

Effect of Interleukin-18 Gene Polymorphisms on Sensitization to Wheat Flour in Bakery Workers

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Lower respiratory symptoms in bakery workers may be induced by wheat flour and endotoxins. We hypothesized that endotoxins from wheat flour may stimulate innate immunity and that interleukin-18 (*IL-18*) gene polymorphisms may affect their regulatory role in innate immune responses to endotoxins. To investigate the genetic contribution of *IL-18* to sensitization to wheat flour, we performed a genetic association study of *IL-18* in Korean bakery workers. A total of 373 bakery workers undertook a questionnaire regarding work-related symptoms. Skin prick tests with common and occupational allergens were performed and specific antibodies to wheat flour were measured by ELISA. Three polymorphisms of the *IL-18* gene (-607A/C, -137G/C, 8674C/G) were genotyped, and the functional effects of the polymorphisms were analyzed using the luciferase reporter assay. Genotypes of -137G/C (GC or CC) and haplotype *ht3* [ACC] showed a significant association with the rate of sensitization to wheat flour. Luciferase activity assay indicated *ht3* [AC] as a low transcript haplotype. In conclusion, the regulatory role of *IL-18* in lipopolysaccharide-induced responses in bakery workers may be affected by this polymorphism, thus contributing to the development of sensitization to wheat flour and work-related respiratory symptoms.

Key Words: Interleukin-18; Genes; Polymorphism; Sensitization; Wheat Flour

INTRODUCTION

Baker's asthma is a common occupational respiratory disease (1-3). According to an epidemiological study on baker's asthma in a Korean population (4), the prevalence of work-related upper and lower respiratory symptoms is 17.1%. Wheat flour is thought to be a major allergen that induces IgE sensitization and leads to the development of respiratory symptoms and asthma in bakery workers. A fair amount of bacterial endotoxins or lipopolysaccharides (LPS), which are found in wheat flour, may also provoke airway inflammation (5).

IL-18 is a pleiotropic cytokine that promotes Th1 or Th2 immune responses depending on the surrounding cytokine microenvironment (6). Functional dysregulation of IL-18 production may contribute to the development of bronchial asthma (7, 8). Moreover, IL-18 plays an important role in the beneficial and lethal LPS responses (9). A recent study also demonstrated the role of IL-18 in homeostatic control of innate immune responses to the lethal inflammation of endotoxins (10).

Because the regulatory role of IL-18 in innate immunity is provoked by endotoxins of wheat flour in the bakery workplace, we investigated the association between *IL-18* gene polymorphisms and work-related lower respiratory symptoms and sen-

sitization to flour allergens in bakery workers. To clarify the role of IL-18 in the development of baker's asthma, we examined functional single nucleotide polymorphisms (SNPs) (-607A/C, -137G/C, 8674C/G) involved in the transcriptional regulation of IL-18 (7, 11).

MATERIALS AND METHODS

Study subjects

In total, 373 bakery workers who participated in a questionnaire survey regarding work-related respiratory symptoms were enrolled from a single industrial site. The questions regarding respiratory symptoms included whether subjects had experienced upper respiratory symptoms such as nasal itching, runny nose, sneezing, or congestion, as well as lower respiratory symptoms, such as cough, sputum, shortness of breath or wheezing. The subjects who indicated that their symptoms were aggravated during the work, but improved after the work or during holidays were defined as having work-related respiratory symptoms. All bakery workers were given a skin prick test (SPT) that included common inhalant allergens (Bencard, Bretford, UK) and wheat flour allergen extract (4). Atopy was defined for subjects with more than one positive response to common inhalant allergens

on the SPT. The total IgE concentration was measured using a UniCAP system (Pharmacia Diagnostics, Uppsala, Sweden) according to the manufacturer's instructions. The presence of a specific IgE, IgG1 and IgG4 antibodies to wheat flour was determined by enzyme-linked immunosorbent assay (ELISA), as described previously (4). Clinical demographics of all study subjects are shown in Table 1. All subjects were classified according to their dust exposure density as previously described (4) and characterized by prevalence of sensitization rate to wheat flour and inflammatory cytokines in Table 2.

ELISA for IL-18

Serum IL-18 level was measured in bakery workers using a Human IL-18 ELISA Kit (MBL, Nagoya, Japan), in accordance with the manufacturer's guidelines.

Table 1. Clinical characteristics of the bakery workers (n = 373)

Parameters	Findings
Age (yr), mean ± SD	35 ± 7.7
Male	210 (56.3)
Working period (yr), mean ± SD	4 ± 3.5
Atopy (+, %)	127 (34.6)
Smoking (+, %)	145 (41.9)
Lower respiratory symptoms (+, %)*	51 (13.7)
Log total IgE (kU/L)	4.4 ± 1.5
Presence of specific IgE to wheat flour	24 (6.4)
Positive skin prick test response to wheat flour	23 (6.3)
Asthma (+, %)	17 (4.6)
Rhinitis (+, %)	41 (11)

*Lower respiratory symptoms, cough, sputum, shortness of breath or wheezing.

Table 2. Prevalence of work-related respiratory symptoms, sensitization rate to occupational allergen and inflammatory cytokine levels according to exposure intensity

Symptoms or signs	Exposure intensity to wheat*			P
	Low	Intermediate	High	
Respiratory symptoms				
Upper (+, %) [†]	42 (11.3)	40 (10.7)	40 (10.7)	0.274
Lower (+, %) [‡]	11 (2.9)	21 (5.6)	19 (5.1)	0.292
Positive skin prick test (%)				
Wheat	6 (1.6)	8 (2.2)	9 (2.4)	0.918
Rye	5 (1.4)	4 (1.1)	3 (0.8)	0.579
Yeast	3 (0.8)	2 (0.5)	9 (2.4)	0.099
α -amylase	0 (0)	1 (0.3)	0 (0)	0.355
Any baker's allergen	11 (3)	13 (3.5)	20 (5.4)	0.5
Smoking status (+, %)	64 (18.5)	63 (18.2)	74 (21.4)	0.434
IgE specific to wheat flour	6 (1.6)	10 (2.7)	8 (2.1)	0.643
IgG1 specific to wheat flour	7 (1.9)	36 (9.7)	33 (8.9)	< 0.001
IgG4 specific to wheat flour	4 (1.1)	20 (5.4)	28 (7.5)	< 0.001
log IL-8 (pg/mL) [§]	4.7 ± 1.3	5.1 ± 1.3	5 ± 1.4	0.122
log IL18 (pg/mL) [§]	5.4 ± 0.4	5.6 ± 0.4	5.6 ± 0.4	0.006
log MPO (ng/mL) [§]	4.7 ± 0.5	4.8 ± 0.6	4.8 ± 0.6	0.803

*Exposure intensity, geometric mean (range): Low, 0.01(0.00-0.35); Intermediate, 1.16 (0.02-5.97); High, 3.04 (0.07-11.27) mg/m³; [†]Upper respiratory symptoms; nasal itching, runny nose, sneezing, or congestion; [‡]Lower respiratory symptoms, cough, sputum, shortness of breath or wheezing; [§]Presented as mean ± standard error. Bold character indicates significance. P values were applied by Pearson's chi-square test for categorical variable and ANOVA for continuous variable. A positive skin prick test is defined as an A/H ratio ≥ 1. A positive result for specific IgE antibodies is defined as a level greater than three standard deviations of the level in non-atopic healthy controls, or equal to the mean plus.

IL-18 SNP genotyping

Genomic DNA was prepared from peripheral blood samples of all participants using a Puregene DNA purification kit (Qiagen, Germantown, MD, USA) according to the manufacturer's protocol. Genotyping of -607A/C (rs1946518) and -137G/C (rs187238) was performed using the SNaPshot assay according to the manufacturer's instructions (ABI PRISM SNaPshot Multiplex Kit; Applied Biosystems, Foster City, CA, USA). Genotyping of 8674C/G (rs5744247) was screened using the TaqMan fluorogenic 5' nuclease assay (Applied Biosystems). Nucleotide sequence information on primers used for SNP genotyping are described in Table 3. Linkage disequilibrium between all pairs of biallelic loci was measured using Lewontin's D'(|D'|) and r² in a linkage disequilibrium (LD) block (12).

Preparation of the plasmid construct for the promoter activity assay

The 752-bp fragment of the promoter of the human *IL-18* gene, including -607A/C and -137G/C, was prepared by PCR amplification using each haplotype (*ht1* [CG], *ht2* [AG], and *ht3* [AC]) human genomic DNA as templates using a pair of primers (forward primer, 5'-CTGAATTTTGGTATCCCTCTCCC-3'; reverse primer, 5'-GGAGACAATTCCTTGCTGACTG-3'). Each of the PCR products was gel purified using an agarose-gel purification kit (iNtRON, Daejeon, Korea) and ligated into the TOPO vector (Invitrogen Corporation, Carlsbad, CA, USA). These DNA plasmids were then cut with *KpnI* and *XhoI* restriction enzymes (Takara, Shuzo, Japan) and ligated into the *KpnI-XhoI* site of the pGL3-Basic Luciferase Reporter Vector (Promega, Madison, WI, USA) using T4 DNA ligase (Elpis Biotech, Daejeon, Korea). All

Table 3. Primer sequences for SNP genotyping

rs Number	Method	Primer sequences	
rs1946518	SNaPshot assay	Forward primer	TAAATATTTGCCCTCTTACCTGAA
		Reverse primer	ATTTTCATAAACTTCTTATCTGCTG
		Genotyping primer	GGATACCATCATTAGAATTTTAT
rs187238	SNaPshot assay	Forward primer	TTTCTTAAAGTCAGAAAGAGATACTCAG
		Reverse primer	AATGGAAGTCGAAATAAAGTGG
		Genotyping primer	GTAATATCACTATTTTCATGAAAT
rs5744247	Taqman assay	Forward primer	CCTTCAGCCTCTCACCCATAG
		Reverse primer	CACCTGAGGATGCCATAAACACAT
		VIC probe	AAGGATGAAGCGAGTCTCT
		FAM probe	AAGGATGAAGCCAGTCTCT

constructs were verified by direct sequencing. DNA plasmids were prepared from these constructs using the Endo Free Plasmid Maxi Kit (Qiagen, Valencia, CA, USA) and then analyzed for quantity and integrity using UV spectrometry and agarose-gel electrophoresis.

Transient transfection and luciferase activity

A549 (human alveolar basal epithelial cells, ATCC CRL-185) and BEAS-2B (human bronchial epithelial cell, ATCC CRL-9609) cells were maintained in RPMI 1640 (Gibco, Invitrogen Corporation) supplemented with 1% penicillin-streptomycin, 0.1 mM MEM non-essential amino acid (Gibco, Invitrogen Corporation) and 10% fetal bovine serum (FBS, Gibco, Invitrogen Corporation) in a 5% CO₂ incubator at 37°C. Cells were plated, and, after being grown to 70%-80% confluence, cells were transiently transfected by lipofectamine (Invitrogen Corporation) according to the manufacturer's protocol. Briefly, 1 × 10⁵ cells were seeded in 12-well plates and transfected with 1 µg of reporter construct, 5 ng of *Renilla* plasmid DNA, and 5 µL LipofectAMINE (Invitrogen Corporation). At 48 hr after transfection, the cells were lysed and assayed for firefly luciferase activity according to the manufacturer's instructions (Promega). Transfection and luciferase assays were repeated three times according to the method described above.

Statistical analysis

Genotype distributions at each polymorphism were evaluated for departures from Hardy-Weinberg equilibrium using the chi-squared goodness-of-fit test. Statistical analyses were performed using SPSS version 12 software (SPSS Inc., Chicago, IL, USA). Differences in clinical characteristics between groups were examined using Student's *t* tests and analysis of variance (for continuous variables) or the chi-squared test (for categorical variables). Risk factors were determined by logistic regression analysis after adjustment for age and gender covariates. Haplotypes of the *IL-18* gene were analyzed using Haploview v4.0 (13).

Ethics statement

The protocols used in this study were reviewed and approved by the Ajou University Institute Review Board. Written informed

consent was obtained from each participant.

RESULTS

Clinical characteristics of the subjects

Table 1 shows the clinical demographics of study subjects. The mean age of the 373 workers was 35 ± 7.7 yr, and the mean working period in the bakery was 4 ± 3.5 yr. Atopy was detected in 34.6% of the subjects. Of the 373 workers, 51 (13.7%) complained of work-related lower respiratory symptoms, including cough, sputum, shortness of breath, or wheezing. The prevalence of wheat flour sensitization was 6.3% using the SPT and 6.4% by the presence of IgE specific to wheat flour. When the workers were categorized into three groups based on the extent of exposure to flour dust (low: 0.01 mg/m³, intermediate: 1.16 mg/m³, high: 3.04 mg/m³), in accordance with a previous study (4), serum IL-18 levels showed a significant rise with increased intensity of exposure to wheat flour (*P* < 0.05, data not shown).

Prevalence of sensitization rate to wheat flour, and inflammatory cytokine levels according to exposure intensity

When the workers were categorized into three groups based on the extent of exposure to flour dust (low; 0.01 mg/m³, Intermediate; 1.16 mg/m³, High; 3.04 mg/m³), in accordance with the previous study (4), the prevalence of work-related respiratory symptoms did not differ among workers with low, intermediate, and high exposure to flour dust. The prevalence of IgG specific to wheat flour increased with increasing intensity of exposure to wheat flour dust. In accordance with this finding, serum IL-18 level showed a significant increase (Table 2).

Genotype and haplotype frequencies of IL-18 polymorphisms in bakery workers

Three functional SNPs (-607A/C; rs1946518, -137G/C; rs187238, 8674C/G; rs5744247) were targeted in this study. The magnitude of LD between *IL-18* SNPs, -137G/C and 8674C/G, showed a high value, with a pairwise LD value of *D'* = 1 and *r*² = 0.569. Among three SNPs of the *IL-18* gene, the promoter polymorphisms at -137G/C showed a significant association with the

Table 4. Prevalence of work-related respiratory symptoms, rate of sensitization to wheat flour, and inflammatory cytokine levels according to the IL-18 genotype (A) and haplotype (B)

A.

Parameters	-607A/C			-137G/C			8685C/G		
	AA (%)	AC + CC (%)	<i>P</i>	GG (%)	GC + CC (%)	<i>P</i>	CC (%)	CG + GG (%)	<i>P</i>
Age (yr)	35.6 ± 7.9	34.7 ± 7.7	0.329	35.2 ± 7.7	34.8 ± 7.7	0.66	34.2 ± 8	35.4 ± 7.6	0.145
Sex (male)	53 (49.5%)	157 (59.2%)	0.087	53 (49.5%)	157 (59.2%)	0.087	78 (59.5%)	132 (54.5%)	0.353
Working period	4.4 ± 3.7	3.8 ± 3.4	0.139	4.2 ± 3.6	3.4 ± 3	0.062	3.6 ± 3.4	4.2 ± 3.5	0.155
Atopy	38 (35.8%)	89 (34.2%)	0.768	38 (35.8%)	89 (34.2%)	0.768	48 (36.9%)	79 (33.3%)	0.489
Smoking status (+, %)	26 (63.4%)	55 (57.9%)	0.547	26 (63.4%)	55 (57.9%)	0.547	50 (41.3%)	95 (42.2%)	0.871
Log total IgE (kU/L)	4.5 ± 1.5	4.4 ± 1.5	0.466	4.3 ± 1.4	4.5 ± 1.6	0.333	4.5 ± 1.5	4.4 ± 1.4	0.488
Work-related respiratory symptoms									
Lower (+)	14 (13.1)	37 (14)	0.824	35 (12.6)	15 (16.3)	0.366	20 (15.3%)	31 (12.8%)	0.51
Positive skin prick test to wheat flour	9 (8.4)	14 (5.4)	0.277	11 (4)	12 (13)	0.002	9 (6.9%)	14 (5.9%)	0.693
Specific IgE to wheat flour	8 (7.5)	16 (6)	0.609	14 (5)	9 (9.8)	0.102	8 (6.1%)	16 (6.6%)	0.85
Log IL-18 (pg/mL)*	5.4 ± 0.4	5.6 ± 0.4	0.005	5.5 ± 0.4	5.5 ± 0.4	0.159	5.6 ± 0.4	5.5 ± 0.4	0.081

*Presented as the mean ± standard error. Bold character indicates significance. *P* values were applied by Pearson's chi-square test for categorical variables and t-test for continuous variables.

B.

Parameters	<i>ht1</i> [CGC]			<i>ht2</i> [AGG]			<i>ht3</i> [ACC]		
	+/+ or +/-	-/-	<i>P</i>	+/+ or +/-	-/-	<i>P</i>	+/+ or +/-	-/-	<i>P</i>
Age (male, +)	34.8 ± 7.6	35.6 ± 7.9	0.389	35.5 ± 7.5	34.2 ± 8.1	0.099	34.8 ± 7.7	35.2 ± 7.7	0.671
Sex	156 (59.5%)	53 (49.5%)	0.084	131 (54.6%)	78 (60.5%)	0.322	55 (59.8%)	154 (55.6%)	0.544
Working period	3.8 ± 3.4	4.4 ± 3.7	0.148	4.2 ± 3.5	3.6 ± 3.4	0.136	3.4 ± 3	4.2 ± 3.6	0.057
Atopy	88 (34.2%)	38 (35.8%)	0.809	78 (33.2%)	48 (37.5%)	0.421	36 (39.1%)	90 (33.2%)	0.313
Smoking status (+, %)	102 (42.7%)	41 (39.8%)	0.635	95 (42.6%)	48 (40.3%)	0.73	31 (35.2%)	112 (44.1%)	0.168
Log total IgE (kU/L)	4.4 ± 1.5	4.5 ± 1.5	0.482	4.3 ± 1.4	4.5 ± 1.5	0.364	4.5 ± 1.6	4.4 ± 1.4	0.359
Work-related respiratory symptoms									
Lower (+)	36 (13.7%)	14 (13.1%)	1	31 (12.9%)	19 (14.7%)	0.635	15 (16.3%)	35 (12.6%)	0.382
Positive skin prick test to wheat flour	14 (5.4%)	9 (8.4%)	0.344	14 (5.9%)	9 (7%)	0.659	12 (13%)	11 (4%)	0.005
Specific IgE to wheat flour	15 (5.7%)	8 (7.5%)	0.635	15 (6.3%)	8 (6.2%)	1	9 (9.8%)	14 (5.1%)	0.133
Log IL-18 (pg/mL)*	5.6 ± 0.4	5.4 ± 0.4	0.006	5.5 ± 0.4	5.6 ± 0.4	0.075	5.5 ± 0.4	5.5 ± 0.4	0.152

*Presented as the mean ± standard error. *P* values were applied by Fisher's exact test for categorical variables and t-test for continuous variables.

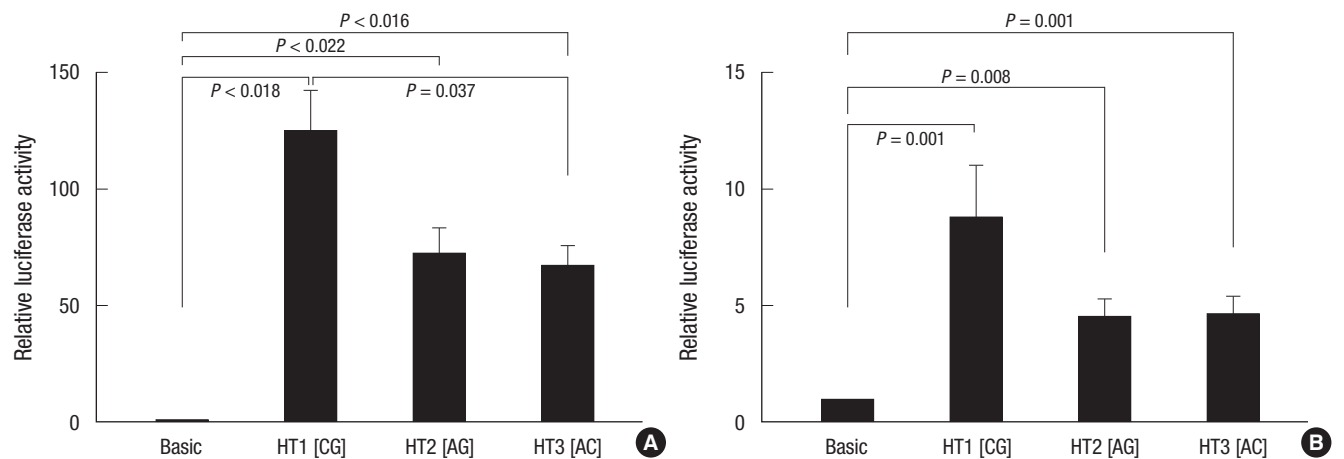


Fig. 1. Effects of promoter polymorphisms on transcription activity of the human *IL-18* gene. (A) Promoter activity in a human alveolar basal epithelial cell line (A549). (B) Promoter activity in a human bronchial epithelial cell line (BEAS2B). Relative luciferase activity is represented as the ratio of activity to luciferase activity in cells transfected with the empty control vector pGL3-Basic. The luciferase activity assay was performed in three independent experiments (n = 9). Values represent the mean ± SD of nine data points in each group. Statistical differences were evaluated using an ANOVA.

rate of sensitization to wheat flour by the SPT (Table 4). The variant genotype (GC or CC) frequency of the -137G/C polymorphism was significantly higher in the positive SPT to wheat flour

compared to that of the wild-type genotype (GG) (*P* = 0.002). Among the three haplotypes, haplotype 3, *ht3* [ACC], showed a higher positive SPT to wheat flour (*P* = 0.005). The significance

remained after multiple corrections. When we compared serum IL-18 levels according to *IL-18* polymorphisms in bakery workers, -607C/A and haplotype 1, *ht1* [CGC], showed an association with increased serum IL-18 levels.

Functional effects of IL-18 polymorphisms on transcriptional activity

For analysis of the functional variability of the two promoter SNPs (-607A/C and -137G/C), three plasmid constructs of the *IL-18* promoter containing two SNPs, *ht1* [CG], *ht2* [AG], and *ht3* [AC] were cloned into pGL3-Basic with a firefly luciferase gene reporter. The luciferase activities of *ht1* [CG], which contains the -607 C allele, were significantly elevated compared to those of *ht2* [AG] and *ht3* [AC], which exhibit the -607A allele in two different airway epithelial cell lines (A549 and BEAS2B; Fig. 1). The variant genotype (AC or CC) of -607A/C also showed a significant association with serum IL-18 level; however, other SNPs (-137G/C; 8674C/G) were not associated (data not shown). These findings indicate that the -607 C allele may be important in transcriptional regulation of the *IL-18* gene.

DISCUSSION

Bakery workers exposed to endotoxins of wheat flour, including contaminated LPS, can initiate innate immunity. We hypothesized that IL-18 may affect the innate immunity provoked by endotoxins of wheat flour in the bakery workplace and that polymorphisms in the *IL-18* gene may interfere with the regulatory role of IL-18.

Innate immunity initiated by LPS can progress under controlled regulation, which appears to be dependent on the exposure dose of LPS in mice (9). LPS can provoke a controlled antibacterial host defense program in a low dose, whereas it can induce lethal inflammatory reactions in a high dose when IL-18 may play a regulatory role in innate immunity. At lower doses of LPS, stimulation of reversible IL-18 expression enhanced antibacterial host defenses, while at higher doses of LPS, stimulation of a progressive, irreversible increase in IL-18 levels induced lethal inflammation. These findings indicate that the interactive network between LPS and IL-18 may affect innate immunity.

There has been a report showing increased level of IL-18 in nasal lavage fluid after the inhalation challenge test with flour allergen in bakers with flour-induced occupational airway allergy (14). This finding suggests an active involvement of IL-18 in persistent allergic inflammation found in baker's asthma.

When we checked serum IL-18 levels in bakery workers, increased serum IL-18 levels were found in accordance with increased intensities of exposure to wheat flour. This indicates that serum IL-18 levels may be affected by wheat flour dust in which LPS may be involved. Genetic association studies performed as part of this study demonstrated a significant association between

the genotype of -137G/C (GC or CC) and haplotype *ht3* [ACC] with the rate of sensitization to wheat flour. Bakery workers carrying the -137GC or CC genotype had a tendency for higher serum-specific IgE antibodies to wheat flour, although the significance was not statistically meaningful. This finding indicates that genotyping of the *IL-18* gene polymorphism and SPT to wheat flour may be applied to predict work-related respiratory symptoms in bakery workers.

Functional dysregulation of IL-18 related to polymorphisms has been addressed in bronchial asthma (7, 11). Examination of IL-18 production according to two promoter polymorphisms, -607C/A and -137G/C, demonstrated that alleles -607C and -137G are associated with an increase of IL-18 production in peripheral blood mononuclear cells from healthy donors (15). A cyclic adenosine monophosphate -responsive element protein (CREB) at the -607 position and a histone 4 transcription factor-1 (H4TF-1) nuclear factor at the -137 position were found to be involved in transcriptional regulation of IL-18 (11). This finding was replicated in this study when we compared serum IL-18 level according to IL-18 polymorphisms in bakery workers. As Harada et al. reported that 8674C/G (rs5744247) showed higher transcriptional activity and was significantly correlated with serum IL-18 levels in Americans (7), another functional SNP located in intron 1 was also investigated in this study. A comparison of serum IL-18 levels according to IL-18 polymorphisms in bakery workers demonstrated that -607C/A polymorphisms and the haplotype 1 *ht1* [CGC] affect serum IL-18 levels, which is in agreement with previous reports (7).

These findings suggest that alterations of IL-18 caused by genetic variants may affect the regulatory role of IL-18 in innate immunity initiated by endotoxins from wheat flour dust, finally leading to increased sensitization to wheat flour.

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