

Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The RNA samples were then stored at −80°C until analysis was performed.

Complementary DNA (cDNA) synthesis: The cDNA synthesis was performed using a cDNA synthesis kit (Takara Bio Inc., Otsu, Shiga, Japan) following the manufacturer's instructions. After cDNA synthesis, the housekeeping gene was confirmed using a primer set for the *β-actin* gene: forward 5'-TCT TCC AGC CTT CCT TCC TG-3' and reverse 5'-ACC GTG TTG GCG TAG AGG TC-3'[40]. Only samples positive for the *β-actin* gene were subjected to polymerase chain reaction (PCR) for amplification of each immuno-inhibitory molecule.

PCR amplification for immuno-inhibitory molecules: A total of 18 representative samples (3 samples for each molecule) were selected for the amplification of PD-1, PD-L1, TIM-3, GAL-9, LAG-3, and CTLA-4 molecules. The primer sets used for immuno-inhibitory molecule amplification are shown in Table 1. In the PCR reaction, 5 µl of a single cDNA sample was added to 45 µl of the reaction mixture, which comprised 5 µl of ×10 Ex Taq buffer (Takara Bio Inc.), 4 µl of deoxynucleotide solution (Takara Bio Inc.), 4 µl (10 µM concentration) of forward and reverse primers (Hokkaido System Science Company, Japan), 10 µl of 5 M betaine, 0.25 µl of Ex Taq polymerase (Takara Bio Inc.), and 17.75 µl of deuterium-depleted water. PCR amplification was performed under the following thermal cycle conditions: initial denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at the optimal temperature for 30 sec, extension at 72°C for 1 min, and a final synthesis at 72°C for 7 min, using the GeneAmp PCR System 9700 (Applied Biosystems, USA). The amplified PCR products were separated by electrophoresis and visualized under ultraviolet light.

DNA cloning and sequencing: A total of 18 PCR products (3 samples for each molecule) were extracted for sequencing by using the FastGene gel/PCR Extraction kit (Nippon Genetics Company, Japan). The extracted PCR products were ligated into the pGEM-T Easy vector (Promega Corporation, USA), and the plasmid

was transformed into the DH5α *Escherichia coli* strain, plated on a Luria-Bertani (LB) agar (Thermo Fisher Scientific, USA), and cultured in LB broth (Thermo Fisher Scientific, USA). Plasmid DNAs from the positive clones were extracted from the LB culture by using the FastGene Plasmid Mini kit (Nippon Genetics Company). The plasmids were amplified using the GeneAmp PCR System 9700 (Thermo Fisher Scientific, USA). The quality of the plasmid preparation for each gene of the bacterium was verified using NanoDrop 8000 analytic equipment (Thermo Fisher Scientific, USA), and sequencing analysis for the bacterium was carried out using the CEQ8000 DNA analysis system (Beckman Coulter Inc Company, USA).

Homology analyses: The obtained sequences were analyzed using the Bio-Edit software and basic local alignment search tool application. In addition, the signalP 4.1 server (Technical University of Denmark [DTU], Copenhagen, Denmark), TMHMM server version 2.0 (DTU), and NetNGlyc 1.0 server (DTU) were used to predict the cleavage sites, transmembrane helices, and possible N-linked glycosylation sites, respectively, in the deduced protein sequences of the PD-1, PD-L1, TIM-3, GAL-9, LAG-3, and CTLA-4 genes.

Real-time PCR: To evaluate *PD-L1* mRNA expression in response to infectious diseases, expression analysis was conducted for yak diagnosed with BVDV infection[37]. Total RNA was extracted from fresh peripheral blood mononuclear cells (PBMCs) by using TRIzol® reagent, and cDNA was synthesized as described above. Quantitative real-time PCR for *PD-L1* mRNA was performed in the LightCycler 480 System II (Roche Diagnostics, Mannheim, Germany) using SYBR Premix Dimer Eraser (Takara Bio Inc.) following the manufacturer's instructions. The primers used were 5'-GGG GGT TTA CTG TTG CTT GA-3' and 5'-GCC ACC TCA GGA CTT GGT G AT-3' for *yak PD-L1* and 5'-CGC ACC ACT GGC ATT GTC AT-3' and 5'-TCC AAG GCG ACG TAG CAG AG-3'for *bovine actin*. The cycling conditions consisted of initial template denaturing at 95°C for 30 s, followed by amplification of the template for 45 cycles of 95°C for 5 s, 55°C (*PD-L1*) or 60°C (*actin*) for 30 s, and 72°C for 30 s. A final melting curve analysis was performed from 65°C to 95°C at a rate of 0.1°C/s (continuous acquisition), with a final cooling to 40°C over 10 s. Each amplification procedure was performed in triplicate, and the values for *PD-L1* mRNA expression were expressed as a ratio obtained by dividing the concentration of *PD-L1* mRNA by that of *actin* mRNA. Data were analyzed by one-way analysis of variance followed by the Student's *t* test. Differences between groups were considered significant if probability values (P) < 0.05 were obtained.

Results

Amino acid (aa) alignments of the immuno-inhibitory molecules

In total, full-length of 6 immuno-inhibitory protein genes were cloned and identified from both Mongolian native cattle and yak (Fig. 1–6). The lengths of amplified proteins were 843 bp encoding 280 aa for TIM-3, 972 bp encoding 323 aa for GAL-9, 1551 bp encoding 516 aa for LAG-3, 666 bp encoding 221 aa for CTLA-4, 849 bp encoding 282 aa for PD-1, and 870 bp encoding 298 aa sequences for PD-L1.

The identities of the amino acid sequences were 71.6%–100% and 72.8%–99.6% for PD-1, 82.5%–100% and 82.9%–99.7% for PD-L1, 73.4%–100% and 72.0%–96.5% for TIM-3, 94.1%–100% and 94.1%–100% for GAL-9, 80.2%–99.6% and 70.8%–99.4% for LAG-3, and 95.9%–100% and 95.9%–99.5% for CTLA-4 from Mongolian native cattle and yak, respectively, compared to the corresponding sequences from other animal species.

Prediction for structure of the immuno-inhibitory molecules

The amino acid sequences of the immuno-inhibitory molecules from Mongolian native cattle and yak were passed through the protein-structure prediction server at the Center for Biological Sequence Analysis at DTU.

According to the predictions, the amino acid residues of PD-1 at positions 1–20, 21–170, 171–191, and 192–282 corresponded to the signal peptide, extracellular domain, transmembrane region, and intracellular domain, respectively (Fig. 1). Additionally, the amino acid residues of PD-L1 at positions 1–24, 25–238, 239–259, and 260–289 corresponded to the signal peptide, extracellular domain containing IgV and IgC domains, transmembrane region, and intracellular domain, respectively (Fig. 2). The amino acid residues of TIM-3 at positions 1–23, 24–191, 192–214, and 215–280 were found to correspond to the signal peptide, extracellular domain containing Ig and mucin domains, transmembrane region, and intracellular domain, respectively (Fig. 3). The amino acid residues of GAL-9 at positions 1–148, 149–197, and 198–323 were found to correspond to the N-terminal carbohydrate-recognition domain, a linker peptide, and the C-terminal carbohydrate-recognition domain, respectively (Fig. 4).

The amino acid residues of LAG-3 at positions 1–23, 24–433, 434–456, and 457–516 were found to correspond to the signal peptide, the extracellular domain containing 4 regions characterized as domains 1–4, the transmembrane domain, and the intracellular domain, respectively (Fig. 5). Finally, the amino acid residues of CTLA-4 at position 1–35, 36–161, 162–182, and 185–221 were found to correspond to the signal peptide, extracellular domain, transmembrane region, and intracellular domain, respectively (Fig. 6).

Predicted functional domains and motifs of the immuno-inhibitory molecules

All cysteine residues evaluated for both Mongolian native cattle and yak, specifically 4 aa for GAL-9, 6 aa for PD-1, 8 aa for TIM-3, LAG-3, and CTLA-4, and 10 aa for PD-L1, were intact in their respective locations with the exception of 1 aa substitution at position 9 in TIM-3 for yak (Y). The potential N-linked glycosylation sites in all immuno-inhibitory molecules were entire such as, 2 aa for TIM-3, 1 aa for GAL-9, 2 aa for LAG-3, 3 aa for CTLA-4, 1 aa for PD-1, and 3 aa for PD-L1. A potential tyrosine-kinase phosphorylation motif (HPVENIY) was conserved in the intracellular domain of TIM-3 for all bovine species. In addition, a possible cleavage site for metalloproteases at position 404–413 with 10 aa and an immuno-inhibitory motif at position 473–478 with 6 aa containing "KTGELE" were found for LAG-3.

Amino acid substitutions in the immuno-inhibitory molecules

Amino acid alignment showed that immuno-inhibitory molecules among bovine species were highly homologous to one another. There was no amino acid substitution in PD-1, PD-L1, GAL-9, and CTLA-4 from Mongolian native cattle compared to the Holstein and Hereford breeds of cattle. For TIM-3 of Mongolian native cattle, 3 aa substitutions (1 for the extracellular domain and 2 for the intracellular domain) and no amino acid substitutions were found compared to those of Holstein and Hereford breeds of cattle, respectively. In addition, 2 aa substitutions (1 for the extracellular domain and 1 for the intracellular domain) were found in the amino acid residues of LAG-3 for Mongolian native cattle compared to those for Holstein and Hereford breeds of cattle.

Amino acid alignment of the immuno-inhibitory molecules from yak and cattle revealed 8 aa substitutions (one for the signal peptide, four for the extracellular domain, one for the transmembrane region, and two for the intracellular domain) for TIM-3, 2 aa substitutions for LAG-3 (both for the extracellular domain), 1 aa substitution for the intracellular domain of

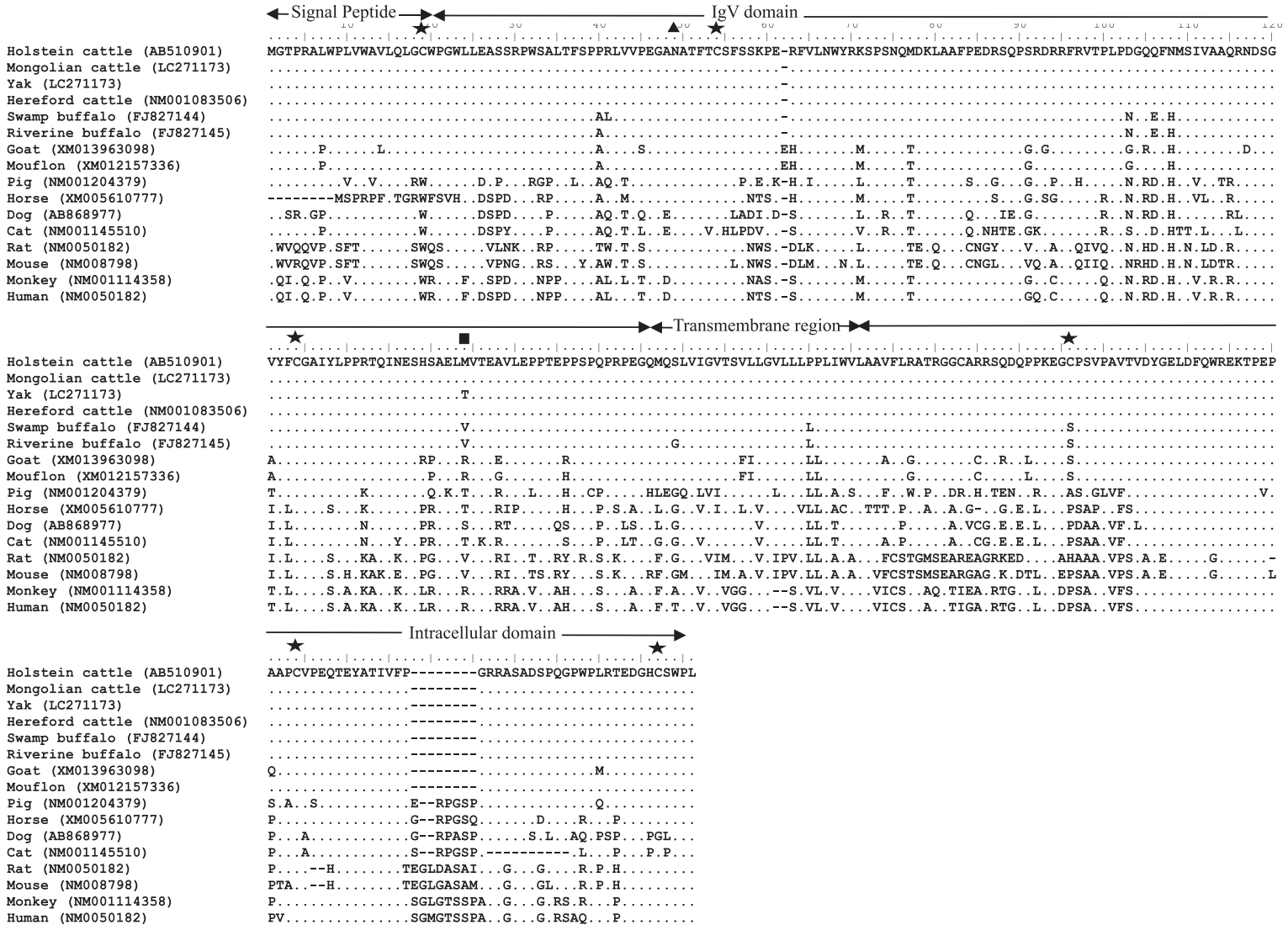

Fig. 1. Alignment of deduced amino acid sequences of PD-1 from Mongolian native cattle and yak compared to other species. A solid star indicates a cysteine residue, and a solid triangle indicates a potential N-linked glycosylation site. A solid square indicates an amino acid difference between yak and cattle species.

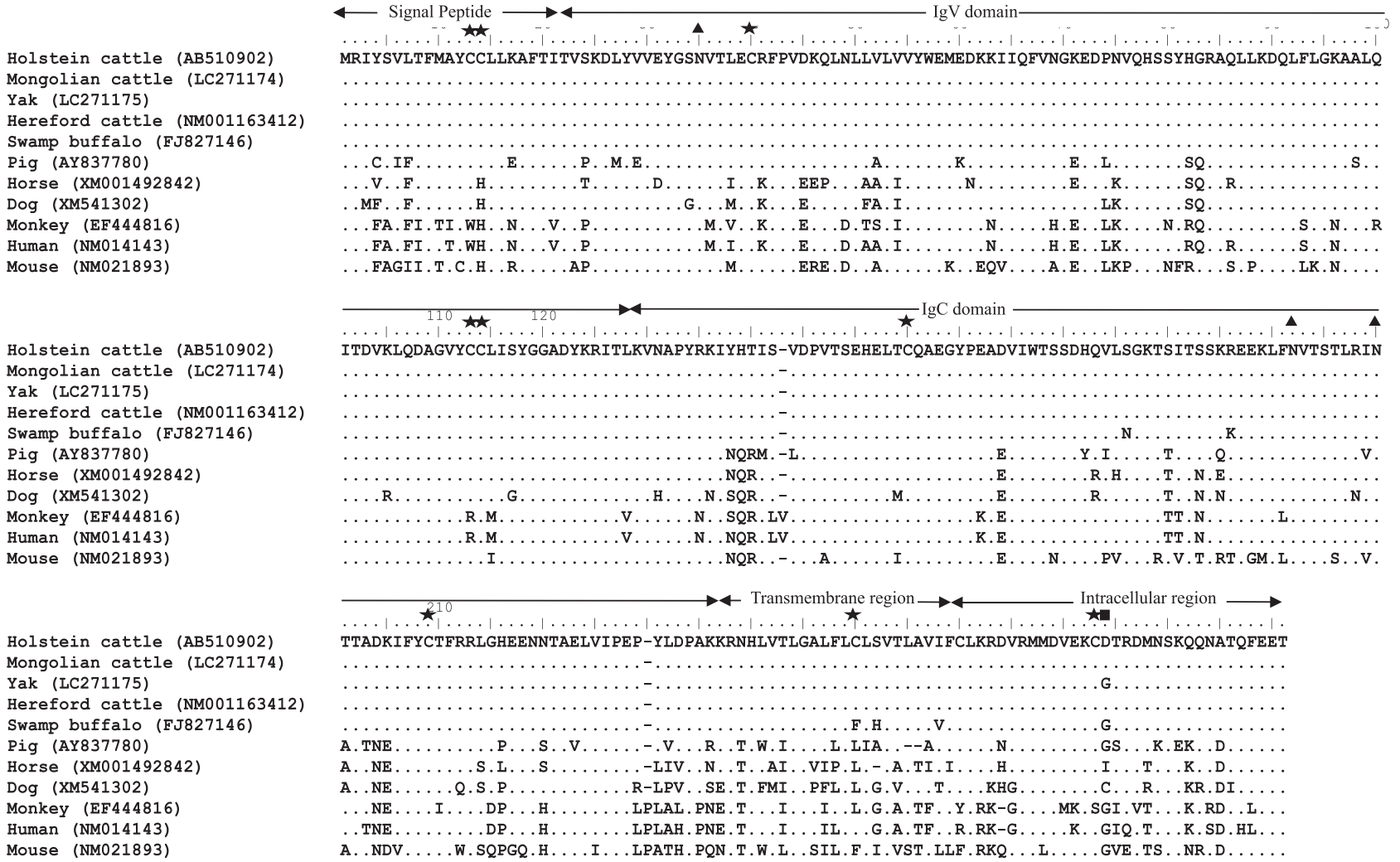

Fig. 2. Alignment of deduced amino acid sequences of PD-L1 from Mongolian native cattle and yak compared to other species. A solid star indicates a cysteine residue, and a solid triangle indicates a potential N-linked glycosylation site. A solid square indicates an amino acid difference between yak and cattle species.

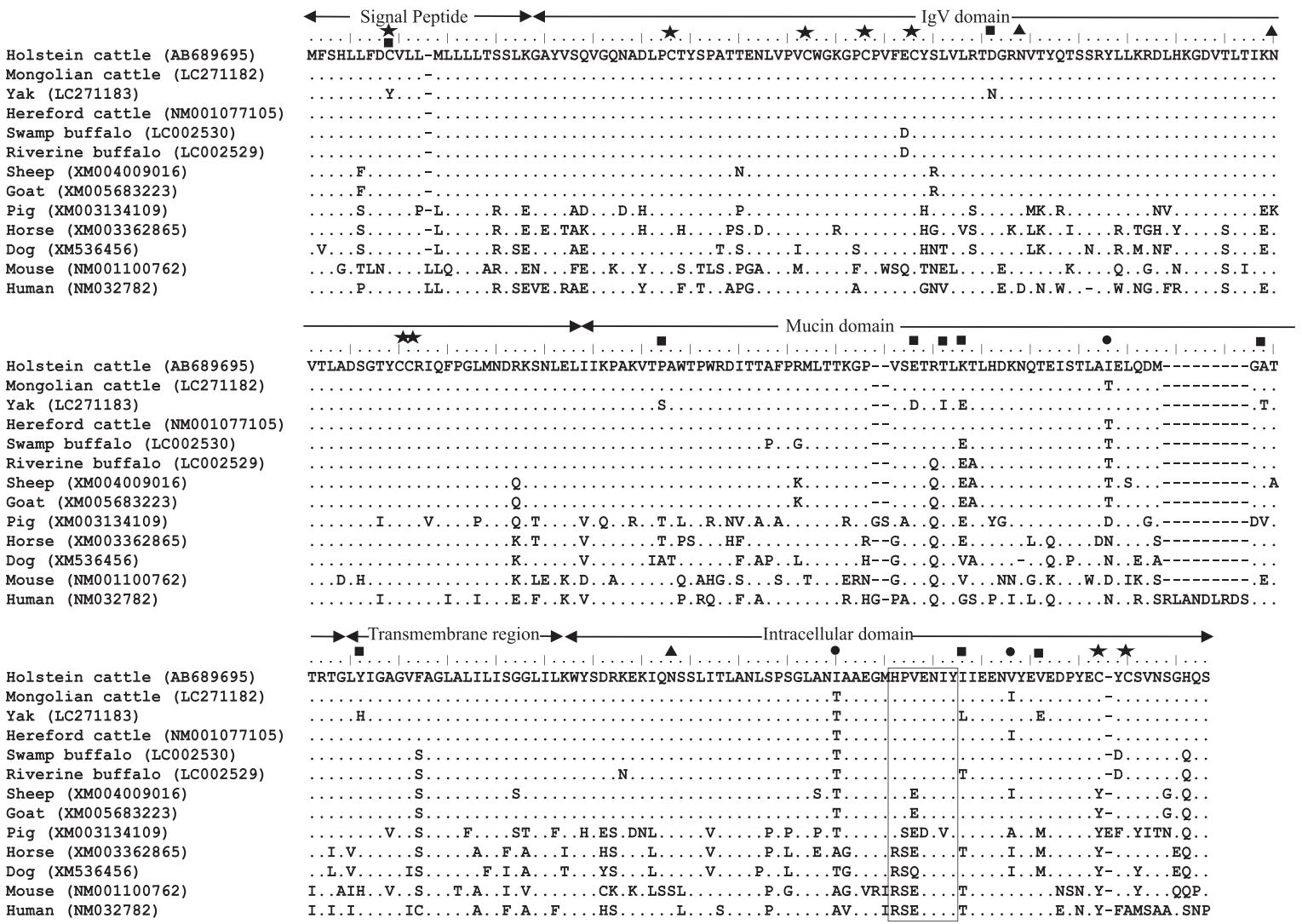

Fig. 3. Alignment of deduced amino acid sequences of TIM-3 from Mongolian native cattle and yak compared to other species. A solid star indicates a cysteine residue, and a solid triangle indicates a potential N-linked glycosylation site. A solid square indicates an amino acid difference between yak and cattle species. A solid circle indicates an amino acid substitution between Mongolian native cattle and other cattle species. The tyrosine-kinase phosphorylation motif is enclosed in a box.

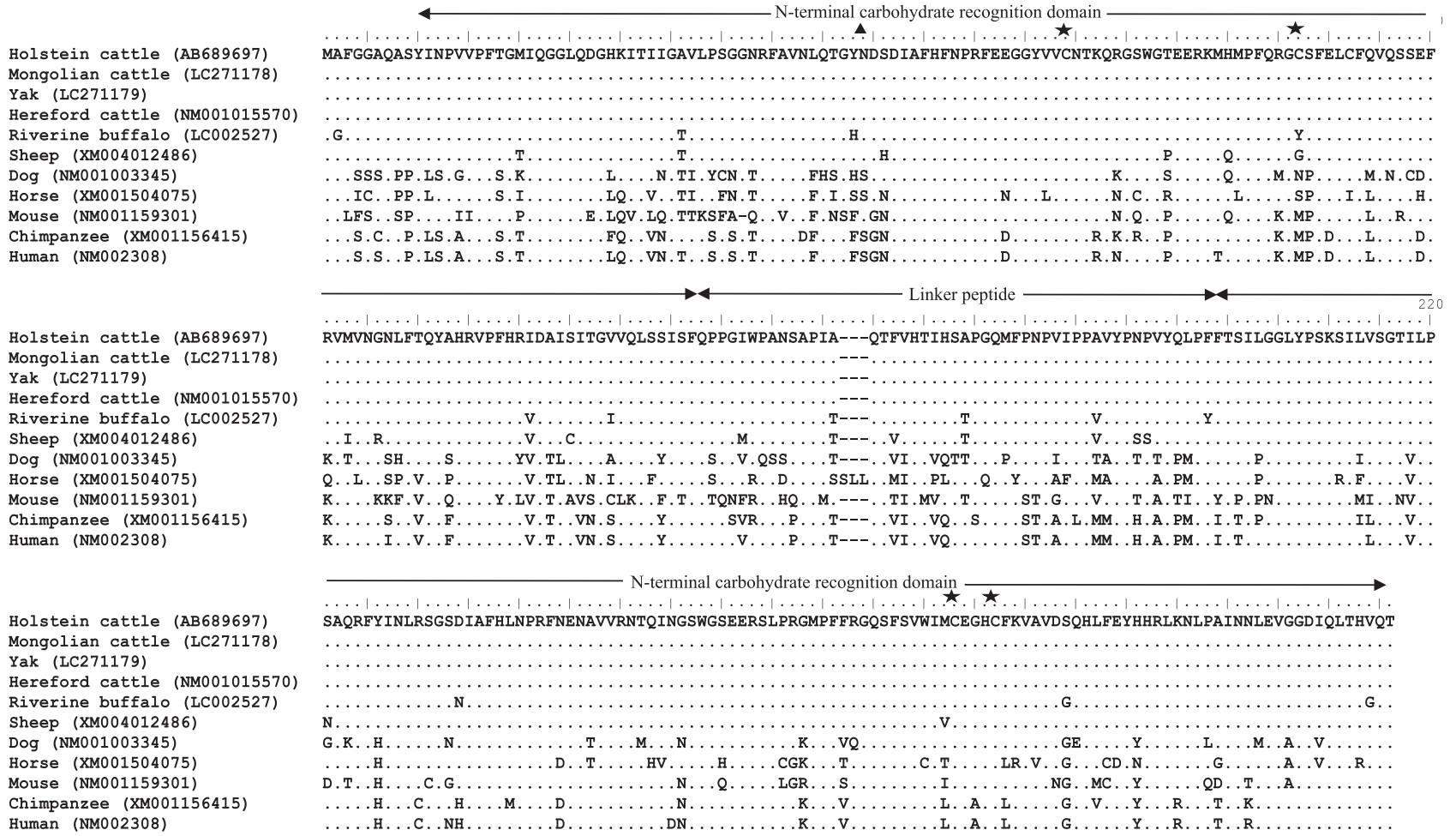

Fig. 4. Alignment of deduced amino acid sequences of GAL-9 from Mongolian native cattle and yak compared to other species. A solid star indicates a cysteine residue, and a solid triangle indicates a potential N-linked glycosylation site.

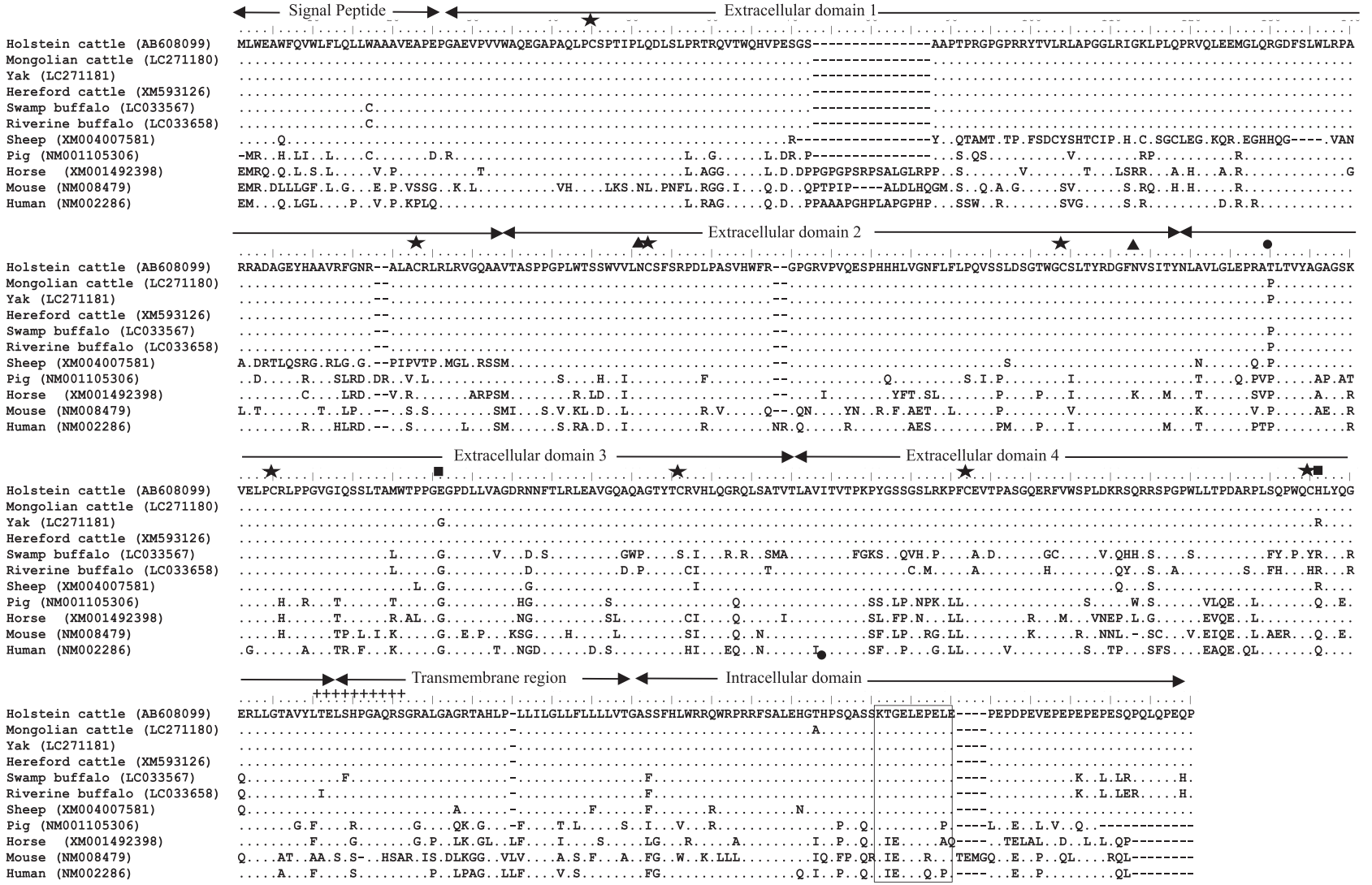

Fig. 5. Alignment of deduced amino acid sequences of LAG-3 from Mongolian native cattle and yak compared to other species. A solid star indicates a cysteine residue, and a solid square indicates an amino acid difference between yak and cattle species. A solid circle indicates an amino acid substitution between Mongolian native cattle and other cattle species. The amino acid residues at positions 404–413, indicated by a plus (+), are possible cleavage sites for metalloproteases. Enclosed in the box is the KTGELE inhibitory motif. A solid triangle indicates a potential N-linked glycosylation site.

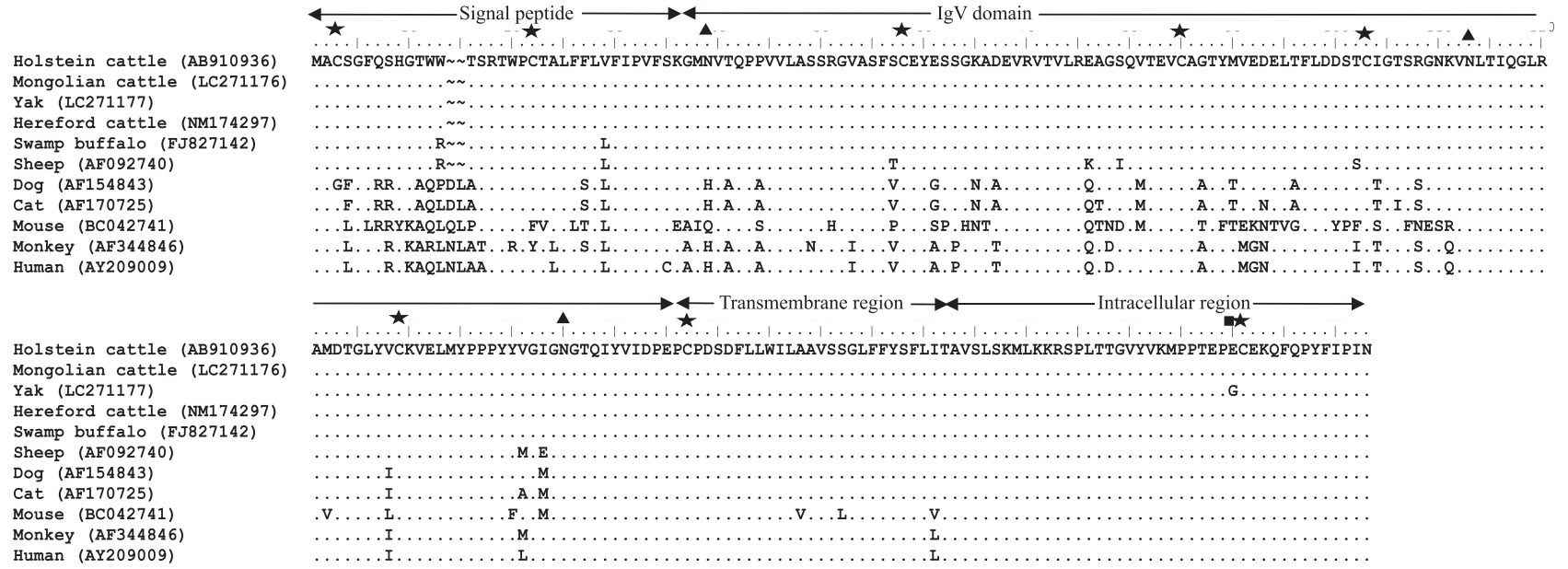

Fig. 6. Alignment of deduced amino acid sequences of CTLA-4 from Mongolian native cattle and yak compared to other species. A solid star indicates a cysteine residue, and a solid triangle indicates a potential N-linked glycosylation site. A solid square indicates an amino acid difference between yak and cattle species.

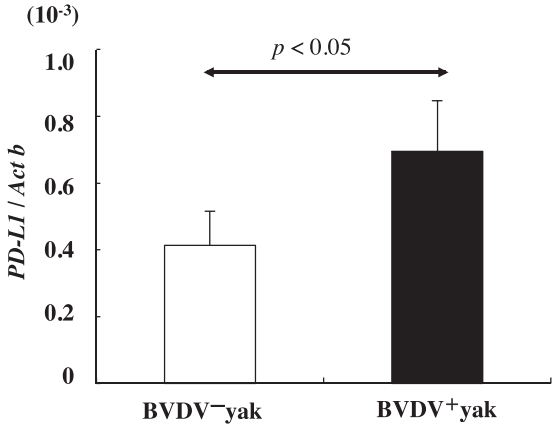

Fig. 7. Analysis of *PD-L1* mRNA expression by quantitative real-time PCR. *PD-L1* mRNA expression was determined in leukocytes from BVDV-infected and uninfected yak. The degree of *PD-L1* mRNA expression is shown as the ratio of the concentration of the PCR products from *PD-L1* mRNA to those from *β-actin* mRNA.

CTLA-4, 1 aa substitution for the extracellular domain of PD-1, and 1 aa substitution for the intracellular domain of PD-L1.

Expression analysis of PD-L1 in BVDV-infected yak

For preliminary application of the genetic information, we examined the PD-L1 mRNA expression levels in leukocytes freshly isolated from BVDV-infected and uninfected yak. Only a limited number of yaks were available for testing. As shown in Fig. 7, PD-L1 expression levels for the infected animals were statistically significantly higher than those for the control yak.

Discussion

Molecular characterization of the immuno-inhibitory molecules PD-1, PD-L1, TIM-3, GAL-9, LAG-3, and CTLA-4 from Mongolian native cattle and yak was performed in this study. The obtained sequences were compared to the corresponding sequences of various animal species including cattle, buffalo, sheep, dog, pig, and mouse. The amino acid sequences of the immuno-inhibitory molecules from the tested animals showed high homologies to those of bovine species, indicating a close relationship between members of the species. The analyzed genes, with the exception of GAL-9, were predicted to encode signal peptides, extracellular domains, transmembrane regions, and intracellular domains. GAL-9 was structurally different and was predicted to consist of N- and C-terminal carbohydrate-binding domains connected by a link peptide, because GAL-9 is a soluble molecule secreted by several cells and was originally characterized as an eosinophil chemoattractant[29].

Cysteine residues are important for protein stability and function, because they are often involved in disulfide bonds that stabilize the protein structure, as well as in binding metallic ions for function[33]. The cysteine residues in the analyzed genes were almost all conserved in their respective locations for all bovine species. However, single amino acid substitutions were found in TIM-3 from yak, GAL-9 and LAG-3 from riverine-type buffalo, PD-1 from both swamp- and riverine-type buffalo, and PD-L1 from swamp-type buffalo. In total, 2 substitutions were found only in LAG-3 from swamp-type buffalo.

N-linked glycosylation sites also play an essential role in folding and quality control for proteins in the endoplasmic reticulum, and a tightly controlled biosynthetic pathway ensures the correct assembly of the glycan[9,20]. Numerous diseases associated with defects in the N-glycosylation pathway highlight its importance in humans[17,19]. Potential N-linked glycosylation sites were found in the amino acid sequences of the immuno-inhibitory molecules identified from Mongolian native cattle and yak, 1 for GAL-9 and PD-1, 2 for TIM-3 and LAG-3, and 3 for CTLA-4 and PD-L1, and were all intact in their respective locations.

The tyrosine-kinase phosphorylation motif (HPVENIY) in the predicted intracellular domain of TIM-3 for Mongolian native cattle and yak was identical to those of other bovine species. In general, the motif for tyrosine-kinase phosphorylation transduces signals through the

phosphorylated tyrosine, by acting as a functional receptor in many important physiological processes including cellular growth, differentiation, and intracellular signaling[25,29,34,45]. The inhibitory motif in the cytoplasmic region (KTGELE/KIEELE) of LAG-3 was conserved for both Mongolian native cattle and yak. This inhibitory motif is known to mediate intracellular signal transduction by down-regulating T cell expansion[22].

Alignment of the deduced amino acid sequences revealed 1 aa substitution for CTLA-4, PD-1, and PD-L1, 2 aa substitutions for LAG-3, and 8 aa substitutions for TIM-3 between the yak and cattle sequences, but no substitution for GAL-9. Immuno-inhibitory molecules and their ligands play a crucial role in the immune system, because they can inhibit communication between adjacent and distant cells, a mechanism that can facilitate disease progression during infection[6,15,40]. Amino acid substitutions in these immuno-inhibitory molecules may alter their functions, but it remains to be determined whether the substitutions observed are functionally important.

Recent studies showed that over-expression of immuno-inhibitory molecules leads to immune dysfunction during chronic infections in animals and humans[12,23,26,40,42,44]. However, there are no reports available on the role of these molecules in disease progression in yak. Thus, the expression level of PD-L1 for yak during natural BVDV infection was investigated using quantitative real-time PCR to obtain preliminary insights into the influence of PD-L1. BVDV has the ability to cause persistent infection in cattle, due to the involvement of immuno-regulatory host factors, including the expression of cytokines, but the exact mechanism of disease progression in infected animals is not well understood[3,7,31]. Although the expression levels of other immuno-inhibitory molecules for yak were not investigated, because the number of BVDV-positive samples from yak was limited, the expression level of PD-L1 for BVDV-infected yak was higher than those of BVDV-uninfected control yak. According to established reports, the negative regulatory PD-1/PD-L1 pathway has been implicated in the induction of effector lymphocyte exhaustion during chronic viral infection, such as infection with HIV, SIV, LCMV, and HTLV-1[5,13,27,43,46]. These data might suggest that PD-L1 is involved in the inhibition of T cell function or immunological tolerance during BVDV infection. Further studies are necessary to determine the roles of immuno-inhibitory molecules for Mongolian native animals during the progression of diseases including BVDV.

This is the first report describing the genetic characteristics of immuno-inhibitory molecules from Mongolian native cattle and yak. This study demonstrates that the amino acid sequences of immuno-inhibitory molecules from Mongolian native cattle and yak are highly homologous to each other and to their counterparts in other bovine breeds. Thus, further detailed investigations of these molecules are necessary to elucidate the differences in the immune responses to chronic infection in these animal species.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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