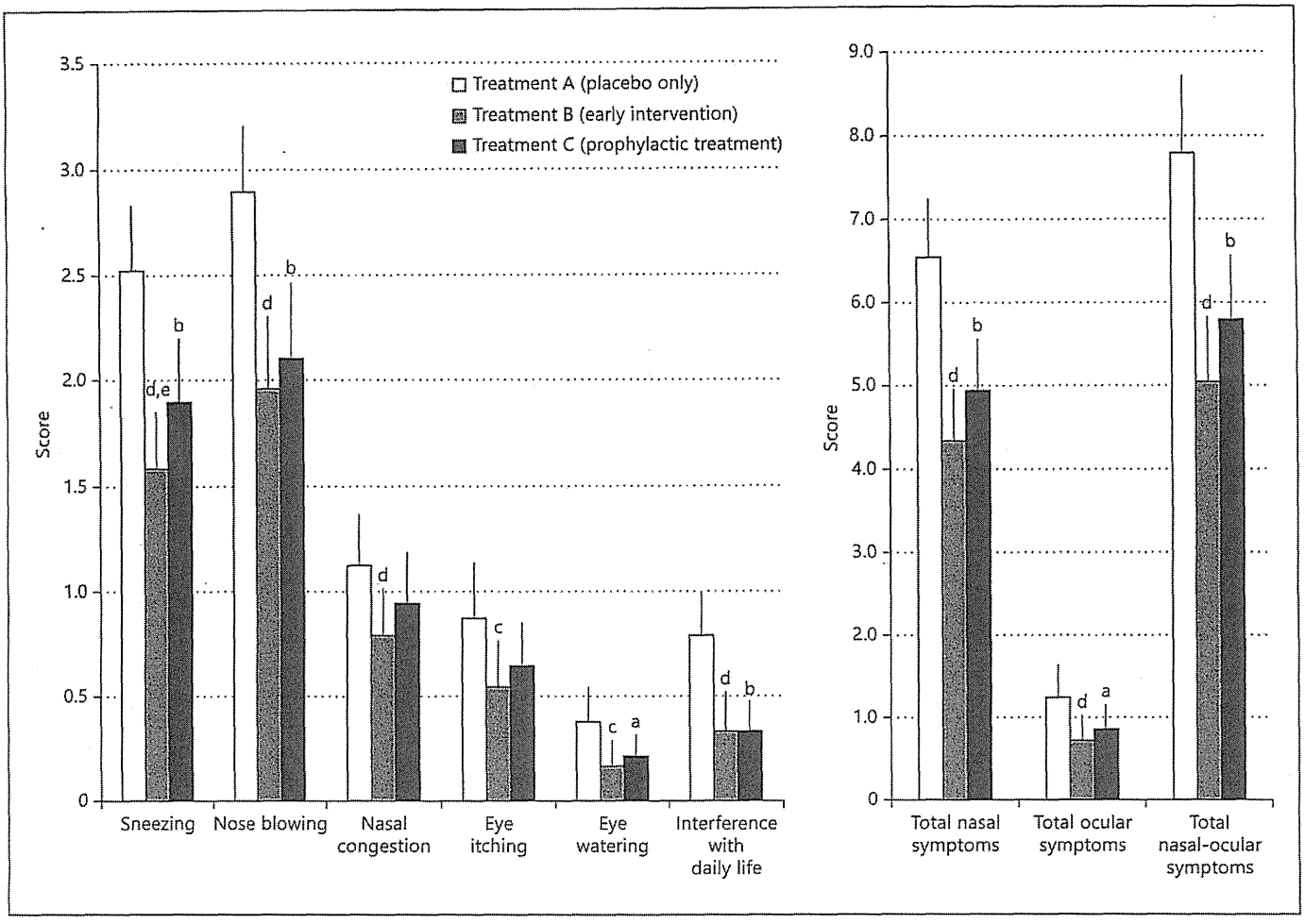

Fig. 3. Symptom scores for 12 h on day 9. Almost all symptoms except nasal congestion and eye itching had significantly lower scores with treatments B and C compared with treatment A. There was no significant difference between treatments B and C for almost all symptoms except sneezing. Values represent means ± 95% confidence intervals. [a] $p < 0.05$, [b] $p < 0.01$: treatment C vs. treatment A; [c] $p < 0.05$, [d] $p < 0.01$: treatment B vs. treatment A; [e] $p < 0.05$: treatment B vs. treatment C.

observed in <5% of patients in each treatment group. There was no significant difference in the rates of all adverse events among the 3 treatment arms.

Discussion

In this study, 1-hour priming exposure on day 8 increased the sensitivity of the nasal mucosa and subsequent 3-hour exposure induced more severe allergic symptoms on day 9. As shown in figure 1a, for treatment A (placebo only), the score for total nasal symptoms at 60 min on day 9 was significantly higher than at 60 min on day 8 ($p < 0.01$). This suggests that the sensitivity of the nasal mucosa was increased by 1-hour priming exposure on day 8. Late-phase symptoms were observed in all patients, and the numbers of sneezes and nose blows after leaving the chamber were almost the same as those observed in the chamber, as previously reported [14]. Thus, it is very important to evaluate both the early-phase and late-phase symptoms. The effect of levocetirizine lasts for as long as 24 h, making it appropriate to determine total nasal symptoms for 12 h as an endpoint.

Treatment B in this study was regarded as a model of early intervention soon after the start of symptoms, whereas treatment C was equivalent to prophylactic treatment beginning 1 week before the start of pollen dispersal. The purpose of 1-hour exposure on day 8 was to induce mild symptoms for priming. Early intervention with levocetirizine after pollen exposure on day 8 effectively

attenuated nasal and ocular symptoms induced by pollen exposure on day 9. Levocetirizine had significant effects on nasal congestion, as well as on sneezing and rhinorrhea, and the effects on all symptoms persisted after pollen exposure.

Repeated nasal exposure to antigen increases the hypersensitivity of the nasal mucosa and worsens nasal symptoms. The detailed mechanism is uncertain, but allergic inflammation in the nasal mucosa may contribute to the induction of hypersensitivity. Inhibition of the development of nasal hypersensitivity during pollen dispersal is important for improvement of nasal symptoms and quality of life of patients with SAR. Nasal allergic inflammation and increased sensitivity are thought to contribute to induction of symptoms in the late phase following an acute-phase reaction to specific pollen allergens [16, 17]. Pollen exposure results in enhanced expression of adhesion molecules [18], increased infiltration of the nasal mucosa by inflammatory cells [19–21], hyperpermeability of epithelial cells [22, 23] and an increased neural sensory response [24, 25]. Levocetirizine has anti-inflammatory effects, including inhibition of eosinophil migration [26] and the expression of intercellular adhesion molecules [27]. Levocetirizine improved all symptoms experienced by patients in the ECC during the full period of pollen exposure (3 h) and also in the late phase after leaving the chamber on day 9, compared with placebo.

Antihistamines are rapidly acting agents for relief of nasal symptoms [28, 29]. The present study showed that levocetirizine is very effective for nasal discharge and sneezing, as well as nasal obstruction, even with 1 day of treatment soon after the start of symptoms. Superior efficacy was not observed with prophylactic treatment compared with early intervention, and at some time points the prophylactic treatment was less effective than the early treatment. Thus, there was less improvement of nasal congestion and a shortened persistence of drug efficacy with prophylactic treatment. Contrary to the expected result for a histamine H1 receptor antagonist as an inverse agonist, levocetirizine was not effective as prophylactic treatment. Thus, treatment with levocetirizine for 8 consecutive days before pollen exposure was not superior to treatment for 1 day soon after pollen exposure.

Seasonal dispersion of cedar pollen around Tokyo normally starts in February. A pollen surveillance system has recently been developed, and an annual forecast of the start date and the probable pollen count is made available to the public in January. However, annual variations are largely due to climate change, and a precise forecast is still difficult. The actual start date of pollen dispersal usually varies from 1 to 2 weeks before or after the forecast. Therefore, to receive prophylactic treatment, patients may have to start taking a drug much earlier than the actual start of pollen dispersal. Thus, our finding that early intervention with an antihistamine soon after onset of symptoms is effective for attenuating these symptoms and has effects equal to or greater than those of prophylactic treatment is of interest.

A limitation of this study was the use of levocetirizine only, and further studies are needed to examine whether other antihistamines have similar effects. Similarly to other second-generation antihistamines, levocetirizine has few side effects and is safe. In this study, the frequency of side effects did not differ among the 3 treatment arms, although there was a trend for a higher rate with consecutive administration of levocetirizine. Overall, our result suggests that taking levocetirizine soon after the start of symptoms is equally or more effective for relief of symptoms compared with prophylactic treatment beginning before pollen dispersal. These results are beneficial from the perspectives of reduction of medical costs and potential reduction of side effects.

Acknowledgements

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This trial has the following clinical registration number: UMIN000006318 (http://www.umin.ac.jp/ctr/index-j.htm).

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ORIGINAL ARTICLE

Determining Minimal Clinically Important Differences in Japanese Cedar/Cypress Pollinosis Patients

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ABSTRACT

Background: Statistically significant results of medical intervention trials are not always clinically meaningful. We sought to estimate the minimal clinically important difference (MCID) (the smallest change in a given endpoint that is meaningful to a patient) during seasonal alteration of Japanese cedar/cypress pollinosis (JCCP).

Methods: Results of a double-blinded, placebo-controlled trial of JCCP patients conducted between 2008 and 2010 were analyzed using an anchor-based method in which a face scale for Japanese rhinoconjunctivitis quality-of-life questionnaire (JRQLQ) was set as an anchor. MICDs were calculated as changes of average scores, including those for naso-ocular symptoms with 5 items in diary cards (T5SS), naso-ocular symptoms with 6 items (T6SS) and QOL with 17 items on the JRQLQ when face scale scores either improved or deteriorated by one point.

Results: In 2009 and 2010, 3,698 and 374, respectively, grains/cm^2 of pollens were dispersed. The MCIDs for T5SS in 2009 and 2010 were 1.426 (0.285 per item) and 1.441 (0.288), respectively. The MCIDs for T6SS were 4.115 (0.686) and 3.183 (0.531) in 2009 and 2010, respectively. The MCIDs for QOL were 10.469 (0.616) and 6.026 (0.354) in 2009 and 2010, respectively.

Conclusions: For T5SS in the diary, T6SS and QOL in JRQLQ, unit differences of 1.5 (0.3 per item), 3.6 (0.6) and 8.2 (0.5), respectively, were considered clinically meaningful by JCCP patients. The MCID for symptoms recorded in the diary was stable irrespective of the dispersed pollen level.

KEY WORDS

face scale, minimal clinically important difference, pollinosis, quality of life, symptom score

INTRODUCTION

In order to evaluate the efficacy of interventions for allergic rhinitis (AR), setting specific endpoints is required. The total nasal symptom score, which is the sum of 4- or 5-point scaled scores for sneezing, rhinorrhea and nasal congestion as recorded in an allergy dairy, is generally used as a primary endpoint in Japan. Secondary endpoints are often defined, including quality of life (QOL), as determined by the Japanese Rhinoconjunctivitis Quality of Life Questionnaire (JRQLQ), the ocular symptom score and the naso-ocular symptoms score (especially for seasonal AR), work productivity, sleepiness, impaired performance and safety.[1-5]

The efficacy of various medical interventions is usually estimated by statistical significance. However, statistically significant differences do not always reflect clinically meaningful differences. For example, a clinical trial of a therapy involving a large patient

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population may result in a statistically significant finding that nevertheless has no clinical relevance.[6] Thus, clinically meaningful differences should be determined.[7] In fact, the minimal clinically important difference (MCID) of endpoints for various therapies for AR has been examined in a few studies.[8,9] For example, Juniper *et al.* interpreted the data obtained using Rhinoconjunctivitis Quality of Life Questionnaire (RQLQ), and set a value of 0.5 change of score from baseline as the MCID.[8] Barnes *et al.* determined, by using the global rating of change scale, that an MCID is 0.4 and 0.55 unit change of the Mini RQLQ and total nasal symptom scores, respectively.[9] However, to our knowledge, determining an MCID for AR has not been done in Japan.

In the self-reported Japanese Rhinoconjunctivitis Quality of Life Questionnaire (JRQLQ), a patient's general state is monitored by a 5-point face scale, depicting facial emotions ranging from "fine" to "crying".[10,11] In the present study, we utilized this face scale with an anchor-based method, and determined the units of total symptom and QOL score changes resulting in 1 face scale unit change, as the MCID.[12] We believe that the present findings may provide a basis for understanding the clinical meaning of results of medical interventions for Japanese cedar/cypress pollinosis (JCCP), or facilitate AR research in Japan.

METHODS

SAMPLE

We calculated MCIDs using an "anchor-based" method.[12] We used data from a randomized, double-blinded, placebo-controlled trial for the efficacy of sublingual immunotherapy for Japanese cedar/cypress pollinosis (JCCP) conducted between 2008 and 2010 in our hospital. This trial was approved by the institutional review board of Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences (Rinri-1204). In this trial, 55 patients with JCCP (17 males and 38 females, age range 23-79 [mean 53.1 ± 11.9] years) were enrolled in 2008, and then received sublingual immunotherapy with active or placebo extract of Japanese cedar pollen (Torii Pharmaceutical, Tokyo, Japan). Naso-ocular symptoms and QOL were monitored in the dispersal season of Japanese cedar and cypress pollen in 2009. Subsequently, 36 of the enrolled patients (10 males and 26 females, age range 31-75 [mean 55.4 ± 9.6] years) continued to receive the same treatment in the 2009-2010 season, and then the identical assessment was performed in the 2010 pollen dispersal season. Prior to participation in the study, all patients provided written informed consent.

NASO-OCULAR SYMPTOMS AND QOL

During the pollen dispersal season, subjects completed the JRQLQ twice a month for a total of 6 times (February 16, March 1 and 16, April 1 and 16, and May 1). The JRQLQ contains 3 sections, as follows: naso-ocular symptoms with 6 items (sneezing, rhinorrhea, nasal congestion, itchy nose, itchy eyes and watery eyes), rhinitis-related QOL with 17 items; and a global status determined by a 5-point face scale depicting emotions ranging from "fine" to "crying".[10] In addition, subjects' daily naso-ocular symptoms were recorded by filling in diary cards. On these cards, the presence and intensity of three nasal symptoms (sneezing, rhinorrhea, and nasal congestion) and two ocular symptoms (watery and itchy eyes) were recorded in a 5-point scale using Okuda's modified classification.[11]

CALCULATION OF MCID

The MCID was determined based on the changes of face scale scores before and after the 6 time points when the JRQLQ was completed. Thus, 5 time periods were investigated for each subject enrolled. Because the subjects were asked to choose the face scale item that best described their general status in the past 1-2 weeks, the average of all the T5SS scores (naso-ocular symptom score with 5 items) recorded in the diary (during 6 time periods: February 1 to 15, February 16 to 28, March 1 to 15, March 16 to 31, April 1 to 15 and April 16 to 30) was calculated (Fig. 1).[10] Data were excluded when there were missing values. The changes of face scale scores were classified into 5 grades: greater than or equal to 2 scale-points improvement, ≤-2; 1 scale-point improvement, -1; no change, 0; 1 scale-point exacerbation, +1; and greater than or equal to 2 scale-points exacerbation, ≥+2. The MCIDs were calculated as changes in the average symptom and QOL scores when the face scale score was either improved or exacerbated by 1 point. The actual calculating formula used is as follows: MCID = $(|a - b| + |c - b|)/2$; a, b and c represent mean changes in the T5SS, T6SS, or QOL scores when the grade of the mean face scale change is -1,0 and +1 during each time period, respectively (Table 1).

MEASUREMENT OF POLLEN DISPERSAL

The daily amount of Japanese cedar and Japanese cypress pollen dispersal was measured from January 20 to May 10 of both 2009 and 2010 using a Durham sampler that was installed on the rooftop of the Okayama University Hospital building.[5]

STATISTICAL ANALYSIS

The nonparametric Mann-Whitney U test was used to compare data between groups. *P* values of less than 0.05 were considered to be statistically significant. Statistical analyses were performed with SPSS software (version 11.0 SPSS, Chicago, IL, USA).

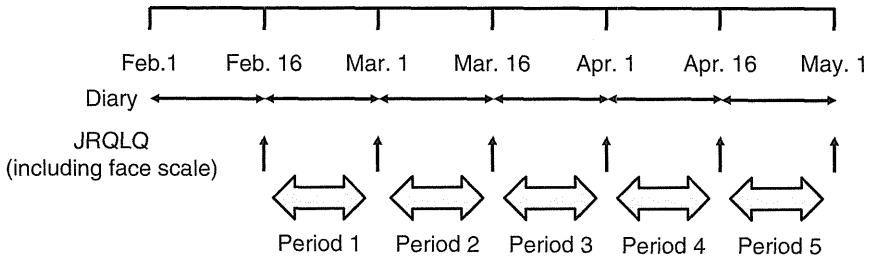

Fig. 1 Calculation of MCID. The MCIDs for symptom and QOL scores were determined based on face scale score changes before and after 6 time points (February 16, March 1 and 16, April 1 and 16, and May 1).

Table 1 Formula to calculate MCID

$$\frac{\left|\left[\begin{array}{l}\text{changes of symptom/QOL}\\ \text{scores at 1 improvement}\\ \text{in face scale score}\end{array}\right]-\left[\begin{array}{l}\text{changes of symptom/QOL}\\ \text{scores at no change in}\\ \text{face scale score}\end{array}\right]\right|+\left|\left[\begin{array}{l}\text{changes of symptom/QOL}\\ \text{scores at 1 exacerbation}\\ \text{in face scale score}\end{array}\right]-\left[\begin{array}{l}\text{changes of symptom/QOL}\\ \text{scores at no change in}\\ \text{face scale score}\end{array}\right]\right|}{2}$$

RESULTS

DISPERSAL OF JAPANESE CEDAR AND CYPRESS POLLEN IN 2009 AND 2010

A total of 3,698 grains/cm^2 of Japanese cedar/cypress pollen were dispersed in 2009. On the other hand, only 374 grains/cm^2 of Japanese cedar/cypress pollen were dispersed in 2010. The amounts of cedar/cypress pollen grains observed in 2009 and 2010 were 228.1% and 23.1%, respectively, of the average amount observed at our hospital from 2001 to 2010, which was 1,621 grains/cm^2.

THE MCID IN T5SS (TOTAL NASO-OCULAR SYMPTOM SCORE WITH 5 ITEMS) RECORDED ON DIARY CARDS

In 2009, the year with high pollen dispersal, 245 eligible diary card samples were analyzed: 11, 23, 114, 72, and 25 samples were classified as ≤-2, -1, 0, +1, and ≥+2, respectively. These improvements and exacerbations, as scored by face scale, lead to a symmetrical decrease and increase, respectively, of T5SS, as recorded on diary cards. Statistically significant differences in the change of T5SS were observed when the face scale score change was +1 (p = 0.001), greater than or equal to +2 (p = 0.026) and greater than or equal to -2 (p = 0.046) (Fig. 2A).

In 2010, the year with low pollen dispersal, 169 eligible diary card samples were analyzed: 7, 13, 107, 31, and 11 samples were classified as ≤-2, -1, 0, +1, and ≥+2, respectively. Statistically significant differences in the T5SS score change were seen for face scale scores of +1 (p = 0.003) and ≥+2 (p < 0.001) (Fig. 2B).

The MCID was calculated based on a 1-point improvement or deterioration of T5SS score recorded in the diary for each time period. In 2009 and 2010, the MCIDs of T5SS were determined to be 1.426 ([|-1.130 - 0.351| + |1.772 - 0.351|]/2: 0.285 per item) and 1.441 ([|-1.462 - 0.009| + |1.419 - 0.009|]/2: 0.288 per item), respectively (Table 2).

MCID IN T6SS (TOTAL NASO-OCULAR SYMPTOM SCORE WITH 6 ITEMS) BY JRQLQ RESULTS

In 2009, 251 eligible JRQLQ samples were investigated; 11, 23, 116, 73, and 28 samples were classified as ≤-2, -1, 0, +1, and ≥+2, respectively. In 2010, 173 eligible samples were classified as ≤-2 (n = 7), -1 (n = 13), 0 (n = 110), +1 (n = 33), and ≥+2 (n = 10). Compared with the T5SS, as determined by the diary recordings, the face scale score changes did correlate with a more robust and significant alteration of T6SS as determined by the JRQLQ (T6SS) in both 2009 and 2010 (p < 0.001, Fig. 3). Based on the calculation shown above, the MCIDs for T6SS by JRQLQ were determined to be 4.115 ([|-4.174 - 0.629| + |4.055 - 0.629|]/2: 0.686 per item) and 3.183 ([|-3.308 - (-0.163)| + |2.788 - (-0.163)|]/2: 0.531 per item) in 2009 and 2010, respectively (Table 2).

MCID OF QOL SCORE BY JRQLQ

In 2009, 255 eligible samples were investigated; 11, 24, 117, 74, and 29 samples were classified as ≤-2, -1, 0, +1, and ≥+2, respectively. In 2010, 179 eligible samples were classified as ≤-2 (n = 7), -1 (n = 14), 0 (n = 112), +1 (n = 34), and ≥+2 (n = 12). Similar to T6SS results, the changes of face scale score significantly correlated with alteration of the QOL score with 17 items as determined by JRQLQ responses, in both 2009 and 2010 (p < .0001, except for one exacerbation in 2010 where the p value was 0.003) (Fig. 4). The MCIDs of

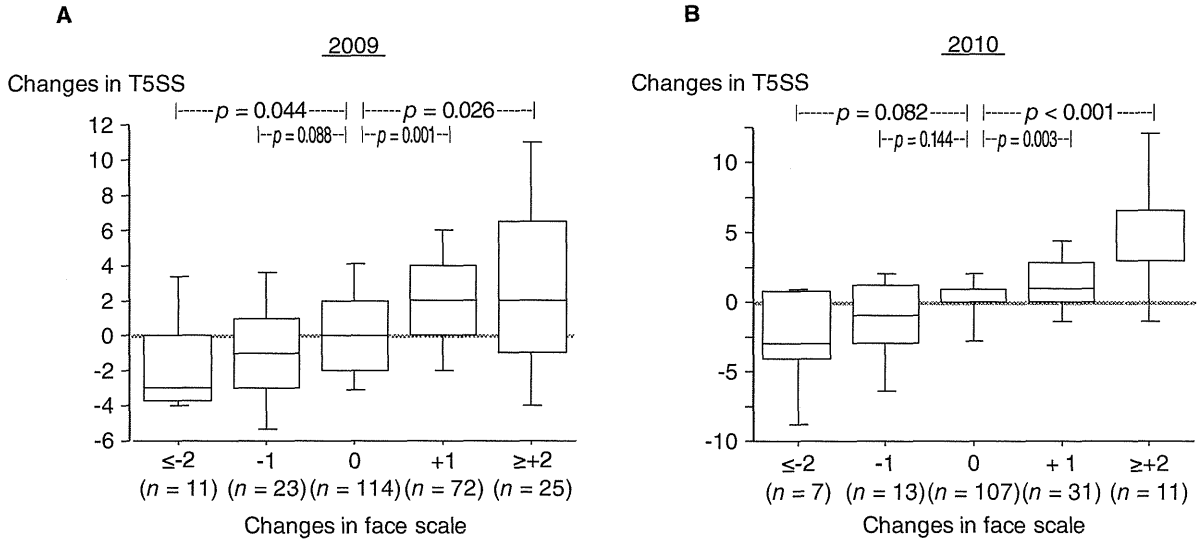

Fig. 2 T5SS changes on the diary cards based on face scale score changes, in 2009 (**A**) and 2010 (**B**). The rectangle includes the range from the 25th to the 75th percentiles, the horizontal line indicates the median, and the vertical line indicates the range from the 10th to 90th percentiles. *P*-values were determined by using the Mann-Whitney U test.

Table 2 Calculated MCID based on the minimal change of face scale

Endpoint type	Year	MCID	
		at total score	per 1 item
Symptom scores			
diary (5 items)	2009	1.426	0.285
	2010	1.441	0.288
JRQLQ (6 items)	2009	4.115	0.686
	2010	3.183	0.531
QOL scores			
JRQLQ (17 items)	2009	10.469	0.616
	2010	6.026	0.354

the QOL score on the JRQLQ were determined to be 10.469 ([|-11.000 - 1.034| + |9.937 - 1.034|]/2: 0.616 per item) and 6.026 ([|-8.400 - (-0.379)| + |3.652 - (-0.379)|]/2: 0.354 per item) in 2009 and 2010, respectively (Table 2).

DISCUSSION

In the present study, we have applied an anchor-based approach to derive the MCIDs for major endpoints in an assessment of Japanese cedar/cypress pollinosis, the major type of allergic rhinitis in Japan. Although a few previous studies had shown such MCIDs in allergic rhinitis,[8,9] we believe that this is the first report calculating the MCIDs for symptoms and QOL scores in Japanese patients with allergic rhinitis.

We calculated MCIDs in two consecutive seasons. In 2009, high pollen dispersal was observed. On the contrary, pollen dispersal was extremely low in 2010. The amount of pollen exposure affects the severity of rhinitis.[5,13,14] For example, we performed a double-blinded placebo-controlled trial to determine whether early interventional treatment with mometasone furoate nasal spray is effective for Japanese cedar/cypress pollinosis in 2010 (total 374 grains/cm^2) and 2011 (total 1,973 grains/cm^2).[5,14] The T5SSs in the placebo group at the peak of Japanese cedar pollen dispersal were 3.12 and 7.33 in 2009 and 2010, respectively. This study advantageously resulted in a comparison of MCIDs during high and low pollen dispersal seasons.

The MCIDs for T5SS in the diary cards in 2009 and 2010 were 1.426 (0.285 per item) and 1.441 (0.288 per item), respectively. This result suggests that a 1.5-unit difference in the 5-point T5SS scale and a 0.3 unit difference in each symptom score were clinically meaningful in this population, regardless of the amount of allergen exposure. These results can be used to evaluate whether differences in symptom scores among treatment groups are clinically meaningful or not. For example, our recent randomized, double-blinded, placebo-controlled trial for Japanese cedar/cypress pollinosis has shown that the average T5SS throughout the study period (February to April) in patients with early interventional treatment with mometasone was 2.3, which was statistically lower than in patients with placebo treatment (score, 5.0; $p < 0.01$) and those with post-onset treatment with mometasone (score, 3.9; $p = 0.03$).[14] Based on the MCIDs calculated in the present study, the efficacy of early interventional treatment with mometasone is not only statistically significant but also clinically meaningful, as compared to post-onset treatment with mometasone or placebo administration.

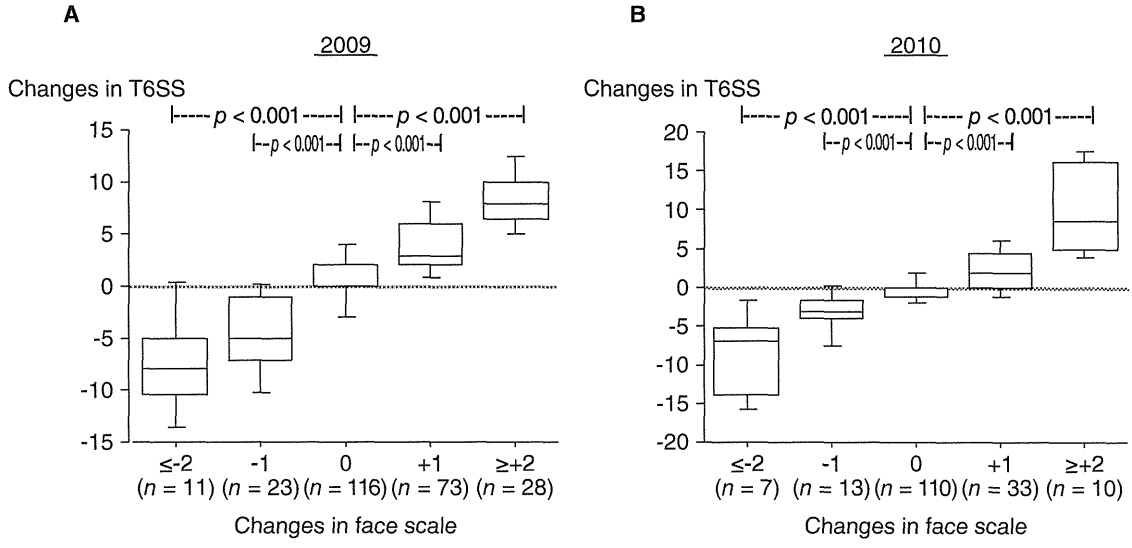

Fig. 3 T6SS changes on the JRQLQ based on face scale score changes, in 2009 (**A**) and 2010 (**B**). The rectangle includes the range from the 25th to the 75th percentiles, the horizontal line indicates the median, and the vertical line indicates the range from the 10th to 90th percentiles. *P*-values were determined by using the Mann-Whitney U test.

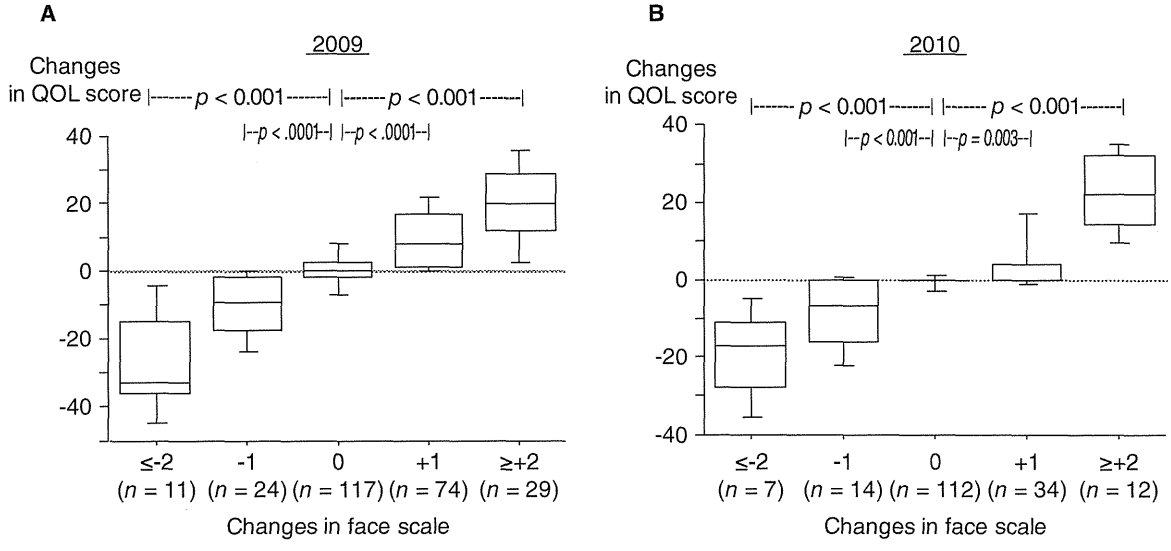

Fig. 4 QOL score changes on the JRQLQ based on the face scale score changes, in 2009 (**A**) and 2010 (**B**). The rectangle includes the range from the 25th to the 75th percentiles, the horizontal line indicates the median, and the vertical line indicates the range from the 10th to 90th percentiles. *P*-values were determined by the Mann-Whitney U test.

The MCIDs for T6SS by JRQLQ were determined to be 4.115 (0.686 in each symptom) and 3.183 (0.531 in each symptom) in 2009 and 2010, respectively. This result suggests that a 3.6-unit difference in T6SS and a 0.6-unit difference in each symptom score were clinically meaningful. However, these MCIDs by JRQLQ responses are relatively variable year to year, and seem to be influenced by the amount of pollen exposure, as compared to T5SS results from the diary cards, by which the MCIDs were almost equal in 2009 and 2010. Although the 5-point scale for nasoocular symptoms is set in both the diary and the JRQLQ, the specific criteria of the scales differ.[10] We think the one of the reasons why there were no significant changes of in T5SS value among the changes in face scale in 2009 and 2010 is that T5SS consists of more precise scale criteria for each symptom. For example, severity of nasal blockade in JRQLQ is simply divided into 5 scales as follows: 0, none; 1, mild; 2, moderate; 3, severe; and 4, very severe. On the other

hand, severity of nasal blockade in the diary is classified into 5 scales as follows: 0, no troublesome nasal blockade; 1, nasal blockade without oral breathing; 2, severe nasal blockade causing occasional oral breathing in a day; 3, severe nasal blockade causing prolonged oral breathing in a day; and 4, completely obstructed all day.

The MCIDs for the sum of QOL scores in JRQLQ results were determined to be 10.469 (0.616 in each item) and 6.026 (0.354 in each item) in 2009 and 2010, respectively. This result suggests that a 8.2-unit difference in the sum of QOL score and a 0.5-unit difference in each item are considered clinically meaningful. Although items, scales or anchors are different, this result is consistent with the report by Juniper showing that a 0.5-unit difference in each item on the RQLQ represents the MCID.[8] These values also can be used to evaluate whether differences in QOL scores among treatment groups are clinically meaningful. For example, we have previously reported a distinct randomized double-blind comparative study of sublingual immunotherapy for Japanese cedar pollinosis. In this study, the mean changes of QOL score from baseline data in February to peak data in the peak pollen dispersal period were 1.10 and 0.58 for the placebo and active treatment groups, respectively, showing that active sublingual immunotherapy significantly alleviated an deterioration of QOL ($p < 0.05$).[15] In addition, the difference of the mean change of QOL score between the two groups (0.52) is more than the estimated value of the MCID for QOL score (0.5). Furthermore, there was no difference in the number of medication used during the season between active and placebo treatment, suggesting that the sublingual immunotherapy is clinically efficacious and meaningful.

Subjects were allowed to use antihistamines and eye drops for rescue medication on demand. All subjects were enrolled in this study irrespective of use of the rescue medications. We investigated whether rescue medication affects MCID. In 2009, rescue medications were used in 133 out of 245 samples. Median change of T5SS at 1-point improvement in face scale was -1.0 and -1.0 in subjects with and without rescue medications, respectively ($p = 0.868$ by Mann-Whitney U test). Median change of T5SS at 1-point exacerbation in face scale was 2.0 and 2.0 in subjects with and without rescue medications, respectively ($p = 0.407$). In fact, MICD for T5SS is 1.496 (0.299 per item) and 1.493 (0.299 per item) in samples with and without rescue medications, respectively. Similar result was seen in 2010. In 2010, rescue medications were used in 56 out of 169 samples. Median change of T5SS at 1-point improvement in face scale was -3.0 and -1.0 in subjects with and without rescue medications ($p = 0.482$). Median change of T5SS at 1-point exacerbation in face scale was 1.0 and 1.5 in subjects with and without rescue medications ($p = 0.256$). MICD for T5SS was 1.885 (0.377 per item) and 1.333 (0.267 per item) in samples with and without rescue medications, respectively, in 2010. These results suggest that taking medications had minimal effect on MICD.

One concern regarding the present study is whether the estimated value of the MCID strictly reflects "minimal" differences, since we used a face scale with a 5-point scale as an anchor. Previous reports used the global rating of change scale with a 15-point scale as an anchor.[8,9] For example, Juniper *et al.* estimated that a change in score of 0.5 per item represents the MCID based on a change of 2 (a little better), 3 (somewhat better), -2 (a little worse) or -3 (somewhat worse) on the global rating of change scale.[8] Future investigations using an independent questionnaire as an anchor that includes the global rating of change scale will be required in order to determine the precise MCID for symptom and QOL scores. Since a 1 scale-point change of face scale score seems to correlate with a substantial alteration, as compared with a 1 scale-point change on the global rating of change scale, the values reported in the present study may represent clinically "meaningful," but not "minimally," important differences.

In conclusion, we have derived the MCIDs for symptom scores in the diary (0.3 unit per item), symptom scores on the JRQLQ (0.6 unit per item) and QOL scores (0.5 unit per item). Among these, we think that MCID for T5SS recorded in the diary seems to be most reliable and reflect the patient's condition because the MCIDs for T5SS in 2009 (0.285 unit per item) and 2010 (0.288 unit per item) were similar as compared with those for T6SS in JRQLQ (0.686 and 0.531 unit per item in 2009 and 2010, respectively) and QOL scores in JRQLQ (0.616 and 0.354 unit per item in 2009 and 2010, respectively). These values can enable physicians to interpret research findings regarding both statistical and clinical significance of allergic rhinitis treatment, especially treatment of Japanese cedar/cypress pollinosis. On the other hand, since the values are the average of the MCIDs of 2009 and 2010 and might be variable in another year depend on the amount of pollen, additional analysis using data from another years with different pollen dispersal will conduce to a more significance of the value.

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Association Study of Matrix Metalloproteinase-12 Gene Polymorphisms and Asthma in a Japanese Population

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Key Words

Matrix metalloproteinase 12 · Asthma · Single-nucleotide polymorphism · Association study · Chemokine

Abstract

Background: Matrix metalloproteinase 12 gene *(MMP12)* has been shown to be associated with asthma in a Caucasian population. In this study, we investigate whether single-nucleotide polymorphisms (SNPs) of *MMP12* are associated with a risk for asthma in a Japanese population. ***Methods:*** We tested for an association between SNPs in *MMP12* and asthma, including its severity, in a Japanese population (630 pediatric and 417 adult patients with atopic asthma and 336 children and 632 adults as controls). The rs652438 A and G variants (N357S) were generated by site-directed mutagenesis and an assay with artificial peptide substrates was used to compare two types of MMP12 activity. The effect of MMP12 inhibition with MMP12-specific small interfering RNA (siRNA) on chemokine secretion from airway epithelial cells was also tested in vitro. ***Results:*** N357S showed a p value <0.05 for childhood and combined (adult plus childhood) asthma in the dominant model [odds ratio (OR) 1.60, 95% confidence interval (CI) 1.00–2.56, $p = 0.047$; OR 1.40, 95% CI 1.04–1.89, $p = 0.028$, respectively]. This risk variant is associated with asthma severity in adult patients. In the functional assay, the minor-allele enzyme showed significantly lower activity than the major-allele enzyme. MMP12-specific siRNA suppressed IP-10 secretion from airway epithelial cells upon stimulation with IFN-β. ***Conclusions:*** Our results suggest that *MMP12* confers susceptibility to asthma and is associated with asthma severity in a Japanese population. *MMP12* may be associated with asthma through inappropriate attraction of leukocytes to the inflamed tissue.

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Introduction

Bronchial asthma is an inflammatory lung disease caused by a combination of genetic and environmental factors [1, 2]. There are lines of evidence that genetic variations influencing tissue remodeling or host defense system are involved in the development of asthma and its symptoms [2].

Matrix metalloproteinases (MMPs) are a family of secreted and membrane-bound endopeptidases. Their physiologic roles in the lung include not only degradation of the extracellular matrix, which is important for cellular infiltration into tissues and tissue repair or remodeling, but also for cytokine or chemokine processing and regulation of their secretion, which is important in the host defense system [3–5]. Some MMPs (e.g. MMP12) are also known to have a protein sequence for bactericidal activity [6].

The results of several studies suggest that MMP12 has important roles in lung diseases, such as asthma and chronic obstructive pulmonary disease [7]. The association between *MMP12* genetic variants and asthma or its clinical phenotype has also been reported in a Caucasian population. For example, Mukhopadhyay et al. [8] reported that a common serine variant (rs652438) was associated with the severity of asthma and Hunninghake et al. [9] showed that the minor allele of a single-nucleotide polymorphism (SNP) in the promoter region of *MMP12* was associated with a positive effect on lung function in children with asthma.

In a mouse model of asthma, Di Valentin et al. [10] showed that, unlike other MMPs (e.g. MMP9 and MMP13), MMP12 was induced not only by short-term allergen exposure but also by intermediate and long-term allergen exposure. Their data suggest that MMP12 plays various roles in both the acute and chronic phases of asthma development.

Although MMP12 is known to be secreted predominantly by macrophages, human bronchial epithelial cells also secrete MMP12 [11]. Airway epithelial cells are known to serve not only as a barrier to the outside but also as coordinators of immune responses in the airways; for example, bronchial epithelial cells participate in the homeostasis of the dendritic cell (DC) network present within the bronchial epithelium through the secretion of chemokines [12]. In addition to the degradation of the extracellular matrix or processing of cytokines or chemokines, MMP12 has also been shown to stimulate airway epithelial cells to enhance the release of several chemokines [13].

Table 1. Characteristics of the study population

	Childhood asthma	Adult asthma	Pediatric control	Adult control
Number	630	417	336	632
Mean age, years	10.5	41.4	9.3	43.7
Age range, years	3–15	16–83	6–12	20–75
Sex, male/female	1/0.61	1/1.05	1/1.02	1/0.37

The main aim of the present study was to investigate whether genetic variations in *MMP12* were associated with atopic asthma in a Japanese population. We also aimed to explore the relationship between *MMP12* polymorphisms and MMP12 enzyme activity. Finally, the effect of our findings on chemokine release from epithelial cells upon inflammatory stimuli was evaluated.

Methods

Subjects

All participants were Japanese, and asthma was diagnosed according to the criteria of the National Institutes of Health [14] by physicians specializing in allergic diseases.

The diagnosis of atopy was based on one or more positive skin scratch test responses to a range of seven common allergens in the presence of a positive histamine control and a negative vehicle control, or the presence of positive (≥0.35 IU/ml) specific IgE against at least one of the measured allergens. The seven allergens for the skin scratch test were house dust mite *(Dermatophagoides pteronyssinus)*, cat dander *(Felis domesticus)*, dog dander *(Canis familiaris)*, orchard grass *(Dactylis glomerata)*, Ambrosia *(Ambrosia artemisiifolia)*, cedar pollen *(Cryptomeria japonica)*, and black mold *(Alternaria alternate)*. Total and specific IgE levels in serum were assayed using the CAP-radioallergosorbent testing method (Pharmacia Diagnostics, Uppsala, Sweden). Eight specific IgEs were measured: house dust mite, dog dander, cat dander, black mold, cedar pollen, orchard grass, egg white and golden, black bellied, or djungarian hamsters *(Mesocricetus auratus/Cricetus cricetus/Phodopus sungorus)*. In this study, we only analyzed data from patients with atopic asthma.

The number of cases of atopic childhood and adult asthma were 630 and 417, respectively, and the number of pediatric and adult controls was 336 and 632, respectively (table 1). The mean ages (range) of the four groups were as follows: childhood asthma, 10.5 (3–15) years; adult asthma, 41.4 (16–83) years; child control, 9.3 (6–12) years, and adult control, 43.7 (20–75) years. The details of these subjects have been described previously [15]. As adult controls, a total of 632 healthy individuals who had neither respiratory symptoms nor a history of asthma-related diseases (mean age 43.7, 20–75 years; male:female ratio = 1.0:0.37) were recruited by physicians' interviews about whether they had been diagnosed with asthma and/or atopic diseases [16]. For pediatric controls and the investigation of total and specific IgE levels, we recruited

Table 2. Locations and allele frequencies of SNPs of the *MMP12* gene

SNP	dbSNP No.	Position[a]	Minor-allele frequency, %[b]	Allele	Location
SNP1	rs2276109	102745791	0.012	A/G	promoter, -82A/G
SNP2	rs10895367	102745130	0.100	G/C	intron 1
SNP3	rs7123600	102741968	0.237	A/G	intron 4
SNP4	rs632009	102738499	0.144	C/T	intron 5
SNP5	rs652438	102736642	0.083	A/G	exon 8, 1082A>G, Asn357Ser

[a] Based on National Center for Biotechnology Information (NCBI).
[b] Based on HapMap data No. 27.

children attending an elementary school in Chiba City, Japan. The clinical characteristics of this population as well as inclusion and exclusion criteria have been described previously [17]. In brief, after the exclusion of questionnaire-assessed asthma and/or atopic dermatitis subjects and those with congenital heart diseases and lung diseases caused by premature birth, 336 children having a complete set of information on the total and eight specific IgE levels, genotypes and environmental factors were assigned to the pediatric group.

The asthma severity in pediatric and adult patients was determined by physicians who were experts in allergic diseases and was defined according to the degree of therapy required to control symptoms at the time of entry into the study in accordance with the Japanese Pediatric Guideline for the Treatment and Management of Asthma 2005 [18] and the criteria of the Global Initiative for Asthma Guidelines 2002 [19], respectively. In pediatric patients, the distribution of subjects (severity data were available in 218 pediatric patients) was as follows: step 1, mild intermittent 9.2% (20 individuals); step 2, mild persistent 38.1% (83 individuals); step 3, moderate persistent 34.9% (76 individuals); step 4, severe persistent 17.4% (38 individuals), and step 5, severe persistent 0.5% (1 individual). In adult patients, the distribution of subjects (severity data were available in 357 adult patients) was as follows: step 1, mild intermittent 4.5% (16 individuals); step 2, mild persistent 55.6% (197 individuals); step 3, moderate persistent 24.9% (88 individuals), and step 4, severe persistent 15.0% (53 individuals).

Written informed consent was received from all participants and the study was approved by the ethics committees of Chiba University Graduate School of Medicine and RIKEN.

SNP Selection

SNP selection was based on data from HapMap No. 27 and performed according to the following criteria: SNPs in *MMP12* with a minor-allele frequency greater than 5% (HapMap JPT population) were identified. The linkage disequilibrium (LD) patterns from the HapMap JPT population were evaluated to avoid selecting SNPs in strong LD (cutoff, $r^2 = 0.8$). Four SNPs were identified from the 5′ end: rs10895367, rs7123600, rs632009, and rs652438. Because the SNP in the promoter region (rs2276109) was previously shown to be associated with asthma phenotypes in a Caucasian population [9], we added this SNP to the above 4 SNPs for genotyping. The location and allele frequency of the 5 selected SNPs are shown in table 2. The structure of *MMP12* and location of the SNPs (minor-allele frequency ≥0.001, based on HapMap data No. 27) and their LD map are shown in figure 1. These SNPs are assumed to capture the majority of the known common variations in the gene.

Genotyping

Genomic DNA was prepared from whole-blood samples using a standard protocol. Whole-genome amplification was performed using the illustra GenomiPhi V2 amplification kit (GE Healthcare, Little Chalfont, UK) according to the manufacturer's standard protocol.

Genotyping was performed using the SNaPshot® method (Applied Biosystems, Foster City, Calif., USA). Multiplex PCR amplification was performed in 10 μl reaction mixture containing 5 ng amplified template DNA, 0.05 U TaKaRa Ex Taq® HS (TaKaRa Bio, Otsu, Japan), 67 mM Tris-HCl (pH 8.8), 16.6 mM $(NH_4)_2SO_4$, 6.7 mM $MgCl_2$, 6.7 μM EDTA, 10 mM 2-mercaptoethanol, 1.5 mM of each dNTP and 0.1–0.2 μM of each PCR primer shown in table 3. The amplification was carried out on GeneAmp® PCR System 9700 (Applied Biosystems) using the following PCR program: initial denaturation step of 1 min at 98°C, 10 cycles of 10 s at 98°C, 30 s at 60°C, and 2 min at 72°C, followed by a final extension of 5 min at 72°C. Post-PCR treatment to remove primers and unincorporated dNTPs was performed with shrimp alkaline phosphatase (SAP; Promega Corporation, Madison, Wisc., USA) and exonuclease I (*Exo*I; New England Biolabs, Ipswich, Mass., USA). The PCR product was incubated with 0.5 U SAP and 1 U *Exo*I for 1 h at 37°C followed by 15 min at 80°C for enzyme inactivation. The SNaPshot reaction was performed in 10 μl reaction mixture containing 0.5 μl SNaPshot ready reaction mix (Applied Biosystems), 2 μl SAP/*Exo*I-treated PCR products and 0.05–0.3 μM each of the SNaPshot primers shown in table 3. SNaPshot primers were designed to anneal adjacent to the mutation of interest and to contain an additional sequence with several (incomplete) repeats of the 'acgt' sequence (indicated by lower case) at the 5′-end to obtain a convenient length for discrimination from other SNaPshot products. The reaction mixture was subjected to an initial step of 96°C for 1 min to activate the enzyme, followed by 30 single-base extension cycles of denaturation at 96°C for 10 s, and annealing and extension at 60°C for

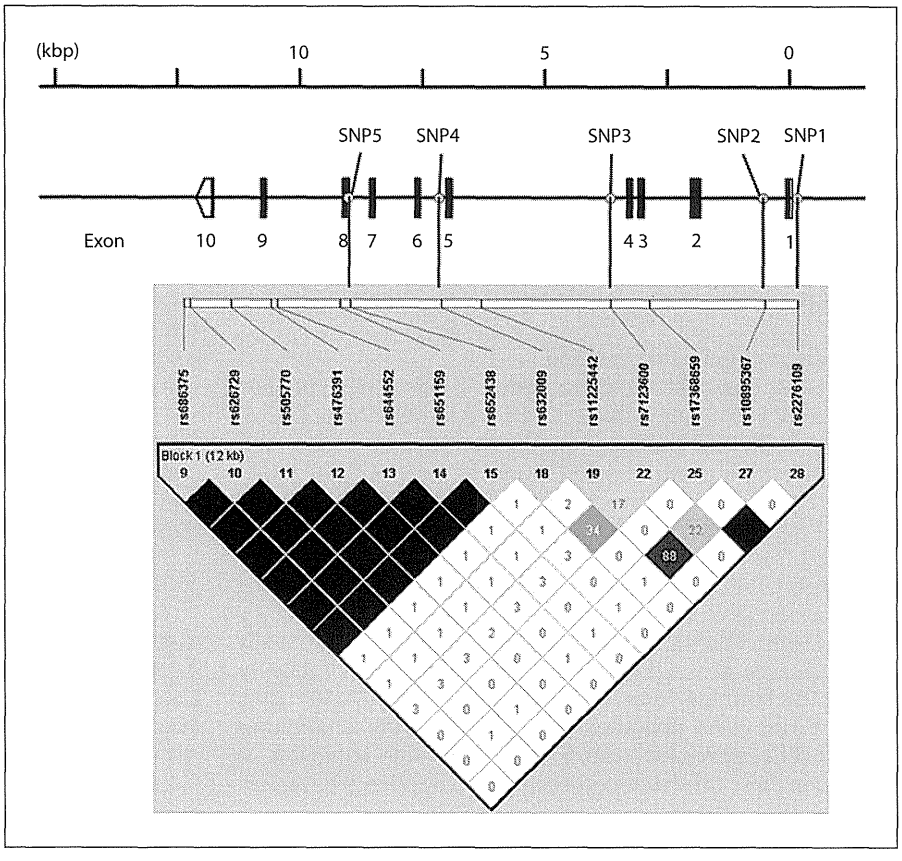

Fig. 1. Structure of the *MMP12* gene and location of (SNPs) and their LD map.

Table 3. Primers for genotyping SNPs in the *MMP12* gene

SNP1 (rs2276109)	PCR forward	5′-TGCTAATTGATCCATTGTCGTCTG
	PCR reverse	5′-TGGCCTGCAGGAGCAGTATTAG
	SNaPshot	5′-ctgactGTGAATATGAATCCTATGAGTGACTCA
SNP2 (rs10895367)	PCR forward	5′-TTCCCTTTACCCTCAGTCATCTCA
	PCR reverse	5′-AGTTGGGACACAAATTTCTTGCTT
	SNaPshot	5′-tgactgactCCCTCAGTCATCTCAGTTATATCTTTACAG
SNP3 (rs7123600)	PCR forward	5′-AAGCTCAGAGGAACCTTAAGAAGCA
	PCR reverse	5′-TCCTCTAATTGTTGGCACACACAG
	SNaPshot	5′-GGTAAAAGCTGTTAATTAGGTGGTAAGTT
SNP4 (rs632009)	PCR forward	5′-GGCATTCAGTCCCTGTATGGTG
	PCR reverse	5′-TCTGCCTCTGGCTCTGACGTT
	SNaPshot	5′-ctgactgactCCCTGTGTGGCAGGTGACACAG
SNP5 (rs652438)	PCR forward	5′-TGTCTTCACAGATGACAAATACTGGTT
	PCR reverse	5′-CTGGAAGGTCAAGGAGCTTTGG
	SNaPshot	5′-tgactGCTCTTGGGATAATTTGGCTCTGGTCTTAAA

30 s. Postextension treatment to remove the 5′-phosphoryl group of the ddNTPs was performed with calf intestine alkaline phosphatase (CIAP; Nippon Gene, Tokyo, Japan). The final mixture (6 μl) was treated with 1 U CIAP for 60 min at 37°C, followed by 15 min at 80°C for enzyme inactivation. The SNaPshot products (1 μl) were mixed with 10 μl HiDi™ formamide (Applied Biosystems) and 0.05 μl GeneScan™ 120 LIZ™ size standard (Applied Biosystems), and electrophoresed in a 50-cm-long capillary using Performance Optimum Polymer 6 (POP6) on an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems). The resulting data were analyzed with GeneMapper® v3.5 Software (Applied Biosystems).

Expression Vector Construction

A cDNA fragment encoding human *MMP12* was obtained from the human multiple tissue cDNA panel (TaKaRa Bio) (A allele at target SNP5_N357S) and amplified using PCR with the following primers: forward, 5′-AAACTGACTCGAGCCGTTTAGAAGTTTACAATG-3′; reverse, 5′-TTAATTACTCGAGTTCTAACAACCAAACCAGCT-3′. The cDNA fragment was digested with *Xho*I and cloned into pcDNA™3.1(+) (Life Technologies, Carlsbad, Calif., USA), which was previously digested with *Xho*I, using DynaExpress DNA Ligation Kit ver. 2 (BioDynamics Laboratory, Tokyo, Japan). The N357S mutation of *MMP12* was introduced by PCR-based site-directed mutagenesis with the primers shown in table 4 (underlining indicates the position of rs652438) using PrimeSTAR® MAX polymerase (TaKaRa Bio). We constructed two MMP12 expression vectors. The nucleotide sequences of *MMP12* in these constructs were confirmed by DNA sequencing.

Cell Culture

HEK293 cells (human embryonic kidney cell line) were cultured in minimum essential medium (Invitrogen, Paisley, UK) supplemented with 2 mM L-glutamine, 1% nonessential amino acids, 10% fetal bovine serum and antibiotics.

Primary normal human small airway epithelial cells (SAECs) as well as all the basal medium (SABM™) and growth supplements [SAGM™ SingleQuots®, which contains bovine pituitary extract, human recombinant epidermal growth factor, hydrocortisone, epinephrine, transferrin, insulin, retinoic acid (RA), triiodothyronine, gentamicin/amphotericin-B (GA-1000) and bovine serum albumin (BSA] were obtained from Lonza (Switzerland). Cells were cultured according to the manufacturer's instructions. SAECs were maintained in SABM supplemented with SAGM SingleQuots.

Transient Transformants

Empty vector pcDNA3.1(+) or MMP12 expression vectors pcDNA3.1-MMP12 were transiently transfected into HEK293 cells using Lipofectamine™ LTX (Invitrogen) in 6-well tissue culture plates according to the manufacturer's instructions. After 24 h of incubation, cells were serum starved, and supernatants were collected after 24 h and stored at −80°C until use.

MMP Activity Assay

Protease activity of MMP12 in the conditioned medium was evaluated with synthetic fluorescence peptide as substrate using SensoLyte® 520 MMP12 Assay Kit *fluorimetric* (AanSpec Inc., Fremont, Calif., USA). Because purified recombinant MMP12 and MMP12 protein in conditioned medium from the HEK293 transformant were spontaneously active, the incubation step with 4-aminophenylmercuric acetate was skipped. Fluorescence of 5-FAM was monitored at excitation/emission wave lengths of 490/520 nm using infinite® F200 (Tecan, Männedorf, Switzerland). BioPlex™ 200 (BioRad Laboratories, Hercules, Calif., USA) was used to measure MMP12 protein concentrations in the medium with Fluorokine® MAP assay kit (R&D Systems, Minneapolis, Minn., USA). Standard proteins included in the kit were used to calculate the MMP12 protein concentration. There was no significant difference in MMP12 protein levels secreted from transient transformants between transfection of the different constructs.

Table 4. Primers for mutagenesis in the *MMP12* cDNA

	Sequence
Forward	5′-ATTAGCA**G**TTTAAGACCAGAGCCAAA
Reverse	5′-TCTTAAA**C**TGCTAATTAACCAGTATT

Small Interfering RNA-Targeted Knockdown of MMP12

SAECs were seeded into 24-well plates and cultured overnight to 40–50% confluency. MMP12-specific siRNA (Mission® siRNA; siRNA ID: SASI_Hs01_00077192; Sigma-Aldrich, St. Louis, Mo., USA) was added at a final concentration of 5 nM with Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. Control cells were transfected with siPerfect® Negative Control (Sigma-Aldrich) or treated with transfection medium alone.

Measurement of Chemokine Secretion from Interferon-β-Stimulated SAEC

The medium was replaced by SABM supplemented with GA-1000, RA, and BSA alone 24 h before stimulation of SAECs. Subsequently, SAECs were incubated for 24 h with 50 ng/ml interferon-β (IFN-β; PeproTech, Rocky Hill, N.J., USA). Cell supernatants were cleared by centrifugation and stored at −80°C until measurement. Chemokine (IP-10, MCP-1, MIP-1β, RANTES and IL-8) was detected using the Procarta® human cytokine assay kit (Panomics/Affymetrix, Fremont, Calif., USA) according to the manufacturer's instructions. For blocking experiments, cells were pretreated with MMP12-specific siRNA or control for 48 h before stimulation.

Statistical Analysis

Pairwise LD in SNPs was estimated as r^2. We calculated allele frequencies and tested their agreement with the Hardy-Weinberg equilibrium using a χ^2 goodness-of-fit test. Because minor-allele frequencies of selected SNPs were low (table 2), subjects carrying one or two copies of the minor allele were analyzed as one group. We compared differences in the allele frequencies and genotype distribution of each polymorphism between the case and control subjects using a 2 × 2 contingency χ^2 test (allele and dominant genotype models). A case-control association study was performed using SNPAlyze® ver. 4.1 program (Dynacom, Chiba, Japan). Combined analyses of childhood and adult asthma were performed by meta-analysis with a general variance-based method under a random effect model. Statistical analysis was performed with SPSS software (ver. 17.0J; SPSS Japan, Tokyo, Japan). p values <0.05 were judged to be significant.

Table 5. *MMP12* genotype frequencies in controls and cases

SNP	Genotype frequency											
	childhood asthma			adult asthma			pediatric control			adult control		
	11[1]	12[2]	22[3]	11[1]	12[2]	22[3]	11[1]	12[2]	22[3]	11[1]	12[2]	22[3]
SNP1	0.966	0.034	0.000	0.946	0.054	0.000	0.961	0.039	0.000	0.958	0.042	0.000
SNP2	0.773	0.212	0.015	0.799	0.189	0.012	0.793	0.189	0.018	0.798	0.186	0.016
SNP3	0.525	0.411	0.064	0.540	0.378	0.082	0.541	0.375	0.084	0.522	0.423	0.056
SNP4	0.775	0.219	0.006	0.741	0.257	0.002	0.757	0.228	0.015	0.766	0.233	0.002
SNP5	0.881	0.106	0.013	0.874	0.119	0.007	0.922	0.078	0.000	0.898	0.099	0.003

[1] Frequency of homozygosity for major allele.
[2] Frequency of heterozygosity.
[3] Frequency of homozygosity for minor allele.

Table 6. SNP association study of the *MMP12* gene

SNP	Allelic frequency comparison						Genotype frequency comparison in dominant model					
	childhood asthma vs. child control		adult asthma vs. adult control		all asthma vs. all control[1]		childhood asthma vs. child control		adult asthma vs. adult control		all asthma vs. all control[1]	
	p value	OR and 95% CI	p value	OR and 95% CI	p value	OR and 95% CI	p value	OR and 95% CI	p value	OR and 95% CI	p value	OR and 95% CI
SNP1	0.690	1.15 0.57–2.32	0.374	1.30 0.73–2.30	0.348	1.24 0.79–1.93	0.687	1.16 0.57–2.34	0.369	1.31 0.73–2.34	0.343	1.24 0.79–1.95
SNP2	0.602	1.08 0.81–1.45	0.877	1.02 0.77–1.36	0.635	1.05 0.86–1.29	0.488	1.12 0.81–1.55	0.986	1.00 0.73–1.37	0.622	1.06 0.85–1.33
SNP3	0.921	1.01 0.82–1.25	0.839	1.02 0.84–1.24	0.827	1.02 0.88–1.18	0.644	1.06 0.82–1.39	0.560	1.08 0.84–1.38	0.458	1.07 0.89–1.29
SNP4	0.409	1.13 0.85–1.50	0.382	1.13 0.86–1.47	0.230	1.13 0.93–1.37	0.549	1.10 0.81–1.50	0.360	1.14 0.86–1.52	0.280	1.12 0.91–1.39
SNP5	**0.015**	1.74 1.11–2.74	0.173	1.29 0.89–1.87	**0.012**	1.46 1.09–1.96	**0.047**	1.60 1.00–2.56	0.220	1.28 0.86–1.88	**0.028**	1.40 1.04–1.89

[1] Combined analyses of childhood and adult asthma were performed by meta-analysis with general variance-based method under a random effect model. Values in boldface indicate statistical significance.

Results

Association between Polymorphisms of the MMP12 *Gene and Childhood Atopic Asthma*

The characteristics of patients with atopic asthma and controls are shown in table 1. As there was a difference in sex ratio between the cases and controls, we performed a χ^2 analysis for sex and genotype frequencies. There was no evidence of an association between sex and genotype frequencies except for SNP2 ($p = 0.045$).

Among the five genotyped SNPs, SNP1 (–82A/G; rs2276109) is the functional promoter SNP which has previously been shown to be associated with childhood asthma and COPD [9], and SNP5 (1082A/G; rs652438), which has the amino acid substitution N375S, has previously been shown to be associated with the clinical asthma phenotype in children and young adults [8].

Table 5 shows the genotype frequency of each polymorphism and table 6 shows the association test results between asthma patients and controls [childhood asthma vs. child control, adult asthma vs. adult control, and all asthma (combined population of pediatric and adult asthma patients) vs. all control (combined population of child and adult controls)].

The odds ratio (OR) for SNP5 was 1.74 [95 % confidence interval (CI) = 1.11–2.74, p = 0.015] for the allelic comparison (A vs. G) and 1.60 (95 % CI = 1.00–2.56, p = 0.047) in the minor-allele dominant model (AA vs. AG+GG) for the pediatric population. ORs for SNP5 were 1.46 (95% CI = 1.09–1.96, p = 0.012) for the allelic comparison and 1.40 (95% CI = 1.04–1.89, p = 0.028) in the dominant model for the combined population. The p values did not reach significance after Bonferroni's correction.

Association between SNP5 and Asthma Severity in Adults

Because SNP5 (Asn357Ser) was reported to be associated with greater asthma severity in a Caucasian population [8], we analyzed whether this variant was also associated with asthma severity in a Japanese population. In pediatric asthma patients, this variant did not show any association with asthma severity (data not shown). In adult patients, however, the carrier frequency for this variant significantly increased linearly with severity, with a p value of 0.01 in linear regression analysis between asthma severity and carrier frequencies for this variant (fig. 2).

Effect of Amino Acid Changes on MMP12 Activity

The amino acid substitution in SNP5 is located in the hemopexin-like domain, which is thought to determine substrate specificity and to be the site for interaction with tissue inhibitor of metalloproteinases [20]. However, until recently, it has not been known whether the amino acid change in SNP5 has any effect on MMP12 activity. To evaluate the functional effect of SNP5 on MMP12 activity, we constructed MMP12 protein expression vectors with different amino acid sequences of MMP12 cDNA and transfected them into human cell lines (HEK293 cells). The two types of amino acid sequence at this SNP site were 357N (major allele) and 357S (minor allele). MMP12 enzyme secreted into the culture medium from the HEK293 transformants and its activity toward an artificial peptide substrate was monitored. As shown in figure 3, the minor-allele enzyme showed slightly but significantly lower proteolytic activity than the major-allele enzyme. This result was similar to that reported by Haq et al. [21]. They showed that MMP12 activity with the major allele of SNP5 was greater than that with the minor allele, although the observed difference in the activity between the major- and minor-allele enzyme was much larger than in the present study.

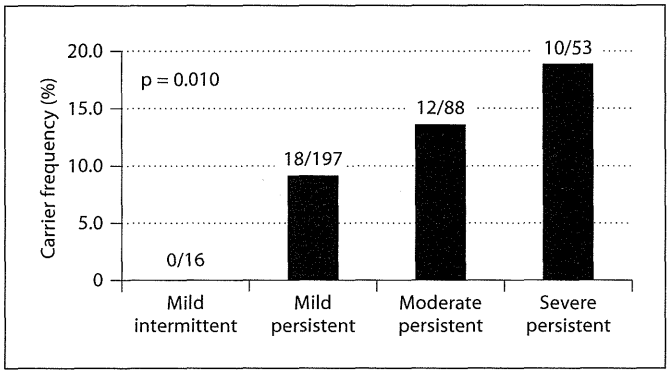

Fig. 2. Association between SNP5 minor-allele carrier frequency and asthma severity in adult asthma patients. Numbers on graph represent number of minor-allele carriers/number of patients in indicated asthma severity group (p value for linear regression analysis).

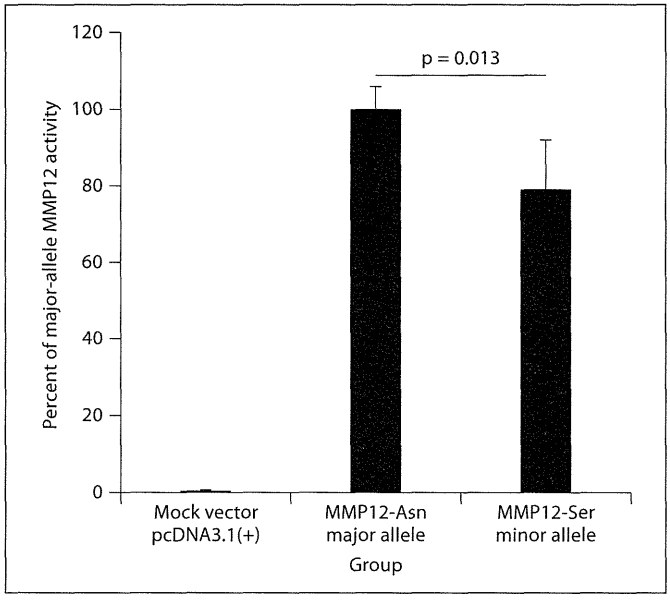

Fig. 3. Comparison of peptide cleavage activity of different alleles of MMP12. Vertical axis indicates relative activity (Sensolyte 520 MMP-12 assay endpoint) compared with the major-allele MMP12 enzyme. The values represent the means ± SD of three independent experiments (p value for the unpaired t test).

MMP12-Specific Knockdown by siRNA Inhibits Chemokine Secretion

In the lung, MMP12 is known to be secreted predominantly by macrophages as well as by other cells, such as bronchial epithelial cells [11]. The known physiologic roles of MMP12 include not only degradation of extracellular matrix but also regulation of secretion and activa-

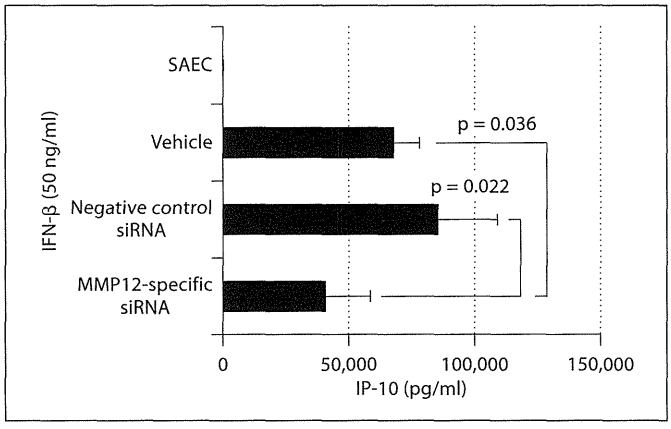

Fig. 4. MMP12 siRNA reduced IP-10 secretion from SAECs following stimulation with IFN-β. SAECs were pretreated with MMP12-specific siRNA, control siRNA, or vehicle for 48 h before stimulation. The medium was replaced 24 h before stimulation by SABM supplemented with GA-1000, RA, and BSA alone. Subsequently, SAECs were incubated for 24 h with or without IFN-β. The concentration of IP-10 in the medium was measured. Data shown are the means ± SDs of three independent experiments (p value for unpaired t test).

tion of cytokines and chemokines [13]. Furthermore, it is also known that airway epithelial cells in patients with asthma are involved in the control of lung inflammation and DC recruitment during allergen stimulation [12].

We thus hypothesized that the decreased activity of MMP12 affects chemokine release from lung epithelial cells upon cytokine stimulation. To evaluate this hypothesis in vitro, we used siRNA technology with MMP12-specific knockdown in airway epithelial cells. We tested several combinations of stimuli (lipopolysaccharide, FSL-1, IFN-β and IFN-λ) on the cells and chemokines (IP-10, MCP-1, MIP-1β, RANTES, and IL-8) secreted upon stimulation. We found that SAECs secreted high levels of IP-10 in response to IFN-β stimulation and also found that SAECs transfected with MMP12-specific siRNA secreted less IP-10 upon IFN-β stimulation than SAECs transfected with negative control (fig. 4). This suggests that endogenous MMP12 activity is, at least in part, required for appropriate secretion of IP-10 under inflammatory conditions.

Discussion

In agreement with our results, Mukhopadhyay et al. [8] reported that a common serine variant in *MMP12* (rs652438) was associated with asthma phenotypes. This variant is also associated with poor outcomes in breast cancer patients [22]. However, the results of the MMP12 activity assay showing that the risk allele (minor allele) enzyme had a lower activity than the major-allele enzyme seem to contradict the findings from an animal model of acute allergic and inflammatory airway disease using MMP12-deficient mice [4]. MMP12-deficient mice showed decreased eosinophil and macrophage recruitment in interleukin 13-induced lung injury [23], reduced macrophage migration and influx in smoke-induced emphysema [24], reduced neutrophil influx to the alveolar space in acute lung injury [25], or reduced release of tumor necrosis factor-α from macrophages after smoke exposure [26]. From these observations, it appears that MMP12 plays unfavorable and pathologic roles in lung inflammation and lung tissue remodeling. On the other hand, using a murine lung fibrosis model mimicking idiopathic pneumonia syndrome after bone marrow transplantation, England et al. [27] reported that MMP12 from tissue cells was important in limiting idiopathic pneumonia syndrome allowing proper remodeling of extracellular matrix and effective repair of tissue injury. In an experimental autoimmune encephalomyelitis model, MMP12 deficiency worsened the outcome, and this was shown to be associated with cytokine and chemokine dysregulation [28]. In addition to the idiopathic pneumonia syndrome model, MMP12 was thought to play a protective role in experimental autoimmune encephalomyelitis. Based on the spatially and chronologically multifaceted nature of MMP12 in inflammation and tissue-remodeling processes [10], we speculate that reduced activity of MMP12 may lead to the development of asthma or its severe phenotype under some circumstances.

IFN-β is known to be an important cytokine for controlling viral infection, such as rhinovirus respiratory infection [29, 30]. Airway epithelial cells exposed to virus or allergen are known to produce higher levels of chemokines such as IP-10, which are involved in the recruitment of T-helper type 1 cells, inflammatory DCs, and other cell types [12, 31]. DCs are well known as critical immune cells which play pivotal roles in both innate and adaptive immune responses [32]. In addition to the DC subset that is present in the lung in the steady state, certain subsets of DCs (e.g. monocyte-derived DCs) are readily recruited upon stimulation with inflammatory mediators [33]. Along with the MMP12 activity of upregulation of active chemokine release, MMP12 was also reported to inactivate some CXC chemokines (e.g. MIP-2α or MIP-2β) and generate antagonists to some CC chemokines (e.g. MCP-

1 or MCP-2), and this activity of MMP12 was thought to be important for terminating neutrophil influx and regulating inflammatory responses [5]. To maintain tissue homeostasis, it is very important that appropriate types of cells (e.g. macrophages, DCs, or neutrophils) are readily and promptly recruited to the inflammatory site [32, 33]. This orchestrated response is critical for the control of inflammation [12, 31], and is also important for inhibiting persistent inflammation, which may lead to pathologic tissue remodeling [34]. Bronchial epithelial cells of asthma patients were shown to have reduced ability to produce IFN-β and IFN-λ in response to viral infection [35]. As shown in figure 4, reduced MMP12 activity leads to reduced secretion of IP-10 from airway epithelial cells upon stimulation with IFN-β. Based on previous reports and our present findings, we speculate that the reduced activity of MMP12 might lead to reduced infiltration of inflammatory cells (e.g. T-helper type 1 cells, inflammatory DCs, or macrophages) into the inflammatory site, where these cells are involved in wound healing or microbial elimination in the early phase of inflammation, through the reduction of active chemokine release and/or insufficient degradation of extracellular matrix. Thus inappropriate recruitment of cells in the early phase of inflammation and also inappropriate termination of cellular influx might lead to chronic inflammation and/or activation of other MMP proteins, and subsequently pathologic tissue remodeling.

There are several other possible causes for the link between this SNP and the asthma phenotype. Because this SNP causes amino acid substitution in the hemopexin-like domain of the MMP12 protein, which is thought to be involved in substrate specificity [20], we cannot exclude the possibility that this nonsynonymous SNP affects the substrate specificity of MMP12 so that MMP12 activity is higher for some physiologic substrates in vivo than the major-allele enzyme, which may lead to differences in patient asthmatic phenotype. In addition, the *MMP12* gene is located at 11q22, and 9 *MMP* genes are known to be clustered in this region on chromosome 11q21–24. Thus SNPs in other *MMP* genes, which are in strong LD with SNP5 in *MMP12,* may be functional and responsible for causing an asthma phenotype.

In conclusion, we investigated the association between *MMP12* polymorphisms and atopic asthma in a Japanese population and also its functional implication. Although the p value of N357S (rs652438) in *MMP12* did not reach significance after Bonferroni's correction, the lower limit of the 95% CI of the OR for this SNP is above 1.0 for the pediatric population and for the total (children + adults) population. In addition, the risk variant of *MMP12* is associated with disease severity in adult asthma patients. In the MMP12 functional assay with artificial peptide substrates, the risk allele (minor allele) enzyme showed significantly lower proteolytic activity than the major-allele enzyme for this substrate. With MMP12-specific siRNA, we showed that reduced MMP12 activity might lead to inappropriate IP-10 secretion from airway epithelial cells upon stimulation with IFN-β. Reduced MMP12 activity may play a pathologic role in the development of asthma and its phenotypes.

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Disclosure Statement

The authors declare no conflict of interest.

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