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Genetic Variants of Human β-Defensin-1 and Chronic Obstructive Pulmonary Disease

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Chronic obstructive pulmonary disease (COPD) is due to interactions between cigarette smoke exposure and other risk factors. Genetic variations of human β-defensin-1 (hBD-1), an endogenous antimicrobial peptide in the airway, were investigated in 60 patients and 213 healthy volunteers by single-strand conformation and restriction fragment length polymorphism analysis and DNA sequencing. Four nucleotide variations in the 5′ and 3′ untranslated regions and two nonsynonymous substitutions in the coding region were identified. Of these, a newly found Ile38 variant was observed in 15.0% of patients but only in 2.8% of healthy individuals and was significantly associated with the disease (OR = 6.1, 95% confidence intervals 2.0–18.3, *P* = 0.0012). More than 80% of those with Ile38 experienced sputum production for more than 3 months during the follow-up period. Genetic variations in hBD-1 may define a high-risk subgroup of COPD where the component of chronic bronchitis is predominant.

Key Words: **COPD; defensin; genetic susceptibility; association study; chronic bronchitis.**

COPD is a disease state characterized by the progressive development of airflow limitation that is not fully reversible (1). It is one of the major causes of premature death in the world and its prevalence and mortality are projected to increase further in the coming decades (2). Cigarette smoking is a major environmental risk factor for the development of COPD. However, only 10 to 20% of cigarette smokers develop clinically significant COPD, suggesting that an interaction with genetic risk factors could affect the disease susceptibility. Family and twin studies have supported this notion (3, 4). In fact, α1-antitrypsin deficiency is the best-documented genetic risk factor (5). Also, other factors such as endogenous protease inhibitors, detoxifying enzymes and inflammatory mediators are expected to be involved in the pathogenesis of COPD (6–11), but their association with this disease has not yet been widely accepted.

Childhood respiratory tract infection has been associated with reduced lung function in adulthood (12) and tracheobronchial infection is known to be a major cause of acute exacerbations in COPD. Although the precise role of respiratory infection in COPD has not been elucidated so far, it is conceivable that chronic airway inflammation triggered by infectious agents is involved, at least in part, in the etiology, pathogenesis and clinical course of the disease (13). Thus, genetic variations involved in the airway mucosal immunity could be candidates for the host-related risk factors that contribute to the development of COPD.

Defensins are small cationic peptides with a broad-spectrum antimicrobial activity directed against gram-negative and -positive bacteria, fungi, and some viruses and act as key effector molecules in the innate immune system (14–16). Based on the positions of six conserved cysteine residues forming three intramolecular disulfide bonds, defensins are divided into two groups, α- and β-defensins. In humans, six α-defensins expressed in leukocytes and two β-defensins in epithelia have been well characterized. Of these, human β-defensin-1 (hBD-1) is constitutively expressed in airway epithelium and considered to play an important role in mucosal immunity in the lung (17–19). In addition, polymorphisms in the untranslated region (UTR) of its gene have been previously reported (20). In this study, variations in promoter and both exons of the hBD-1 gene were examined extensively and their possible associations with COPD were investigated.

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TABLE 1

PCR Primers Used in This Study

Amplified region	PCR primer		Product size (bp)	Annealing temperature (°C)
	Primer name	Nucleotide sequence		
hBD1 promoter	HBD1PRO-U1	5′-CCTCCATGTGATCCAGAAGG-3′	273	56
	HBD1PRO-L1	5′-AGACTGGTGGATTGCACAAC-3′		
hBD1 exon 1	HBD1EX1-U1	5′-ACCCTTGACTGTGGCACCTC-3′	263	56
	HBD1EX1-L1	5′-GGGATGGGAAACTCTAGCAG-3′		
hBD1 exon 2	HBD1EX2-U1	5′-CAAGCCATGAGTCTGAAGTGT-3′	302	53
	HBD1EX2-L1	5′-ACAAGATTCATTTTGGCCCA-3′		
	HBD1EX2-MM[a]	5′-TCAACAGTGGAGGGCAATGT-3′	111	50

[a] In the PCR-RFLP analysis of hBD1 exon 2, HBD1EX2-MM was chosen as a reverse primer, instead of HBD1EX2-L1.

MATERIALS AND METHODS

Subjects. Sixty Japanese patients with COPD were enrolled in this study. Genomic DNA was extracted from peripheral blood leukocytes by a method described elsewhere (21). Informed consent was obtained from all the subjects and this study was reviewed and approved by the local Ethical Committee. Lung function measurements including forced expiratory volume in one second (FEV_1) and forced vital capacity (FVC) were made on each patient under stable conditions. All the subjects were male and had irreversible airflow obstruction with FEV_1/FVC less than 70%. Their signs, symptoms, smoking history and chest radiograph were also assessed and the diagnosis of COPD was made according to the guidelines of the American Thoracic Society (22). Additional clinical information including initial symptoms and age at onset was available only in 38 of 60 COPD patients.

Control 1 and control 2 are independent sets of anonymous blood samples obtained from 94 and 119 unrelated healthy volunteers in the Japanese population, respectively. Their smoking history is unknown.

Polymerase chain reaction (PCR). The hBD-1 gene consists of two exons (20). The sequence of the primers used for the polymerase chain reaction (PCR) is based on the genomic DNA sequence of the hBD-1 gene (GenBank Accession Nos. U50930 and U50931). One primer set was designed to amplify a target from the promoter region; another, a target that included exon 1 and the adjacent intron; and a third, part of the intron and exon 2. These primer sets and their annealing temperatures are shown in Table 1. PCR was carried out in a 25-μl reaction volume containing 100 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), 50 mM of KCl and 1 unit of *Taq* polymerase (AmpliTaq Gold DNA polymerase, Perkin–Elmer Applied Biosystems, Foster City, CA). After the initial denaturation at 94°C for 5 min, the reaction mixture was subjected to 30 cycles of 94°C for 30 s, annealing for 30 s at 56°C, and 72°C for 1 min followed by the final extension at 72°C for 5 min.

Single-strand conformation polymorphism (SSCP) analysis. One microliter of each PCR product was mixed with 7 μl of deionized formamide, denatured at 94°C for 5 min immediately before loading and electrophoresed on a nondenaturing 10% polyacrylamide gel with an acrylamide:bisacrylamide ratio of 49:1 in 45 mM Tris–borate and 1 mM EDTA. A gel containing 5% glycerol was also tested. Electrophoresis was carried out under constant voltage of 300 V/gel, using a minigel electrophoresis apparatus with a constant temperature control system (90 × 80 × 1 mm, AE-6410 and AE-6370; ATTO, Tokyo, Japan). The optimal buffer temperature was chosen from 4 to 25°C and the duration of electrophoresis was 1.5 to 2.5 h depending on the PCR fragments to be separated. Single-strand DNA fragments on the gel were visualized using a silver staining kit (Daiichi Pure Chemicals, Tokyo, Japan). The target representing hBD-1 exon 1 plus the adjacent intron separated well on a gel without glycerol at 13°C for 2 h.

Direct sequencing analysis. Both sense and antisense strands of PCR products were directly sequenced using the same primers used for the PCR amplification. Fluorescence-based automated sequencing of PCR products was performed on ABI PRISM 377 DNA sequencer using the dye-terminator method according to the manufacturer's instructions (ABI PRISM dRhodamine Terminator Cycle Sequencing-Ready Reaction kit; Perkin–Elmer Applied Biosystems).

PCR-based restriction fragment length polymorphism (RFLP) analysis. A novel PCR-RFLP detection system was designed to genotype a G to A substitution at genomic position 1654 (GenBank Accession No. U50931) that changes the code from valine to isoleucine, respectively, at position 38 of the hBD-1 polypeptide. A fragment of 111 bp containing the polymorphic site was amplified with a primer set HBD1EX2U1 and HBD1EX2MM (Table 1). In this PCR, the reverse primer HBD1EX2MM contained a nucleotide mismatch that introduced a *Hinc*II restriction site only on the DNA fragment carrying the code for valine 38. After the PCR, the amplified products were digested with 2 units of *Hinc*II (Takara Shuzo Co., Ltd., Shiga, Japan) for 2 h at 37°C and analyzed on a 4% agarose gel containing 0.5 μg/ml of ethidium bromide. *Hinc*II digestion gave two fragments of 93 and 18 bp only in the product with the sequence for valine 38.

Statistical analysis. Disease association was assessed by the chi-square (χ^2) test. When any expected number in the 2 × 2 contingency table is less than 5, the *P* value was directly calculated by Fisher's exact test. The odds ratio (OR) was defined as the cross-product ratio of the numbers shown in the 2 × 2 table. Age, smoking index, and pulmonary function parameters between patients and controls were compared using unpaired Student's *t* test. *P* values less than 0.05 were considered significant.

RESULTS

Screening for Variations of the hBD-1 Exon 1 and Frequencies of the Variations in the Patients and Controls

Exon 1 of the hBD-1 gene is known to carry three nucleotide substitutions, −52(G/A), −44(C/G) and −20(G/A) in the 5′-UTR from the translation start site (20). Variations of hBD-1 exon 1 were examined by SSCP analysis. As shown in Fig. 1, three independent alleles, 01, 02, and 03 were detected. Direct nucleotide sequence analysis revealed the three aforementioned

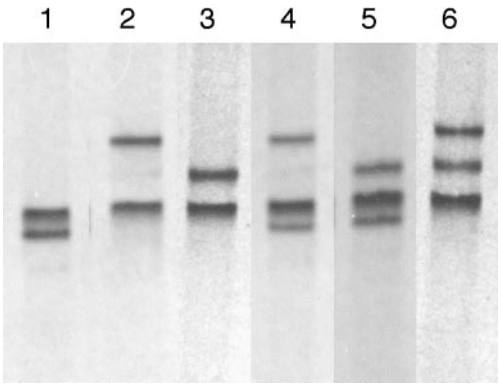

FIG. 1. Genotyping of variations of the hBD-1 exon 1 by the PCR-SSCP method. SSCP patterns of homozygotes and heterozygotes for variations of the hBD-1 exon 1 are shown. Electrophoresis was carried out on a 10% polyacrylamide gel without glycerol at 13°C for 2 h. Lane 1, a homozygote for the 01 allele; lane 2, a homozygote for the 02 allele; lane 3, a homozygote for the 03 allele; lane 4, a heterozygote for the 01 and 02 alleles; lane 5, a heterozygote for the 01 and 03 alleles; lane 6, a heterozygote for the 02 and 03 alleles.

substitutions in exon 1. The three alleles 01, 02 and 03 detected by SSCP analysis were compatible with haplotypes −52G/−44C/−20A, −52A/−44C/−20G and −52G/−44G/−20G, respectively, and no other haplotypes were found. These findings are consistent with a report by other investigators (20).

The frequencies of these alleles and genotypes in the 60 patients with COPD and the 94 normal subjects from control 1 were determined. As summarized in Table 2, distributions of the three alleles were not significantly different between the patients and controls.

Screening for Variations in the hBD-1 Exon 2 and Identification of a Novel Variation Val38Ile

Initial screening for variations of the exon 2 of the hBD-1 gene was performed as above by the SSCP method. However as variations of hBD-1 exon 2 did not separate well by the electrophoretic conditions described under Materials and Methods, exon 2 was investigated directly by nucleotide sequencing. Three nucleotide substitutions, one in the 3′-UTR and two in the coding region were identified. The one in the 3′-UTR is located at position +5 after the stop codon, +5(G/A) and was reported previously (20). In the coding region one was an alanine to valine substitution at position 48, Ala48Val, that was recently reported elsewhere on line (23) and the other, was a newly found variation coding for a valine to isoleucine substitution at position 38, Val38Ile.

TABLE 2

Frequencies of Variations of the hBD-1 Exon 1 in the Patients and Controls

	COPD (n = 60)	Control (n = 94)
	[number of individuals (%)]	
Genotype		
01/01	8 (13.3)	15 (16.0)
01/02	27 (45.0)	36 (38.3)
01/03	11 (18.3)	13 (13.8)
02/02	11 (18.3)	21 (22.3)
02/03	2 (3.3)	8 (8.5)
03/03	1 (1.7)	1 (1.1)
Allele frequency		
01 (−52G/−44C/−20A)	0.45	0.42
02 (−52A/−44C/−20A)	0.43	0.46
03 (−52G/−44G/−20G)	0.12	0.12

TABLE 3

Frequencies of Val38Ile of the hBD-1 Exon 2 in the Patients and Controls

	COPD (n = 60)	Control 1 (n = 94)	Control 2 (n = 119)	Controls (total n = 213)
	[number of individuals (%)]			
Genotype				
Val38/Val38	51 (85.0)	92 (97.9)	115 (96.6)	207 (97.2)
Val38/Ile38*	9 (15.0)	2 (2.1)	4 (3.4)	6 (2.8)
Ile38/Ile38	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Allele frequency				
Val38 allele	0.92	0.99	0.98	0.99
Ile38 allele†	0.08	0.01	0.02	0.01

* P = 0.004 between COPD and control 1; P = 0.01 between COPD and control 2 by Fisher's exact test.

† P = 0.004 between COPD and control 1; P = 0.01 between COPD and control 2 by Fisher's exact test.

Associations between hBD-1 Exon 2 Variations and COPD

hBD-1 exon 2 variations were genotyped by direct sequencing in the 60 patients with COPD and the control 1 group. In the control population allele frequencies of +5G and +5A were 0.60 and 0.40 and those of Ala48 and Val48 were 0.98 and 0.02, respectively, and they were not associated with COPD (data not shown). In contrast, as shown in Table 3, the frequency of individuals with the Ile38 allele in the patient population (15.0%) was much higher than that in the control 1 (2.1%) and the presence of the Ile38 was significantly associated with COPD (P = 0.004). No individuals homozygous for Ile38 were found in either population. To confirm the low frequency of Ile38 in the general population and its positive association with COPD, we genotyped the control 2 group, another set of healthy individuals in the Japanese population, by the PCR-RFLP method and compare them to the patient group analyzed in the same way. Figure 2 shows a representative RFLP pattern. The results are summarized in Table 3. PCR-RFLP results from the patients agreed with those from direct sequencing. The frequency of Ile38 in the control 2 set (3.4%) was compa-

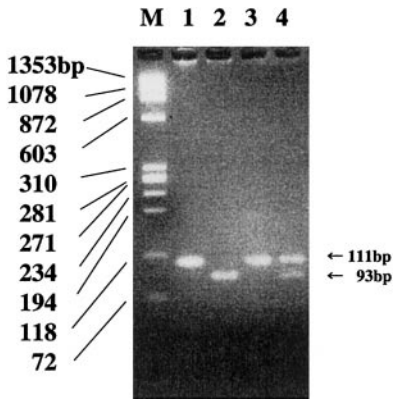

FIG. 2. Genotyping of Val38Ile variation of the hBD-1 Exon 2 by the PCR-RFLP method. Genotyping of Val38Ile in hBD-1 Exon 2 by the PCR-RFLP method. After the PCR reaction, the amplified products were digested with *Hinc*II for 2 h at 37°C and analyzed on an agarose gel containing ethidium bromide. *Hinc*II digestion gave 2 fragments of 93 and 18 bp only if the amplified product codes for valine at position 38 of the hBD-1 protein. M; marker (ϕX-174 *Hae*III). Lanes 1 and 3, products before digestion; lanes 2 and 4, products after digestion; lanes 1 and 2, a homozygote for the Val38 allele; lanes 3 and 4, a heterozygote for the Val38 and Ile38 alleles.

rable to that in control 1. In total, the presence of Ile38 showed a highly significant association with the disease (OR = 6.1, 95% confidence intervals 2.0–18.3, P = 0.0012).

Clinical Features of the Patients with Ile38 and without Ile38

To determine whether patients with Ile38 of the hBD-1 compose a clinically separate subgroup, we compared clinical features of patients with Val38/Ile38 to those with Val38/Val38 (Table 4). First, there were no significant differences between the two groups for age at onset, age at examination, and pulmonary function measurements including FEV_1/FVC and FEV_1% predicted. Next, the number of cigarettes smoked for patients with Ile38 (42 pack-years) appeared to be slightly less than those for patients without Ile38 (68 pack-years), although the difference did not reach the 0.05 significance level (P = 0.08). When we further examined their smoking history, Ile38 was found to be associated with the patients with pack-years less than 20 (P = 0.01). Furthermore, in 38 patients whose detailed clinical information was available, only 9% of the patients without Ile38 experienced chronic cough and sputum production as initial symptoms. In contrast, 33% of the patients with Ile38 had these airway symptoms at the time of onset of the disease and 83% experienced them for more than 3 months during the follow-up period.

Screening for Variations of the hBD-1 Promoter Region

When the SSCP analysis was also performed for the promoter region of the hBD-1 gene, no variant bands were detected in the patients or control 1. Also, by direct sequencing, no nucleotide substitutions were found between the transcription start site and position −294 of the gene (data not shown).

DISCUSSION

In the present study, we carried out screening for variations of hBD-1 and identified two nonsynonymous nucleotide substitutions in the coding region and four nucleotide sequence variations in the 5′ and 3′ UTRs. Of these, a newly identified variation, Val38Ile showed a significant positive association with COPD.

To our knowledge, this is the first report that a variation in defensins is associated with a human disease. Defensins are important effector molecules of innate immunity, an ancient form of host defense (24). It is conjectured that the innate immune system appeared much earlier than the adaptive immune system and has evolved by natural selection to acquire specificities for infectious microorganisms (25).

The frequencies of three nucleotide substitutions in the 5′ UTR and one in the 3′ UTR of the hBD-1 were comparable to those in the German population reported previously (20), except that −52G/−44G/−20G and +5G were less frequently observed in the Japanese population probably because of ethnic differences. Although nucleotide substitutions in the UTR might influence the expression level of the hBD-1 gene, none of them was associated with the disease in this study. Of the two nonsynonymous substitutions identified in this study, the Ile38 showed a highly significant association with COPD. The frequency of individuals with the Ile38 allele in the COPD group was 15% whereas it

TABLE 4

Clinical Features of the Patients with and without Ile-38

	Genotype	
	Val/38Ile38 (n = 9)	Val38/Val38 (n = 51)
Age at examination (years)	68.7 ± 10.3	68.5 ± 9.7
Smoking history (pack-years)	41.7 ± 28.9	68.0 ± 42.3
Pack-years <20 [n (%)]	3*	1
FEV1/FVC (%)	49.2 ± 12.1	44.0 ± 11.3
FEV1 (% predicted)	53.3 ± 18.0	48.2 ± 20.5
Age at onset (years)†	59.8 ± 11.9	64.7 ± 9.2
Initial symptoms [n (%)]†		
Dyspnea	3 (50.0)	26 (81.3)
Chronic cough and sputum	2 (33.3)	3 (9.4)
Dyspnea with wheezing	1 (16.7)	1 (3.1)
None	0 (0.0)	2 (6.3)
Sputum production of more than 3 months [n (%)]†	5 (83.3)	11 (34.4)

* P = 0.01 by Fisher's exact test.

† Information about these items was available only in 38 of 60 COPD patients.

was approximately 2 to 3% in the two independent control groups representing the general Japanese population. Considering the Hardy–Weinberg equilibrium, it is reasonable that all individuals with Ile38 were heterozygous Val38/Ile38 in our analysis. In this study, control subjects with matching smoking history were not provided. Nevertheless, it is meaningful that the smokers susceptible to the disease had the variant more frequently than the general population.

To determine whether this relatively rare variant is associated with specific clinical features of COPD, we analyzed the available medical information. The mean smoking index of the patients with this variant was slightly lower than those without it and the proportion of the patients with a smoking index less than 20 pack-years was significantly higher in patients with Ile38 than in those without this allele. These data indicate that Ile38 may accelerate deleterious effects of cigarette smoking in the development of COPD or the variant acts as an independent risk factor for COPD.

The number of patients with detailed clinical information was small for ethical reasons and the frequency of the Ile38 variant was low. Despite these limitation we found that of the patients with this mutation one third had chronic cough and sputum as initial symptoms and more than 80% experienced sputum production for more than three months during the follow-up period. As these clinical features are more representative of chronic bronchitis than by emphysema, this Ile38 variant of hBD-1 may have a role in the development of chronic bronchitis rather than emphysema in COPD.

Recently it has been reported that the antimicrobial activity of β-defensins is decreased by the high-salt airway surface fluid in patients with cystic fibrosis (CF) (26). The authors argued that reduced activity of the defensins contributes to the characteristic airway colonization by bacteria in CF patients and is involved in its pathogenesis. Thus it is likely that hBD-1 constitutively expressed in epithelia directly kills airborne microorganisms attached to the airway mucosal surface and plays an important role in suppression of airway inflammation. Although how β-defensins kill their target is still unknown, it has been postulated that the mechanism involves interactions of the defensins with target microorganisms followed by their membrane permeabilization (27).

Another possible mechanism by which variants of β-defensins exert their effect in COPD is by acting as a chemoattractant for immature dendritic cells and memory T cells through the CCR6 chemokine receptor (28). If this functions *in vivo,* the release of β-defensins from airway epithelial cells would recruit the members of the adaptive immune system to the airway. The presence of the Ile38 variant might modulate this chemoattractant activity.

The question arises concerning how the mutant hBD-1 impairs the function of defensins in individuals heterozygous for the mutation. According to recent results from X-ray structure analysis β-defensins appear to operate by forming an octameric assembly (27). In such a case, when a single variant peptide is contained as a component of a multimeric protein, the whole structure of the complex can be destabilized and its function disrupted. This phenomenon is known as a dominant negative effect (29). It would be interesting to examine whether the Ile38 variant is acting as a dominant negative mutation.

In conclusion, novel genetic variation in hBD-1 showed a positive association with COPD and may have a role in the development of the disease in which the component of chronic bronchitis is predominant. A larger study including other ethnic populations is needed because this is an infrequent variant. In addition, the precise mechanism by which the hBD-1 variant is implicated in the pathogenesis of COPD should be investigated in the future.

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