The TCCs (3×10^4 cells/well in 96-well flat-bottomed culture plates) were cultured in the presence of a soluble OM peptide mixture (1 μM) and irradiated autologous PBMC (1.5×10^5/well) for 56 hr. Culture supernatants of the TCCs were collected and stored in aliquots at -80℃ until the determination of lymphokine concentrations. Enzyme-linked immunosorbent assay (ELISA) kits for detecting human IL-4 (Biosource Int'l), and IFN-γ (Ohtsuka, Tokyo, Japan) were used for quantification of the lymphokines in the supernatants, according to the manufacturers' instructions.

Intracellular IFN-γ and IL-4 staining

TCCs specific to OM were cultured at a density of 2×10^6 cells /mL in an RPMI 1640 medium for 4 hr at 37℃. During the 4-hr incubation, the cells were stimulated with a combination of 25 ng/mL of phorbol 12-myristate 13-acetate (PMA) (SIGMA) and 2 μg/mL of ionomycin (SIGMA) in the presence of 10 ng/mL of Brefeldin-A (SIGMA). Then the cells were directly stained with an FITC-conjugated anti-CD4 monoclonal antibody (Coulter-Immunotech, Marseille, France) for 15 min at room temperature, and fixed with an FACS Lysing Solution (Becton Dickinson, Mountain View, CA) for 10 min. After washing, they were preincubated with an FACS Permeabilizing Solution (Becton Dickinson) for 10 min and after washing again, they were incubated with FASTIMMUNE IFN-γ FITC/IL-4 PE (Becton Dickinson) for 30 min at room temperature. The cells were then washed twice and resuspended in a phosphate-buffered saline (PBS). Flow cytometric analysis was performed using an FACS Calibur. The results were expressed as the percentage of each cytokine-producing cell population in a total population of CD4 cells.

Cell-surface-marker phenotypes of TCCs

A fluorescein isothiocyanate (FITC)-labeled monoclonal anti-IL-12Rβ1 antibody

(PharMingen, San Diego, CA), a phycoerythrin (PE)-labeled monoclonal anti-IL-12Rβ2 antibody (kindly donated by Dr. F Sinigaglia, Italy), anti-IL-18Rα, and anti-human CCR-3 (R&D Systems, McKinley Place, MN) were used to analyze the phenotype of our TCCs. FITC-labeled anti-Leu4/CD3, anti-Leu3a/CD4 (Becton Dickinson), anti-TCR-α/β-1-WT31 (Becton Dickinson), anti-TCR-γ/δ (Endogen, Woburn, MA), and PE-labeled anti-Leu2a/CD8 (Becton Dickinson) were also used to analyze the phenotypes of our TCCs by double-color staining. The stained cells were analyzed using an FACScan instrument (Becton Dickinson). The forward scatter threshold was set to exclude only debris in the preparation.

Results

Production of IL-4 and IFN-γ in supernatants of TCCs

Cytokine secretion was investigated in the TCCs specific to OM. Since these clones were maintained in the presence of IL-2 and IL-4, they were washed with the culture medium and were then cultured with the OM peptide mixture (1 μM) and irradiated autologous PBMC, but in the absence of IL-2 and IL-4, for 56 hr. Cell proliferation was similar among these clones when examined using a 3H-thymidine uptake (data not shown). Most TCCs secreted both IL-4 and IFN-γ in response to the peptide mixture, as well as OM crude protein (data not shown), but the secretion patterns were variable; an IFN-γ dominant pattern (IFN-γ >> IL-4) was seen in IH3.1 and YT6.1, an IFN-γ >IL-4 pattern in TM1.3,TM1.4 and IH3.3, IL-4> IFN-γ pattern in YN1.5., as shown in Figure 1.

Intracellular IL-4 and IFN-γ staining

Since the patterns of IL-4 and IFN-γ secretion were variable among the TCCs,

intracellular IL-4 and IFN-γ staining was analyzed. As shown in Figure 2, intracellular IFN-γ single-positive cells were predominant in TM1.3, TM1.4, IH3.1 and YT6.1 and IFN-γ and IL-4 double-positive cells were predominant in YN1.1, YN1.5 and IH3.3.

Together with cytokine secretion data, TM1.3, TM1.4, IH3.1, and YT6.1 had a Th1-dominant phenotype and YN1.1, YN1.5, and IH3.3 had a Th0-dominant phenotype.

Cell-surface-marker phenotypes of TCCs.

All TCCs specific to OM exhibited $CD3^+$, $CD4^+$, $CD8^-$, $\alpha\beta TCR^+$ and $\gamma\delta TCR^-$ phenotypes (data not shown). Moreover, all TCCs were IL-12Rβ1-positive (Fig.3). TM1.3, YN1.5, IH3.1, IH3.3 and YT6.1 were IL-12Rβ2-positive and TM1.4 and YN1.1 were IL-12Rβ2-negative(Fig.3). IL18Rα was co-expressed with IL-12Rβ2 in these clones (data not shown).

Discussion

Murine helper T-cells are divided into two subsets, Th1 and Th2 cells. The former produce IL-2 and IFN-γ and the latter produce IL-4 and IL-5 (12). Such dichotomy is also evident in human cells, albeit to a less polarized extent than in murine T-cells (13). Th1 cells cause effective non-immediate-type hypersensitivity reactions whereas Th2 cells promote IgE production leading to immediate-type hypersensitivity.

In our previous study, it was shown that the proliferative responses of PBMCs to Ovalbumin (OVA) in children with AD who are sensitive to hen's eggs were significantly higher than those of healthy children and hen-egg-sensitive children with immediate symptoms. However, in patients with AD there were no significant correlations between the proliferative response to PBMCs and the RAST values (14). We also reported on the high sensitivity and specificity of proliferative responses of

lymphocytes to OVA for the detection of hen-egg allergy in patients with AD (15). These studies indicate that the molecular basis of non-immediate-type hypersensitivity, as seen in AD patients who are sensitive to hen's eggs is different from that of immediate-type hypersensitivity to hen's eggs.

Chicken OM has been reported to be the most important allergenic protein in egg-white than other egg-white proteins (2,3) and consists of three tandem homologous domains (16). Hence, we previously established several OM-specific TCCs in non-polarizing conditions from 4 patients with egg-white allergy to investigate molecular basis of hen-egg allergy (9). In this paper, we characterized these TCCs in regard to helper T-cell phenotypes. Patients TM and YN presented immediate-type hypersensitivity symptoms, and their levels of total IgE and CAP-RAST for egg-white and OM were high. On the other hand, patients IH and YT presented non-immediate symptoms, and their levels of total IgE and CAP-RAST for egg-white and OM were low. These clinical manifestations suggested that Th1 and Th2 cells mainly contribute to the pathogenesis of hen-egg allergy in the former two patients and the latter two patients, respectively. All of the twenty-four Der pI-specific TCCs from a patient with severe atopic disease were reported to have the Th2-type character (17). This may indicate that imbalance between Th1 and Th2 in a patient's condition influences the subtypes of these TCCs. Hence we expected that Th1 clones would be established from IH and YT and that Th2 clones would be from TM and YN.

In the present study, we analyzed IFN-γ and IL-4 production patterns by stimulation with OM, intracellular IFN-γ and IL-4 staining in 7 T-cells clones.
The results were further confirmed by the cell-surface marker of Th1 cells. TM1.3, TM1.4, IH3.1, and YT6.1 had a Th1-dominant phenotype and YN1.1, YN1.5, and IH3.3 had a Th0-dominant phenotype. IH3.1 and YT6.1 had a Th1-dominant phenotype in accord with the fact that they were established from patients who

presented non-immediate-type hypersensitivity. However, TM1.3 and TM1.4 were established from patient TM who presented immediate-type hypersensitivity symptoms, but both had a Th1-dominant phenotype. YN1.1 and YN1.5 were from patient YN who also presented immediate-type hypersensitivity symptoms but both had a Th0 phenotype. Our results indicate that the cell phenotype of TCCs does not always reflect clinical manifestation corresponding to immediate or non-immediate hypersensitivity. The phenotypes of TCCs could not be simply predicted, so that establishment of further TCCs and their characterizations are now under way.

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Figure legends

Fig. 1.

Cytokine production patterns of our TCCs. T-cells were cultured in the presence of an OM peptide mixture (1mM each). After incubation for 56 hours, culture supernatants were collected immediately, and the cytokines were measured by ELISA. Net cytokine concentration in culture supernatants is expressed as the mean value of duplicate cultures.

Fig. 2.

Intracellular IFN-γ and IL-4 staining. TCCs specific to OM were cultured at a density of 2 x 10^6 cells per mL in an RPMI 1640 medium with PMA and ionomycin in the presence of Brefeldin-A for 4 hr at 37℃. After PMA stimulation, the cells were directly stained with an FITC-conjugated anti-CD4 monoclonal antibody. After permeabilization, the cells were stained with IFN-γ FITC/IL-4 PE. Flow cytometric analysis was performed using an FACS Calibur.

Fig. 3.

Flow cytometric analysis of our TCCs. FITC-labeled monoclonal antibody, anti-IL-12R β1, and a PE-labeled monoclonal antibody, anti-IL-12R β2, were used. Stained cells were analyzed using an FACScan instrument.

Fig. 1

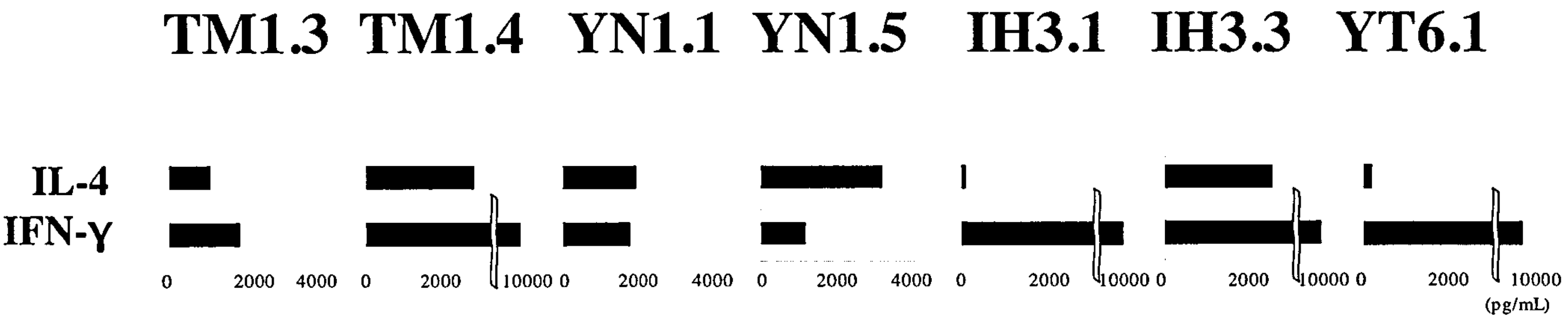


Fig. 2

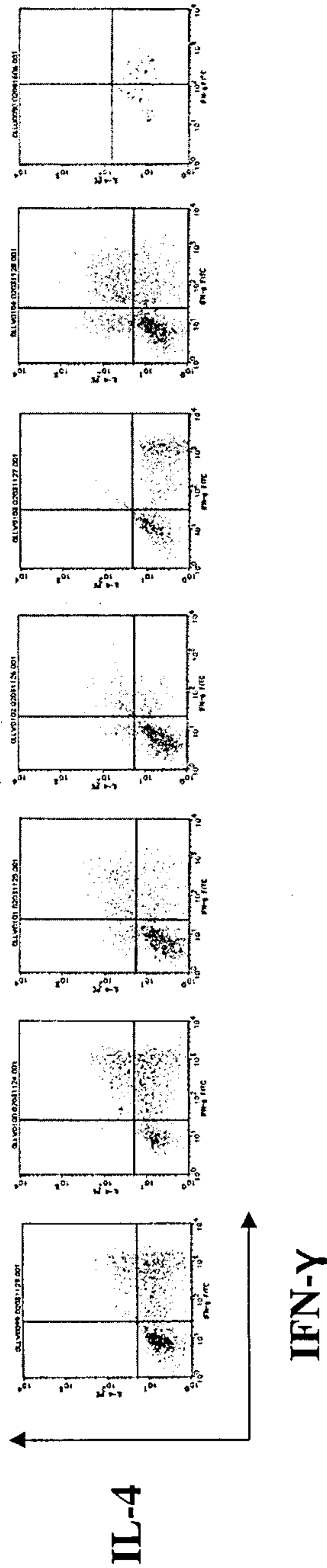
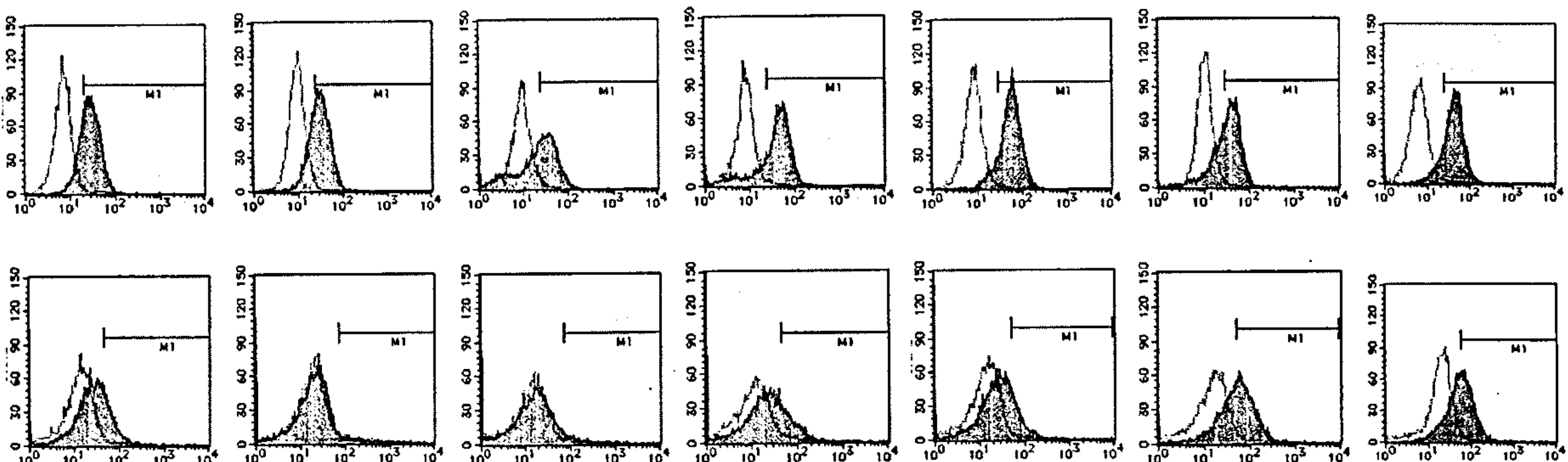


Fig. 3



IL-12B promoter polymorphism associated with asthma and *IL-12B* transcriptional activity

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Short running title is "Asthma and *IL-12B* promoter polymorphism".

ABSTRACT

Background: The interleukin-12B gene (*IL-12B*) encodes the p40 chain of interleukin-12 (IL-12), which promotes cell-mediated Th1 responses and the production of interferon-gamma (IFN-γ) that downregulates IgE production. Chromosome 5q31-33 near the *IL-12B* locus is significantly linked to asthma, as determined by a genome-wide search in the Japanese population.

Methods: We sequenced exons 1-8 including parts of the introns and promoter region of *IL-12B* in asthmatic patients and healthy controls. We examined plasma IL-12 concentrations, IL-12 production by Derf1-stimulated peripheral blood mononuclear cells (PBMCs) and the *IL-12B* transcriptional activity.

Results: *IL-12B* promoter polymorphism existed as $^{-2703}$CTCTAA/GC and $^{-2403}$T/C alleles, which were linked to each other. Homozygosity for haplotype 1 ($^{-2703}$CTCTAA /$^{-2403}$T) was associated with asthma susceptibility in Japanese children (P<0.001). Both plasma IL-12 concentrations and IL-12 production by Derf1-stimulated PBMCs in the subjects with homozygotes for haplotype 1 were lower than those with homozygotes for haplotype 2 ($^{-2703}$GC /$^{-2403}$C) (P<0.001). The transcriptional activity of the

construct with haplotype 1 was lower than that of the construct with haplotype 2, and the *IL-12B* transcriptional activity was influenced by the $^{-2403}$T/C allele rather than by the $^{-2703}$CTCTAA/GC allele.

Conclusion: Homozygosity for haplotype 1, which is associated with reduced *IL-12B* transcriptional activity and reduced IL-12 production, is a predisposing factor for asthma susceptibility in Japanese children.

Key words: asthma, IgE, interferon-gamma, interleukin-12B promoter polymorphism

INTRODUCTION

Interleukin-12 (IL-12) is a heterodimeric molecule that is composed of two disulfide-linked subunits, p35 and p40. It is produced by macrophages, B cells and other antigen-presenting cells (APCs), [1, 2] and plays important roles in interferon-gamma (IFN-γ) production by T cells and natural killer (NK) cells.

Genome-wide linkage screens, in which the genetic factors of the diseases can be identified, have been performed for asthma and recognized many regions linked to asthma.[3] Asthma is associated with Th2 cytokines, such as IL-4, IL-5, IL-9, IL-13, which are mapped to chromosome 5q31-33. Polymorphisms of the IL-4 receptor α chain and IL-13 are associated with asthma.[4-6] Yokouchi *et al.* have reported significant evidence for linkage of asthma to 5q31-33 near the *IL-12B* locus but not the IL-4 and IL-13 loci in the Japanese population.[7] Therefore, *IL-12B* is one of the candidate genes for asthma. Several polymorphisms have been identified in *IL-12B* [8, 9], including a single-nucleotide polymorphism in the 3' untranslated region, which has been associated with the susceptibility to type 1 diabetes and atopic dermatitis [10, 11] but not to asthma and allergic rhinitis in the Japanese population.[12] Recently, it has been

reported that the polymorphism exists in the *IL-12B* promoter region.[13, 14]

In this study, we sequenced exons 1-8 including parts of the introns and region 3 kb upstream from the transcriptional start site of *IL-12B* in 30 patients. Furthermore, we investigated *IL-12B* promoter polymorphism in 111 asthmatic patients and 78 controls, and examined the relationship between *IL-12B* polymorphism and asthma prevalence, IL-12 production and *IL-12B* transcriptional activity. We showed that *IL-12B* promoter polymorphism is associated with asthma and influences IL-12 production and *IL-12B* transcriptional activity.

METHODS

Patients and control subjects

One hundred and eleven asthmatic patients and 78 controls were enrolled in this study. Asthma was diagnosed on the basis of the American Thoracic Society guidelines. All the asthmatic patients showed total IgE levels above 200 IU/mL or specific sensitization to major allergens such as house dust and mite. The mean age±SD of the asthmatic patients was 5.6±2.9 years, and their mean IgE±SD level was 906.8±1347.4 IU/mL. All the controls had a negative history of atopic diseases. Their plasma IgE levels were lower than 150 IU/mL and their specific IgE levels were negative. The mean age ±SD of the controls was 4.7±3.4 years, and their mean IgE±SD level was 52.3±52.4 IU/mL. Informed consent was obtained from all the subjects or their parents.

Detection of IL-12B polymorphism

Genome DNA was extracted from neutrophils with a Sepa-gene kit (Sanko Junyaku, Tokyo, Japan). Exons 1-8 including parts of the introns and region 3 kb upstream from the transcriptional start site of *IL-12B* were amplified and sequenced using an ABI 3100

DNA sequencer (Applied Biosystems, CA, USA). We also sequenced previously identified with polymorphisms in the introns.[9] The conditions for the PCR were 94℃ for 1 min, 60℃ for 1 min, and 72℃ for 1 min. The primers used are shown in Table 1.

Cell preparation

Peripheral blood mononuclear cells (PBMCs) were separated from the heparinized blood of the subjects by gradient centrifugation in Ficoll-Paque (Pharmacia, Uppsala, Sweden). PBMCs were suspended at a density of 1×10^6/mL in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mmol/L L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin.

Cell culture

PBMCs (1×10^6/mL) were cultured in the presence or absence of 5 IU/mL recombinant human IL-12 (R&D Systems, Inc, Wiesbaden, Germany) or 5 μg/mL *Derf1* (Asahi, Tokyo, Japan) for 24 hr in a final volume of 1 mL in a round-bottom tube

(Falcon 2059, Becton Deckinson Labware, Franklin Lakes, NJ, USA) at 37℃ in a humidified atmosphere containing 5% CO_2.[15]

Assays for cytokines

Plasma samples isolated from heparinized blood and the supernatants of the cell culture were stored at −30℃ until assay. IL-12 concentrations in the plasma and the supernatants of the *Derf1*-stimulated cell culture were measured with a human IL-12+p40 ELISA kit (Bio Source International, CA, USA); the minimum detection limit was 7.81 pg/mL. This ELISA kit recognized both natural and recombinant human IL-12, as well as the free p40 subunit. IFN-γ concentrations in the supernatants of the IL-12-stimulated cell culture were measured with a human IFN-γ ELISA kit (Ohtsuka, Tokyo, Japan); the minimum detection limit was 15.6 pg/mL.[16]

IgE assay

Plasma IgE levels were determined by chemiluminescent enzyme immunoassay. Specific IgE antibodies for house dust, mite, cedar pollen, cow's milk, and hen's egg

were measured by using a Uni-Cap assay kit (Pharmacia, Uppsala, Sweden). A specific IgE level above 3.5 IU/mL was considered positive.

Cell lines and reagents

Mouse macrophagic cell line RAW264 (RIKEN Cell Bank, Tsukuba, Japan) was cultured in RPMI 1640 supplemented with 10% FCS, 2 mmol/L L-glutamine, 100 U/mL penicillin and 100 μ g/mL streptomycin.

Plasmids for luciferase assay

The -2808/-2303 distal enhancer region of *IL-12B* was obtained from genomic DNA by PCR amplification with the primers 5'-ttccggtcgACATGTTGATAAACCTCTTCTCC-3' and 5'-ttgccggatcCGTAGCTCACAAGGGGACATCAAAGATGAC-3'. The conditions for the PCR were 94℃ for 1 min, 60℃ for 1 min, and 72℃ for 1 min. *Sal*I and *Bam*HI restriction endonuclease sites embedded in the PCR primers allowed the release of a 512-bp insert. The amplified PCR product was subcloned into the luciferase reporter plasmid PicaGene Promoter Vector 2 (PGV-P2; Toyo Ink Mfg. Co.,