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Citation
Japanese Journal of Veterinary Research, 65(2): 65-74

Issue Date
2017-05

DOI
10.14943/jjvr.65.2.65

Doc URL
http://hdl.handle.net/2115/66478

Type
bulletin (article)

File Information
65-2 065-074.pdf
Molecular characterization of the lymphocyte activation gene-3 (LAG-3, CD223) of swamp- and riverine-type water buffaloes (Bubalus bubalis)

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Received for publication, September 21, 2016; accepted, March 15, 2017

Abstract
The present study was conducted to characterize LAG-3 of swamp- and riverine-type water buffaloes by DNA sequencing, homology and phylogenetic analysis. Bubaline LAG-3 sequence contained an open reading frame of 1551 nucleotide, encoding a polypeptide of 516 amino acids. Nucleotide and amino acid sequence homology of LAG-3 revealed 76–96% and 61–94% identity in water buffalo to that of other mammals, respectively. LAG-3 protein sequence of water buffalo contained four extracellular domains, a transmembrane domain and different conserved regions. There were three N-glycosylation sites, two sequence motifs: ‘RGD’ and ‘WXC’ motif and five cysteine residues located at different positions of extracellular region. Likewise, the possible serine phosphorylation site and the ‘KTGELE’ inhibitory motif were found in the intracellular region of bubaline LAG-3. However, one highly conserved cysteine residue in mammalian LAG-3 was replaced by tyrosine in both swamp- and riverine-type water buffaloes. Phylogenetic analysis generated high bootstrap value between the two types of water buffalo which further confirmed the degree of relationship between bubaline species. This was the first report that describe the genetic characteristic of LAG-3 in swamp- and riverine-type water buffaloes.

Key Words: LAG-3; swamp-type water buffalo, riverine-type water buffalo, phylogenetics

Introduction

T-lymphocyte–associated protein 4 (CTLA-4, CD152), Lymphocyte activation gene-3 (LAG-3), and programmed cell death 1 (PD-1, CD279) are inhibitory receptors such as cytotoxic

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doi: 10.14943/jjvr.65.2.65
function at multiple levels to ensure appropriate T-cell homeostasis, activation, and differentiation. These inhibitory molecules also contribute to cell extrinsic regulation by controlling T$_{reg}$ homeostasis and function, mediating induced T$_{reg}$ development, and mitigating dendritic cell differentiation and function. LAG-3 (CD223) is a member of the immunoglobulin superfamily and has been identified as a membrane protein and is expressed on various immune cells. LAG-3 is involved in the down-regulation of immune responses during the progression of chronic diseases, as well as in facilitating immune evasion by several pathogens causing chronic infections and tumors. Human and murine LAG-3 were mainly expressed in activated T-cells and natural killer (NK) cells while bovine LAG-3 was mainly expressed in CD4$^+$ and CD8$^+$ T-cells. The complete nucleotide sequence of LAG-3 was found to be between 500-2,000 bp in some representative animals.

Comparative in-depth study of the molecular structures of certain genes involves in the cell mediated immune responses between two closely related species is important to determine differences with regards to their reaction to pathogens. Transcriptional studies of these important proteins are also vital to elucidate their expression in the event of infection. Molecular analysis could provide valuable information to be used to study the role of LAG-3 and other inhibitory receptor molecules in an organism’s immune system. A pre-investigated query gene with well-established functions or roles can be used to study the function of a similar gene from the same species or related organisms. A study conducted about the murine LAG-3 sequence wherein the removal of a highly conserved motif found on the cytoplasmic region completely abolished the function of the gene. On the other hand, the over expression of LAG-3 that was observed in cattle infected with bovine leukemia virus and mice with lymphocytic choriomeningitis virus implies its apparent involvement in negatively regulating T cell proliferation during chronic disease progression.

There are still no studies conducted regarding the genetic characteristic of LAG-3 in bubaline species. Fortunately there is already existing data in the sequence of LAG-3 in cattle. Cloning and sequencing of bovine LAG-3 have an open reading frame of 1,551 nucleotides, encoding a polypeptide of 515 amino acids in length. Since both bovine and bubaline are closely related species, bovine LAG-3 sequence served as the reference for the determination of the genetic characteristic of bubaline LAG-3. The present study aimed to establish the LAG-3 sequence in water buffalo. This study can contribute to the understanding of the structure of the immune responses and provide clues for immunotherapeutic strategies. Thus, it can help to improve the health of water buffaloes to certain diseases. Moreover, it can increase the productivity and efficiency of the agriculturally significant animal. On the other hand, the differences that may arise between the LAG-3 of swamp- and riverine-type water buffaloes can contribute to their speciation. This present study was conducted in order to determine and describe the sequence of the LAG-3 in swamp- and riverine-type water buffaloes.

Materials and Methods

Clinical samples: Ten representatives from each type, Murrah buffalo (riverine-type) and Carabao (swamp-type) were selected for the collection of samples. The age of the selected animals were one year and above. Murrah buffalo and Carabao were raised on communal confinement and in backyard housing, respectively. Blood was collected in sterile tubes with lithium heparin to prevent coagulation. After collection, the samples were prepared for subsequent peripheral blood mononuclear cell (PBMC) culture.
**PBMC isolation and cultivation:** Bubaline PBMCs were isolated from collected heparinized venous blood by density gradient centrifugation with percoll (Sigma-Aldrich, St. Louis, MO, USA). In a 50 ml tube, 10 ml of fresh blood and 40 ml of EDTA PBS pH 7 was added and was centrifuged at 1,790 × g for 15 min. After centrifugation, 10 ml of buffy coat was transferred in a 15 ml tube containing 4 ml of 60% percoll solution. The tube was again centrifuged for 20 min at 1,030 × g. PBMC was then collected and transferred in another 15 ml tube with 15 ml EDTA PBS. The cell suspension was subjected to centrifugation at 150 × g for 10 min. The solution was washed with EDTA PBS twice after centrifugation and the supernatant was discarded. Suspension of the cell was done in 1 ml of Rowell Park Memorial Institute medium 1640 (RPMI) (Gibco BRL, Grand Island, NY, USA) at room temperature. Concanavalin A was added RPMI with PBMC and cultured for 12–24 hr. The culture was transferred to a 5 ml centrifuge tube and was centrifuged at maximum speed for 10 min. Cell lysis solution was added after the supernatant was discarded. The solution was transferred in another centrifuge tube and was centrifuged twice at 19,320 x g for 1 min.

**RNA extraction:** Total RNA was extracted from cultivated PBMC using TRIzol® reagent (Invitrogen, Carlsbad, Ca, USA) according to the manufacturer’s instructions. First, the PBMCs were homogenized through the addition of 1 ml TRIzol® Reagent. After homogenization, different phases of the sample were separated. The samples were incubated for 5 min at room temperature to permit complete dissociation of the nucleoprotein complex. Then, 0.2 ml of chloroform was added. The tube was shaken vigorously by hand for 15 seconds and was incubated for 2–3 min at room temperature. After incubation, the sample was centrifuged at 12,000 × g for 15 min at 4°C to be able to separate the different phases. The aqueous phase was transferred into a new tube for RNA isolation.

For the isolation of RNA, the aqueous phase was added with 0.5 ml of 100% isopropanol to the aqueous phase, per 1 ml of TRIzol® and was incubated at room temperature for 10 minutes to precipitate the RNA. The aqueous phase was centrifuged at 12,000 × g for 10 min at 4°C. The pellet was washed with 1 ml of 75% ethanol per 1 ml of TRIzol® Reagent. The sample was briefly subjected to vortex and was centrifuged at 7,500 × g for 5 min at 4°C. After centrifugation, the wash was discarded and the RNA pellet was air dried for 5–10 min. RNA pellet was resuspended in 50 μl RNase-free water. RNA samples were refrigerated at −20°C.

**Complementary DNA (cDNA) synthesis:** The cDNA synthesis was done using cDNA Synthesis Kit (Takara Bio Inc.) following the manufacturer’s procedure. RNA primer mixture was prepared using 1 μl of random hexamers, 1 μl of dNTP, 3 μl of extracted RNA templates and 5 μl of RNase free DW. The mixture was incubated in a PCR machine at 65°C for 5 min and was cooled immediately on ice.

Reaction mixture was prepared containing 4 μl of 5x PrimeScript Buffer, 0.5 μl of RNase Inhibitor, 1 μl of PrimeScript RTase and 10 μl of RNase free DW. The total reaction mixture was first incubated at 30°C for 10 min. The temperature was then raised to 50°C for 45 min and raised further to 95°C for another 4 min. After incubation, total reaction mixture was stored at 4°C until subsequent reaction.

**β-actin gene amplification:** The β-actin primer sequences: forward 5’-CGC ACC ACC GGC ATC GTG AT-3’ and reverse 5’-TCC AGG GCC ACG TAG CAG AG-3’. A total volume of 18.1 μl that contains 12.5 μl Top Taq, 1.6 μl MgCl₂, 0.5 μl of 10 pmol forward and reverse primers and 3.0 μl of DNA template were prepared.

The β-actin primer set (F/R) PCR profile cycle was: initial denaturation for 5 min at 94°C, 35 cycles of denaturation step for 30 sec at 94°C, annealing step for 30 sec at 55°C, and extension
step for 30 sec at 72°C followed by final elongation step for 5 min at 72°C that completed the reaction. Amplicon size was \( \sim 227 \) base pairs. Only samples positive for \( \beta \)-actin gene was further subjected to PCR.

**LAG-3 gene amplification:** The LAG-3 primer pair is: forward 5'-ATG CTG TGG GAG GCT TGG TT-3' and 5'-TCA GGG TTG CTC TGG CTG CA-3'. PCR was carried out in a total volume of 20.0 \( \mu l \) containing 6.9 \( \mu l \) of DDW, 4.0 \( \mu l \) of 10x PCR buffer, 1.4 \( \mu l \) of MgCl2, 1.6 \( \mu l \) of dNTPs, 0.5 \( \mu l \) each of 10 pmol primers, 0.1 \( \mu l \) of Taq polymerase, and 5.0 \( \mu l \) of DNA template. A negative control containing DDW instead of DNA template was used.

The PCR cycle profile for the LAG-3 primer set was 1 cycle of initial denaturation at 94°C for 5 min, 40 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 1 min and extension at 72°C for 1 min, followed by another extension step at 72°C for 5 min to complete the reaction. Amplicon size was 1,551 bp.

All PCR products were analyzed using 2% agarose gel and viewed under short UV illumination.

**DNA sequencing:** PCR products were sent to First Base, Malaysia for DNA sequencing.

**Homology analysis:** Homology analysis of LAG-3 nucleotides of swamp- and riverine-type water buffalo were done using Basic Local Alignment Search Tool (BLAST) program. CLUSTAL X program was used for the multiple sequence alignment. The locations of signal peptide cleavage site, transmembrane helicase, and N-Glycosylation sites in the deduced amino acid sequences were determined using Signal P4.1, TMHMM v.2.0, and NetNGlyc 1.0

**Phylogenetic analysis:** Phylogenetic analysis was performed using the MEGA 5.0 program. The evolutionary history was inferred using the neighbor-joining method.

### Results

**Homology analysis**

The sequence of LAG-3 of both types of water buffaloes encompassed 1,551 nucleotides encoding 516 amino acids. The LAG-3 nucleotide sequences of both types of water buffaloes were 95% identical. The sequence identity of water buffalo LAG-3 nucleotide sequence with other mammalian species is 76–96% while its deduced amino acid sequence is 61–94% (Table 1). High homology in LAG-3 sequences among different mammals with reference to bubaline species

<table>
<thead>
<tr>
<th>Species</th>
<th>Swamp-type (LC033567)</th>
<th>Riverine-type (LC033568)</th>
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<tbody>
<tr>
<td></td>
<td>Nucleotide</td>
<td>Amino Acid</td>
</tr>
<tr>
<td>Cattle (NM001245949)</td>
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<td>91</td>
</tr>
<tr>
<td>Goat (XM005681013)</td>
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<td>Sheep (XM004007581)</td>
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<td>Pig (NM001105306)</td>
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<td>76</td>
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<td>Human (NM002286)</td>
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</tr>
<tr>
<td>Mouse (NM008479)</td>
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</table>

(GenBank Accession Number)
mammalian species including both types of water buffaloes was revealed and may imply that this gene have little divergence in bubaline species.

There were nine amino acid substitutions between the LAG-3 sequences of bubaline species and 26 amino acid substitutions between swamp-type and Artiodactyl species located at different positions (Fig. 1). The predicted signal peptide cleavage site by SignalP was located at amino acid residue 23 and 24. The amino acid residues 1-23 represent a leader peptide region. Amino acid residues 24-434, 435-473, 432-441 and 474-536 corresponds to extracellular domain, transmembrane domain, possible cleavage sites by metalloproteases and cytoplasmic region, respectively (Fig. 1).

The extra loop in Domain 1 of swamp- and riverine-type water buffalo LAG-3, similar to that of the cattle, pig, sheep and goat, showed 15 amino acid deletions. Five cysteine residues found in the extracellular region of bubaline LAG-3 are well conserved among the mammalian species except for one cysteine residue in Domain 1 which was substituted in sheep (V). Interestingly, the highly conserved second cysteine residue found in Domain 4 of mammalian LAG-3, a part of the ‘WXC’ (Tryptophan-X-Cysteine, where X can be any amino acid residue) structural motif, is replaced by a tyrosine (Y) in swamp- and a histidine (H) residue in riverine-type water buffalo. In addition, LAG-3 consists three possible N-linked glycosylation sites; these are NCS, NVS

![Fig. 1. Deduced amino acid sequences alignment of LAG-3 gene from swamp- and riverine-type water buffaloes compared with other species.](image-url)
and NST (Fig. 1). N-glycosylation sites are comprised of three adjacent amino acid residues, Asn-X-Thr/Ser, where X can be any amino acid residue except for Pro\(^{29}\).

The ‘RGD’ motif found in Domain 1 is conserved in the LAG-3 sequences of the bubaline species including cattle.

The intracellular region of LAG-3 contains three characteristic regions: Serine residue, KTGELE motif, EP (Glutamic Acid-Proline) repeated motif. The serine residue located at amino acid residue 487 is conserved in all mammalian species (Fig. 1). The KTGELE motif is found in amino acid residues 500–506 is conserved in LAG-3 of the animals belonging to order Artiodactyla. Residues 501 and 502 corresponding to amino acids TG replaced to the IE within human, murine and horse LAG-3 ‘KIEELE’ motif.

**Phylogenetic analysis**

The evolutionary relationship was inferred using neighbor-joining method by the MEGA5 software. Phylogenetic analysis of mammalian LAG-3 revealed two clusters: Family Bovidae and Family Muridae (Fig. 2). The first group consists of bubaline, bovine, ovine and caprine species while mouse is part of the second group. The results further confirmed the degree of relationship between bubaline species. Additionally, the close relationship between water buffalo and other species of order Artiodactyla were emphasized. However, the LAG-3 sequence of both types of water buffalo are most distant to that of the human and murine species. Consequently, a mammalian phylogeny constructed using 26 gene fragments of 164 mammals shows similar results.

**Discussion**

LAG-3 has emerged as an important immune-molecule in T cell biology. Essentially, it maintains lymphocyte homeostatic balance through various inhibitory mechanisms\(^{9}\). The understanding of the structure of the immune response relies on the comparative molecular analysis of the immune receptors\(^{22}\). Additionally, transcriptional studies of immune receptor molecules are imperative to fully understand their expression during the events of disease and infection\(^{15,20}\).

High homology found in bubaline LAG-3 sequences possibly connotes conserved biological activity of the gene in the mammalian species. Thus, it is highly probable that LAG-3 sequences

![Fig. 2. Evolutionary relationship of LAG-3 sequences of different mammalian species. Bootstrap values are based on 1000 repetitions and are shown at the nodes supporting a particular cluster.](image-url)
of bubaline species have almost if not exactly the same function as the murine and bovine LAG-3. Bubaline LAG-3, like CD4, has four extracellular immunoglobulin-like domains containing conserved structural motifs throughout\(^1\). Clustered at the base of the extra loop in the first domain of the extracellular region of LAG-3 are most of the residues essential for adhesion to MHC II proteins. The second domain is either involved in the binding or in positioning the Domain 1 for interaction with MHC II. Thus, this domain is important for successful binding between LAG-3 and MHC II. However, both the third and fourth domains were irrelevant in LAG-3–MHC II interactions\(^10\). All of the inhibitory receptors contain single transmembrane spanning regions and cytoplasmic tails ranging from 35 to 178 amino acids in length\(^5\).

The replacement of cysteine by tyrosine and histidine in swamp-type and riverine-type may be a unique region in the LAG-3 of water buffaloes. The ‘WXC’ signature motif is found in an equivalent position only in CD4 protein but not in any other immunoglobulin superfamily domain. Although, there are no strong validations about the regulatory functions of this motif, it may suggests how the gene may have originated. The presence of the ‘WXC’ motif in an unusual position suggests that LAG-3 has evolved by gene duplication from a pre-existing two-domain immunoglobulin light chain-like structure\(^25\). Single cysteine to tyrosine transition appears to interfere with the receptor binding activity of the growth inhibitory function of myostatin, in cattle. This, however, is possibly a result of the disruption of a significant cysteine knot structure that is present only in myostatin\(^2\). This distortion of cysteine residue in bubaline could also interfere with immuno-inhibitory function of LAG-3. Cysteine residues are subjected to oxidative posttranslational modification and play an important role in protein structure and cell response\(^3\). This particular substitution in bubaline LAG-3 could be the immunological basis of disease resistance/tolerance and susceptibility in both types of water buffalo.

Meanwhile, N-linked glycosylation sites are the regions where sugar molecule known as glycan attaches to the nitrogen atom of the asparagine residue. This linkage is important for both the structure and function of the proteins\(^11,21\). Interestingly, the glycosylation site in domain 3 of swamp-type has replaced phenylalanine (F) by serine (S) and this could have a significant effect on biological function of LAG-3. On the other hand, metalloprotease cleavage in LAG-3 controls the efficiency of T cell proliferation and cytokine production. Thus, its presence is significant to the effect of LAG-3 during down regulation\(^17\).

Similar with cattle, bubaline LAG-3 has serine. Previous study suggests that this site is a possible phosphorylation site\(^26\). Phosphorylation occurs when a phosphate group attaches to an organic molecule, most commonly protein. This modification is an important mechanism for regulating protein activity. Hence, the presence of a possible serine phosphorylation site in LAG-3 may play an important role in LAG-3 function\(^6\).

KTGELE/KIEELE conserved motif is significant to LAG-3 signaling during down regulation of T cell expansion. However, study conducted regarding the functions of the motifs in the cytoplasmic tail of LAG-3 showed that the absence of the EP repeated motif had no effect on LAG-3 function, although it may help the KTGELE/KIEELE motif in preventing co-receptor activity. The EP amino acid repeated motif, that is quite unique in human LAG-3, is the binding motif between LAG-3 and LAG-3–associated proteins (LAP)\(^12\). Although EP motif is found within the bubaline LAG-3, it is short and interrupted by other amino acid residues. LAP’s connection to LAG-3 function has not yet been confirmed and its importance has yet to be determined\(^26\).

Generally, genetic characteristic of LAG-3 in water buffaloes may have similar function to that of other closely related species. Nevertheless, the substitutions in conserved motif and other sites of LAG-3 in water buffaloes may have an effect
in T cell homeostasis. If so, this could be one of the initial steps to the understanding of immune response and developing immuno-therapeutic strategies in water buffaloes diseases. The present study was first report that describes the genetic sequence of LAG-3 in swamp- and riverine-type water buffalo. However, further studies are required to determine the effect of these variations since these may have impacts to the inhibitory function of LAG-3.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

We thank Dr. Arnel N. del Barrio, Philippine Carabao Center (PCC) Executive Director, for the support to finish the study. Special thanks to all the staff of the Biosafety and Environment Section of PCC for their technical support. This research was supported by JSPS KAKENHI, grants from the Science and Technology Research Promotion Program for Agriculture, Forestry, Fisheries and Food Industry, Japan (grant 26058B) and the NARO, Bio-oriented Technology Research Advancement Institution (the special scheme project on regional developing strategy: grant 16817557).

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