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# Effect of Toll-like receptor 4 gene polymorphisms on work-related respiratory symptoms and sensitization to wheat flour in bakery workers

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**Background:** Bakery workers are exposed to flour allergens and endotoxins, which interact to induce allergic responses and respiratory symptoms. We hypothesized that Toll-like receptor 4 (TLR4) may be involved in the development of work-related respiratory symptoms and sensitization to wheat flour.

**Objective:** To investigate the genetic contribution of TLR4 to respiratory symptoms and sensitization to wheat flour in bakery workers, we performed a genetic association study of TLR4 in Korean bakery workers.

**Methods:** A total of 381 workers completed a questionnaire regarding work-related symptoms. Skin prick tests with common and occupational allergens were done, and specific antibodies to wheat flour were measured by enzyme-linked immunosorbent assay. Two single-nucleotide polymorphisms (SNPs) of the TLR4 gene (−2027A>G and −1608T>C) were genotyped, and the functional effects of the polymorphisms were analyzed using the luciferase reporter and electrophoretic mobility shift assay.

**Results:** Homozygotes for the −2027G and −1608C alleles exhibited a lower prevalence of work-related lower respiratory symptoms than carriers of the −2027AA/AG ( $P = .007$ ) and −1608TT/TC ( $P = .021$ ) genotypes. Furthermore, haplotype analysis indicated that workers with the haplotype 2, ht2 [GC], had fewer work-related lower respiratory symptoms ( $P = .021$ ). The ht2 [GC] construct showed lower promoter activity than the haplotype 1, ht1[AT], in both BEAS-2B ( $P = .001$ ) and U937 cells ( $P = .007$ ).

**Conclusion:** Bakery workers carrying the TLR4 variants are at lower risk of developing work-related chest symptoms. This finding suggests that the TLR4 gene may be involved in allergic sensitization to wheat flour as well as endotoxin-induced respiratory symptoms in endotoxin-allergen-exposed workers and that carriers of TLR4 variants are less affected by environmental exposure.

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## INTRODUCTION

Baker's asthma is one of the most common occupational respiratory diseases, with reported incidence rates of between 1 and 10 cases per 1,000 person-years.<sup>1,2</sup> Work-related respiratory symptoms and sensitization to flour are usually included in the definition of baker's asthma,<sup>3</sup> which is mediated

through the classical immunoglobulin (Ig) E allergic pathway, and only 30% of bakers with work-related symptoms are reportedly sensitized.<sup>4</sup>

Bakery flour dust is a complex mixture of proteins and other organic materials, including bacterial endotoxins.<sup>5</sup> Bacterial endotoxins, or lipopolysaccharides (LPS), which are ubiquitous in domestic and work environments, are known to induce airflow obstruction and bronchial hyperresponsiveness by inhalation.<sup>6</sup> Lipopolysaccharides alone can contribute to activation of airway neutrophils, which secrete inflammatory cytokines, including interleukin-8 (IL-8, CXCL-8) or myeloperoxidase (MPO).<sup>7</sup> Additionally, several studies have demonstrated that endotoxins affect the process of Th2 priming and augment inflammatory responses to allergen exposure.<sup>8,9</sup>

Toll-like receptor 4 (TLR4), the cell-surface endotoxin receptor, plays a central role as a first line of defense against microbial attack, and it is abundantly expressed on innate immune cells, such as human monocytes/macrophage, mast cells, and bronchial epithelial cells.<sup>10,11</sup> Several functional variants of TLR4 have shown reduced responsiveness to

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endotoxins in Western populations. Subjects with G299/I399 show fewer respiratory symptoms than those carrying the D299/T399 wild type at moderate endotoxin concentrations,<sup>12,13</sup> and its functional relationship has been confirmed experimentally.<sup>14</sup> However, the association between TLR4 variants and lung function has been controversial.<sup>15,16</sup> Moreover, in Asian populations, no functional *TLR4* polymorphisms related to asthma have yet been discovered.<sup>17–20</sup>

Given that the bakery workplace is characterized by both endotoxin and allergen exposure, we investigated the association of TLR4 polymorphisms with work-related respiratory symptoms and sensitization to flour allergens to further clarify the role of TLR4 in the development of baker's asthma. In a Korean population, we detected no known nonsynonymous single nuclear polymorphism (SNP) such as G299/I399. To identify biologically functional SNPs, we determined the nucleotide sequence of the promoter region of TLR4, which may be involved in the transcriptional regulation of the TLR4 gene. We also evaluated the functional effects of TLR4 polymorphisms through *in vitro* functional studies.

## METHODS

### Study Subjects

A questionnaire survey was used to screen for work-related respiratory symptoms in 381 workers from a single industrial site in Seongnam City, Korea. All workers were asked to complete the questionnaire regarding respiratory symptoms, including whether they 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 at work, but improved after work or during the holidays, were defined as having work-related respiratory symptoms. Subjects who suffered from current respiratory infections or other chronic diseases such as chronic heart failure and had taken daily medications to influence immune functions, such as systemic corticosteroids, were excluded from the study. The protocols used in this study were reviewed and approved by the Ajou University Institute Review Board. Informed consent was obtained from each participant.

The workers were categorized into 3 groups based on the extent of exposure to flour dust. The job category of the study subjects with exposure intensity was listed as was reported in our previous publication.<sup>20</sup> The group with the highest wheat dust exposure levels (geometric mean, 3.04 mg/m<sup>3</sup>) consisted of workers in the mixing, weighing, and sieving departments. The intermediate-exposure group, which consisted of workers engaging in kneading, baking, and decoration, experienced a geometric mean exposure of 1.16 mg/m<sup>3</sup>. The remaining workers, who did not work in the main production area, were considered the low-exposure group, experiencing a geometric mean exposure of 0.01 mg/m<sup>3</sup>.

### Skin Prick Test and Measurement of Specific Antibodies to Wheat Flour

All 381 workers were administered a skin prick test (SPT) that included common inhalant allergens, such as tree mixture, grass mixture, mugwort, ragweed, cat fur, dog fur, *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, and *Alternaria* (Bencard, Bretford, United Kingdom), and a wheat flour allergen extract (collected from the workplace and prepared as described previously),<sup>18</sup> egg (Bencard),  $\beta$ -amylase (from *Aspergillus* spp.; Sigma-Aldrich, St. Louis, Missouri), and storage mite (*Tyrophagus putrescentiae*; Allergopharma, Reinbek, Germany). The results of SPTs are reported as the ratio of mean wheal diameter of allergen to histamine (A/H ratio). For A/H  $\geq 1$ , the reaction was defined as positive. Atopy was defined for subjects with more than 1 positive response to common inhalant allergens on the SPT.

The presence of specific IgE antibody to wheat flour was determined by ELISA. Specific IgE antibody was measured in 50- $\mu$ L aliquots of serum from each worker and from each of 60 unexposed healthy controls with negative responses to common and occupational allergens on SPT using biotin-labeled goat antihuman IgE antibody (1:1,000 dilution; Vector Laboratories, Inc., Burlingame, California). A positive cutoff value was derived from the mean + 3 standard deviations of the unexposed controls.

### ELISA for IL-8 and MPO

The serum IL-8 (Pierce Biotechnology, Inc., Rockford, Illinois) was measured in exposed workers by ELISA (Bioxytech MPO-Enzyme Immunoassay; OXIS Health Products, Inc., Portland, Oregon), as was the serum MPO level, in accordance with the manufacturers' guidelines.

### TLR4 SNP Genotyping

Genomic DNA was prepared from peripheral blood samples using a Puregene DNA Purification Kit (Qiagen, Germantown, Maryland) according to the manufacturer's protocol. By direct sequencing of the promoter regions of the TLR4 gene, we found 2 polymorphisms: –2027A>G and –1608T>C. Additional samples were genotyped for these 2 SNPs by using the SNaPshot ddNTP primer extension method (Applied Biosystems, Foster City, California). The sequences of the primers used for amplification, and extension to detect TLR4 polymorphisms were as follows: for the –2027A>G, forward 5'-TCACAGCTTGGTTTTTGACA-3', reverse 5'-TTGTAAAGCTTTTAGGACAGTGTCT-3', and extension 5'-CCTGATTTAAACAGGAATATTATG-3'; for the –1608T > C, forward 5'-TACCATTGCGTATCTT-TATTTTAATACA-3', reverse 5'-AAATGGTGTACAG-GAGTTCTCATT-3', and extension 5'-TTACAGACCA-GAAAGTAATAATAAG-3'.

### In Vitro Promoter Activity Assay

A 2,108-bp fragment (from –2080 to +11) of the human TLR4 gene was prepared by polymerase chain reaction, using

three haplotypes (ht1 [AT] and ht2 [GC]) of genomic DNA as the template, each of which was separately subcloned into the *KpnI*–*SacI* sites of the pGL3-Basic luciferase reporter vector (Promega, Madison, Wisconsin).

U937 (human myelomonocytic cell, ATCC CRL–1593.2) and BEAS-2B (human bronchial epithelial cell, ATCC CRL-9609) cells were maintained in RPMI–1640 (Gibco, Invitrogen Corporation, Auckland, New Zealand) supplemented with 1% penicillin–streptomycin, 0.1 mM modified Eagle medium nonessential amino acid (Gibco, Invitrogen Corporation), and 10% fetal bovine serum (Gibco, Invitrogen Corporation) in a 5% CO<sub>2</sub> incubator at 37°C. Cells were plated and grown to 70% to 80% confluence and then were transiently transfected by lipofectamine (Invitrogen, Carlsbad, California) according to the manufacturer's protocol. Briefly,  $1 \times 10^5$  cells for Bease2B and  $4 \times 10^5$  cells for U937 were seeded in 12-well plates and transfected with 1 µg reporter construct, 5 ng *Renilla* plasmid DNA, and 5 µL Lipofectamine (Invitrogen). At 48 h 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 in triplicate according to the method described earlier.

### Electrophoretic Mobility Shift Assay

U937 cells were washed with ice-cold phosphate-buffered saline and then suspended in ice-cold hypoosmotic buffer (10 mM 4-[2-hydroxyethyl]-1-piperazine ethanesulfonic acid [HEPES]/KOH, pH 7.9, 2 mM MgCl<sub>2</sub>, 0.1 mM ethylenediaminetetra-acetic acid, 10 mM KCl, 1 mM dithiothreitol [DTT], 1 mM phenylmethanesulfonylfluoride, 1 mM NaVO<sub>3</sub>, 10 mM NaF, and complete protease inhibitor cocktail; Roche, Basel, Switzerland). The cells were incubated on ice for 10 minutes and for an additional 15 minutes in the presence of 0.2% Nonidet P-40. After centrifugation at  $15,000 \times g$  for 30 seconds, the pellet was resuspended in cold saline buffer containing 50 mM HEPES/KOH (pH 7.9), 50 mM KCl, 300 mM NaCl, 0.1 mM ethylenediaminetetra-acetic acid, 10% (v/v) glycerol, 1 mM DTT, 1 mM phenylmethanesulfonylfluoride, and protease inhibitors. The mixture was incubated on ice for 30 minutes. After centrifugation at  $15,000 \times g$  for 30 min at 4°C, the supernatant was stored at –80°C until use. The oligonucleotides used for electrophoretic mobility shift assay (EMSA) are shown in the legend of Figure 1.

The double-stranded oligonucleotides were annealed by incubation at 95°C for 5 minutes, followed by gradual cooling to room temperature. The double-stranded DNA probes were radiolabeled at the 5'-ends with DNA polymerase I (Promega) and radiolabeled Cytidine 5'-[α-<sup>32</sup>P]-triphosphate [α-<sup>32</sup>P]CTP. The reaction mixtures (10 µL), which contained 5 µg of nuclear extract, 10 mM HEPES (pH 7.9), 8 ng poly(dI:dC), 1 mM MgCl<sub>2</sub>, 30 mM KCl, 1 mM DTT, 1 mM NaVO<sub>3</sub>, 10 mM NaF, and 10 mM 5% glycerol, were preincubated on ice for 20 minutes and then incubated for 20 minutes on ice in the presence of 0.2 pmol of a radiolabeled probe. The reaction mixtures were then subjected to electro-

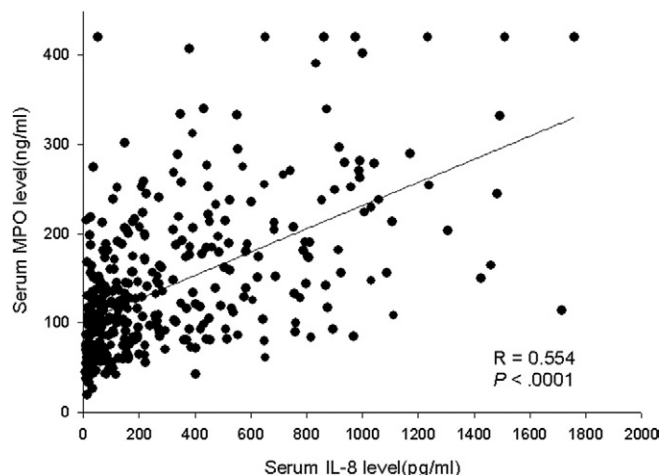


Figure 1. Correlation between the serum levels of MPO and IL-8 in bakery workers.

phoresis on native 6% polyacrylamide gels. For competition experiments, unlabeled blunt-ended competitor oligonucleotides were added to binding reaction mixtures before adding radiolabeled oligonucleotide probes. The gels were dried, and radioactivity was detected using a Fuji FLA3000 scanner (Fuji Photo Film, Tokyo, Japan).

### Statistical Analysis

Genotype distributions at each polymorphism were evaluated for departures from Hardy–Weinberg equilibrium using the  $\chi^2$  goodness-of-fit test. The two SNPs (–2027A>G and –1608T>C) were in Hardy–Weinberg equilibrium in the study group. Statistical analyses were performed using SPSS version 12 software (SPSS Inc., Chicago, Illinois). Differences in clinical characteristics between groups were examined using a Student's *t* test and an analysis of variance (ANOVA; for continuous variables) or Fisher's exact test (for categorical variables). Risk factors were determined by logistic regression, and Pearson's correlation analysis was used to assess the correlations between 2 variables. Haplotypes of the *TLR4* gene were analyzed using Haploview v4.0.<sup>21</sup> The magnitude of linkage disequilibrium between *TLR4* promoter SNPs, –2027A/G and –1608T>C, showed a high value, with a pairwise linkage disequilibrium value of  $D' = 1$  and  $r^2 = 0.58$ .

## RESULTS

### Study Subjects

The mean age of the 381 workers was  $34.92 \pm 7.72$  years, and the mean exposure period in the bakery was  $3.96 \pm 3.48$  years (Table 1). Atopy was detected in 35.1% of the subjects, whereas the prevalence rates of self-reported physician-diagnosed asthma and rhinitis were 4.7% and 10.8%, respectively. The workers were categorized into 3 groups based on the extent of exposure to flour dust based

Table 1. Clinical Characteristics of the Bakery Workers (*n* = 381)

|                                 |               |
|---------------------------------|---------------|
| Age (years), mean (min, max)    | 34.9 (20, 58) |
| Sex (%)                         |               |
| Female                          | 165 (43.3)    |
| Male                            | 216 (56.7)    |
| Working period (years)          | 4.0 (0.1, 16) |
| Atopy (+, %)                    | 131 (35.1)    |
| Smoking (+, %)                  | 147 (42)      |
| Asthma (+, %)                   | 18 (4.7)      |
| Rhinitis (+, %)                 | 41 (10.8)     |
| Exposure intensity <sup>a</sup> |               |
| Low                             | 111 (29.1)    |
| Intermediate                    | 126 (33.1)    |
| High                            | 144 (37.8)    |

Dust exposure intensities among the 3 groups were statistically significant after ANOVA followed by Bonferroni correction.

<sup>a</sup> 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/m<sup>3</sup>.

on our previous study. The dust exposure intensity among the 3 groups was significant.

#### Prevalence of Work-Related Respiratory Symptoms, Sensitization Rate to Wheat Flour, and Inflammatory Cytokine Levels According to Exposure Intensity

The prevalence of work-related respiratory symptoms was not different among workers with low, intermediate, and high exposure to flour dust (Table 2). In addition, the intensity of exposure showed no significant association with a specific IgE antibody or SPT results.

No significant differences in serum IL-8 or MPO levels were observed in bakery workers according to the intensity of flour dust exposure (Table 2). Moreover, a significant correlation was observed between IL-8 and MPO levels in the sera of workers (Fig 1).

#### Prevalence of Work-Related Respiratory Symptoms, Sensitization Rate to Wheat Flour, and Inflammatory Cytokine Levels According to TLR4 Polymorphisms

Workers with –2027GG and –1608CC genotypes showed lower prevalence rates of work-related lower respiratory symptoms than carriers of –2027AA/AG (*P* = .007) and –1608TT/TC genotypes (*P* = .021) (Table 3A).

In the haplotype analysis, carriers of the haplotype 2, ht2 [GC] showed a lower rate of work-related lower respiratory symptoms (*P* = .021), whereas those with the type 1, h1 [AT] haplotype were more likely to have symptoms than the others (*P* = .007) (Table 3B). Serum IL-8 and MPO levels in bakery workers did not differ according to the *TLR4* genotype (Table 3A) or haplotype (Table 3B).

The *TLR4*–2027GG genotype remained significant (OR = 0.163, 95% CI = 0.036–0.731, *P* = .018) in the multivariate model for predicting work-related lower respiratory symptoms in the context of other potential risk factors, such as a specific IgE to wheat flour, positive SPT to wheat flour, atopy, and prior diagnosis of asthma or rhinitis after adjusting for age and sex (data not shown). None of the target *TLR4* SNPs showed significant associations with upper respiratory symptoms.

#### Combined Effects of TLR4 Polymorphisms and IgE to Wheat Flour on Work-Related Lower Respiratory Symptoms

Subjects were divided into 3 groups according to the *TLR4* haplotype and the presence of specific serum IgE antibodies to wheat flour (Table 4). In total, 40% of workers with both the h1 [AT] and the specific IgE to wheat flour reported having lower respiratory symptoms, whereas these symptoms were observed in 3.8% of workers without either of these factors, with a 17 times higher odds ratio (OR) (group I versus group III). Among the workers carrying the h1 [AT],

Table 2. The Prevalence of Work-Related Respiratory Symptoms, Sensitization Rate to Occupational Allergen, and Inflammatory Cytokine Levels According to Exposure Intensity

|                              | Exposure intensity to wheat |                |                | <i>P</i> |
|------------------------------|-----------------------------|----------------|----------------|----------|
|                              | Low                         | Intermediate   | High           |          |
| Respiratory symptoms         |                             |                |                |          |
| Upper (+, %)                 | 43 (11.3)                   | 40 (10.6)      | 40 (10.6)      | .2       |
| Lower (+, %)                 | 11 (2.9)                    | 22 (5.8)       | 21 (5.5)       | .247     |
| Positive skin prick test (%) |                             |                |                |          |
| Strong wheat                 | 6 (1.6)                     | 8 (2.1)        | 9 (2.4)        | .927     |
| Rye                          | 5 (1.3)                     | 4 (1.1)        | 3 (0.8)        | .573     |
| Yeast                        | 3 (0.8)                     | 2 (0.5)        | 9 (2.4)        | .097     |
| α-Amylase                    | 0 (0)                       | 1 (0.3)        | 0 (0)          | .359     |
| Any baker's allergen         | 11 (2.9)                    | 14 (3.7)       | 20 (5.3)       | .551     |
| Specific IgE to wheat flour  | 6 (1.6)                     | 11 (2.9)       | 8 (2.1)        | .485     |
| IL-8 (pg/mL) <sup>a</sup>    | 238.86 ± 28.12              | 325.21 ± 31.51 | 314.25 ± 30.62 | .114     |
| MPO (ng/mL) <sup>a</sup>     | 132.73 ± 7.28               | 142.1 ± 6.98   | 142.67 ± 7.18  | .571     |

*P* values were applied by Pearson's  $\chi^2$  test for categorical variables and ANOVA for continuous variables.

A positive skin prick test is defined as an A/H ratio  $\geq 1$ .

A positive result for specific IgE antibodies is defined as a level greater than 3 standard deviations of the level in nonatopic healthy controls.

<sup>a</sup> Presented as means  $\pm$  standard error.

Table 3. The Prevalence of Work-Related Respiratory Symptoms and Sensitization Rate to Wheat Flour and Inflammatory Cytokine Levels According to TLR4 Genotype (A) and Haplotype (B)

| <b>A.</b>                               |                             |                |          |                         |                    |          |
|-----------------------------------------|-----------------------------|----------------|----------|-------------------------|--------------------|----------|
|                                         | <b>TLR4-2027 A&gt;G</b>     |                |          | <b>TLR4-1608 T&gt;C</b> |                    |          |
|                                         | <b>AA+AG (%)</b>            | <b>GG (%)</b>  | <b>P</b> | <b>TT+TC (%)</b>        | <b>CC (%)</b>      | <b>P</b> |
| Work-related respiratory symptoms       |                             |                |          |                         |                    |          |
| Upper (+)                               | 104 (32.5)                  | 19 (32.8)      | 1.000    | 108 (31.2)              | 11 (39.3)          | .402     |
| Lower (+)                               | 52 (16.1)                   | 2 (3.4)        | .007     | 54 (15.5)               | 0 (0)              | .021     |
| Positive skin prick test to wheat flour |                             |                |          |                         |                    |          |
|                                         | 22 (7)                      | 1 (1.8)        | .226     | 22 (6.5)                | 0 (0)              | .395     |
| Specific IgE to wheat flour (%)         |                             |                |          |                         |                    |          |
|                                         | 20 (6.2)                    | 5 (8.6)        | .562     | 21 (6)                  | 3 (10.7)           | .408     |
| IL-8 (pg/mL) <sup>a</sup>               | 299.15 ± 19.19              | 283.74 ± 46.03 | .754     | 297.92 ± 18.86          | 286.92 ± 52.6      | .872     |
| MPO (ng/mL) <sup>a</sup>                | 141.61 ± 4.68               | 129.23 ± 8.02  | .186     | 140.07 ± 4.42           | 139.59 ± 11.81     | .976     |
| <b>B.</b>                               |                             |                |          |                         |                    |          |
|                                         | <b>ht-1[AT]</b>             |                |          | <b>ht-2[GC]</b>         |                    |          |
|                                         | <b>ht-1/ht-1+ht-1/- (%)</b> | <b>-/- (%)</b> | <b>P</b> | <b>ht-2/ht-2</b>        | <b>ht-2/-+/-/-</b> | <b>P</b> |
| Work-related respiratory symptoms       |                             |                |          |                         |                    |          |
| Upper (+)                               | 101 (32)                    | 18 (31.6)      | 1.000    | 11 (39.3)               | 108 (31.3)         | .403     |
| Lower (+)                               | 52 (16.4)                   | 2 (3.5)        | .007     | 0 (0)                   | 54 (15.6)          | .021     |
| Positive skin prick test to wheat flour |                             |                |          |                         |                    |          |
|                                         | 22 (7.1)                    | 0 (0)          | .034     | 0 (0)                   | 22 (6.5)           | .395     |
| Specific IgE to wheat flour             |                             |                |          |                         |                    |          |
|                                         | 20 (6.3)                    | 4 (7)          | .772     | 3 (10.7)                | 21 (6.1)           | .408     |
| IL-8 (pg/mL) <sup>a</sup>               | 299.71 ± 19.41              | 287.5 ± 46.68  | .807     | 286.92 ± 52.6           | 298.73 ± 18.9      | .862     |
| MPO (ng/mL) <sup>a</sup>                | 141.97 ± 4.73               | 130 ± 8.12     | .206     | 139.59 ± 11.81          | 140.19 ± 4.44      | .970     |

+, Presence.

P value was applied by Fisher's exact test.

Upper respiratory symptoms: nasal itching, runny nose, sneezing, or congestion; lower respiratory symptoms: cough, sputum, shortness of breath, or wheezing

<sup>a</sup> Presented as means ± standard error.

Table 4. Combined Effect of TLR4 Haplotype and Specific IgE Response to Wheat Flour on Work-Related Lower Respiratory Symptoms

| <b>Work-related lower respiratory symptoms (+, %)</b> | <b>P</b> | <b>OR (95%CI)</b>    |
|-------------------------------------------------------|----------|----------------------|
| Group I (n = 20)                                      | .001     | 17 (3.193–90.509)    |
| Group II (n = 298)                                    | .044     | 4.417 (1.038–18.805) |
| Group III (n = 53)                                    | .005     | 1                    |

Workers in group I had at least one ht1[AT] and IgE antibody to wheat flour.

Workers in group II had at least one ht1[AT] and no IgE antibody to wheat flour.

Workers in group III had neither ht1[AT] nor IgE antibody to wheat flour (used as the reference value).

the OR for those with specific IgE to wheat flour was almost 4 times higher than that of those without the specific IgE (group I versus group II). In workers without the specific IgE to wheat flour, the workers having the h1 [AT] increased the OR by 4.417 times (group II versus group III).

### Functional Effects of TLR4 Polymorphisms on Transcriptional Activity

To evaluate whether the *TLR4*–2027A>G and –1608T>C polymorphisms are associated with altered promoter activity

in human monocytic cells and bronchial epithelial cells, we used a luciferase reporter gene with the *TLR4* promoter and compared the reporter activities of plasmids containing either common alleles (–2027A and –1608T; ht1 [AT]) or rare alleles (–2027G and –1608C; ht2 [GC]). Constructs that contained rare alleles exhibited significantly lower luciferase activities than those containing common alleles in both the U937 (Fig 2A) and BEAS-2B (Fig 2B) cell lines.

To assess whether the –2027A>G and –1608T>C loci serve as transcription factor binding sites, gel shift assays

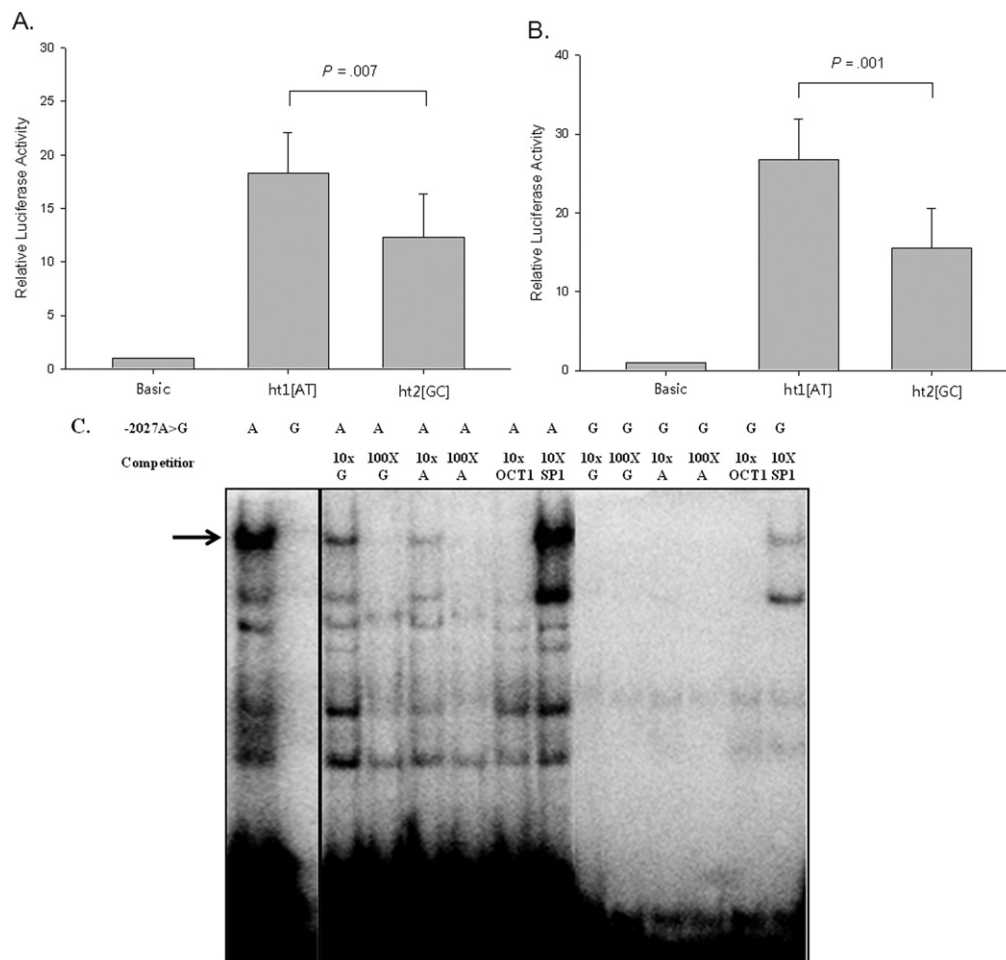


Figure 2. Relative luciferase activity (ratio of luciferase production driven by the reporter construct to that of the promoter-less control vector, pGL3-Basic) in (A) U937 ( $n = 9$ ) and (B) BEAS-2B cells ( $n = 9$ ). Bars indicate the mean values of 3 independent experiments, and the standard deviation is indicated by error bars. Statistical analysis was performed using a Mann–Whitney *U*-test. (C) EMSA for the *TLR4* -2027A>G polymorphism using U937 nuclear extracts. The following double-stranded oligonucleotides were used as probes: for -2027A/G, 5'-GACTTAGCAT[a/g]CATAATATTC-3'; for NF- $\kappa$ B, 5'-AGTTGAGGGGACTTTCCAGG-3'; for OCT1, 5'-TGTCGAATGCAATCACTAGA-3'; for SP1, 5'-ATTCGATCGGGGCGGGGCGAGC-3'.

were performed with nuclear extracts from U937 cells using double-stranded oligonucleotide probes that corresponded to *TLR4* -2027A/G and -1608T>C. Among -2027A, -2027G, -1608T, and -1608C probes, one shifted band was noted for the -2027A probe in U937 cells (Fig 2C). The shifted band produced by the -2027A probe disappeared in the presence of the unlabeled -2027A probe but remained visible in the presence of the unlabeled -2027G probe.

## DISCUSSION

We hypothesized that *TLR4* may be involved in the pathogenesis of baker's asthma, which develops in work environments with continuous exposure to flour allergens and endotoxins.

Endotoxin exposure is associated with respiratory symptoms and diseases in many workplace settings, especially where workers are exposed to considerable amounts of en-

dotoxins, such as poultry or pig farms.<sup>22,23</sup> The concentrations of IL-8 and neutrophil number reportedly increased in bronchoalveolar and nasal lavage fluids of healthy subjects after exposure to endotoxin-contaminated swine dust.<sup>24</sup> In recent years, the suggestion was made that the genetic effects of *TLR4* polymorphisms on the biological responses to endotoxin exposure should be considered as controlling factors for work-related respiratory diseases under the concept of a gene–environment interaction.<sup>25</sup> Several studies have been performed to investigate the effects of *TLR4* gene polymorphisms on occupational asthma induced by organic dust in, for example, swine farms or laboratories.<sup>26,27</sup>

In this study, we found that workers exhibiting variants of the *TLR4* promoter at -2027 A>G and -1608 T>C had lower work-related lower respiratory symptoms. Furthermore, an *in vitro* functional study indicated that the construct with ht2 [GC] showed significantly lower promoter activity in U937

cells than those with ht1 [AT], which was replicated in bronchial epithelial cells, suggesting that ht2 [GC] is associated with decreased TLR4 gene expression. This result was further supported by those of an EMSA study, which showed reduced intensity of the complex in probes for –2027G compared with –2027A. A survey of a transcription factor database (<http://www.cbrc.jp/research/db/TFSEARCH.html>) suggested that the A to G conversion of the –2027A polymorphism may generate a putative transcription factor-binding motif for Oct-1, which was not confirmed by a supershift assay in this study. Our results provided supporting evidence that TLR4 may play a critical role in the development of baker's asthma in several ways.

First, endotoxins may induce respiratory symptoms directly in exposed workers through the TLR4 pathway.<sup>28</sup> We observed a strong correlation between IL-8 and MPO levels, which suggested that TLR4 may be involved in the innate immune response to the endotoxin of flour dust in exposed workers. Among the exposed workers, *TLR4* polymorphisms were associated with a lower prevalence of work-related respiratory symptoms. The results of the current study correspond with those of earlier studies indicating that *TLR4* variants are related to endotoxin hyporesponsiveness.<sup>12,13</sup> The protective effects of *TLR4* functional variants on work-related lower respiratory symptoms in bakery workers may reflect reduced inflammatory responses to endotoxins in the lung.

Second, TLR4 also may be involved in the regulation of allergic sensitization to wheat flour. TLR4 signaling was shown to be required to modulate allergic responses in several animal model studies.<sup>29,30</sup> *TLR4*-deficient mice showed decreased ovalbumin-induced eosinophilic inflammation in the lung, allergen-specific IgE levels, and Th2 cytokine production compared with wild-type controls. We observed a significantly lower sensitization rate to wheat flour on SPT in carriers of *TLR4* variants. This implies that TLR4 signaling may be involved in the sensitization process to flour allergens when workers are exposed to allergens and endotoxins simultaneously in the form of organic dust.

Furthermore, we found a combined effect of the *TLR4* haplotype and the specific IgE response to wheat flour on work-related lower respiratory symptoms. Several studies demonstrated that the IgE-mediated response is mainly associated with work-related symptoms in bakery workers.<sup>31,32</sup> In the current study, TLR4 ht1 [AT] appeared to multiply the risk of exhibiting work-related lower respiratory symptoms along with the specific IgE antibody to wheat flour in a synergistic manner.

We were limited in this study because we could not verify the exact level of endotoxin exposure in the workplace, although it could be estimated based on previously published data.<sup>5</sup> A recent investigation indicated that endotoxin exposure was positively associated with bronchial hyperresponsiveness, while inversely associated with atopy and IgE to grass pollen in agricultural workers.<sup>16</sup> Considering the moderate level of endotoxin exposure in bakery workers, the level

of endotoxin exposure in the current study population may have been more effective in augmenting allergic processes than extremely high endotoxin concentrations.

In addition, we did not know when the sensitization to wheat flour actually occurred, because this was a cross-sectional study. A previous longitudinal study<sup>33</sup> indicated that the incidence of positive SPT to occupational allergens in apprentice bakers increases with the duration of exposure and that positive SPT to occupational allergens at the commencement of training was a significant risk factor for asthma. Further longitudinal genetic studies are required to elucidate the genetic effects of *TLR4* on allergic sensitization to new allergen exposure.

This study's findings have several important implications. We attempted to clarify the genetic effects of *TLR4* on work-related respiratory symptoms and sensitization to flour allergens in the context of environmental interaction to assess the risk of baker's asthma. The current study suggested a potential new mechanism by which the TLR4 pathway may induce baker's asthma. This is the first study to investigate the functional SNPs of *TLR4* in relation to baker's asthma in an Asian population.

In conclusion, our findings suggest that TLR4 may contribute to the development of lower respiratory symptoms by occupational endotoxin exposure and may augment allergic sensitization to wheat flour in bakery workers. *TLR4* polymorphisms may be an important factor in predicting the risk of occupational asthma in environments associated with endotoxin and allergen exposure.

## REFERENCES

1. Cullinan P, Cook A, Nieuwenhuijsen MJ, et al. Allergen and dust exposure as determinants of work-related symptoms and sensitization in a cohort of flour-exposed workers: a case-control analysis. *Ann Occup Hyg.* 2001;45:97–103.
2. Brisman J, Jarvholm B, Lillienberg L. Exposure-response relations for self reported asthma and rhinitis in bakers. *Occup Environ Med.* 2000; 57:335–340.
3. Brant A. Baker's asthma. *Curr Opin Allergy Clin Immunol.* 2007;7: 152–155.
4. Houba R, Heederik D, Doekes G. Wheat sensitization and work-related symptoms in the baking industry are preventable: an epidemiologic study. *Am J Respir Crit Care Med.* 1998;158:1499–1503.
5. Domanska A, Stroszejn-Mrowca G. Endotoxin in the occupational environment of bakers: method of detection. *Int J Occup Med Environ Health.* 1994;7:125–134.
6. Reed CE, Milton DK. Endotoxin-stimulated innate immunity: a contributing factor for asthma. *J Allergy Clin Immunol.* 2001;108:157–166.
7. Michel O, Nagy AM, Schroeven M, et al. Dose-response relationship to inhaled endotoxin in normal subjects. *Am J Respir Crit Care Med.* 1997;156:1157–1164.
8. Alexis NE, Lay JC, Almond M, Peden DB. Inhalation of low-dose endotoxin favors local t(h)2 response and primes airway phagocytes *in vivo*. *J Allergy Clin Immunol.* 2004;114:1325–1331.
9. Eisenbarth SC, Piggott DA, Huleatt JW, et al. Lipopolysaccharide-enhanced, Toll-like receptor 4-dependent t helper cell type 2 responses to inhaled antigen. *J Exp Med.* 2002;196:1645–1651.
10. Kaisho T, Akira S. Toll-like receptor function and signaling. *J Allergy Clin Immunol.* 2006;117:979–987.

11. von Scheele I, Larsson K, Palmberg L. Budesonide enhances Toll-like receptor 2 expression in activated bronchial epithelial cells. *Inhal Toxicol.* 2010;22:493–499.
12. Werner M, Topp R, Wimmer K, et al. Tlr4 gene variants modify endotoxin effects on asthma. *J Allergy Clin Immunol.* 2003;112:323–330.
13. Arbour NC, Lorenz E, Schutte BC, et al. Tlr4 mutations are associated with endotoxin hyporesponsiveness in humans. *Nat Genet.* 2000;25:187–191.
14. Lundberg A, Wikberg LA, Ilonen J, Vaarala O, Bottcher MF. Lipopolysaccharide-induced immune responses in relation to the tlr4(asp299gly) gene polymorphism. *Clin Vaccine Immunol.* 2008;15:1878–1883.
15. Raby BA, Klimecki WT, Laprise C, et al. Polymorphisms in Toll-like receptor 4 are not associated with asthma or atopy-related phenotypes. *Am J Respir Crit Care Med.* 2002;166:1449–1456.
16. Smit LA, Heederik D, Doekes G, Lammers JW, Wouters IM. Occupational endotoxin exposure reduces the risk of atopic sensitization but increases the risk of bronchial hyperresponsiveness. *Int Arch Allergy Immunol.* 2009;152:151–158.
17. Noguchi E, Nishimura F, Fukai H, et al. An association study of asthma and total serum immunoglobulin e levels for Toll-like receptor polymorphisms in a Japanese population. *Clin Exp Allergy.* 2004;34:177–183.
18. Yoon HJ, Choi JY, Kim CO, et al. Lack of Toll-like receptor 4 and 2 polymorphisms in Korean patients with bacteremia. *J Korean Med Sci.* 2006;21:979–982.
19. Hang J, Zhou W, Zhang H, et al. Tlr4 asp299gly and thr399ile polymorphisms are very rare in the Chinese population. *J Endotoxin Res.* 2004;10:238–240.
20. Hur GY, Koh DH, Kim HA, et al. Prevalence of work-related symptoms and serum-specific antibodies to wheat flour in exposed workers in the bakery industry. *Respir Med.* 2008;102:548–555.
21. Schaid DJ, Rowland CM, Tines DE, Jacobson RM, Poland GA. Score tests for association between traits and haplotypes when linkage phase is ambiguous. *Am J Hum Genet.* 2002;70:425–434.
22. Larsson BM, Larsson K, Malmberg P, Martensson L, Palmberg L. Airway responses in naive subjects to exposure in poultry houses: comparison between cage rearing system and alternative rearing system for laying hens. *Am J Ind Med.* 1999;35:142–149.
23. Dosman JA, Lawson JA, Kirychuk SP, et al. Occupational asthma in newly employed workers in intensive swine confinement facilities. *Eur Respir J.* 2004;24:698–702.
24. Larsson BM, Palmberg L, Malmberg PO, Larsson K. Effect of exposure to swine dust on levels of il-8 in airway lavage fluid. *Thorax.* 1997;52:638–642.
25. Martinez FD. Gene–environment interactions in asthma: with apologies to William of Ockham. *Proc Am Thorac Soc.* 2007;4:26–31.
26. Senthilselvan A, Dosman JA, Chenard L, et al. Toll-like receptor 4 variants reduce airway response in human subjects at high endotoxin levels in a swine facility. *J Allergy Clin Immunol.* 2009;123:1034–1040.
27. Pacheco K, Maier L, Silveira L, et al. Association of Toll-like receptor 4 alleles with symptoms and sensitization to laboratory animals. *J Allergy Clin Immunol.* 2008;122:896–902.
28. Michel O. Role of lipopolysaccharide (lps) in asthma and other pulmonary conditions. *J Endotoxin Res.* 2003;9:293–300.
29. Dabbagh K, Dahl ME, Stepick-Biek P, Lewis DB. Toll-like receptor 4 is required for optimal development of th2 immune responses: role of dendritic cells. *J Immunol.* 2002;168:4524–4530.
30. Phipps S, Lam CE, Kaiko GE, et al. Toll/il-1 signaling is critical for house dust mite-specific helper t cell type 2 and type 17 [corrected] responses. *Am J Respir Crit Care Med.* 2009;179:883–893.
31. Quirce S, Fernandez-Nieto M, Escudero C, et al. Bronchial responsiveness to bakery-derived allergens is strongly dependent on specific skin sensitivity. *Allergy.* 2006;61:1202–1208.
32. van Kampen V, Rabstein S, Sander I, et al. Prediction of challenge test results by flour-specific ige and skin prick test in symptomatic bakers. *Allergy.* 2008;63:897–902.
33. Walusiak J, Hanke W, Gorski P, Palczynski C. Respiratory allergy in apprentice bakers: do occupational allergies follow the allergic march? *Allergy.* 2004;59:442–450.

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