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Molecular variations in Th1-specific cell surface gene *Tim-3*

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Abbreviations: AHR, airway hyperreactivity; HAVCR, hepatitis A virus cellular receptor; SNP, single nucleotide polymorphism; TIM, T-cell immunoglobulin domain and mucin domain

Abstract

The family of T-cell immunoglobulin domain and mucin domain (TIM) proteins is identified to be expressed on T cells. A member of Tim family, *Tim-3* (T cell immunoglobulin mucin 3) is selectively expressed on the surface of differentiated Th1 cells. *Tim-3* might have an important role in the induction of autoimmune diseases by regulating macrophage activation and interacts with *Tim-3* ligand to regulate Th1 responses. To determine the variation sites in the coding and promoter region of human *Tim-3* gene, we performed variation scanning by direct sequencing using the genomic DNA isolated from the patients with asthma or allergic rhinitis and healthy controls without asthma and allergic rhinitis. We identified four single nucleotide polymorphisms (SNPs) including one novel SNPs (-1541C >T) and two variation sites (-1292_-1289delTAAA and -1282_-1278dupTAAAA) in the coding and promoter region of human *Tim-3* gene in both the patients and healthy groups.

Keywords: asthma; hypersensitivity; polymorphism, rhinitis; Tim-3

Introduction

Naive T helper cells differentiate into Th1 or Th2 cells by multiple factors, which include cytokine profile, type of antigen, delivery route of antigens, transcription factors and signaling pathways (O'Garra 2000; Ho and Glimcher 2002). The balance between Th1 and Th2 cells is critical in the immune response to pathogens, tumor antigens and allergens. Th1 and Th2 cells cross-regulate the differentiation of partner subset. The predominant induction of Th2 cells inhibits autoimmune diseases and the predominant induction of Th1 cells can regulate induction of asthma and allergic diseases (Lack *et al*., 1994; Nicholson *et al*., 1995; Hofstra *et al*., 1998).

Recently, the family of T-cell immunoglobulin domain and mucin domain (TIM) proteins was identified to be expressed on T cells (McIntire *et al*., 2001). All three of this gene family encodes cell surface glycoproteins with common structural motifs, including a signal peptide, Ig domain, mucin domain, transmembrane region and intracellular tail with phosphorylation sites. The predicted structure of TIM-1 contains two tyrosine residues and includes a conserved tyrosine kinase phosphorylation motif on the cytoplasmic region. TIM-2 also has two extracellular N-linked putative glycosylation sites and an intracellular tyrosine kinase phosphorylation motif (McIntire *et al*., 2001). TIM-3 (also known as HAVCR-2, hepatitis A virus cellular receptor 2) encodes the integral membrane glycoprotein with multiple extracellular putative glycosylation sites and an intercellular tyrosine phosphorylation motif. The human homologue of *Tim-1*, which lies at human chromosome 5q33.2, encodes for the cellular receptor for hepatitis A virus (Feigelstock *et al*., 1998). TIM-1 was considered as a membrane protein that is associated with the development of Th2 biased immune responses and may be selectively expressed on Th2 cell (McIntire *et al*., 2001; Umetsu *et al*., 2002). More recently, Kumanogoh *et al*. reported that TIM-2 interacts with the class IV semaphorin Sema4A, which enhances the activation and differentiation of T cells (Kumanogoh *et al*., 2002). TIM-3 was not expressed on naive T cells, B cells, macrophages or dendritic cells. TIM-3 was only present at the highest level in Th1 cells and has an

essential role in Th1 responses and macrophage activation (Monney *et al*., 2002). Comparison of the sequence of the coding regions for the three Tim genes in the two strains of mice showed sequence variants in *Tim-1* and *Tim-3* (McIntire *et al*., 2001). The results of above studies led us to identify the polymorphism of *Tim-3* genes in a Korean.

In this study, we scanned the variation sites of human *Tim-3* gene using the genomic DNA isolated from 24 controls without asthma and allergic rhinitis, 24 asthma and 26 allergic rhinitis patients by direct sequencing.

Materials and Methods

Subjects and DNA samples

Blood samples were obtained from 24 unrelated asthma patients, 26 unrelated allergic rhinitis patients and 24 unrelated controls without asthma and allergic rhinitis. The asthma and allergic rhinitis patients were recruited from our outpatient clinic at Chonbuk National University Hospital and Wonkwang Medical Hospital, respectively. The healthy controls were recruited from the general population who took a comprehensive medical testing at Wonkwang University Hospital. The all subjects used in this study were Korean living in the same area. Genomic DNA was extracted from leukocytes of peripheral blood by a standard phenol-chloroform method or by Invisorb spin blood Maxi kit (Invitek, Germany) according to manufacturer's direction.

PCR and sequencing analysis

The promoter and coding regions of *Tim-3* were partially amplified using the seven primer pairs (Table 1). PCR was carried out in a 25 μl reaction volume containing 50 ng of genomic DNA, 0.5 μM of the primers, 0.2 mM of dNTP, 1.5 mM of $MgCl_2$, 10 mM of Tris-HCl (pH 8.3) and one unit of Ex Taq polymerase (TaKaRa). PCR was run for 30 cycles of denaturation at 95°C for 30 s, annealing at melting temperature of the each primer pair for 40 s, and extension at 72°C for 1 min or 2 min. Both sense and antisense strands of PCR products were directly sequenced using the same primers used for the PCR amplification. Five primers (Tim-3-PF1: 5'-TTAAGAG-CCTTGACCAAGTTCA-3', Tim-3-PF2; 5'-ATACAAAA-TTATCCGGGGTGGT-3', Tim-3-PF3; 5'-TGTGAGCT-CACTTCCCTTTTAT-3', Tim-3-PF4; 5'-GAGAGCCAT-GCATGTATTATCT-3', and Tim-3-PF5; 5'-CATGAC-TGAGTAGCGTTTTCTT-3') were additionally used to sequence the promoter region. PCR products purified by PCR purification kit (Millipore) were used as template DNA for cycle sequencing. The PCR for sequencing was performed using BigDye Terminator cycle sequencing and analyzed using an ABI 3100 Prism Automated DNA sequencer (Applied Biosystems) by according to the manufacturer's instruction.

Statistical analysis

The polymorphisms of *Tim-3* gene were identified in 74 unrelated Korean individuals by general direct sequencing method (Kim *et al*., 2003). The asthma or allergic rhinitis patients and healthy controls were compared using case-control association analyses. Allele carrier frequency was defined as the percentage of the individuals carrying the allele among the total number of the individuals. Fisher's exact test or x^2 test from 2×3 contingency table was applied to analyze the comparison of the frequency of discrete variables between unrelated asthma or rhinitis patients and unrelated healthy controls. *P*-value less than 0.05 were considered to indicate statistical significance. The reference sequence is based on the sequence of human chromosome 5 clones CTB-120L21.

Table 1. Primer sequences for amplifying the coding and promoter regions of the Tim-3.

Amplified region	Primer name	Primer sequence (5→3')	Product size (bp)
Promoter and Exon 1	Tim3-1F/Tim3-1R	TTAAGAGCCTTGACCAAGTTCA/TACAATGGCCATCCTTGTATCT	1990
Exon 2	Tim3-2F/Tim3-2R	GCAGTTTTCCTGAAATGGAGTA/GAAGTCAGAGATGAGAACAATC	491
Exon 3	Tim3-3F/Tim3-3R	CCTTTGATTCCCTGAAATAAGC/TGAATTCAGAGCCAGCTAAAGA	203
Exon 4	Tim3-4F/Tim3-4R	TTGGCTATTTTTCCTCTCTCTC/GACAAGTACGGAGTAGAATTCA	221
Exon 5	Tim3-5F/Tim3-5R	GGATGGTCCAATTCACAAATCT/GAATTTGTTATCAGAGGGAGAG	280
Exon 6	Tim3-6F/Tim3-6R	GCTTCCAGTGAACCTATTTGAT/TCTAAGGCACACGTTGAGAG	228
Exon 7	Tim3-7F/Tim3-7R	TGTTACCTGGGAAATGCTTAAC/AGGCAATGACATGCCTGTTTAA	440

Results and Discussion

The genomic size of human *Tim-3* is about 23 kb and consists of 301 amino acids, including a signal peptide, an IgV domain, a mucin domain, a transmembrane region and a cytoplasmic region. To determine the variation sites in the coding and promoter region of human *Tim-3* gene, we performed variation scanning by direct sequencing using the genomic DNA isolated from 24 unrelated asthma patients, 26 unrelated allergic rhinitis patients and 24 unrelated healthy controls. We identified a total of four single nucleotide polymorphisms (SNPs), -1541C>T, -1516G>T (rs10053538), -574T>G (rs10515746) and 4259G>T (rs1036199), and two variation sites, -1292_-1289delTAAA and -1282_-1278dupTAAAA, in the coding and promoter region of human *Tim-3* gene (Table 2; Figure 1).

Among the SNPs identified in this study, three SNPs, -1541C>T (position is calculated from the translation start site), -1516G>T (rs10053538) and -574T>G (rs10515746), were located in promoter region and one SNP, 4259G>T (rs1036199), was located in exon 3. Interestingly, -1516G>T (rs10053538) located at the putative transcriptional binding site of p300. T allele of the both -1767T>G and -882T>C located in promoter region was changed to G and C, respectively, on the all of genomic DNA used in this study (data not shown). We found two different sequences between our sequence and NCBI reference sequence (human chromosome 5 clone CTB-120L21). The allele T (-1767 and -882) of reference sequence was substituted to allele G and C, respectively on the all of genomic DNA samples isolated from 74 Korean. A variation site of promoter region, 1572insC, was also newly identified on our sequence (data not shown). These results led us to think that it might be the sequencing error of the reference sequence or it may be a specific nucleotide sequence of Korean. The coding region of human *Tim-3* gene was consisted of seven exons but only one SNP are identified in exon 3 (Table 2). The change of allele at 4259G>T (rs1036199) of exon 3 is resulted inamino acid substitution; arginine to leucine. A varia-

Table 2. Genotype and allele frequencies of the *Tim-3* variations between the patients with asthma or allergic rhinitis and controls.

Position	Genotype/Allele[a]	Control (*n*)	Asthma (*n*)	Rhinitis (*n*)	Freq.[b]	*P*[c]	
						vs. asthma	Rhinitis
-1541C>T[d]	CC	23	24	26	0.007	-	-
	CT	1	0	0			
	TT	0	0	0			
-1516G>T (rs10053538)	GG	18	19	23	0.095	0.942	0.465
	GT	6	5	3			
	TT	0	0	0			
-574T>G (rs10515746)	TT	0	0	0	0.074	0.470	0.995
	TG	2	5	4			
	GG	22	19	22			
4259G>T (rs1036199)	GG	0	0	0	0.028	–	–
	GT	0	0	4			
	TT	24	24	22			
-1292_-1289delTAAA[d]	Genotype1	0	0	0	0.122	0.942	0.990
	Genotype2	6	5	7			
	Genotype3	18	19	19			
	Allele1	6	5	7		0.762	1.000
	Allele2	42	43	45			
-1282_-1278dupTAAAA[d]	Allele1	6	5	7	0.122	0.762	1.000
	Allele2	42	43	45			

[a]Genotype 1, 2 and 3 mean the homozygous with reference sequence, the heterozygous by reference and variation sequence and the homozygous with variation sequence, respectively; Allele 1 and 2 mean the reference sequence and the variation sequence, respectively. [b]Frequencies of rare alleles, [c]Fisher's exact test or x^2 test from 2 × 3 contingency table was applied, [d]The novel SNP (-1541C>T; agcactttgggaggc **c/t** gaggcgggtggatcgc) and variation sites (-1292_-1289delTAAA; ctccatctcaaaaaaataaataaa **taaa/del** taaaaataaaataaaataaaatgagaaattgattat and -1282_-1278dupTAAAA; ctcaaaaaaataaataaataaataaaaataaaataaaa **taaaa/dup** tgagaaattgattatgatctc) identified in this study.

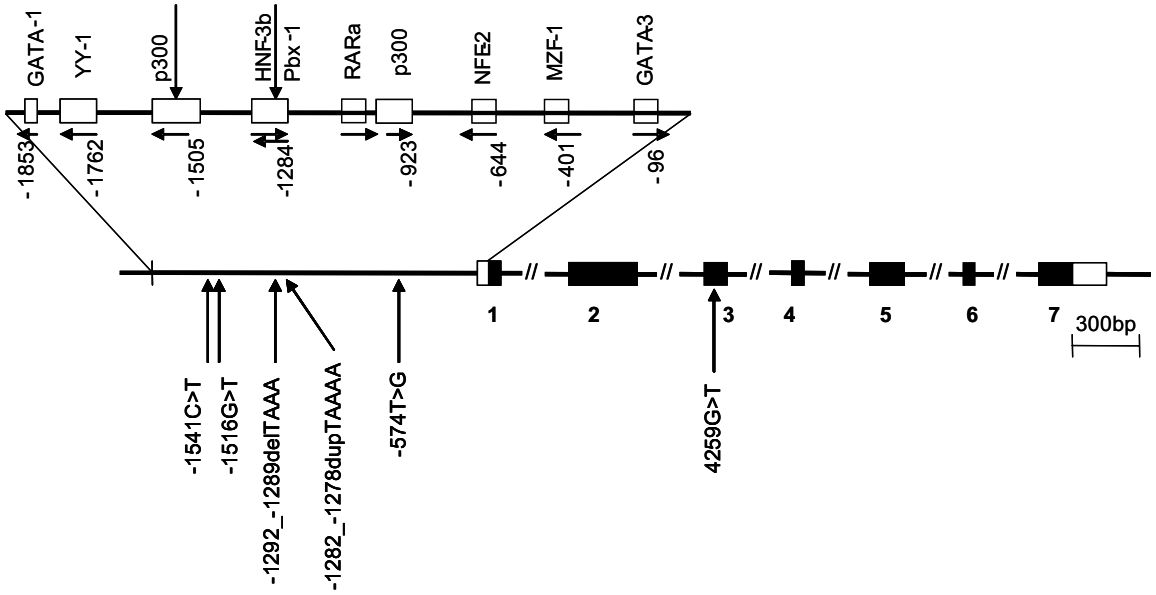

Figure 1. Locations of each single nucleotide polymorphisms (SNPs) and variation sites in *Tim-3*. Coding exons are marked by black blocks and 5'- and 3'-UTR by white blocks. The positions of SNPs were calculated from the translation start site. Putative transcription factor sites are searched at www.cbrc.jp/research/db/TFSEARCH.html. The reference sequence is based on human chromosome 5 clone CTB-120L21.

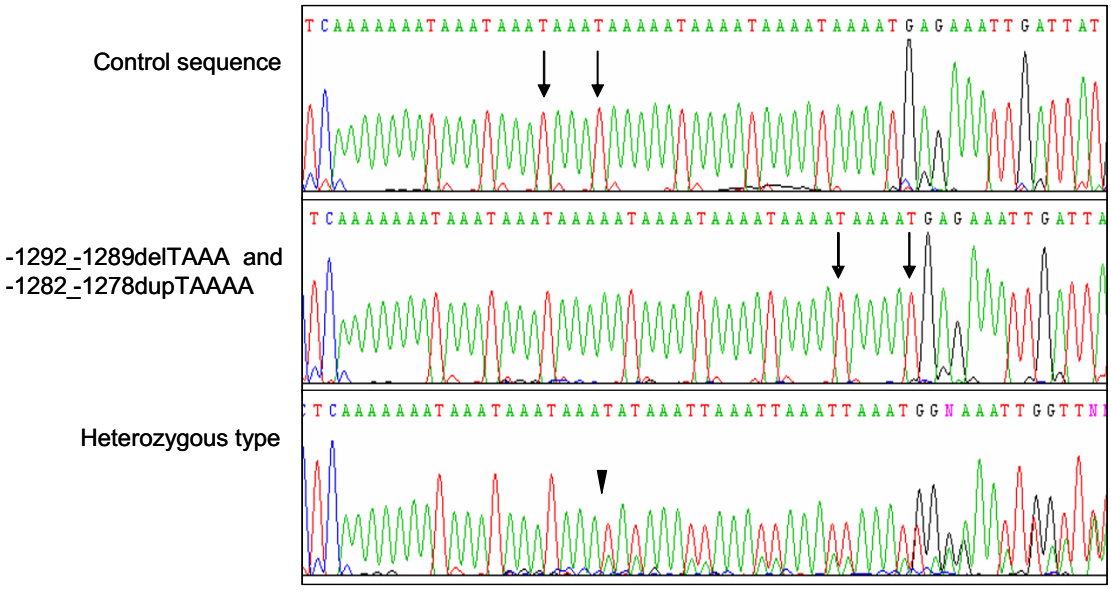

Figure 2. DNA sequence chromatograms of -1292_-1289delTAAA and -1282_-1278dupTAAAA variation sites of *Tim-3* promoter region. PCR products purified by PCR purification kit (Millipore, USA) were used as the template DNA for cycle sequencing. Sequence analysis was performed by BigDye Terminator cycle sequencing using an ABI 3100 Prism Automated DNA sequencer (Applied Biosystems). The arrows indicate variation sites.

tion site, -1292_-1289delTAAA was combined with -1282_-1278dupTAAAA variation (Figure 2). These variations were found the all of experimental samples (n = 74) for this study and located at the putative transcriptional binding site of HNF-3b, which is hepatic nuclear factor 3 beta (Figure 1). The homogeneous variation occurred to high frequency (75.7%), while -1292_-1289delTAAA and -1282_ -1278dupTAAAA variations in some cases concurrently existed as shown in Figure 2. The P values of each SNPs or variation sites were not significant between controls and asthma or allergic rhinitis patients (Table 2).

McIntire *et al*. identified major polymorphisms in *TIM-3* that was strongly associated with Th1/Th2 differentiation and the expression of airway hyperreactivity (AHR) in congenic mice (McIntire *et al*., 2001). They compared the TIM-3 amino acid sequence predicted from the BALB/c that develop Th2 biased immune responses with enhanced AHR and HBA (DBA/2) that develop reduced IL-4 responses, and found amino acid differences at seven positions. In this study, we identified only one polymorphism from the coding region of human *Tim-3* gene. This result suggests that the polymorphic sites of *Tim-3* gene in human differ from that of mice. GG genotype at 4259G>T (rs1036199) was changed to TT in all genomic DNA of both healthy controls and asthma patients (Table 2). Although it is not yet known whether these newly described polymorphisms are associated with some functions, it will be important in future studies to determine whether or not this polymorphism affects the levels of TIM-3 production. It would also be interesting to analyze the allele frequency of these variances with large sample size in patients with autoimmune and allergic diseases.

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