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Biochemical and Biophysical Research Communications 344 (2006) 300–307
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A functional polymorphism in *MMP-9* is associated with childhood atopic asthma

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Received 14 March 2006
Available online 27 March 2006

Abstract

Although MMP-9 has been suggested to be important in inflammation and in the connective tissue remodeling associated with asthma, the genetic influences of the polymorphisms of *MMP-9* are unclear. To examine whether polymorphisms in *MMP-9* are associated with childhood atopic asthma, we identified a total of 17 polymorphisms and conducted an association study with asthma ($n = 290$) and controls ($n = 638$). 2127G>T and 5546G>A (R668Q) were significantly associated with the risk of childhood atopic asthma ($p = 0.0032$ and 0.0016, respectively). In haplotype analysis, we also found a positive association with a haplotype ($p = 0.0053$). *MMP-9* was expressed in cultured human bronchial epithelial cells, and the mRNA expression level was upregulated by dsRNA. Furthermore, the promoter SNP −1590C>T, in strong linkage disequilibrium with 2127G>T, enhanced the transcriptional level of *MMP-9*. Thus, the *MMP-9* gene might be involved in the development of asthma through functional genetic polymorphisms.

Keywords: *MMP-9*; Bronchial asthma; Polymorphisms; Linkage disequilibrium; Haplotype; Association study; Reporter gene assay

Bronchial asthma is a complex disorder caused by a combination of genetic and environmental factors [1,2]. MMPs, a family of zinc- and calcium-dependent endopeptidases, exhibit degradation activity against many of the components of the extracellular matrix and play a crucial role in the migration of inflammatory cells through the basement membrane [3,4]. An MMP-9 is referred to as a geratinase that contains fibronectin type II-like repeats within its catalytic domain, resulting in higher binding affinity to gelatin and elastin [5], and is an important mediator of inflammation in a murine model of asthma and in immune complex-mediated lung injury [6]. Several recent studies have shown increased levels of MMP-9 in

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doi:10.1016/j.bbrc.2006.03.102

bronchoalveolar lavage (BAL) fluid, in bronchial biopsies, in alveolar macrophages from asthmatic patients, and in the plasma of acute severe asthmatic patients [7–10]. Localization of MMP-9 within the airways of asthma has been also reported using immunohistochemistry [11,12]. Several studies of MMP-9 using mouse models of asthma have been conducted [13–16]. Kumagai et al. reported an increase in the amount of MMP-9 in BAL after antigen inhalation in mice sensitized with ovalbumin (OVA), which was accompanied by the infiltration of lymphocytes and eosinophils. A synthetic MMP inhibitor and tissue inhibitor of metalloproteinase (TIMP)-1 inhibit cellular infiltration to the airway lumen [13]. The pathologic role of MMP-9 has been examined in experimentally induced asthma in mice that lack *MMP-9* [14–16]. As compared with wild-type mice, allergen challenged *MMP-9*-deficient mice showed significantly less peribronchial mononuclear cell infiltration of the airways and fewer lymphocytes in the BAL fluid [14]. Vermaelen et al. [15] reported that the specific absence of MMP-9 activity inhibits the development of allergic inflammation through impairing the recruitment of dendritic cells (DCs) into the airways and the local production of proallergic chemokines by DCs. A recent study showed the opposite effect, the enhancement of allergen-induced airway inflammation in *MMP-9*-deficient mice [16]. These findings suggest that the MMP-9 might exert an effect in the pathogenesis of bronchial asthma.

MMP-9 is located on human chromosome 20q11.1-13.1, a position associated with bronchial hyper-responsiveness and specific sensitization [17,18]. Although the biological role of MMP-9 in asthma has been intensively studied, only two genetic association studies have been conducted [19,20]. Zhang et al. [21] identified 10 variants in the *MMP-9* gene using a Caucasian population and found that a promoter polymorphism, −1562C>T, had a functional impact on transcription [22]. Holla et al. [19] examined the −1562C>T variant, but could not find an association with asthma susceptibility. A recent study investigated the four polymorphisms, −1702T>A, −1562C>T, R279Q, and 6C>T, and reported no association with bronchial asthma in children [20]. In other diseases, Krex et al. [23] surveyed the *MMP-9* gene and found 11 SNPs that were not associated with intracranial aneurysms in Caucasians. Hirakawa et al. [24] identified 11 SNPs in Caucasians and 19 SNPs in African Americans and evaluated the association between SNPs and end-stage renal disease. They could not find significant differences between patients and controls for those SNPs. To investigate whether variants of *MMP-9* were related to childhood atopic asthma in a Japanese population, we resequenced the *MMP-9* gene, carried out linkage disequilibrium (LD) mapping, and conducted an association study with regard to the LD pattern.

Bronchial epithelial cells are an important source of MMP-9 and its expression is upregulated by proinflammatory stimuli such as tumor necrosis factor (TNF)-α [25]. Airway epithelial cells play an essential role in host defense against infection [26], and respiratory infections influence both the development and severity of asthma [27,28]. Toll-like receptors (TLRs) are key molecules in innate host defense [29], and double-stranded RNA (dsRNA), the TLR3 ligand, produced by RNA viruses such as rhinovirus and RS virus during replication in infected cells is a potent stimulus for anti-viral innate immune responses [30]. Poly(I:C) is thought to mimic the effects of dsRNA [30], which induces the synthesis of cytokines and chemokines by bronchial epithelial cells [31,32]. A recent study showed that mRNA for TLR1–6 is present in human bronchial epithelial cells [33]. We further investigated whether *MMP-9* mRNA expression in cultured normal human bronchial epithelial cells was upregulated by dsRNA and other TLR ligands, and whether the susceptibility variant affected the transcriptional efficiency of *MMP-9* in bronchial epithelial cell line BEAS-2B.

Materials and methods

Study subjects. All Japanese subjects with asthma were recruited from clinics and the patients were diagnosed according to the criteria of the National Institutes of Health (National Heart, Lung, and Blood Institute, National Institutes of Health, 1991) and demonstrated at least 12% improvement in their FEV_1 measurement after β_2-agonist inhalation. The diagnosis of atopic asthma was based on one or more positive skin scratch test responses to seven common aeroallergens in the presence of a positive histamine control and a negative vehicle control. The seven aeroallergens were house dust, Felis domesticus dander (Feld), Canis familiaris dander, Dactylis glomerata, Ambrosia, Cryptomeria japonica, and Alternaria. Two hundred ninety pediatric atopic asthma patients were recruited (mean age 9.9, 4–15 years; male:female ratio = 1.53:1.0; mean serum IgE level, 1108 IU/ml; Dermatophagiodes pteronyssinus or Dermatophagiodes farinae RAST positive 89%). A total of 638 healthy individuals who had neither respiratory symptoms (mean age 43.8, 20–75 years; male:female ratio = 2.67:1.0) were recruited by physicians' interviews about whether they had been diagnosed with asthma and/or atopic diseases [34]. As there was a large age difference between the cases and controls, we performed linear regression analysis between age and allele frequencies of genotyped SNPs. *R*-squares of all SNPs were less than 0.001 ($R^2 < 0.001$), so there was no evidence of association between age and allele frequencies. All individuals were Japanese and gave written informed consent to participate in the study (or, for individuals less than 16 years old, their parents gave consent), according to the rules of the Process Committee at the SNP Research Center, The Institute of Physical and Chemical Research (RIKEN).

Screening for polymorphisms and genotyping. To identify polymorphisms in the human *MMP-9* gene, we sequenced all thirteen exons, including a minimum of 100 bases of the flanking intronic sequence, 2 kb of the 5′ flanking region, and a 0.2 kb continuous 3′ flanking region of the 13th exon from 24 control subjects. Seventeen primer sets were designed on the basis of the *MMP-9* genomic sequence from the GenBank database (Accession No. AL162458). The sequences were analyzed and polymorphisms were identified using the SEQUENCHER program (Gene Codes Corporation, Ann Arbor, MI). The polymorphism 1216G>A was genotyped by use of the TaqMan system (Applied Biosystems, Foster City, CA). For the 2127G>T, 4841C>G, 5546G>A, and 7588T>C polymorphisms, genotyping was performed by Invader assay as described [35].

Cell culture and reagents. The simian virus 40 (SV-40)-transformed human bronchial epithelial cell line, BEAS-2B, was obtained from the American Type Culture Collection (Rockville, MD) and normal human bronchial epithelial cells (NHBE) were obtained from Clontics (Walkersville, MD). Those cells were cultured in bronchial epithelial basal medium (BEBM™) (Cambrex, East Rutherford, NJ) supplemented with 50 μg/ml bovine pituitary extract, 0.5 μg/ml hydrocortisone, 0.5 ng/ml human epidermal growth factor, 0.5 μg/ml epinephrine, 10 μg/ml transferrin, 5 μg/ml

insulin, 0.1 ng/ml retinoic acid, 6.5 ng/ml triiodothyronine, 50 μg/ml gentamicin, and 50 ng/ml amphotericin-B (all materials from Camblex). Macrophage-activating lipopeptide-2 (MALP-2) (*S*-[2,3-bisAcyloxypropyl]-cysteine-GNNDESNISFKEK) was purchased from Alexis biochemicals (Lausen, Switzerland). Palmitoyl-3-cysteine-serine-lysine-4 (Pam3CSK4) and polyinosinic acid:polycytidylic acid [poly(I:C)] were purchased from InvivoGen (San Diego, CA). Lipopolysaccharides escherichia coli 055:B5 (LPS) was purchased from Sigma–Aldrich (St. Louis, MO).

Quantification of MMP-9 gene expression using real-time RT-PCR. We used MALP-2, Pam3CSK4, poly(I:C), and LPS. NHBE cells were exposed to PamCSK4 (20 and 200 ng/ml), MALP-2 (0.01 and 1 μg/ml), poly(I:C) (0.1 and 10 μg/ml), and LPS (1 and 100 ng/ml). We treated cultured NHBE cells with the indicated ligands for 4, 12, and 24 h. Total RNA was isolated using Nucleospin RNA II (MACHEREY-NAGEL GmbH KG, Düren, Germany). We prepared cDNA from 5 μg of total RNA and synthesized it using SuperScriptIII reverse transcriptase (Invitrogen, Carlsbad, CA). We quantified mRNA using SYBR® Premix Ex Taq™ (TAKARA Bio, Japan) and an ABI Prism 7900 sequence detector (Applied Biosystems) in accordance with the manufacturers' instructions. The primers for *MMP-9* were: 5′-TTCTGCCCGGACCAAGGATA-3′ and 5′-CATTCACGTCGTCCTTATGCA-3′. To determine exact copy numbers of the target genes, quantified concentrations of subcloned PCR fragments of *MMP-9* were serially diluted and used as standards in each experiment. Data were normalized with the glyceraldehyde-3-phosphate dehydrogenase level in each sample.

Luciferase assay. Three concatenated copies of the 29 bps DNA fragments were cloned into pGL3-basic vector (Promega, Madison, WI) in the 5′–3′ orientation. The DNA fragments were: for −1590C, 5′-GGCGTGGTGGCGCACGCCTATAATACCAG-3′ and −1590T, 5′-GGCGTGGTGGCGCATGCCTATAATACCAG-3′. We then transfected subconfluent BEAS-2B cells (5×10^4) cultured in 24-well plates with 0.125 μg of each construct and 0.0025 μg of pRL-TK *Renilla* luciferase vector (Promega, Madison, WI), an internal control for transfection efficiency, using 0.75 ml of FuGENE 6 transfection reagent (Roche Diagnostics, Basel, Switzerland). After 24 h, we lysed the cells and measured firefly and *Renilla* luciferase activities in a luminometer using the Dual-Luciferase Reporter Assay System (Promega). The relative luciferase activity of the *MMP-9* reporter construct is represented as the ratio of the firefly luciferase activity to that of *Renilla*. Each experiment was repeated three times, and each sample was studied in triplicate as described [34].

Statistical analysis. We calculated allele frequencies and tested agreement with Hardy–Weinberg equilibrium using a χ^2 goodness of fit test at each locus. We then compared differences in allele frequencies and genotype distribution of each polymorphism between case and control subjects by using a 2×2 contingency χ^2 test with one degree of freedom (dominant model, recessive model, and allele frequency), and calculated odds ratios (ORs) with 95% confidence intervals (95% CI). We applied Bonferroni correction and multiplied the p values by 15 (the number of variants × the number of tests). Corrected p values of less than 0.05 were judged to be significant. Pairwise LD was calculated as $|D'|$ and r^2 by using the SNPAlyze statistical package (Dynacom, Chiba, Japan) as described [34,36]. Haplotype frequencies for multiple loci were estimated using the expectation–maximization method with SNPAlyze software [34,36]. Haplotype frequencies in cases and controls were evaluated both by the whole distribution with the χ^2 test and by permutation test and χ^2 tests with one haplotype against others (haplotype-wise test). Comparison in reporter assays was performed with Student's t test. A p value of less than 0.05 was considered statistically significant.

Results

Association of MMP-9 polymorphisms with asthma susceptibility

We identified 17 single nucleotide polymorphisms in *MMP-9*, four in the 5′ flanking region, one in the 3′ untranslated region, and six in the intron (Table 1 and Fig. 1). Thirteen polymorphisms were contained in the

Table 1
Locations and allele frequencies of polymorphisms

SNP	Location	Amino acid	Allele frequency[a] (%)						
			Japanese	Caucasian[b]	Caucasian[c]	Caucasian[d]	African American[d]	JSNP (IMS-JST)[e]	NCBI[f]
−1831 T/A	5′-Flanking region		17						rs3918241
−1590 C/T	5′-Flanking region		17	19		14	10		rs3918242
−347 G/A	5′-Flanking region		6						—
−319 C/A	5′-Flanking region		6						—
1216 G/A	Intron 2		44						rs3918251
1404 C/A	Intron 2		41						rs13040572
1408 C/A	Intron 2		41						—
2127 G/T	Intron 4		17		23			070716	rs2274755
2660 G/A	Exon 6	R279Q	40	35	41	65	67		rs2664538
2664 C/T	Exon 6	D280D	4						—
2826 C/T	Intron 6		15						rs3918254
3010 A/G	Intron 6		17					016721	rs2236416
4841 C/G	Exon 10	P574R	40		3	6	16	070717	rs2250889
5268 A/C	Exon 11	G607G	15		47	58	40	070718	rs13969
5546 G/A	Exon 12	R668Q	21		30	15	18	070719	rs2274756
7400 G/A	Exon 13	V694V	22	21	14	17	14	070720	rs13925
7588 T/C	Exon 13	3'UTR	13		1			070721	rs9509

Positions are numbered according to their position relative to the published MMP-9 gene-containing clone (GenBank AL162458). Position 1 is the A of the initiation codon.

[a] Frequency of right indicated allele.
[b] Allele frequencies are from Ref. [21].
[c] Allele frequencies are from Ref. [23].
[d] Allele frequencies are from Ref. [24].
[e] JSNP, number from the Japanese SNP database (http://snp.ims.u-tokyo.ac.jp/).
[f] NCBI, number from the dbSNP of NCBI (http://www.ncbi.nlm.nih.gov/SNP/).

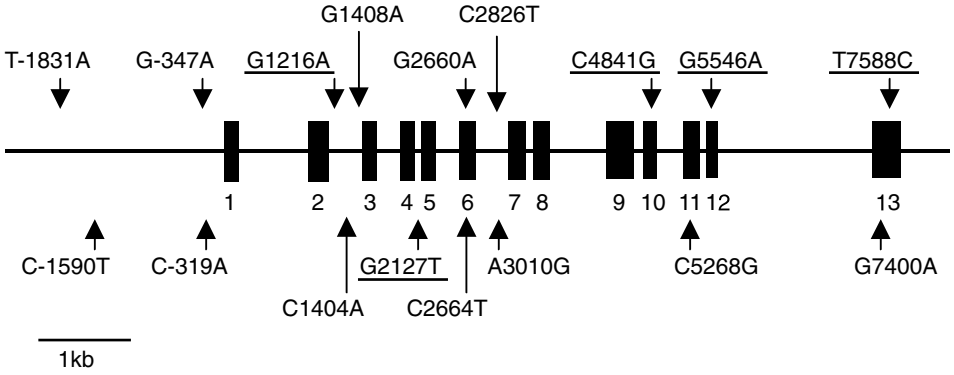

Fig. 1. A graphical overview of polymorphisms identified in relation to the exon/intron structure of the human *MMP-9* gene. Thirteen exons are shown in this figure by black boxes with their numbers, and positions for polymorphisms are relative to the translation start site (+1). Underlined polymorphisms were genotyped in the whole samples.

public databases available at websites; NCBI dbSNP (http://www.ncbi.nlm.nih.gov/SNP/) and IMS-JST JSNP DATABASE (http://snp.ims.u-tokyo.ac.jp/). Three non-synonymous substitutions, 2660G>A (R279Q), 4841C>G (P574R), and 5546G>A (R668Q), were found in *MMP-9*. We also found −1590C>T, 2127G>T, R279Q, P574R, G607G, R668Q, V694V, and 7588T>C, which were reported in other populations (Table 1) [21,23,24]. −1562C>T in promoters reported in recent studies [19–22,24] was designated −1590C>T in this study. Pairwise LD among fourteen SNPs with a frequency >0.10 was measured by different parameters, $|D'|$ and r^2 (Table 2). 2127G>T was in complete LD ($D' = 1.00$ and $r^2 = 1.00$) with −1831T>A, −1590C>T, and 3010A>G. 4841C>G was in complete LD with 1404C>A, 1408C>A, and 2660G>A. 5546G>A was in complete LD with 7400G>A. 7588T>C was in complete LD with 2826C>T and 5268A>C. We finally selected five polymorphisms, 1216G>A, 2127G>T, 4841C>G, 5546G>A, and 7588T>C for association studies.

All of these loci were in Hardy–Weinberg equilibrium in the control group. To test the association between each gene and childhood atopic asthma, we compared differences in allele frequency and genotype distribution of each polymorphism between case and control subjects by using contingency χ^2 tests with one degree of freedom (dominant model, recessive model, and allele frequency). Odds ratios (ORs) with 95% confidence intervals (95% CI) were also calculated. After Bonferroni correction, we found a significant association between the intronic polymorphism at 2127G>T and childhood atopic asthma ($p = 0.0032$, corrected $p = 0.048$). The non-synonymous polymorphism 5546G>A was also significantly associated with child atopic asthmatics ($p = 0.0016$, corrected $p = 0.024$) (Table 3).

Haplotype frequencies in MMP-9 gene

We further analyzed the haplotype structure using two SNPs, 2127G>T and 5546G>A, and identified three common haplotypes covering more than 99.9% of the

Table 2
Pairwise linkage disequilibrium for all possible two-way comparisons among 13 polymorphisms in *MMP-9* with 24 Japanese subjects

	SNP:	1	2	3[a]	4	5	6[a]	7	8	9	10[a]	11	12[a]	13	14[a]
	D':	−1831 T/A	−1590 C/T	1216 G/A	1404 C/A	1408 C/A	2127 G/T	2660 G/A	2826 C/T	3010 A/G	4841 C/G	5268 A/C	5546 G/A	7400 G/A	7588 T/C
	SNP:	5′	5′	Intron 2	Intron 2	Intron 2	Intron 4	Exon 6	Intron 6	Intron 6	Exon 10	Exon 11	Exon 12	Exon 13	Exon 13
r^2	SNP 1		1.00	0.43	1.00	1.00	1.00	1.00	0.50	1.00	1.00	0.20	1.00	1.00	0.14
	SNP 2	1.00		0.43	1.00	1.00	1.00	1.00	0.50	1.00	1.00	0.20	1.00	1.00	0.14
	SNP 3	0.03	0.03		1.00	1.00	0.43	1.00	1.00	0.43	1.00	1.00	0.23	0.27	1.00
	SNP 4	0.13	0.13	0.84		1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.58	0.61	1.00
	SNP 5	0.13	0.13	0.84	1.00		1.00	1.00	1.00	1.00	1.00	1.00	0.58	0.61	1.00
	SNP 6	1.00	1.00	0.03	0.13	0.13		1.00	0.50	1.00	1.00	0.20	1.00	1.00	0.14
	SNP 7	0.13	0.13	0.84	1.00	1.00	0.13		1.00	1.00	1.00	1.00	0.62	0.65	1.00
	SNP 8	0.01	0.01	0.13	0.13	0.13	0.01	0.11		0.50	1.00	1.00	0.03	0.15	1.00
	SNP 9	1.00	1.00	0.03	0.13	0.13	1.00	0.13	0.01		1.00	0.20	1.00	1.00	0.14
	SNP 10	0.13	0.13	0.84	1.00	1.00	0.13	1.00	0.11	0.13		1.00	0.62	0.65	1.00
	SNP 11	0.00	0.00	0.15	0.14	0.14	0.00	0.13	1.00	0.00	0.13		0.08	0.19	1.00
	SNP 12	0.76	0.76	0.01	0.06	0.06	0.76	0.07	0.00	0.76	0.07	0.01		1.00	0.15
	SNP 13	0.76	0.76	0.02	0.07	0.07	0.76	0.08	0.01	0.76	0.08	0.02	1.00		0.15
	SNP 14	0.00	0.00	0.13	0.12	0.12	0.00	0.11	1.00	0.00	0.11	1.00	0.01	0.01	

[a] Polymorphisms were genotyped in this case–control study.

Table 3
Association between polymorphisms of *MMP-9* and childhood atopic asthma

Locus	Allele 1/2	Genotype	Atopic asthma (%)	Control (%)	*p* value[a]	*p* value[b]	*p* value[c]	OR (95% CI)[c]
SNP 3	G/A	11	99 (36)	228 (37)	NS	NS	NS	
1216		12	145 (53)	291 (47)				
		22	30 (11)	99 (16)				
SNP 6	G/T	11	177 (63)	452 (71)	NS	NS	0.0032	1.46 (1.13–1.88)
2127		12	88 (31)	170 (27)			0.048[d]	
		22	16 (6)	15 (2)				
SNP 10	C/G	11	138 (50)	289 (46)	NS	NS	NS	
4841		12	127 (46)	288 (46)				
		22	14 (5)	55 (9)				
SNP 12	G/A	11	164 (58)	429 (68)	NS	NS	0.0016	1.48 (1.16–1.88)
5546		12	102 (36)	189 (30)			0.024[d]	
		22	16 (6)	16 (3)				
SNP 14	T/C	11	153 (57)	386 (61)	NS	NS	NS	
7588		12	105 (39)	214 (34)				
		22	13 (5)	31 (5)				

NS, not significant.
[a] Dominant model.
[b] Recessive model.
[c] Allele 1 vs. allele 2.
[d] *p* value corrected with Bonferroni correction (raw *p* values were multiplied by 15).

Table 4
Frequencies of haplotypes and odds ratios in the control group and childhood atopic asthma group

	Haplotype		Atopic asthma		Control		χ^2 (df = 1)	*p* value	Permutation *p* value
	SNP 6 2127 G/T	SNP 12 5546 G/A	Number of allele	Ratio	Number of allele	Ratio			
1	G	G	439	0.77	1045	0.83	7.73	0.0054	0.0060
2	T	A	120	0.21	199	0.16	7.78	0.0053	0.0070
3	G	A	11	0.02	22	0.02	0.080	0.78	0.74
Total allele			570		1266				

population in both case and control groups (Table 4). The frequency pattern of the haplotype differed between the control and childhood atopic asthma groups ($\chi^2 = 8.00$, $p = 0.018$, df = 2). In haplotype-wise tests, we obtained *p* values both by χ^2 tests and by permutation tests (1000 permutations). The *p* values thus obtained were similar (Table 4). The frequency of the G-G haplotype (haplotype 1) in the control group was 83% and that in the affected individuals was 77%. The difference was statistically significant ($\chi^2 = 7.72$, $p = 0.0054$, permutation $p = 0.0060$ [haplotype 1 vs. others]). The frequency of the T-A haplotype (haplotype 2) in the controls was 16%, whereas that in the affected individuals was 21%. The difference was statistically significant ($\chi^2 = 7.78$, $p = 0.0053$, permutation $p = 0.0070$ [haplotype 2 vs. others]).

Expression and reporter gene analysis in MMP-9 gene

We examined whether dsRNA and other TLR agonists contributed to the regulation of *MMP-9* expression in normal human bronchial epithelial (NHBE) cells. The results showed that only poly(I:C) treatment significantly enhanced *MMP-9* mRNA expression, whereas the others had no significant effect (Fig. 2).

We next attempted to identify the functional SNPs affecting the *MMP-9* expression level in human bronchial cell line BEAS-2B. Variants −1831T>A and −1590C>T in the promoter were in complete LD with 2127G>T. To investigate the functional effects of the associated variants, we examined transient expression of the −1831T>A and −1590C>T luciferase reporter constructs. Polymorphism −1590C>T, in the promoter of *MMP-9*, affected relative luciferase activity ($p < 0.001$ for comparison between allele −1590C and −1590T by Student's *t* test, 0.25 [s.d. 0.01] vs. 0.87 [s.d. 0.07], respectively) (Fig. 3). The −1590T construct had 3.48-fold higher luciferase reporter activity than the −1590C construct. These results suggested that the −1590T allele might affect the increased transcriptional activity of the *MMP-9* gene in vivo. Variant −1831T>A had little effect on transcriptional activity (data not shown).

Discussion

To determine the role of the *MMP-9* gene in the pathogenesis of childhood asthma, we investigated the sequence

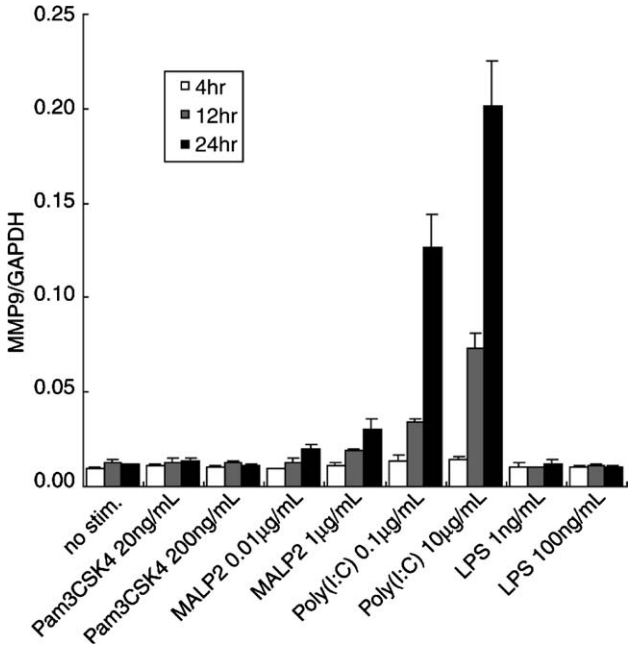

Fig. 2. Induction of *MMP-9* gene in response to TLR ligands in normal human bronchial epithelial cells. The data normalized to *GAPDH* are presented as means ± SD in duplicate assays. Results are from one experiment, representative of three separate experiments.

variations of *MMP-9* and their effects on the expression of the gene. We identified 17 polymorphisms (four variants are novel) and conducted LD mapping of the gene region. From LD mapping, we selected five essential polymorphic sites and performed further analyses with them. Several genetic variations at the *MMP-9* locus in a Caucasian and an African American population were reported for the *MMP-9* gene [21,23,24]. Although we found 17 SNPs in this study, including six variants in the coding region, we could not find G15G, A20V, E82K, E572K, and C674C, which were identified in the Caucasian and African American populations [21,23,24] in the coding region. While the distribution of genetic variants of *MMP-9* in Japanese was different from that in the Caucasian and African American populations, the common LD structure of the *MMP-9* locus might be helpful for further genetic analyses of other diseases in whose pathogenesis MMP-9 might be involved.

Several genetic studies have surveyed the *MMP-9* gene as a potential candidate gene for asthma or pulmonary emphysema. In a Czechoslovakian population, an association study was conducted using −1590C>T polymorphism with 138 Caucasian subjects [19]. In a German population, an association study of four polymorphisms, including −1590C>T, was conducted with 231 asthmatic children [20]. Both studies failed to find any association of the surveyed polymorphisms for asthma susceptibility. Failure to replicate genetic associations in complex disease occurs commonly [37,38]. Sample size ($n = 138$ in a Czechoslovakian population) might explain the conflicting results. Another possible explanation for the discordance with our study is unrecognized differences in environmental exposures such as the proportion of microbes [39]. In addition, epistatic interactions, influence of the genotype at one locus on the effect of a mutation at another locus, may reflect the contradictory interethnic results.

A recent report showed an association between the promoter polymorphism −1590C>T and upper lung dominant emphysema in patients with COPD in a Japanese population [40]. The −1590C>T variant is in an important regulatory element that results in higher activity of the T-allelic promoter [22]. In this study, we found a significant association between 2127G>T, in complete LD with −1590C>T, and childhood atopic asthma, and confirmed the higher promoter activity of the −1590T allele in cultured human bronchial epithelial cells. Recent reports have also shown that the level of MMP-9 in BALF correlates with numbers of neutrophils, macrophages, and bronchial epithelial cells in acute asthma and exfoliation of bronchial epithelial cells [7–10]. The rhinovirus has emerged as the most important cause of these acute episodes [41], and dsRNA produced by RNA viruses is a potent stimulus for anti-viral innate immune responses, resulting in production of inflammatory cytokines and chemokines [30–32]. Here we showed that a related variant influenced the transcriptional activity and poly(I:C) upregulated the *MMP-9* mRNA expression in

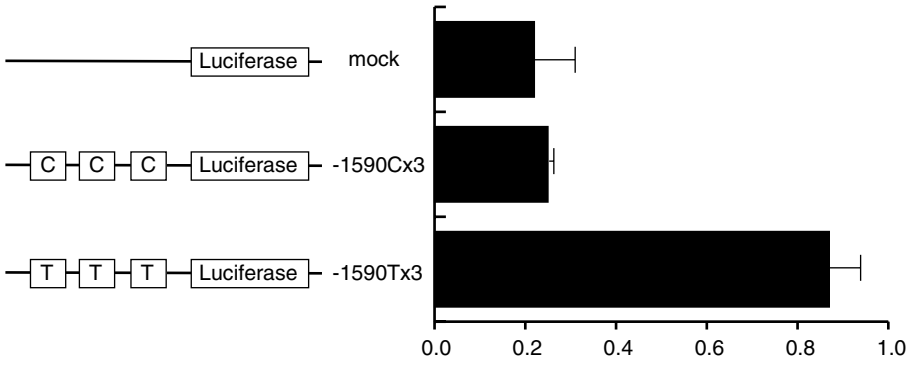

Fig. 3. Effect of −1590C>T polymorphism on the transcriptional activity of the human *MMP-9* promoter in a bronchial epithelial cell line. Each experiment was conducted in triplicate for each sample, and the results are expressed as means ± SD for three independent experiments. $p < 0.001$ by Student's t test.

normal bronchial epithelial cells, whereas the other treatments had no significant effect. Respiratory viral infections can trigger exacerbations of asthma and COPD. The susceptible allele, which contributes to enhance *MMP-9* expression, might cause an excess amount of MMP-9 during respiratory viral infections and play an important role in asthma or pulmonary emphysema.

The proteolytic activities of MMPs are precisely controlled during activation from their precursors and inhibition by endogenous inhibitor, tissue inhibitor of metalloproteinase (TIMP), and an airway imbalance between levels of MMP-9 and TIMP-1 is involved in matrix remodeling [3,6]. It was reported that *TIMP-1* polymorphism, 539C>T (Ile158Ile), was significantly associated with asthma [42]. To investigate whether polymorphisms in *TIMP-1* are associated with childhood atopic asthma would be helpful to clarify the role of the protease/antiprotease balance in asthma.

We showed here a significant association between asthma susceptibility and two polymorphisms, including nonsynonymous polymorphism, 5546G>A (R668Q). In this study, we did not examine the functional effects of 3010A>G and 7400G>A polymorphisms, which are in linkage disequilibrium with the two related variants, 2127G>T and 5546G>A, respectively. One study showed that only 22% of the transcription factor binding site (TFBS) regions are located in 5′ termini of protein-coding genes while 36% lie within or immediately 3′ to well-characterized genes [43]. Furthermore, polymorphisms in mRNA were shown to contribute to transcript stability in a previous study [34]. 3010A>G, 5546G>A, and 7400G>A might affect the expression level or mRNA stability of the *MMP-9* gene. The functions of these linked polymorphisms, however, remain to be elucidated.

Our data strongly support the important role of *MMP-9* in asthma. Further investigation of the connection between genotypes and the functional role of MMP-9 in the airway epithelium during allergic events may provide additional targets for therapeutic interventions and would be helpful to clarify the etiology of asthma.

Acknowledgments

We thank all the participants in the study. We are grateful to members of The Rotary Club of Osaka-Midosuji District 2660 Rotary International in Japan for supporting our study. We also thank Hiroshi Sekiguchi, Miki Kokubo, and Aya Jodo for technical assistance and Chinatsu Fukushima for providing patients' data.

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