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Technical report

# Molecular cloning of canine interleukin-31 and its expression in various tissues

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ABSTRACT

The newly discovered cytokine, interleukin-31 (IL-31), belongs to the short-chain cytokine group. It was reported that transgenic expression of IL-31-induced pruritus, similar to atopic dermatitis, in mice, further, excessive amounts of IL-31 was also expressed in the skin from human patients with atopic dermatitis as compared to that from normal people. In this study, canine IL-31 was molecularly cloned from concanavalin A-stimulated canine peripheral blood mononuclear cells (PBMCs), and its nucleotide sequence was determined. Canine IL-31 contains 4 alpha-helix structures characteristic of the IL-31 family, and the amino acid identity of canine IL-31 with those of human or mouse is 54% and 28%, respectively. Furthermore, we detected low levels of canine IL-31 in the thymus, testis, spleen, and kidneys, but not in the skin of atopic dogs.



The cytokine interleukin (IL)-31, which was newly discovered in 2004, is predominantly expressed in T helper (Th)2 cells (Dillon et al., 2004). Stimulation of T cells with anti-CD3 and anti-CD28 antibodies immediately upregulates IL-31 mRNA expression (Dillon et al., 2004). Microarray analysis has showed that IL-31 induces certain chemotactic genes, such as CXCL1, CCL17 (thymus and activation-regulated chemokine [TARC]), CCL19 (macrophage inflammatory protein [MIP]3β), CCL22 (monocyte-derived chemokine [MDC]), CCL23 (MIP3), and CCL4 (MIP1β) (Dillon et al., 2004).

Transgenic mice that overexpress IL-31 showed characteristic skin diseases, pruritus and alopecia (Dillon et al., 2004). The subcutaneous injection of IL-31 into mice triggers infiltration by the inflammatory cells, neutrophils, eosinophils, lymphocytes, and macrophages, and results in epidermal thickening and dermal acanthosis. In NC/Nga mice, with atopic dermatitis (AD) due to natural causes, IL-31 is overexpressed in skin lesions and correlates with the pruritus (Takaoka et al., 2005, 2006). In human AD patients, the expression of IL-31 mRNA is considerably higher in skin lesions than in non-lesional skin, and the expression in non-lesional skin is greater than that in normal skin from healthy patients (Sonkoly et al., 2006). Another study has reported that $CD45RO^{+}$ (memory) cutaneous lymphocyte antigen (CLA)-positive T cells in the skin of AD patients express IL-31 mRNA and protein (Bilsborough et al., 2006). Neis et al. reported that IL-31 mRNA overexpression in the skin of patients with AD or allergic contact dermatitis is correlated with IL-4 and IL-13 mRNA expression, but not with interferon (IFN)-γ mRNA expression (Neis et al., 2006).

Type I hypersensitivity against environmental allergens is considered to be the main mechanism of canine AD, and the levels of Th2-mediated cytokines such as IL-4 are increased in the skin lesions of dog with AD (Nuttall et al., 2002). Moreover, infiltration by inflammatory cells, lymphocytes and neutrophils, is an important mechanism underlying the aggravation of the skin lesions; the overexpresssion of chemotactic genes such as CCL17/TARC

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**Table 1**
List of oligonucleotide primers.

| Primer name | Nucleotide sequence | Target gene |
|---|---|---|
| YTM11 | 5′-TGTGCCTGCAGATACTTTTGA-3′ | Canine IL-31 |
| YTM12 | 5′-GGTTCGACCAGATAGCCTTG-3′ | |
| YTM17 | 5′-TCTGGCTCTAGAAGCTGTTGC-3′ | Canine IL-31 |
| YTM14 | 5′-ACGCCGAGAACTGCTGTAAA-3′ | |
| VTM21 | 5′-TCACCATGCTCTCCCACAC-3′ | Canine IL-31 |
| YTM22 | 5′-CTGAGGTCCAGAGTTTAGTGACTT-3′ | |
| YTM33 | 5′-TCAACGGATTTGGCCGTATTGG-3′ | Canine GAPDH |
| YTM34 | 5′-TGAAGGGGTCATTGATGGCG-3′ | |
| YTM251 | 5′-GGCTGACAAGGTGGTACAAGACTTC-3′ | Canine TARC |
| YTM252 | 5′-CAGATGGACTTGCCTTGGACAG-3′ | |
| YTM255 | 5′-CCCAATGAGCAGGATGAGTT-3′ | Canine β2 microglobulin |
| YTM256 | 5′-CAGTTGTCTCGGTCCCACTT-3′ | |

(Maeda et al., 2005), CCR4 (Maeda et al., 2002b), and CCL28/mucosae-associated epithelial chemokine (MEC) (Maeda et al., 2008) contributes to the aggravation of skin lesions in the dogs with AD. In this study, canine IL-31 cDNA was molecularly cloned from concanavalin A (ConA)-stimulated peripheral blood mononuclear cells (PBMCs) and the expression of IL-31 mRNA was assessed in various tissues.

To clone canine IL-31, the primers, YTM11, YTM12, YTM17, and YTM14 were synthesized on the basis of the portions of canine genomic sequences that were considered to correspond to regions of canine IL-31 sequence (Table 1). Canine PBMCs were collected by centrifugation of peripheral blood samples from a healthy adult dog on Lymphoprep density gradient (AXIS-SHIELD, Oslo, Norway). The cells were cultured in RPMI1640 (SIGMA, Tokyo, Japan) supplemented with 10% FCS, antibiotics, and 50 μM 2-mercaptoethanol per ml at 37 °C in a humidified atmosphere of 5% $CO_2$ in air. PBMCs at a density of $2 \times 10^6$ cells/ml were stimulated with 5 μg of ConA (SIGMA) per ml for 24 h. Total RNA was extracted from the stimulated cells by using TRI reagent (Molecular Research Center Inc., Cincinnati, OH) according to the manufacturer's instructions. After treatment with *DNase*I (Applied Biosystems, Tokyo, Japan), reverse transcription of the total RNA was performed to generate cDNA, which was subsequently used as a template for PCR. Reverse transcriptase-polymerase chain reaction (RT-PCR) was conducted using the SuperScript First-Strand Synthesis System (Invitrogen Life Technologies, Tokyo, Japan) and oligo $(dT)_{15}$ primer. The resultant cDNA was amplified by PCR with KOD-Plus (TOYOBO, Osaka, Japan), according to manufacturer's instructions. After incubation at 95 °C for 2 min, PCR amplification was performed with 40 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s, and polymerization at 72 °C for 30 s followed by polymerization at 72 °C for 10 min. The amplified products obtained using primers YTM11 and YTM12, and YTM17 and YTM14 produced bands of approximately 130 and 450 bp bands, respectively (Fig. 1A). These 2 bands were cloned into TA vector using TOPO-TA-cloning kit (Invitrogen Life Technologies) and their nucleotide sequences were determined by BigDye Terminator v3.1Cycle Sequencing Kit (Applied Biosystems) using ABI 377 automated DNA sequencer. These 2 clones were shown to be similar to human and mouse IL-31 cDNAs. On the basis of the obtained sequences, the primers YTM21 and YTM22 were used to clone full-length canine IL-31. Canine IL-31 cDNA contained a 480-bp coding region and predicted amino acid sequences of canine IL-31 have 159 amino acids, showing 54% and 28% similarity to human and mouse IL-31, respectively (Fig. 1B and C). Amino acid analysis performed using the SignalP 3.0 software revealed that the signal peptides at the N-terminus of canine IL-31 are similar to those of human and mouse IL-31. Using the PredictProtein software, we found that canine IL-31 contains 4 alpha-helix loops, which is a characteristic feature of the IL-31 family, and has 2 *N*-glycosylation sites at amino acids 66–69 and 83–86 of amino acids. These features indicate that the nucleotide sequence determined in this study encodes canine IL-31.

Next, PBMCs isolated from 2 normal dogs were cultured in the presence of ConA. After cDNA was generated from each sample, as described above, it was subjected to real time PCR amplification with a QuantiTect SYBR Green PCR Kit (QIAGEN, Valencia, CA) according to the manufacturer's protocol. The primers used for assaying IL-31 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were based on canine nucleotide sequences (Table 1). Each assay was performed using duplicate samples. Predenaturation at 95 °C for 15 min was followed by 45 cycles of PCR amplification consisting of denaturation at 94 °C for 15 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. PCR and fluorescence intensity detection were performed with the StepOne PCR system (PerkinElmer, Waltham, MA). The PCR cycle number at the threshold was represented as $C_T$. The difference between $C_T$ for the target and $C_T$ for the internal control, i.e., $\Delta C_T$, was calculated. In the case of skin samples, $C_T$ values for the internal controls for each set of stimulated and unstimulated samples were compared, and only minor differences were found. The value of $2^{-\Delta C_T}$ was considered to represent the amount of target mRNA relative to the amount of internal control (Mizuno et al., 2003). Baseline levels of IL-31 expression in freshly isolated canine PBMCs from both dogs were below detectable limits. Stimulation with ConA profoundly induced IL-31 expression at 6 h in the PBMCs obtained from both dogs; this expression gradually decreased, as observed at 12 and 24 h (Fig. 2A). These results indicate that IL-31 is predominantly induced in activated T cells, which is consistent with the results of a previous study on mice (Dillon et al., 2004).