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Experimental & Molecular Medicine (2015) 47, e157; doi:10.1038/emm.2015.10

www.nature.com/emm

ORIGINAL ARTICLE

Effects of *MBL2* polymorphisms in patients with diisocyanate-induced occupational asthma

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Diisocyanate (DI) is the most common cause of occupational asthma (OA) in Korea. Mannose-binding lectin (MBL) initiates the lectin complement activation pathway following oxidative stress and plays an important role in the regulation of inflammatory processes. To determine whether there is a genetic association between *MBL2* polymorphisms and DI-OA, 99 patients with DI-OA, 99 asymptomatic exposed controls (AECs) and 144 unexposed normal controls were enrolled in this study. Three polymorphisms (−554 G>C, −431A>C and −225 G>C) in the *MBL2* promoter were genotyped, and serum MBL levels were determined by enzyme-linked immunosorbent assay. Functional variabilities in the promoter polymorphisms were analyzed by a luciferase reporter assay and electrophoretic mobility shift assay (EMSA). A significantly higher frequency of haplotype (ht) 2 [CAG] was noted in the DI-OA group compared with the AEC group ($P=0.044$). The patients with DI-OA carrying ht2 [CAG] had significantly lower PC_{20} methacholine levels ($P<0.001$) than the non-carriers. The serum MBL levels were significantly higher in the DI-exposed subjects (both the DI-OA patients and AECs) carrying ht1 [GAG] ($P=0.028$). Luciferase activity was significantly enhanced in ht1 [GAG] compared with ht2 [CAG] in human hepatocarcinoma cells (Hep3B) ($P=0.002$). The EMSA showed that a −554G probe produced a specific shifted band compared with the −554C probe. These findings suggest that decreased serum MBL levels due to polymorphisms of the *MBL2* gene may increase susceptibility to the development of DI-OA in DI-exposed individuals.
Experimental & Molecular Medicine (2015) **47,** e157; doi:10.1038/emm.2015.10; published online 10 April 2015

INTRODUCTION

Diisocyanates (DIs) are highly reactive chemicals and well-known triggers of occupational asthma (OA).[1] Toluene DI, 4,4-methylenediphenyl DI and hexamethylene DI are DIs that constitute a group of low-molecular weight agents used in various manufacturing industries, such as painting, construction and wood-working.[2,3] In particular, toluene DI is the most common cause of OA in Korea with a prevalence of 2.9–13% among exposed workers.[4,5]

DI-induced OA (DI-OA) is characterized by chronic airway inflammation with remodeling and hyperresponsiveness.[6] The pathogenic mechanisms of DI-OA have not yet been elucidated; however, several immunological and non-immunological mechanisms have been suggested.[2,7] Oxidative stress, which plays an important role in the pathogenesis of airway inflammation, has been suggested to be a pathogenic mechanism of DI-OA.[8] It has been demonstrated that inflammation driven by increased oxidative stress occurs in the airways of asthmatic patients.[9] In cases of DI-OA, inhaled DI causes tissue injury, activating both inflammatory and bronchial epithelial cells, which generate reactive oxygen species (ROS) and nitrogen species that cause oxidative stress.[10] Increased production of ROS has also been noted in macrophages, antigen-presenting cells, neutrophils and eosinophils within the lungs of patients with asthma, which can further exacerbate asthma by increasing airway hyperresponsiveness and triggering inflammation.[11]

Studies have suggested that oxidative stress can induce mannose-binding lectin-mediated complement activation.[12,13] Because complement activation is involved in innate defense mechanisms, it could play an important role in the regulation of airway inflammation. The presence of mannose-binding lectin (MBL) in the bronchoalveolar lavage fluid of children with airway infections and not in that of controls suggests its role in pulmonary defense.[14] It has been shown that oxidative stress can decrease functional airway MBL in chronic obstructive pulmonary disease patients.[15] Similarly, nebulized administration of human plasma-derived MBL at clinically achievable

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Received 15 September 2014; revised 29 October 2014; accepted 12 December 2014

concentrations significantly reduced inflammation and improved efferocytosis in a smoking mouse model, restoring pulmonary macrophage efferocytic ability and reducing airway inflammation, suggesting that it might play an important role in attenuating inflammation induced by oxidative stress.[15]

MBL is an important serum protein of the innate immune system produced mainly by hepatocytes. It selectively recognizes the carbohydrate patterns of infectious agents or infected cells and then opsonizes antigens and activates the lectin complement pathway system.[16] MBL triggers opsonophagocytosis and is involved in the modulation of inflammation by initiating the lectin complement pathway.[17] The human MBL 2 (*MBL2*) gene is located on chromosome 10q11.2-q21 and encodes MBL.[18] Six single nucleotide polymorphisms (SNPs) of *MBL2* have been associated with altered serum MBL levels in population studies.[19,20] In addition, our previous study has suggested that *MBL2* gene polymorphisms can alter serum MBL levels in Korean adult asthmatics.[21] However, it is not known whether an alteration in the MBL serum level due to *MBL2* genetic variants could have an influence on DI-OA.

On the basis of these observations, we investigated genetic polymorphisms of the *MBL2* gene and serum MBL levels associated with *MBL2* variants in patients with DI-OA. A case–control study was performed, in which three groups were evaluated as follows: DI-OA, asymptomatic exposed controls (AECs) and normal controls (NCs). The functional effects of *MBL2* polymorphisms were evaluated using a luciferase reporter assay and electrophoretic mobility shift assay (EMSA).

MATERIALS AND METHODS

Study subjects

Ninety-nine patients with DI-OA were enrolled from a pool of volunteers who had been exposed to DI in their workplaces, which included spray painting, furniture polishing, and the musical instrument and car industries. The DI-OA phenotype was determined by positive responses to the DI-bronchial challenge test, which was performed at the Ajou University Medical Center, Suwon, South Korea. Ninety-nine AECs who worked in environments similar to those of the patients with DI-OA and 144 unexposed NCs were recruited from the pool of volunteers. Written informed consent was obtained from all subjects, and the Institutional Review Board of Ajou University Hospital approved the study. Skin-prick tests were performed with 55 common aeroallergens (Bencard Co., Bredford, UK), and atopy was defined as one or more positive reactions to common inhalant allergens. Total IgE concentrations were measured using an ImmunoCAP system (Thermo Scientific, Uppsala, Sweden) according to the manufacturer's instructions.

DI-bronchial challenge test

The DI-bronchial challenge test was performed according to a protocol previously described.[4,22]

Detection of IgE and IgG antibodies specific to DI-human serum albumin conjugate

Serum-specific IgE and IgG levels were determined by enzyme-linked immunosorbent assay as previously described.[23] A vapor-type DI-human serum albumin (HSA) conjugate was prepared from 80% of 2, 4-DI isomers based on a recently described DI vapor-phase exposure system.[23] Specific DI binding was calculated as the difference in optical density between mock-conjugated and DI-albumin wells. Positive cutoff values were based on the mean ± two standard deviations of the absorbance values obtained from tests with unexposed healthy controls.

DNA extraction, SNP identification and genotyping

Total genomic DNA was isolated from peripheral blood samples using a Puregene DNA Purification Kit (Gentra, Minneapolis, MN, USA) according to the manufacturer's protocol. On the basis of previous findings[21] and the SNP sequencing results, we chose three SNPs in the *MBL2* promoter (−554 G>C, −431A>C and −225 G>C). Samples were genotyped for the three SNPs by a TaqMan allelic discrimination assay using TaqMan probes (Applied Biosystems, Foster City, CA, USA). The sequences of the primers and probes used for SNP genotyping have been previously reported.[21]

Measurement of human serum MBL level

The serum MBL level was measured using a human MBL DuoSet ELISA Development Kit (R&D Systems, Minneapolis, MN, USA). Serum samples were stored at −80 °C prior to measurement.

Cell culture and preparation of *MBL2* constructs for promoter assays

Hep3B cells (human hepatocarcinoma cells) were maintained in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco), 100 U ml^{-1} penicillin G sodium and 100 μg ml^{-1} streptomycin sulfate (Gibco), and cultured at 37 °C in humidified 95% air and 5% CO_2. A 1239-bp fragment of the human *MBL2* gene was prepared by PCR amplification using human genomic DNA as a template (forward primer: 5′-GCTAGGCTGCTGAGGTTTCTT-3′ and reverse primer: 5′-GGGCTGGCAAGACAACTATTAG-3′). The PCR products, haplotype (ht) 1 [$G_{-554}A_{-431}G_{-225}$] and ht2 [$C_{-554}A_{-431}G_{-225}$], were used as templates for the cloning construct. Each PCR product was gel-purified with an agarose gel purification kit (GeneAll Biotech, Seoul, Korea) and ligated into a TOPO vector (Invitrogen, Carlsbad, CA, USA). The plasmid was digested with *Kpn*I and *Xho*I (Takara, Shuzo, Japan) and ligated into a pGL3-basic luciferase reporter vector (Promega, Madison, WI, USA) using T4 DNA ligase (Elpis Biotech, Daejeon, Korea). All constructs were confirmed by restriction enzyme analysis and DNA sequencing. Plasmid DNAs were prepared from these constructs using an Endo Free Plasmid Maxi Kit (Qiagen, Hilden, Germany), and concentrations and purity were assessed by UV spectrophotometry and agarose gel electrophoresis.

Transient transfection for luciferase activity assessment

Transfection of Hep3B cells was performed. Detailed transfection protocols have been described in our previous study.[24] Hep3B cells were transfected using a microporator (model MP-100; Digital Biotechnology, Seoul, Korea) and an MPK-1096 solution kit (Digital Biotechnology) according to the manufacturer's instructions. Briefly, 12-well plates were filled with 1000 μl of Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Plates were incubated in a humidified incubator at 37 °C and 5% CO_2. Hep3B cells (2×10^5 cells per well), reporter plasmid DNA containing *MBL2* (1 μg) and *Renilla* plasmid DNA (5 ng) were suspended in 10 μl Solution R (MPK-1096). The incorporation tube was filled with 3 ml Solution E

Table 1 Clinical characteristics of the study subjects

				P-*value*	
	DI-OA (n = *99*)	*AEC* (n = *99*)	*NC* (n = *144*)	*DI-OA* vs *AEC*	*DI-OA* vs *NC*
Age (year)[a]	42.46 ± 9.71	41.63 ± 8.73	33.19 ± 13.54	0.524	**<0.001**
Sex (male/total)	65/99 (65.66%)	60/99 (60.61%)	58/144 (40.28%)	0.461	**<0.001**
Atopy (presence/total)	30/72 (41.67%)	15/52 (28.85%)	11/78 (14.1%)	0.143	**<0.001**
FEV1 (%)[a]	87.24 ± 21.64	99.98 ± 18.36	97.8 ± 7.11	**0.002**	**<0.001**
Log PC_{20} methacholine (pg ml^{-1})[a]	1.29 ± 1.12/68	3.07 ± 0.52/18	NA	**<0.001**	NA
Total IgE (IU ml^{-1})[a]	274.49 ± 420.51	266.29 ± 433.75	87.95 ± 112.42	0.903	**0.001**
Specific IgE to DI-HSA (positive/total)	14/64 (21.88%)	8/93 (8.6%)	1/34 (2.94%)	**0.019**	**0.013**
Specific IgG to DI-HSA (positive/total)	18/64 (28.13%)	16/93 (17.2%)	2/34 (5.88%)	0.103	**0.009**
Serum MBL level (μg l^{-1})[a]	571.54 ± 432.86	637.92 ± 471.37	425.36 ± 186.55	0.412	0.208

Abbreviations: AEC, asymptomatic exposed control; DI-OA, diisocyanate-induced occupational asthma; FEV1, forced expiratory volume in 1 s; HSA, human serum albumin; NA, not applicable; NC, normal control; *n*, number of subjects.
The values in bold indicate significant *P*-values. Each *P*-value <0.05 was considered to be significant.
[a]This value is presented as the mean ± s.d.

(MPK-1096). The cell and DNA mixtures were inserted into a microporator pipette station. The Hep3B cells were shocked using the following settings: 850 V, 60-ms pulse width and one pulse. The samples were transferred to preincubated 12-well plates and incubated for 24 h. The cells were lysed with 100 μl per well of passive lysis buffer (Promega). A 10-μl aliquot from each well was assayed for luciferase activity using a Dual-Luciferase Reporter Assay Kit (Promega) and a Clarity Luminescence microplate reader (BioTek, Winooski, VT, USA). pGL3-control (Promega) and promoterless pGL3-basic vectors were used as positive and negative controls, respectively. Transfection efficiency was determined by measuring *Renilla* activity using a Dual-Luciferase Reporter Assay Kit (Promega) after co-transfection of both the reporter construct and the *Renilla* control vector into the cell line.

Nuclear extract preparation and EMSA

Nuclear extracts were prepared from Hep3B cells, and protein concentrations were determined using the Bradford assay as previously described.[25] Samples were stored at −80 °C until use. The double-stranded oligonucleotide sequences for *MBL2* −554G and −554C were 5′-GCAAGCCTGTGTAAAACACCA-3′ and 5′-GCAAGCCTGT CTAAAACACCA-3′, respectively. Competitor oligonucleotide nuclear factor-κB (NF-κB) (5′-AGTTGAGGGGACTTTCCCAGG-3′) and activating protein-1 (AP-1) (5′-TTCCGGCTGAGTCATCAAGCG-3′) were used as probes for the EMSA. Oligonucleotides and their complementary strands were designed and purchased (Bioneer, Daejeon, Korea). Preparation of double-stranded oligonucleotides, radiolabeled DNA probes and reaction mixtures was performed as previously described. For the competition experiments, unlabeled blunt-ended competitor oligonucleotides were added to the binding reaction mixtures before addition of the radiolabeled oligonucleotide probe. The gels were dried, and radioactivity was detected using a FLA-7000 scanner and Multi Gauge software ver. 3.0 (Fuji Photo Film Co., Ltd., Tokyo, Japan).

Statistical analysis

Statistical analyses were performed using SPSS (Statistical Package for the Social Sciences) version 12.0 software (SPSS Inc., Chicago, IL, USA). Genotype frequency was examined for the subject groups using a χ^2 test, and differences in genotype and haplotype frequencies were examined using a logistic regression analysis with co-dominant, dominant and recessive models after accounting for age and sex as co-variables. Differences in clinical characteristics among the groups were examined using the independent *t*-test for continuous variables and the χ^2 test for categorical variables. Statistical significance was established at $P<0.05$.

RESULTS

Clinical characteristics of the study subjects

The clinical characteristics of the study population are summarized in Table 1. The mean age of the patients with DI-OA was 42.46 years (±9.71) and that of the unexposed NCs was 33.19 years (±13.54) ($P<0.001$). The prevalence of males was significantly higher in the DI-OA group than in the NC group (65.66% male DI-OA patients and 40.28% male NCs) ($P<0.001$). In addition, the atopy rate was also significantly higher for the patients with DI-OA (41.67% for the DI-OA patients and 14.1% for the NC group) ($P<0.001$). The serum total IgE level of the patients with DI-OA was 274.49 IU ml^{-1} (±420.51), and that of the NCs was 87.95 IU ml^{-1} (±112.42) ($P=0.001$). The patients with DI-OA had a higher prevalence of serum-specific IgE and IgG to DI-HSA conjugates than those in the control groups (21.88% serum-specific IgE to DI-HSA and 28.13% serum-specific IgG to DI-HSA) ($P=0.013$ and $P=0.009$, respectively). No significant differences were observed in the serum MBL levels between the patients with DI-OA and those in the control groups (Table 1). However, the DI-exposed workers (both the DI-OA and AEC subjects) tended to have higher serum MBL levels than those in the NC group (610.05 ± 455.08 vs 425.36 ± 186.55 μg l^{-1}; data not shown).

MBL2 genotype and haplotype frequencies

We selected three *MBL2* SNPs (−554 G>C, −431A>C and −225G>C) for this study. Linkage disequilibrium analyses were performed among the three SNPs. Four common haplotypes, ht1 [GAG], ht2 [CAG], ht3 [CAC] and ht4 [CCG], were constructed utilizing the EM algorithm.

Table 2 Allele and genotype frequencies of *MBL2* gene polymorphisms in study subjects

				[a]P-*value*		
	DI-OA (n = *99*)	*AEC* (n = *99*)	*NC* (n = *144*)	*DI-OA* vs *AEC*	*DI-OA* vs *NC*	*DI-exposed* vs *NC*
– 554 G/C (rs11003125)						
GG	33 (33.33%)	30 (30.3%)	37 (25.69%)	0.789	0.281	0.541
GC	40 (40.4%)	46 (46.46%)	76 (52.78%)	0.711	0.811	0.964
CC	26 (26.26%)	23 (23.23%)	31 (21.53%)	0.702	0.166	0.308
q	0.465	0.465	0.479	0.979	0.454	0.530
– 431A/C (rs11003124)						
AA	88 (88.89%)	75 (75.76%)	114 (79.17%)	**0.016**	0.214	0.239
AC	8 (8.08%)	24 (24.24%)	26 (18.06%)	0.999	0.396	0.090
CC	3 (3.03%)	0 (0%)	4 (2.78%)	**0.016**	0.080	0.606
q	0.071	0.121	0.118	0.081	0.058	0.303
–225 G/C (rs7096206)						
GG	81 (81.82%)	70 (70.71%)	113 (78.47%)	0.169	0.554	0.716
GC	17 (17.17%)	27 (27.27%)	27 (18.75%)	0.550	0.490	0.450
CC	1 (1.01%)	2 (2.02%)	4 (2.78%)	0.060	0.314	0.925
q	0.096	0.157	0.122	0.064	0.258	0.876

Abbreviations: AEC, asymptomatic exposed control; DI-OA, diisocyanate-induced occupational asthma; NA, not applicable; NC, normal control; *n*, number of subjects; q, minor allele frequency.
Logistic regression analysis was applied to control for age and sex as covariates.
The values in bold indicate significant *P*-values. Each *P*-value <0.05 was considered to be significant.
[a]Each *P*-value was calculated using the co-dominant, dominant and recessive models.

Tables 2 and 3 show the genotype and haplotype frequencies, respectively, of each *MBL2* SNP in the study subjects. A significantly higher frequency of the AA genotype at −431 A>C was noted in the DI-OA group compared with the AEC group ($P=0.016$, both in the co-dominant and recessive analysis models), but no significant differences among the study groups were noted in the genotype frequencies of the −554 G>C and −225 G>C polymorphisms. A higher frequency of ht2 [CAG] was noted in the DI-OA group in the recessive analysis model compared with the AEC group ($P=0.044$ in the recessive analysis model), but no significant differences in ht1 [GAG], ht3 [CAC] or ht4 [CCG] were noted among the study groups.

Serum MBL levels according to *MBL2* genotype and haplotype in DI-exposed workers

Significant differences in serum MBL levels were observed according to the *MBL2* polymorphisms in the DI-exposed workers (both the DI-OA and AEC subjects). Subjects carrying ht1 [GAG] showed significantly higher serum MBL levels than those with other haplotypes (743.85 ± 404.11 vs $553.33 \pm 465.55\ \mu g\ l^{-1}$, $P=0.028$; Figure 1).

PC_{20} methacholine levels according to *MBL2* haplotype 2 [CAG] in patients with DI- OA

PC_{20} methacholine levels were assessed according to *MBL2* polymorphism in the patients with DI-OA. The patients with DI-OA carrying ht2 [CAG] showed significantly lower PC_{20} methacholine levels than those of the patients with other genotypes and haplotypes (1.32 ± 1.92 vs $6.07 \pm 8.14\ pg\ ml^{-1}$, $P<0.001$; Figure 2). Other clinical parameters, including atopy, baseline FEV1% and other immunological findings, were not significantly different according to *MBL2* genotype or haplotype frequency (data not shown).

Effects of *MBL2* polymorphisms on transcriptional activity between haplotype 1 [GAG] and haplotype 2 [CAG]

We performed a luciferase reporter assay using the haplotype constructs ht1 [GAG] and ht2 [CAG] to determine the transcriptional effects of the *MBL2* −554 G>C polymorphisms. A construct composed of the *MBL2* sequence and a luciferase reporter gene was transfected into Hep3B cells. Reporter activities were compared between ht1 [GAG] and ht2 [CAG] in *MBL2*. Luciferase activity was significantly enhanced in the ht1 [GAG] construct compared with ht2 [CAG] in the Hep3B cells ($P=0.002$, Figure 3).

Electrophoretic mobility shift assay (EMSA)

To determine whether the −554 G>C polymorphism altered transcription factor binding affinity, we performed an EMSA with nuclear extracts from Hep3B cells using double-stranded oligonucleotide probes that corresponded to −554 G>C. The −554G probe produced a specific band that differed from that of the −554C probe (Figure 4).

DISCUSSION

DI-OA is a complex disease influenced by multiple genetic and environmental factors. Oxidative stress has been described as a trigger that aids in the development of DI-OA.[8] Moreover, it can aggravate airway inflammation by inducing

Table 3 Haplotype frequencies of three single nucleotide polymorphisms of *MBL2*

				[a]P-*value*		
	DI-OA (n = *99*)	*AEC* (n = *99*)	*NC* (n = *144*)	*DI-OA* vs *AEC*	*DI-OA* vs *NC*	*DI-exposed* vs *NC*
ht1 (GAG)						
+/+	33 (33.33%)	30 (30.3%)	37 (25.69%)	0.789	0.281	0.541
+/−	40 (40.4%)	46 (46.46%)	76 (52.78%)	0.711	0.811	0.964
−/−	26 (26.26%)	23 (23.23%)	31 (21.53%)	0.702	0.166	0.308
ht2 (CAG)						
+/+	12 (12.12%)	4 (4.04%)	9 (6.25%)	0.070	0.160	0.732
+/−	50 (50.51%)	63 (63.64%)	90 (62.5%)	0.520	0.424	0.816
−/−	37 (37.37%)	32 (32.32%)	45 (31.25%)	**0.044**	0.124	0.486
ht3 (CAC)						
+/+	1 (1.01%)	2 (2.02%)	4 (2.78%)	0.483	0.768	0.661
+/−	9 (9.09%)	13 (13.13%)	11 (7.64%)	0.235	0.613	0.880
−/−	89 (89.9%)	84 (84.85%)	129 (89.58%)	0.550	0.490	0.450
ht4 (CCG)						
+/+	3 (3.03%)	0 (0%)	4 (2.78%)	0.441	0.692	0.235
+/−	1 (1.01%)	4 (4.04%)	3 (2.08%)	0.914	0.446	0.303
−/−	95 (95.96%)	95 (95.96%)	137 (95.14%)	0.999	0.396	0.090

Abbreviations: AEC, asymptomatic exposed control; DI-OA, diisocyanate-induced occupational asthma; NC, normal control; *n*, number of subjects.
Logistic regression analysis was applied to control for age and sex as covariates.
The values in bold indicate significant *P*-values. Each *P*-value <0.05 was considered to be significant.
[a]Each *P*-value was calculated using the co-dominant, dominant and recessive models.

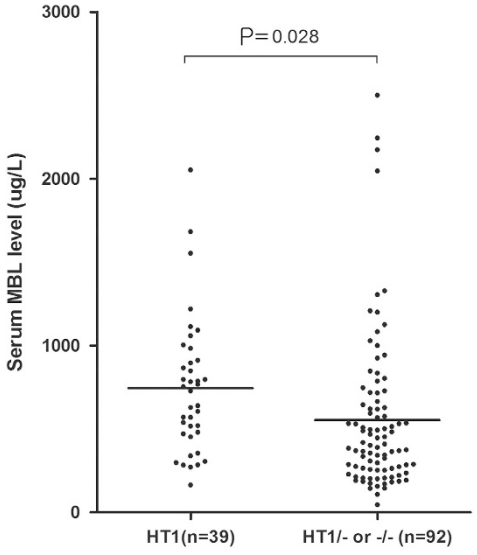

Figure 1 Serum MBL levels according to haplotype ht1 [GAG] of the *MBL2* gene in the DI-exposed group (both the DI-OA and AEC groups).

pro-inflammatory mediators, stimulating bronchospasms, enhancing bronchial hyperresponsiveness and increasing mucus production.[26,27] However, the specific mechanism has not yet been determined. ROS generated during oxidative stress can lead to compromised cellular functioning and increased inflammation by damaging nucleic acids, lipids, proteins and mitochondria.[28] ROS may act as signaling modifiers of transcription factors, such as NF-κB and AP-1, in epithelial cells, which can activate genes encoding many pro-inflammatory cytokines and adhesion molecules, thus inducing the inflammatory response.[29,30] Oxidative-stress-induced MBL-complement activation plays an important role in modulating the inflammatory response; however, the role of MBL in asthma remains obscure.[12,13] Protective as well as deleterious roles of MBL have been shown in different studies of asthmatic patients.[31–33] MBL is a key regulator of inflammation and provides a first line of defense through complement activation.[34] Accumulation of MBL in bronchoalveolar lavage fluid during pulmonary inflammation has been reported, whereas no MBL was detected in the non-disease group.[14] In addition, MBL levels in bronchoalveolar lavage fluid were significantly higher in patients with acute pulmonary inflammation compared with those with chronic inflammation, suggesting that MBL can play a defensive role against airway inflammation.[14] Low serum MBL levels due to *MBL2* genetic variants may fail to counteract the inflammation induced by oxidative stress, resulting in DI-OA. Therefore, we hypothesized that *MBL2* gene polymorphisms affect serum MBL levels and increase susceptibility to DI-OA.

Several association studies of the *MBL2* gene have been conducted in various ethnic groups and in both children and adult patients with asthma. Aittoniemi *et al.*[32] have demonstrated that the carriage of a promoter region variant, −221 G>C, which causes low MBL expression, is a significant risk factor for asthma in Finnish populations. Similarly, MBL

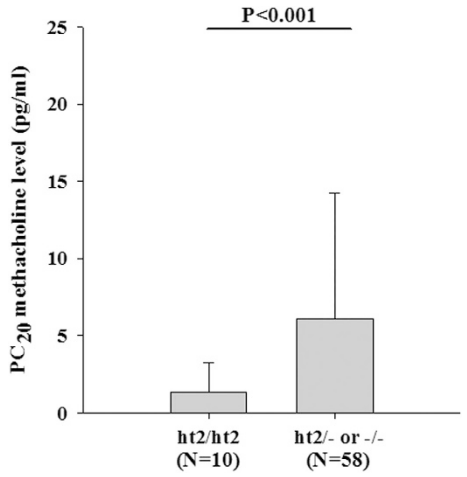

Figure 2 PC_{20} methacholine levels according to the *MBL2* haplotype in patients with DI-OA.

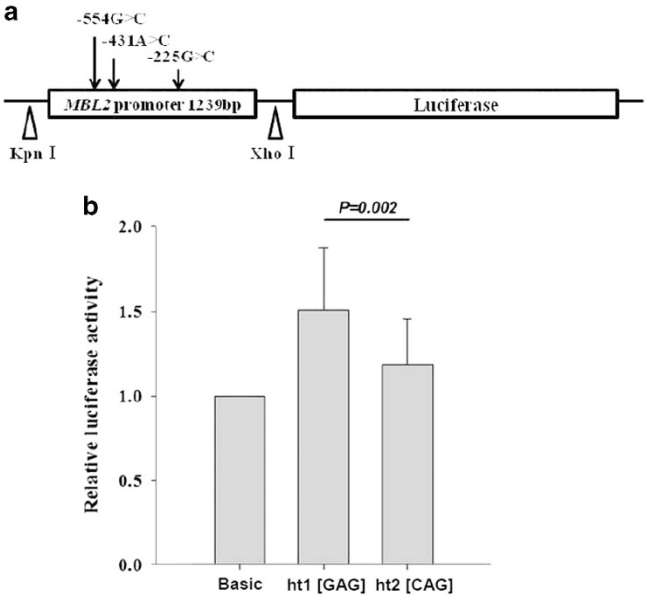

Figure 3 Effect of the *MBL2* –554 G>C polymorphism on transcriptional activity. (**a**) Schematic representation of the reporter gene construct containing the *MBL2* promoter region with –554 G>C polymorphisms. (**b**) Transfection of *MBL2* –554 G>C polymorphisms (ht1 [GAG] and ht2 [CAG]) into Hep3B cells. The data represent the mean values of three independent experiments ± s.d. (total, $n=9$).

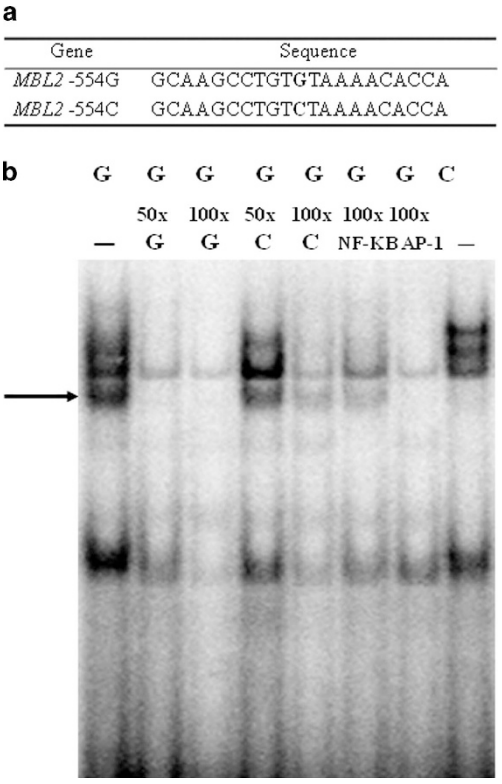

Figure 4 Electrophoretic mobility shift assay of *MBL2* –554 G>C polymorphisms using nuclear extracts obtained from human hepatocarcinoma cells (Hep3B). (**a**) Oligonucleotide sequences were used as a probe and competitor (–554G and –554C, respectively). The boldface characters indicate –554 G>C. (**b**) Differential binding of nuclear proteins to –554 G>C alleles. Transcription factor binding to the –554G probe was assessed by a competitive binding assay. Competitors were present at 50- and 100-fold molar excess compared with the probe. NF-κB: nuclear factor-κB; AP-1: activator protein-1.

insufficiency has been reported to contribute to the development and maintenance of airway hyperresponsiveness during chronic fungal asthma.[31] Oxidative stress has been shown to decrease functional airway MBL in chronic obstructive pulmonary disease by increasing susceptibility to degradation.[15] To our knowledge, this is the first study to investigate a possible association between DI-OA and promoter polymorphisms of *MBL2*. In this study, we noted a significant difference in the frequency of ht2 [CAG] between the DI-OA and AEC groups. We also found severe airway hyperresponsiveness in the DI-OA patients carrying ht2 [CAG], indicating that they were more susceptible to the development of this symptom. Airway hyperresponsiveness in patients with DI-OA is one of the most important clinical features and has been used for diagnostic assessment.[35–37] We noted a significantly higher prevalence of serum-specific IgE to DI-HSA in the DI-OA subjects compared with the AEC subjects. In our previous study, we have shown that the level of serum-specific IgE to DI-HSA is significantly higher in DI-OA patients compared with unexposed healthy controls.[38] Serum IgE levels are usually elevated in asthmatic patients, adversely affecting asthma pathogenesis.[39]

In vitro experiments have shown that DI can induce oxidative stress in the lungs and affect the function of lung tissue.[40,41] Hodge *et al.*[42] have demonstrated that MBL facilitates the reduction in lung inflammation induced by cigarette smoking in a mouse model. Our results also support these previous findings. The functional study of *MBL2* promoter polymorphisms in the Hep3B cell line showed differential transcriptional regulation by dual-luciferase activity. The decreased promoter activity observed for *MBL2* ht2 [CAG] compared with ht1 [GAG] suggests that *MBL2* expression is

downregulated in liver cells in the presence of *MBL2* ht2 [CAG]. Thus, in subjects with ht2 [CAG], the protective role of MBL against oxidative stress induced by DI is reduced, which may lead to DI-OA.

In addition, the EMSA results revealed that − 554G-specific DNA binding was more prominent compared with − 554C and that this binding may be important in the transcriptional regulation of the *MBL2* gene. This finding was further confirmed by a competition assay using a − 554G probe. The candidate transcription factor AP-1, which was suggested by the transcription factor database, also competed specifically with the − 554G band. This finding indicates that the − 554G allele may bind with the transcription factor AP-1 for transcriptional regulation of the *MBL2* promoter, whereas the − 554C allele binds weakly, producing less significant transcriptional regulation. Transcription factor AP-1 is well known as a redox-sensitive transcription factor, and it can be activated by oxidative stress in inflammatory diseases.[43–45] In our previous study, we have shown that serum MBL levels in asthmatics are significantly higher compared with healthy non-asthmatics; however, there are no significant differences in genotype and haplotype frequencies between these groups.[21] MBL levels have been reported to be elevated in individuals with inflammatory disease compared with healthy controls, suggesting that increased MBL levels in asthmatics might be an acute response to inflammation.[46] However, in the present study, there were no marked differences in serum MBL levels between the DI-OA patients and NCs, which might have been due to the high prevalence of ht2 [CAG] in the DI-OA group, causing the low expression of *MBL2*.

In the present study, endogenous expression of *MBL2* was limited only to the Hep3B cells. A previous functional study performed using various tissues has concluded that *MBL2* transcription is limited to liver tissue. In addition, extra-hepatic transcription of *MBL2* can be detected in the small intestine and cord blood at low mRNA levels, and *MBL2* mRNA is not detected in human mononuclear cells. This study has several limitations. There was a small number of study subjects in each group. Although ROS have been widely considered as important mediators of DI-OA, data are lacking for the determination of the role of ROS in DI-OA. The presence of MBL in the bronchoalveolar lavage fluid of asthmatic patients has been previously reported; however, there is a lack of data regarding DI-OA subjects.[14] Another limitation of this study is the weak association between ht2 [CAG] distribution and serum MBL levels. Although we identified a significantly higher ht2 [CAG] frequency in the patients with DI-OA, no differences in serum MBL levels were observed in the patients with ht2 [CAG] distribution. Therefore, further studies are required to elucidate the functional role of *MBL2* in the pathogenesis of DI-OA.

In conclusion, reduced serum MBL levels caused by genetic variants of *MBL2* could lead to enhanced susceptibility to DI-OA. Low serum MBL levels may fail to neutralize the detrimental effects of oxidative stress due to DI exposure, which may contribute to the development of DI-OA.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This research was supported by a grant from the Korea Health Technology R&D project through the Korea Health Industry Development Institute (KHIDI) funded by the Ministry of Health & Welfare, Republic of Korea (HI14C0065).

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