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Characterization of CTLA-4, PD-1 and PDL-1 of swamp and riverine type water buffaloes

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ABSTRACT

Characterization of CTLA-4, PD-1 and PDL-1 genes from swamp and riverine type water buffaloes was done by molecular cloning, sequencing and phylogenetic analysis. The cloned cDNA of CTLA-4, PD-1 and PDL-1 contained an open reading frame of 666, 849 and 870 nucleotides, encoding a polypeptide of 221, 282 and 298 amino acids, respectively. Nucleotide sequence homology of both CTLA-4 and PDL-1 had 99.8% in swamp and riverine type, which gives the identical polypeptide. Meanwhile, PD-1 genes of swamp and riverine type water buffaloes had 99.2% of homology in nucleotide sequence, which has substitution of two amino acid residues. The hexapeptide motif, phosphatidylinositol 3′-kinase and potential glycosylation sites were conserved within the tribe Bovinae. Phylogenetic analysis confirmed the degree of relationship between the bubaline species and justify the distinctness of each breeds by the bootstrap value generated.

1. Introduction

CTLA-4 and PD-1 are receptors that negatively regulate T-cell activation. Ligation of both CTLA-4 and PD-1 blocked CD3/CD28-mediated up-regulation of glucose metabolism and Akt activity, but each, accomplished this regulation using separate mechanisms. Together, these data suggest that CTLA-4 and PD-1 inhibit T-cell activation through distinct and potentially synergistic mechanisms [1].

Programmed Death 1, or PD-1, is a Type I membrane protein of 268 amino acids. PD-1 is a member of the extended CD28/CTLA-4 family of T-cell regulators. The recent identification and characterization of additional CD28 and B7 family members including programmed death-1 (PD-1), programmed death ligand-1 (PDL-1) (B7-H1), and PDL2 (B7-DC) has added to the complexity and greater appreciation of how surface molecules control T-cell activation and peripheral tolerance. CD28/B7 interactions mediate co-stimulation and significantly enhance peripheral T-cell responses. CTLA-4, in contrast, interacting with the same B7 molecules, results in decreased T-lymphocyte activity and regulates the immune response. Similarly, PD-1 interactions with PDL-1 and PDL-2 down-modulate T-cell immune responses. Despite these similarities, the regulatory roles of the CTLA-4 and PD-1 pathways are distinct [2].

Two ligands for PD-1, PD-1 ligand 1 (PDL-1; B7-H1) and PD-1 ligand 2 (PDL2; B7-DC), have been identified based on the similarity to other B7 family molecules. PDL-1 is expressed on T cells, B cells, macrophages, dendritic cells, and some non-immune cells and is up-regulated after their activation. PDL-2 is regulated more tightly and is expressed mainly on activated macrophages and dendritic cells. PD-1 ligand 1 and 2 (PDLs) expressed on antigen-presenting cells have been shown to induce T-cell anergy or apoptosis via PD-1 on T cells, whereas PDL1 expressed

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Table 1
Cytokine primers used in this study.

Gene	Product size (bp)	Primers	Annealing temperature (°C)
CTLA-4	666	5′-ATG GCT TGC TCT GGA TTC CA-3′ 5′-TCA ATT GAT GGG AAT AAA ATA AGG C-3′	55
PD-1	513	5′-ATG GGG ACC CCG CGG GCG CT-3′ 5′-GAT GAC CAG GCT CTG CAT CT-3′	60
	504	5′-AAT GAC AGC GGC GTC TAC TT-3′ 5′-TCA GAG GGG CCA GGA GCA GT-3′	60
PDL-1	528	5′-ATG AGG ATA TAT AGT GTC TT-3′ 5′-GCC ACT CAG GAC TTG GTG AT-3′	62
	537	5′-GGG GGT TTA CTG TTG CTT GA-3′ 5′-TTA CGT CTC CTC AAA TTG T-3′	62

on peripheral tissues directly suppresses self-reactive lymphocytes [3].

Novel members of the B7-CD28 superfamily have recently been discovered and they seem to be particularly important for regulating the responses of previously activated T cells. Super-imposition of inhibitory signals like those delivered by CTLA-4 and programmed death (PD)-1-PD-1 ligand (PDL-1) pathway leads to a complex network of positive and negative co-stimulatory signals, the integration of which modulates immune responses [4].

There are two types of water buffalo, the swamp and the riverine type buffalo. Native water buffalo or carabao that is found in the Philippines and in the South and Southeast Asian regions belongs to swamp type water buffaloes, whereas the rest which mainly found in India, Europe and the Americas are of riverine type to include the Bulgarian Murrah buffalo. Swamp and riverine type water buffalo have different chromosome number which is 48 and 50, respectively. Interestingly, their crossbreed has 49 chromosome complement [5–7].

Recent studies have been done on water buffalo cytokines. These studies were conducted to understand more of the differences in the immunological activities between the riverine and the swamp type water buffaloes. Comparison was made based on the result of molecular cloning and sequencing of cytokines with the existing sequences of other mammalian species in the GenBank. Transcriptional activity assessment of water buffalo IFNγ and TNFα were also studied [22–24].

This present study was conducted to identify and analyze the possible differences in genetic structure and contribution to immunological activities of CTLA-4, PD-1 and PDL-1 genes in riverine and swamp water buffaloes. Sequence homology assessment and phylogenetic analyses were done to elaborate the distinctness of each species and to initiate research on the immunological basis behind the claim that swamp type buffalo is more disease-resistant than its riverine counterpart [8,9].

2. Materials and methods

2.1. Isolation of water buffalo RNA and RT-PCR

Blood samples were collected from the Philippine Carabao Center Genepool in the Philippines. At least five blood samples from each water buffalo types, Philippine carabao – swamp type (*Bubalus carabanensis*) and Bulgarian Murrah Buffalo – riverine type (*Bubalus bubalis*), were collected as RNA source. Total RNA was isolated from the buffy coat of heparinized blood using the TRIzol reagent (Invitrogen, USA).

Synthesis of cDNA was carried out using Moloney murine leukemia virus reverse transcriptase (MMLV-RT, Takara, Japan) and random 9mer primers (Takara, Japan). A total volume of 12 μl reaction mixture containing 11 μl of RNA and 100 pmol random 9mer primers was heated at 65 °C for 5 min. Reverse transcription was performed after addition of 8 μl of reaction mixture (5× buffer for MMLV-RT, 20 nmol dNTPs (Takara, Japan), 20 U of RNAse inhibitor (Promega, USA), 0.5 μl DEPC treated water and 200 U of MMLV-RT) for 1 h at 37 °C. After reverse transcription, the reaction mixtures were heated to 70 °C for 15 min in order to inactivate MMLV-RT.

CTLA-4, PD-1 and PDL-1 cDNAs were amplified by PCR using the designed primers (Table 1). PCR was carried out in total volume of 20 μl reaction buffer containing 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 1.25 mM $MgCl_2$, 0.2 mM dNTPs, 5 U of *Taq* polymerase (Takara), 5 M Betain, 10 pmol each of the primers, and 3 μl of cDNA. Cycling conditions for PCR were 40 cycles of 1 min at 94 °C, 1 min at 64 °C annealing temperature and 1 min at 72 °C, followed by the final extension for 5 min at 72 °C. Resultant PCR products were separated on 2% agarose gel containing ethidium bromide (0.5 μg/ml), and visualized under a UV light.

2.2. Cloning and sequencing of CTLA-4, PD-1 and PDL-1 genes

The amplified bands corresponding to each target gene were excised from the gel and purified using the Geneclean kit (Bio 101, USA). The purified cDNA fragments were ligated into the pGEM-T easy vector (Promega, USA), and transformed into a competent *E. coli* strain DH-5α. In each experiment, eight to 10 plasmid clones containing the cDNAs were sequenced using the BigDye terminator cycle sequence kit and an automated DNA sequencer (PRISM, 310 Genetic Analyzer, Applied Biosystems).

2.3. Phylogenetic analysis

Sequence data analyses were performed using the BLAST search of the National Center for Biotechnology