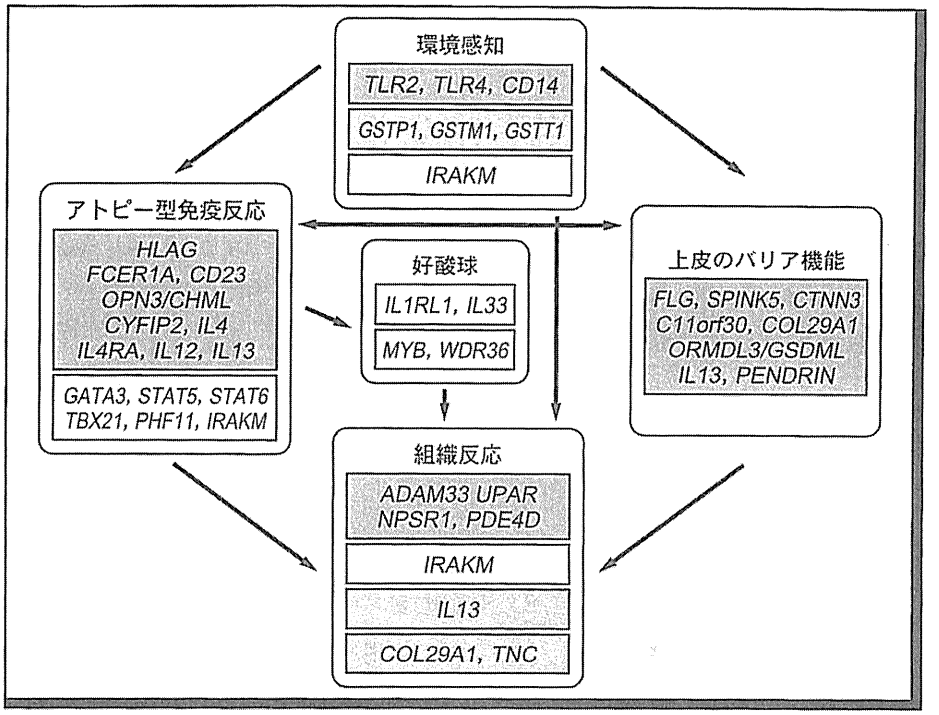

図3　アレルギー疾患に関連する遺伝子の機能的分類（Holloway）

① 環境感知：環境の危険因子を感知するために必要な遺伝子群を含むグループ.

② アトピー型免疫反応：リンパ球の分化，機能に関連する遺伝子群，アトピー性感作，好酸球の増殖，機能に関係する遺伝子も含んでいる.

③ 上皮のバリア機能：最近のゲノム研究から同定されてきた遺伝子が多い．上皮細胞で発現している.

④ 組織反応：組織の炎症，リモデリングに関与する遺伝子群を含むグループ.

（文献 15 より）

られるものは，100%喘息を発症したという結果が出ている。この研究はドイツ人でのもので，日本人ではどうなのかは不明である。

おわりに

ここ数年での研究で，以前よりは信頼性の高い疾患感受性遺伝子が迅速に同定できる時代になってきたと言えるが，遺伝子多型の効果が限定的であることも明白となった。その要因の１つは「喘息」という疾患の多様性にあるという点に注目が集まっている。近年，発症時期，アトピーの状態，呼吸機能などの多くの臨床的パラメータを投入し，クラスター解析などで喘息を分類することで，新しい喘息のサブタイプが提唱されるようになっている[19,20]。また，より病態と直結する endotype という指標を設定することで，喘息の臨床像，サブタイプの病態をより明確に定義できるとの提言もなされている[21]。今後は，喘息の新しいサブタイプ（表現型）ないし endotype を対象として，それらに特異的に関連する遺伝子や遺伝子多型の同定が試みられる時代となることが予想される。

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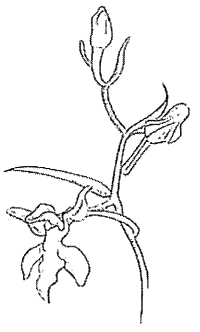

多因子遺伝性疾患としてのアレルギー疾患
—遺伝子解析の意義と限界—

鈴木洋一*

はじめに

多因子遺伝子性疾患の遺伝学的，遺伝子解析の研究は，ゲノム DNA の配列の個人差（一塩基多型〔SNP〕など）を利用して疾患との関連を見出すところから始まる研究手法である。真の関連であれば，遺伝子が病態に関与することとその遺伝子の個人差によって疾患発症リスクが異なるという 2 つの意味をもってくる。本稿では，アレルギー疾患のうち，遺伝子に関する情報の最も多い小児喘息，次に知見の多いアトピー性皮膚炎を中心にこれまでの研究成果をまとめ，遺伝子解析研究の意義と限界について考えてみたい。

I アレルギー疾患の遺伝子解析

アトピー（特異 IgE が陽性），総 IgE 値，喘息，アトピー性皮膚炎などと有意な相関を示したとする遺伝学的研究の報告は，2006 年の Orber の総論[1]の時点で 120，その後の 2 年間で 53 の遺伝子が追加されている。これまでの研究の総括から，大部分の遺伝子多型の表現型に及ぼす影響が単一遺伝子病のようには大きくないこと，病気の定義（表現型の定義）の問題，遺伝子多型の効果が環境要因などの影響を受けるため，遺伝子解析研究の結果は再現性が乏しいということが共通認識になってきた[2]。

Daley ら[3]は，喘息，アトピー，アトピー性喘息，気道過敏性などと相関が陽性に報告されたことのある 93 の遺伝子について，再検討を行った。調べた遺伝子多型の数で補正した場合，有意と判断される遺伝子はなく，遺伝子単位での補正で，4 種の表現型と相関しているとみなされたものは 12 遺伝子（13%）となった。筆者らも日本人の小児気管支喘息，成人気管支喘息，アトピー，総 IgE 値を対象として，23 の最も有望な候補遺伝子を選んで検討したが，多重比較を考慮しない基準でも，いずれかの表現型と相関が示唆されたものは 11 遺伝子となり 50%を下回った[4]。また，観察された多型のオッズ比（OR）でみると，Daley らは 1.4 以下，筆者らの研究でも *IL13* の SNP の OR が 3.01 である以外は 1.28～1.56 の範囲であった。

II 小児期発症喘息の遺伝子解析

多因子遺伝子病の遺伝学的または遺伝子解析による疾患感受性遺伝子の同定は，1990 年ごろより，技術的に可能となってきた。当初は，連鎖解析の手法を拡張した「罹患同胞対解析」と，生物学的情報から関与が予想される遺伝子，すなわち候補遺伝子を取り上げ，症例と対照について遺伝子多型を調べ疾患との相関をみる「候補遺伝子の相関研究」の 2 つの方法論がとられた。前者のアプローチから，初めて大きな成果を上げたのは，2002 年の *ADAM33* 遺伝子の同定であった[1]。その頃になると，アレルギー疾患の感受性への遺伝子多型の影響にあまり大きいものはなく，十分な

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表 1　小児（期発症）気管支喘息と喘息関連表現型に関する GWAS 研究

遺伝子	染色体部位	表現型	人　種	規　模	再現性	研究者，発表年
ORMDL3	17q12-12.1	小児期発症喘息	白人	994 喘息，1,243 対照	複数研究で再現	Moffatt 2007*
FCERIA *RAD50*	1q23 5q23	Total IgE アトピー性喘息	ヨーロッパ人	1,530 全体	4 つの独立サンプル（9769 人）	Weidinger 2008*
IL1RL1 *WDR36* *MYB* *IL33*	2q12 5q22 6q23 9q24	血中好酸球数 喘息	アイスランド人	9,392 全体 7,996 喘息，44,890 対照	好酸球値 12,118 ヨーロッパ人 5,212 東アジア人	Gudbjartsson 2009*
PDE4D	5q12	小児喘息	白人	359 喘息 846 対照	4,342 喘息患者含む 18,891 人の白人	Himes 2009*
TLE4	9q21.31	小児喘息	メキシコ人	492 小児患者と両親	177 小児患者と両親	Hancock 2009*

* 文献は 2）Holloway ら，2010 を参照のこと。

統計学的検出力を得るために，数百，数千例のサンプルが必要であることが認識されてきた。罹患者の同胞や両親のサンプルが必要となる連鎖解析は，症例対照研究に比べて，サンプル収集において不利であり，しだいに候補遺伝子の症例対照研究が主流のアプローチとなっていった。

ゲノムワイド相関解析（genome wide association study：GWAS）は，ゲノム上の数十万個の SNP を一気に調べ，相関を検討するところから遺伝子のスクリーニングを開始する研究法である。GWAS による遺伝子の同定のアレルギー疾患分野における最初の大きな成功例は，2007 年の小児喘息における *ORMDL3* 遺伝子の報告[2]である。それ以降，アレルギー疾患の遺伝子解析は，GWAS による研究がブームとなっている。表 1 には，小児気管支喘息に関連する研究をまとめている。GWAS では，ゲノムワイドの有意差（$P<5\times10^{-8}$）を求められるため，候補遺伝子の症例対照研究より 1 桁以上多い症例数，対照数を要求され，研究発表論文内での再現性も伴っていることが多く，得られた結果の信頼性，再現性は GWAS 時代以前に行われた研究より良好と考えられる。

昨年，喘息については，白人集団に関する大規模なメタアナリシス的な研究が発表された[5]。この研究は，23 の研究，全体で 10,365 人の喘息患者と，16,110 人の対照者をまとめて解析したものである。表 2 は，全喘息患者，全対照者，小児期発症の患者とそれに対する対照者，遅発性喘息患者と，それに対する対照者における約 58 万の SNP のタイピングについて，全喘息・全対照者の比較で，ゲノムワイドの有意水準をクリアした領域を示している。*GSDMA*，*GSDMB*，*ORMDL3* は 1 つの疾患感受性座とみなされ，実質的に 6 つの遺伝子座が有意であり，それに次ぐものとして 3 つの領域があるという状況である。小児期発症喘息のほうが，遺伝子多型との相関は強く出る傾向にあり，とくに *ORMDL3/GSDMB/GSDMA* は，小児期発症喘息に特有な遺伝子座とみられる。Wu ら[6]は，メキシコ人の小児気管支喘息のサンプルに対して，ゲノムワイドにタイピングを行い，これまでの一度でも有意と報告された遺伝子 230 以上の SNP について絞って検討を行った。遺伝子単位の有意差検定で *P* 値 0.009 以下の遺伝子として，*IL11RL1*，*TGFB1*，*DPP10*，*IL18R1* の 4 つの遺伝子が再現されたとされ，白人での結果（表 2）とは異なっている。黒人の 2 つの集団の GWAS から *P* 値 10^{-5}以下で有意とされた成人喘息の感受性遺伝子，*ADRA1B*，*PRNP*，*DPP10* が白人の集団では再現できないという報告[7]もあり，人種差は遺伝子解析の結果に大きく影響している。

アジア人については，今年になって，Illumina 社の BeadChip を用いてゲノムワイドの一次スクリーニングから始めた日本人小児気管支喘息の GWAS の結果が発表された[8]。本研究では，15

表 2　ヨーロッパ系民族の大規模 GWAS 研究の結果

遺伝子	SNP rs 番号	小児期発症喘息 OR (95%CI)	P	遅発性喘息 OR (95%CI)	P	全喘息 OR (95%CI)	P
ゲノムワイドに有意（全喘息で $P<7.2\times10^{-8}$）							
IL18R1	rs3771166	0.85(0.81～0.90)	1.1×10^{-8}	0.94(0.85～1.04)	1.9×10^{-1}	0.87(0.83～0.91)	3.4×10^{-9}
HLA-DQ	rs9273349	1.14(1.08～1.22)	1.9×10^{-5}	1.26(1.16～1.37)	3.9×10^{-8}	1.18(1.13～1.24)	7.0×10^{-14}
IL33	rs1342326	1.27(1.17～1.38)	1.6×10^{-8}	1.12(0.99～1.26)	6.8×10^{-2}	1.2(1.13～1.28)	9.2×10^{-10}
SMAD3	rs744910	0.89(0.84～0.93)	8.1×10^{-6}	0.94(0.87～1.0)	1.4×10^{-1}	0.89(0.86～0.92)	3.9×10^{-9}
GSDMB	rs2305480	0.76(0.72～0.81)	6.4×10^{-23}	1.03(0.94～1.13)	4.9×10^{-1}	0.85(0.81～0.90)	9.6×10^{-8}
GSDMA	rs3894194	1.26(1.19～1.33)	3.0×10^{-17}	1.02(0.94～1.11)	6.0×10^{-1}	1.17(1.11～1.23)	4.6×10^{-9}
IL2RB	rs2284033	0.92(0.87～0.97)	1.6×10^{-3}	0.86(0.80～0.94)	4.2×10^{-4}	0.89(0.86～0.93)	1.2×10^{-8}
追加的な座位（全喘息で $P<5\times10^{-7}$）							
SLC22A5	rs2073643	0.89(0.84～0.93)	7.6×10^{-6}	0.94(0.87～1.0)	1.5×10^{-1}	0.9(0.87～0.94)	2.2×10^{-7}
IL13	rs1295686	0.85(0.79～0.90)	3.3×10^{-7}	0.94(0.85～1.04)	2.6×10^{-1}	0.87(0.83～0.92)	1.4×10^{-7}
RORA	rs11071559	0.88(0.81～0.95)	1.0×10^{-3}	0.78(0.69～0.88)	5.7×10^{-5}	0.85(0.80～0.90)	1.1×10^{-7}

OR：odds ratio　95%CI：OR の 95%信頼区間　　(Moffatt ら[5]，2010)

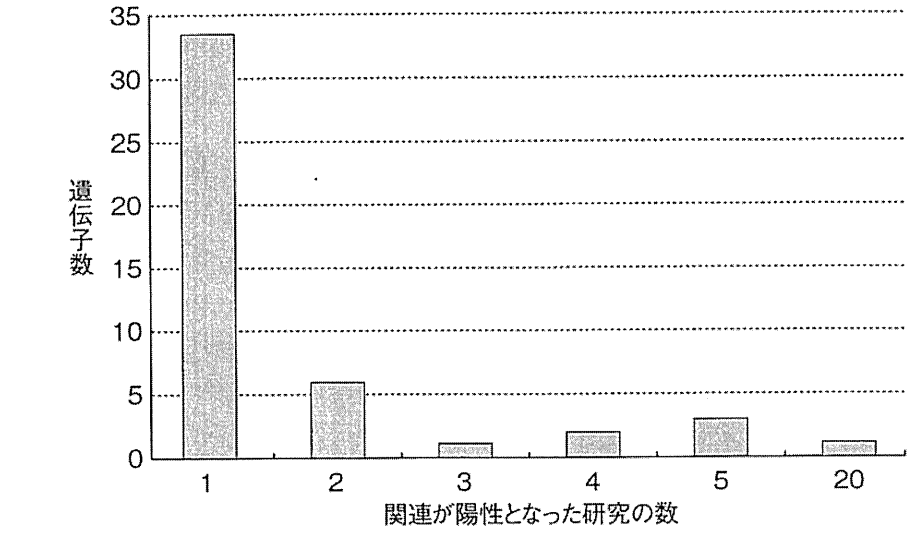
歳未満の喘息と診断された小児 978 名と，対照としてアレルギー疾患がない成人 2402 名とを比較している。Cochran-Armitage トレンドテストでゲノムワイドでの有意差（$P<1\times10^{-8}$）を示した 5 つの SNP は 6 番染色体の HLA 領域のものであった。3 つの SNP について，さらに 1 つの日本人，1 つの韓国人の独立の症例対照集団での再現性の検討も行っている。その結果，*HLA-DPA1* と *HLA-DPB1* の中間に位置する SNP がメタアナリシスで，$P=2.3\times10^{-10}$を示し有意と考えられた。この SNP のアレルの OR は 1.40 であった。他の民族で喘息の感受性遺伝子とされた遺伝子の再現性をみると，*P* 値が 0.05 以下を有意とする緩い基準としてみた場合，*ORMDL3/GSDMB/GSDMA*，*IL5/RAD50/IL13*，*HLA-DR/DQ*，*SMAD3* が有意の相関ということになったが，*PEDE4D*，*TLE4*，*DENND1B*，*IL18R1*，*IL2RB* は有意ではなかった。

本研究から，日本人（東アジア人）の小児気管支喘息の主要な感受性遺伝子は *HLA-DPA1/DPB1* 領域にあり，やはり他の民族での結果とやや異なる様相を示している。

Ⅲ アトピー性皮膚炎の遺伝子解析

アトピー性皮膚炎の遺伝子解析に関する情報は，2010 年，Barnes がまとめている[9]。候補遺伝子的アプローチからは，2009 年の 6 月時点で，111 の研究発表があったとしている。調べられた 81 の遺伝子のうち，半分以上にあたる 46 で少なくとも 1 回，陽性の結果が得られている。そのうち，15 の研究では，相関が再現されなかった。13 の研究では，最低 1 つの研究で再現されている（図）。

アトピー性皮膚炎のリスクに関係する遺伝子のなかで，最も頻回に調べられ，再現されたものは filaggrin（*FLG*）遺伝子である。*FLG* の機能喪失型の変異がアトピー性皮膚炎のリスクを大きく上げることは，2006 年に初めて報告された[9]。filaggrin は角化細胞から 40 万の分子量をもつ profilaggrin として分泌され，その後 10～12 個の分子量 3 万 7000 の filaggrin 蛋白となり，表皮におけるバリア機能と保湿機能に非常に重要な役割を担っている。ヨーロッパ人では，R501X，2282del4 の 2 種類の変異が一般集団で約 4%のアレルにみつかるという。アトピー性皮膚炎の患者では，前者は 18%，後者は 48%のアレル頻度



ADAM33	*IL12B**	*CD14**	*TANTES**	*CMA1*	*IL4**	*FLG**
*BDNF**	*IL12RB1*	*DRFB1*		*IL13**	*ILRA**	
BFL1	*IL5*	*GSTP1*			*SPINK5**	
CARD12	*IRF2*	*IL18**				
*CARD15**	*NAT2**	*NOD1*				
COL29A1	*PHF11*	*TIM1*				
*CSF2**	*SCCE*					
CSTA	*SMPD2*					
CTLA4	*SOCS3*					
CYSLTR1	*ST2*					
*EOTAXIN**	*TAP2*					
FCER1B	*TGFB1*					
GATA3	*TIM4*					
GSTT1	*TLR2*					
HNMT	*TLR9*					
*IL10**	*TOLLIP*					
	VEGF					

* 追試で関連が陰性となった遺伝子

結果が陰性だった遺伝子：*C3*, *CCR4*, *CMA1*, *DEFA4*, *DEFA5*, *DEFA6*, *GPRA*, *IFNG*, *IL13RA*, *IL1B*, *IL1RN*, *IL6*, *IL8*, *IL8RA*, *IL8RB*, *IRAKM*, *KLK7*, *LMP2*, *LMP7*, *MCP1*, *MHC2TA*, *MIP1A*, *NALP1*, *NALP12*, *NALP3*, *NGFB*, *NPSR1*, *PDYN*, *SETDB2*, *STAT6*, *TAP1*, *TARC*, *TIM3*, *TLR6*, *TNFA*

図　アトピー性皮膚炎に関連すると報告された遺伝子（Barnes[9], 2010）
相関が有意と報告した研究報告の数を横軸に，それぞれの遺伝子の数を縦軸に表わしている。

であったという[9]。24 の研究をまとめた大規模なメタアナリシスでも 2 つの変異は有意とされ，OR は 3 をこえている[9]。日本人では，134 人の対照と 127 人のアトピー性皮膚炎の変異の同定が行われ，表 3 に示すような変異がみつかっている[10]。アトピー性皮膚炎の患者では，S2554X と S2889X が多いことが示されており，R501X はみつからなかった。調べられた変異を合わせた場合，対照と患者のそれぞれ 3.7%，26.7%が変異をもっていた。対照と患者の関連の検定の P 値は 1.2×10^{-7}で，優性モデルでの OR（その 95%信頼区間）は，9.42（3.63～24.45）と高い値を示している。また，喘息のあり無しでアトピー性皮膚炎を分けた場合でも，疾患との相関は有意であり OR はあまり変化していない。一方，137 名の喘息患者との関連を調べてみると，表 4 に示したように，アトピー性皮膚炎のある喘息患者では 22%に変異があり，アトピー性皮膚炎のない喘息患者では 5.9%であり，前者と対照の相関の $P=0.012$，OR は 7.37（1.77～30.67），後者と対照の相関は，$P=0.556$，OR＝1.6（0.50～5.22）と明らかに対比をなしている。これらのデータから，*FLG* 遺伝子の変異は，アトピー性皮膚炎の発症により強く関係していると考えられる。*FLG* は喘息の発症，アレルギー性鼻炎，特異 IgE の上昇との関連の報告[9]もあり，アレルギー疾患関連遺伝子のなかで，遺伝子の個人差が表現型に与える変化が現時点で最も明確な遺伝子である。

アトピー性皮膚炎に関する GWAS 解析からの研究報告は少ない。2009 年にドイツ人についての解析[11]，2011 年に中国人での解析[12]が報告さ

表 3　日本人における *FLG* 変異とアトピー性皮膚炎との相関

genotype	R501 Con	R501 AE	3221delA Con	3221delA AE	S1695X Con	S1695X AE	Q1701X Con	Q1701X AE	S2554X Con	S2554X AE	S2889X Con	S2889X AE	S3296X Con	S3296X AE	K4022X Con	K4022X AE	合計 Con	合計 AE	合計 AE AS+	合計 AE AS−
AA	134	172	133	163	133	172	134	169	133	162	132	152	134	166	134	169	129	126	53	73
																	96.3%	73.3%	72.3%	73.7%
Aa	0	0	1	9	1	0	0	3	1	10	2	20	0	6	0	3	5	41	18	23
																	3.7%	23.8%	24.7%	23.2%
aa	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	2	3
																	0.0%	2.9%	2.7%	3.0%
total	134	172	134	172	134	172	134	172	134	172	134	172	134	172	134	172	134	172	73	99

AE：アトピー性皮膚炎　AS：喘息　Con：コントロール　　(Osawa ら[10], 2010)

表 4　日本人における *FLG* 変異と喘息との相関

genotype	R501 Con	R501 AS	3221delA Con	3221delA AS	S1695X Con	S1695X AS	Q1701X Con	Q1701X AS	S2554X Con	S2554X AS	S2889X Con	S2889X AS	S3296X Con	S3296X AS	K4022X Con	K4022X AS	合計 Con	合計 AS	合計 AS AE+	合計 AS AE−
AA	134	137	133	137	133	137	134	137	133	133	132	132	134	136	134	136	129	126	14	112
																	96.3%	92.0%	77.8%	94.1%
Aa	0	0	1	0	1	0	0	0	1	4	2	5	0	1	0	1	5	11	4	7
																	3.7%	8.0%	22.2%	5.9%
aa	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
																	0.0%	0.0%	0.0%	0.0%
total	134	137	134	137	134	137	134	137	134	137	134	137	134	137	134	137	134	137	18	119

AE：アトピー性皮膚炎　AS：喘息　Con：コントロール　　(Osawa ら[10], 2010)

れている。前者では，11 番染色体の 11q13.5 の領域を，後者では *FLG* 以外に新しい 2 か所の領域（5q22.1，20q13.33）を見出している。

Ⅳ 遺伝子解析研究の意義と限界

GWAS を用いる遺伝子解析は，事前の生物学の知識に基づかない新たな疾患関連遺伝子の同定が可能で，候補遺伝子的アプローチより，労力がかかるものの，パラダイムシフトを起こしうる可能性がある研究法である。

みつかってきた遺伝子の多型からアレルギー疾患の発症予測は可能であろうか？　多くのアレルギー関連遺伝子の多型は表現型に対する影響が小さいため，表現型の発現を十分表現するためには，多くの多型が必要となる。あるシミュレーションでは，相対リスクとして 1.5 の因子が 50 個あると，臨床診断の指標である area under the ROC curve の値が 0.8 となるという[13]。これから推定すると，OR にして 1.5 以下がほとんどのアレルギー関連遺伝子多型を利用するとすれば，アレルギー疾患の発症を十分に予測するために 50 をこえる数が必要ともいえ，現時点では，発症予測のために十分な数の遺伝子多型はみつかっていない。

現在，アレルギー疾患の発症予測に最も役立つ可能性のある遺伝子は *FLG* 遺伝子であろう。前述のように，この遺伝子の変異をもつと，アトピー性皮膚炎になるリスクは数倍以上に高くなり，喘息のリスクにもなっている。日本人における，正確な遺伝子変異のリスクの評価，環境要因との関連についてのコホート研究が今後望まれる。

現時点で臨床に役立つ情報が遺伝子解析から得られたとは言い難い状況であるが，*ADAM33* や *ORMDL3* など，候補遺伝子アプローチでは見出されなかったであろう遺伝子の同定が病態の理解の進歩と新たな治療法開発への可能性を示した意義は大きい。しかし，意義のある研究結果を出すためには，サンプル数が数千人，予算は数億円以上の大規模なプロジェクトが必要である。

Key Points

❶アレルギー疾患の感受性に関連する遺伝子多型は，一般にその効果が小さく，ORが2以下のものがほとんどである。

❷filaggrin 遺伝子の変異は例外的に影響が大きく，アトピー性皮膚炎に対し少なくともORが3以上あり，喘息へのリスクファクターともなる。

❸遺伝子解析は，アレルギー疾患の病態に関係する新しい遺伝子の同定のため重要な研究手法である。

❹遺伝子解析による情報は，アレルギー疾患の治療の選択，予後の予測に役立つまでにはなっていない。

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お知らせ（1）

第7回日本統合失調症学会

会　期：2012年3月16日（金），17日（土）
会　場：愛知県産業労働センター（ウインクあいち）
会　長：尾崎紀夫（名古屋大・院・精神医学・親と子どもの心療学分野）
テーマ：統合失調症患者・家族のニーズを適える研究成果を目指して
特別講演：統合失調症と双極性障害の共通病態：ゲノム医学の観点から（Michael O'Donovan　Cardiff University），認知リハビリテーション：治療意欲を考慮して（Alice Medalia Columbia University），死後脳解析（Sabine Bahn University of Cambridge）
教育講演：貝淵弘三（名古屋大・神経情報薬理学），鍋島俊隆（名城大学比較認知科学研究所），定藤規弘（自然科学研究機構生理学研究所）
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Letters to the Editor

食物アレルギーの乳幼児へのアミノ酸調整粉末投与の際はビオチンの補充を

千葉大学大学院医学研究院公衆衛生学
鈴木洋一

ビオチンは，水溶性のビタミンであり，ヒトでは4種類あるカルボキシラーゼの補酵素として働いている．先天代謝異常症であるホロカルボキシラーゼ合成酵素(HLCS)欠損症や，ビオチン摂取量の不足によって，カルボキシラーゼ活性が低下すると複数の代謝経路の代謝異常が起こり，マルチプカルボキラーゼ欠損症と言われる症状を呈する．栄養性のビオチン欠乏症は，従来起こりにくく，臨床的に問題となるのは稀と考えられてきた．しかし，近年，食物アレルギーの治療のためアミノ酸調整粉末のみで数カ月栄養した乳児を中心に，マルチプルカルボキシラーゼ欠損症の症状を示したとする論文，学会発表が散見されるようになった．現在市販されているアミノ酸調整粉末には，ビオチンが添加されていないためにビオチン欠乏症が起こるのである．筆者は，マルチプルカルボキシラーゼ欠損症の鑑別診断のため，HLCS遺伝子の遺伝子診断を行ってきたことから，興味を持ち，近年のアミノ酸調整粉末栄養によるビオチン欠乏症の報告例について文献検索を行った．

ビオチン欠乏症の診断が確実で論文等になっている報告等を藤本らが自件例とともに2005年にまとめている[1]．表は2005年以降の症例報告も含めてまとめたものである．学会発表のみで情報が不足しているため表に含めることが出来ないビオチン欠乏症の報告もあり，2008年の小児科学会では，虫本ら7例の報告，他に2例の症例報告があった．2009年には1例，2010年はすでに3例の学会報告がなされている．これらのことは，食物アレルギーの低年齢化と有病率の上昇によって，アミノ酸調整粉末で栄養される乳児が増加していることを反映しているのではないかと推定される．新生児-乳児消化管アレルギー疾患研究会が最近発表した「新生児-乳児消化管アレルギー診断治療指針」においても食物アレルギーの治療として，アミノ酸調整粉末の利用が薦められている．ガイドラインの発表でさらに利用者が増えることも考えられる．

ミルクへのビオチンの添加は，現在，食品衛生法で認められていない．アミノ酸調整粉末へのビオチンの添加が認められるまで，消化管アレルギーの治療に当たる医師は，アミノ酸調整粉末の利用の際にビオチン欠乏症の予防に注意すべきことを提案したい．

筆者は，平成22年度の厚生労働省の難治性疾患克服研究事業「ビオチン代謝異常症の鑑別診断法と治療方法の開発」の研究において，アミノ産調整粉末投与によるビオチン欠乏症の発生状況の全国調査を予定している．アレルギー学会の会員諸氏の調査への協力を願う次第である．
(ビオチン代謝異常のホームページ　http://www.m.chiba-u.ac.jp/class/pubheal/biotin)

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利益相反（conflict of interest）に関する開示：著者全員は本論文の研究内容について他者との利害関係を有しません．
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表　治療用ミルクによるビオチン欠乏症の報告

No	年	年齢	性	ミルク	血清ビオチン (ng/ml)	有機酸尿	報告者	文献
1	1991	3カ月	男	EF	ND	+	阿部	1)
2	1996	11カ月	男	EF	1.5	+	樋口	1)
3	1996	5カ月	男	EF	1.4	+	樋口	1)
4	1998	14日	男	S-22	2.4	+	高野	1)
5	2001	4歳	女	EP	NT	NT	西原	1)
6	2001	11カ月	男	NOB	1.7	NT	西原	1)
7	2002	4歳	男	EF	1.6	+	細谷	1)
8	2002	5カ月	女	EF	1.8	+	細谷	1)
9	2004	5カ月	男	EF	1.6	+	藤本	1)
10	2005	4カ月	女	?	1.6	+	河場	2)
11	2007	4カ月	女	EF	ND	+	真々田	3)
12	2009	5カ月	女	NMA1	0.4	+	後藤	4)
13	2009	5カ月	男	MHP	NT	NT	加瀬	5)

EF：エレンタールフォーミュラ，S-22：有機酸代謝治療用ミルク，MHP：エピトレス，NMA1：ニューMA-1，NOB：のびやか，NT：施行せず，ND：検出感度以下

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ORIGINAL ARTICLE

Association of the *MMP9* gene with childhood cedar pollen sensitization and pollinosis

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Matrix metalloproteinase 9 (*MMP9*) gene has been shown to be involved in the pathogenesis of allergic rhinitis (AR) and asthma. Previous studies suggested that single-nucleotide polymorphisms (SNPs) of the *MMP9* gene conferred a risk for childhood asthma. However, whether the SNPs confer a risk for AR has not been previously investigated. The objective of this study was to investigate whether SNPs of the *MMP9* gene are associated with risk of seasonal AR (pollinosis), perennial AR and allergen sensitization. A total of 670 school children were recruited in Japan and genotyped for functional polymorphism in the promoter (–1590C/T: rs3918242) and three amino-acid substitutions (R297Q: rs17576; P574R: rs2250889; R668Q: rs17577). Serum levels of total and specific IgE were determined. Disease status and other clinical characteristics of the subjects were investigated using a questionnaire. Associations between the *MMP9* SNPs and both AR and serum IgE levels were evaluated. –1590C/T showed significant association with cedar pollinosis (corrected *P* (*Pcor*)=0.039). R668Q was in strong linkage disequilibrium (LD) with –1590C/T and showed significant association with cedar pollinosis (*Pcor*=0.023) and serum cedar pollen-specific IgE level (*Pcor*=0.022). A haplotype associated with –1590T and 668Q showed a significant association with cedar pollinosis, orchard grass pollinosis and cedar pollen-specific IgE (*Pcor*=0.0012, *Pcor*=0.0059 and *Pcor*=0.0041, respectively). R297Q and P574R were in weak LD with the rest of the SNPs and did not show significant association with disease. Compared with wild-type MMP9 protein (279R–574P–668R), a variant enzyme (279R–574P–668Q) that showed association with pollinosis had lower activity. However, lower enzyme activity was not associated with disease risk because another variant (279Q–574R–668R) showed lower enzyme activity but was not associated with pollinosis. The –1590T allele and its corresponding haplotype was associated with higher promoter activity and with pollen-specific IgE levels and pollinosis, suggesting that –1590C/T may have more impact on sensitization and disease development than R668Q. Our results suggest that the *MMP9* gene confers susceptibility to cedar pollinosis in Japanese children. The *MMP9* gene may be associated with pollinosis through sensitization processes.
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Keywords: allergic rhinitis; association; cedar pollinosis; haplotype; matrix metalloproteinase; *MMP9* gene; serum IgE; SNP

INTRODUCTION

The main symptoms of allergic rhinitis (AR) are nasal congestion caused by mucosal edema; runny nose caused by hypersecretion; and repetitive sneezing. Pollinosis is a seasonal type of AR caused by an allergic reaction to pollen. Japanese cedar pollen is the most common causative allergen for pollinosis in Japan. According to a recent study, the prevalence rates of AR and cedar pollinosis in 2006 were 27.2 and 8.0%, respectively; both of which were higher than the rates in 1996.[1]

Matrix metalloproteinases (MMPs) are a family of enzymes that not only degrade the extracellular matrix but also mediate activation of other proteases and secretion of cytokines, thereby affecting inflammatory processes.[2,3] MMP9, also known as gelatinase B, was shown to be an important mediator of inflammation in a murine model of asthma[4,5] and in immune complex-mediated lung injury.[6] In a mouse asthma model, loss of the *MMP9* gene was found to inhibit the development of allergic inflammation by impairing the recruitment of dendritic cells (DCs) into the alveoli and the local production of proallergic chemokines by DCs.[7] MMP9 levels in bronchoalveolar lavage and in the plasma are positively associated with allergen challenge[8] and severity of disease[9,10] in asthmatic patients, suggesting the involvement of MMP9 in asthma pathogenesis in humans. Compared with what is known about the role of MMP9 in asthma, knowledge regarding AR is limited. It has been demonstrated that nasal provocation with allergen induces release of MMP9 during the late-phase inflammatory response.[11] Lim *et al.*[12] reported that airway remodeling associated with long-term allergen challenge can occur in the nasal mucosa and the lung, and that expressions of MMP9 and tissue inhibitors of metalloproteinase 1 (Timp-1) were increased in

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subepithelial regions. Shimizu *et al.*[13] reported that the attenuating effect of tranilast on MMP9 production from nasal fibroblasts induced by inflammatory stimulation may underlie the therapeutic mode of action of this agent in patients with allergic diseases including AR.

The *MMP9* gene is located at chromosome 20q,[14] where linkage to bronchial hyperresponsiveness[15] and specific sensitization[15,16] has been reported. In a previous association study of the *MMP9* gene with asthma in the Japanese population,[17] single-nucleotide polymorphisms (SNPs) 2127G/A (rs2274755) and 5546G/A (R668Q; rs17577), which were in strong linkage disequilibrium (LD), were shown to be significantly associated with atopic childhood asthma. The SNP 2127G/A was in complete LD with a promoter SNP (−1590C/T, rs3918242) in which the T allele showed higher promoter activity than the C allele in a promoter assay in a bronchial epithelial cell line.[17] There are two more SNPs that change the amino-acid sequence of MMP9: R279Q (rs17576) and P574R (rs2250889). R279Q was shown to be associated with aortic pulse wave velocity and serum MMP9 level.[18] However, the effects of these three amino-acid changes on enzyme activity or function at the molecular level have not been reported previously. To our knowledge, no studies have been conducted to investigate whether the *MMP9* SNPs that showed association with asthma confer a risk for AR or allergic sensitization (atopy).

To investigate the association between the *MMP9* gene and both AR and sensitization to common aero-allergens, we genotyped the functional promoter SNP and three potentially functional coding SNPs, and evaluated the symptoms of AR and serum total/specific IgE levels in Japanese school children. We also evaluated the effect of the amino-acid changes on MMP9 enzyme activity.

MATERIALS AND METHODS

Subjects

Japanese elementary school children in Chiba and Yamanashi prefectures were recruited for this study. A total of 473 school children aged 6–12 years were enrolled in Chiba city in Chiba prefecture, details of which were described previously,[19] and 260 school children within the same age range were enrolled in Hokoto city in Yamanashi prefecture. Blood samples were collected for serum IgE measurement and DNA preparation from 410 children in Chiba and 260 children in Yamanashi in July and August 2006.

Total and specific serum IgE levels were assayed using the CAP-radioallergosorbent test (Pharmacia Diagnostics, Uppsala, Sweden). Eight specific IgEs were measured house dust mite (*Dermatophagouides pteronyssinus*), dog (*Canis familiaris*) dander, cat (*Felis domesticus*) dander, black mold (*Alternaria alternata*), cedar (*Cryptomeria japonica*) pollen, orchard grass (*Dactylis glomerata*), egg white, and golden, black bellied, or djungarian hamsters (*Mesocricetus auratus/Cricetus cricetus/Phodopus sungorus*). Atopy was defined as the presence of ($\geqslant 0.35\,\mathrm{IU\,ml^{-1}}$) specific IgE positive against at least one of the assessed allergens. To assess the status of allergic diseases, questionnaires based on the International Study of Asthma and Allergies in Childhood[20] were used. Subjects with symptoms of AR in any month from February to May and positive serum IgE to cedar pollen (class 1 and higher) were defined as having cedar pollinosis. Subjects with symptoms of AR in any month from May to July and positive serum IgE to orchard grass pollen were defined as having orchard grass pollinosis. Mite-positive perennial AR was diagnosed in children who had symptoms throughout the duration of a year and positive serum mite-specific IgE. Children who were negative for IgE specific to any assessed allergen and had no allergic diseases were assigned to the non-atopic control group. This study was approved by the ethics committee of Chiba University Graduate School of Medicine.

SNP selection and genotyping

In a previous association study by Nakashima *et al.*,[17] 2127G/A (rs2274755) and 5546G/A (R668Q: rs17577) showed significant association with asthma in Japanese children. We selected these as candidate SNPs for AR. In the same study, −1590C/T (rs3918242) was shown to be a functional SNP and in complete LD with 2127G/A in 24 individuals. This SNP, however, was not genotyped in all samples. In light of the importance of this SNP, we genotyped −1590C/T in the present study. We also included two non-synonymous SNPs, R279Q (rs17576) and P574R (rs2250889), because of their possible effect on enzyme activity and susceptibility to AR.

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, Buckinghamshire, UK) according to the manufacturer's standard protocol.

Genotyping was performed using the SNaPshot method (Applied Biosystems, Foster City, CA, USA). Multiplex PCR amplification was performed in a 10-μl aliquot of reaction mixture containing 5 ng amplified template DNA, 0.025 U TaKaRa ExTaq HS (TaKaRa Bio Inc., Otsu, Japan), 1 μl 10× Ex buffer, 200 μM of each dNTP and 0.5 μM each of the PCR primer pairs shown in Supplementary Table 1. Amplification was carried out using a GeneAmp PCR System 9700 (Applied Biosystems) according to the following program: initial denaturation at 95 °C for 3 min followed by 40 cycles at 95 °C for 20 s, 60 °C for 30 s and 72 °C for 1 min, with final extension at 72 °C for 5 min. Post-PCR treatment to remove primers and unincorporated dNTPs was performed with SAP (shrimp alkaline phosphatase; TaKaRa Bio Inc.) and *Exo*I (New England Biolabs, Ipswich, MA, USA). PCR products were incubated with 0.5 U SAP and 1 U *Exo*I for 1 h at 37 °C, followed by incubation for 15 min at 80 °C to induce enzyme inactivation. The SNaPshot reaction was performed in 10 μl reaction mixture containing 0.5 μl SNaPshot Ready Reaction Mix, 2 μl SAP/*Exo*I-treated PCR products and 0.1 μl SNaPshot primers, as shown in Supplementary Table 1. SNaPshot primers were designed to anneal adjacent to the SNP 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 to discriminate it 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 35 single-base extension cycles of denaturation at 96 °C for 10 s and annealing and extension at 60 °C for 30 s. Post-extension treatment to remove the 5′-phosphoryl group of the ddNTPs was performed with CIAP (calf intestine alkaline phosphatase; TaKaRa Bio Inc.). 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 and 0.05 μl GeneScan-120 LIZ size standard (Applied Biosystems) and electrophoresed using a 50-cm length capillary with 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 *MMP9* was obtained from normal human small airway epithelial cells using reverse transcriptase PCR and the following primers: forward, 5′-CCC AAG CTG GCT AGC GAC ACC TCT GCC CTC ACC ATG A-3′; reverse, 5′-CCC TCT AGA CTC GAG GTT GGT CCC AGT GGG GAT TTA-3′ (both primers include the 15-bp homology extension for In-Fusion cloning into vector pcDNA3.1). The cDNA fragment was cloned into pcDNA3.1 (+) (Life Technologies, Carlsbad, CA, USA) and digested with *Nhe*I–*Xho*I using the In-Fusion Advantage PCR Cloning Kit (TaKaRa Bio Inc.). The Q279R, R574P and R668Q mutations were introduced by PCR-based site-directed mutagenesis using PrimeSTAR MAX polymerase (TaKaRa Bio Inc.). We constructed four MMP9 expression vectors containing four different haplotypes of *MMP9*: pcDNA3.1–MMP9-H1 (279R–574P–668R: type 1), pcDNA3.1–MMP9-H2 (279Q–574R–668R: type 2), pcDNA3.1–MMP9-H3 (279R–574P–668Q: type 3) and pcDNA3.1–MMP9-H4 (279Q–574R–668Q: type 4). The nucleotide sequences of *MMP9* in these constructs were confirmed by DNA sequencing before transfection of cells.

Stable transformants

HEK293 (human embryonic kidney cell line) cells were cultured in minimum essential medium supplemented with 2 mM L-glutamine, 1% non-essential amino acids, 10% fetal bovine serum and antibiotics. Empty vector pcDNA3.1 or MMP9 expression vectors pcDNA3.1–MMP9 were transfected into the cells

using FuGENE6 transfection reagent (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. Stable transformants were obtained after 2–3 weeks by selection with 600 μg ml^{-1} Geneticin (G-418).

Enzyme activity

Stable transformant cell lines were maintained with 10% fetal calf serum in minimum essential medium. For the enzyme assay, 5×10^6 cells were seeded in a 25-cm^2 flask with minimum essential medium without fetal calf serum. After 24 h of incubation at 37 °C, medium was recovered and stored at −20 °C until use. MMP9 proenzyme secreted into the culture medium was activated with 1 mM 4-aminophenylmercuric acetate before adding to the assay mixture. Protease activity of MMP9 in the conditioned medium was evaluated with synthetic fluorescence peptide as a substrate using SensoLyte 520 MMP9 Assay Kit *fluorimetric* (AanSpec Inc., Fremont, CA, USA). Fluorescence of 5-carboxy-fluorescein was monitored at excitation/emission wavelengths of 490 and 520 nm using infinite F200 (TECAN, Männedorf, Switzerland). BioPlex 200 (Bio-Rad Laboratories, Hercules, CA, USA) was used to measure MMP9 protein concentrations in the medium with Fluorokine MAP assay kit (R&D Systems, Inc., Minneapolis, MN, USA). Standard proteins included in the kit were used to calculate MMP9 protein concentration.

Statistical analysis

We used r^2 as an estimator of the strength of pairwise LD of SNPs.[21] A 2×2 contingency χ^2 test of independence was performed to test an association between genotypes and the disease in a dominant model. Haplotype inference was performed with an expectation–maximization algorithm implemented in SNPAlyze ver.4.1 (DYNACOM, Mobara, Japan). In single SNP association studies, significant values were corrected for number of SNPs and phenotypes tested (Bonferroni's correction). In the haplotype-wise test, significant values were corrected for multiple comparisons by multiplying the *P*-value by the number of haplotypes and phenotypes. The effects of genotypes on $\log_{10}$-transformed total serum IgE levels were evaluated using analysis of variance. Statistical analysis was performed with SPSS software (version 15.0J; SPSS Japan, Tokyo, Japan). Because the number of specific IgE values out of the detection limit (0.34–100.0 U ml^{-1}) was not negligible, we conducted tobit regression analyses using the AER add-on package in R (http://www.r-project.org/) to evaluate the effect of SNPs on allergen-specific IgE values. Tobit regression analysis allows for modeling a continuous variable in which censored values at a specific value were not negligible.[22] A corrected *P* (*Pcor*)-value <0.05 was considered statistically significant.

RESULTS

Association between polymorphisms of the *MMP9* gene and AR

Basic characteristics of the children in this study are shown in Table 1. We measured total and specific serum IgE levels in 670 schoolchildren in Chiba and Yamanashi prefectures. Cedar pollinosis, orchard grass pollinosis and mite-positive perennial AR were diagnosed according to symptoms and serum IgE levels as described above. Of 54 children with orchard grass pollinosis, 48 also met the criteria for cedar pollinosis. A total of 104 children were found to have cedar-only pollinosis and six had orchard grass-only pollinosis. Children who showed no symptoms of AR, asthma, atopic dermatitis or food allergy and were negative for all measured specific IgE were included in the non-atopic control group. There was no difference in age and sex ratio between children with seasonal or perennial AR and the control group.

We genotyped five *MMP9* SNPs in all subjects. The location of these SNPs is shown in Figure 1, with SNP and LD data from the HapMap database.[23] Three SNPs, −1590C/T (SNP1; rs3918242), 2127G/A (SNP2; rs2274755) and 5546G/A (SNP5; rs17577), were in strong LD and had previously been shown to be associated with childhood asthma.[17] SNP5 is an amino-acid substitution, R668Q. SNP3 and SNP4 are also amino-acid substitutions (R297Q (SNP3; rs17576) and P574R (SNP4; rs2250889)). LD between SNP3 and SNP4 was strong (r^2=0.711); however, these two SNPs had minimal LD with any of the other SNPs. All SNPs were in Hardy–Weinberg equilibrium in the case and control groups. Table 2 shows the genotype frequency of each polymorphism and association test results for the patients with AR and the non-atopic controls. Because SNP1 and SNP2 were in almost complete LD, the genotype results were almost the same. Both SNPs showed significant association with cedar and orchard grass pollinosis. In a dominant model (CC vs CT+TT), the odds ratio (OR) of SNP1 was 0.436 (95% confidence interval (CI)=0.252–0.752, *P*=0.0026, *Pcor*=0.039) for cedar pollinosis, and 0.234 (95% CI=0.097–0.566, *P*=0.00071, *Pcor*=0.011) for orchard grass pollinosis. ORs of SNP2 (GG vs GT+TT) were 0.419 (95% CI=0.242–0.726, *Pcor*=0.025) for cedar pollinosis and 0.234 (95% CI=0.097–0.566, *Pcor*=0.011) for orchard grass pollinosis. The ORs of SNP5 (GG vs GA+AA) for cedar pollinosis and orchard grass pollinosis were 0.430 (95% CI=0.255–0.726, *Pcor*=0.023) and 0.241 (95% CI=0.107–0.541, *Pcor*=0.0049), respectively. Neither SNP3 nor SNP4 showed significant association with the two types of pollinosis.

ORs of SNP1 and SNP5 for the 48 patients with cedar and orchard grass pollinosis were 0.183 (95% CI=0.067–0.499, *Pcor*=0.0051) and 0.206 (95% CI=0.085–0.499, *Pcor*=0.0033), respectively. Diagnoses of cedar pollinosis and orchard grass pollinosis were significantly associated (χ^2 test, $P=1.29\times10^{-7}$).

The results of the association test between SNPs and mite-positive perennial AR are also shown in Table 2. Although raw *P*-values for SNP1 and SNP5 were <0.05, none of the SNPs showed significant association with the disease after correction for multiple testing. ORs of SNP1 and SNP5 for this type of AR were 0.484 (95% CI=0.228–1.028, *P*=0.056) and 0.458 (95% CI=0.221–0.938, *P*=0.031), respectively. Among the 51 mite-positive perennial AR patients, 31 (61%) also met the diagnostic criteria for cedar pollinosis. ORs of SNP1 and SNP5 for this group were 0.302 (95% CI=0.108–0.848, *P*=0.018) and 0.351 (95% CI=0.140–0.884, *P*=0.023), respectively. ORs of SNP1 and SNP5 for mite-positive perennial AR-only patients were 0.848 (95% CI=0.312–2.29, *P*=0.742) and 0.648 (95% CI=0.240–1.75, *P*=0.390), respectively. Diagnoses of cedar pollinosis and mite-positive perennial AR were not significantly associated (χ^2 test, *P*=0.303).

Table 1 Characteristics of the study population

	Whole study population	*Cedar pollinosis*	*Orchard grass pollinosis*	*Mite-positive perennial AR*	*Non-atopic control*	*Atopic*[a]
Number	670	152	54	51	108	495
Age, mean (years)	9.37	9.42	9.44	9.29	9.23	9.36
Age, range (years)	6–12	6–12	6–12	6–12	6–12	6–12
Sex (male:female)	1.06:1.0	1.41:1.0	2.6:1.0	1.83:1.0	1.0:1.30	1.0:0.86
Mean total IgE (log(IU ml^{-1}))	2.10	2.53	2.67	2.51	1.31	2.36

Abbreviation: AR, allergic rhinitis.
[a]Positive for at least one specific IgE, regardless of allergic disease status.

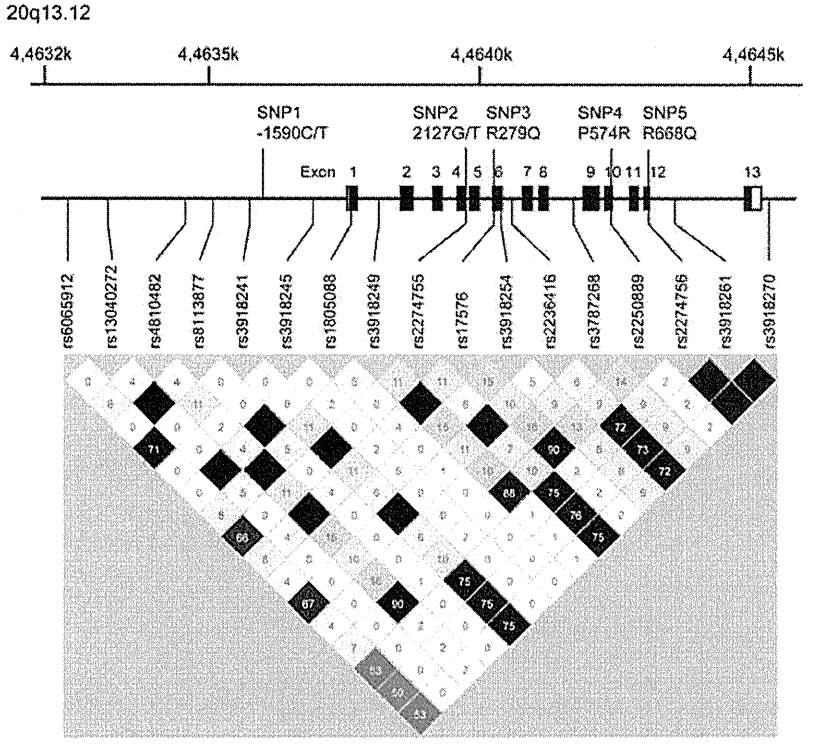

Figure 1 Structure and location of SNPs of the *MMP9* gene. Exons are shown by squares. Filled squares are coding exons. Five SNPs genotyped in this study are shown above the gene map, and HapMap SNPs and their LD status are shown underneath. Data used for LD are based on Japanese data in HapMap data Phase III/Rel3, May 10 on NCBI B36 assembly, dbSNP b126. The nucleotide position is based on February 2009 (GRCh37/hg19) assembly. SNP1 was not found in HapMap database but was known to be in complete LD with rs2918241 and SNP2.[17] Numbers in diamonds in the LD map are $r^2 \times 100$.

Table 2 Association between SNPs of *MMP9* and cedar pollinosis

	Genotype frequency												*Pcor*		
	Cedar pollinosis			*Orchard grass pollinosis*			*Mite-positive perennial AR*			*Control*					
SNP	*11*[b]	*12*[c]	*22*[d]	*11*[b]	*12*[c]	*22*[d]	*11*[b]	*12*[c]	*22*[d]	*11*[b]	*12*[c]	*22*[d]	*Cedar pollinosis vs control*[a]	*Orchard grass pollinosis vs control*	*Mite-positive perennial AR vs control*
−1590C/T (SNP1)	0.783	0.204	0.013	0.870	0.111	0.019	0.765	0.235	0.000	0.611	0.343	0.046	**0.039**	**0.011**	0.844
2127G/T (SNP2)	0.789	0.191	0.020	0.870	0.111	0.019	0.725	0.275	0.000	0.611	0.343	0.046	**0.025**	**0.011**	>1
R279Q (SNP3)	0.421	0.493	0.086	0.463	0.426	0.111	0.480	0.460	0.060	0.509	0.435	0.056	>1	>1	>1
P574R (SNP4)	0.520	0.408	0.072	0.574	0.333	0.093	0.608	0.333	0.059	0.556	0.417	0.028	>1	>1	>1
R668Q (SNP5)	0.737	0.237	0.026	0.833	0.130	0.037	0.725	0.275	0.000	0.546	0.398	0.056	**0.023**	**0.0049**	0.466

Abbreviations: AR, allergic rhinitis; MMP9, matrix metalloproteinase 9; SNP, single-nucleotide polymorphism.
[a]*P*-values for association between an SNP and a phenotype in the dominant model. *Pcor*-values are calculated by multiplying raw *P*-value by 15 (that is, number of SNPs tested×number of diseases).
[b]Homozygous for major allele.
[c]Heterozygous.
[d]Homozygous for minor allele.
Significant *Pcor*-values (<0.05) are in boldface.

Because the numbers of subjects with orchard grass pollinosis and mite-positive perennial AR were similar, the association of SNP1, SNP2 and SNP5 with mite-positive perennial AR may be weaker than that with orchard grass (with or without cedar) pollinosis.

Association between SNPs and serum IgE levels

In the analysis of serum IgE levels, all individuals were included, irrespective of the presence of atopy or disease status. We first examined the distribution of log-transformed total serum IgE [log(-total IgE)] in the 670 school children (data not shown). The rate of values lower than the cutoff was low and the shape of the distribution was almost normal; therefore, we evaluated the association between SNPs and log(total IgE) using analysis of variance. By contrast, because censored values were not negligible, we evaluated the association between SNPs and log-transformed specific IgE values with tobit regression analysis. The effects of SNPs on the IgE values were adjusted for age and sex, and the results of the tests are shown in Table 3. We excluded SNP2 from this analysis because it was thought to be a

Table 3 Association between SNPs and serum IgE levels

			log(total IgE)			*log(cedar IgE)*			*log(orchard grass IgE)*			*log(mite IgE)*		
SNP	*Allele*	*Number of subjects*	*Mean*	*s.d.*	*Pcor*[a]	*Mean*	*s.d.*	*Pcor*[b]	*Mean*	*s.d.*	*Pcor*[b]	*Mean*	*s.d.*	*Pcor*[b]
−1590C/T (SNP1)	C/C	470	2.13	0.68		0.43	0.95		−0.15	0.60		0.48	1.02	
	C/T+T/T	199	2.04	0.67	0.517	0.23	0.90	0.066	−0.25	0.49	0.613	0.34	0.98	>1
R279Q (SNP3)	R/R	308	2.07	0.68		0.42	0.98		−0.19	0.56		0.41	0.99	
	R/Q+Q/Q	359	2.12	0.68	0.737	0.33	0.90	>1	−0.17	0.59	>1	0.46	1.03	>1
P574R (SNP4)	P/P	341	2.07	0.68		0.41	0.96		−0.19	0.56		0.44	1.00	
	P/R+R/R	328	2.13	0.68	>1	0.34	0.91	>1	−0.17	0.59	>1	0.44	1.02	>1
R668Q (SNP5)	R/R	439	2.14	0.68		0.45	0.95		−0.14	0.61		0.48	1.02	
	R/Q+Q/Q	231	2.03	0.67	0.173	0.22	0.90	**0.022**	−0.26	0.48	0.257	0.36	0.99	>1

Abbreviation: SNP, single-nucleotide polymorphism.
[a]Raw *P*-values of coefficients of SNPs for log-transformed total IgE value are calculated as a general linear model with age and sex as covariates. *Pcor*-values are calculated by multiplying raw *P*-values by 16 (that is, number of SNPs tested×number of IgE values).
[b]Raw *P*-values of coefficients of SNPs for log-transformed specific IgE values are calculated using a tobit regression model with age and sex as covariates. *Pcor*-values are calculated by multiplying raw *P* tobit values by 16 (that is, number of SNPs tested×number of IgE values).
Significant *Pcor*-values (<0.05) are in boldface.

Table 4 Association between haplotype and allergic rhinitis (RA)

		Frequency				*Pcor*[a]		
Haplotype	*SNP1–5*	*Cedar pollinosis*	*Orchard grass pollinosis*	*Mite-positive perennial AR*	*Control*	*Cedar pollinosis vs control*	*Orchard grass pollinosis vs control*	*Mite-positive perennial AR vs control*
Haplotype 1	CGGCG	0.566	0.612	0.575	0.510	>1	>1	>1
Haplotype 2	CGAGG	0.254	0.225	0.217	0.214	>1	>1	>1
Haplotype 3	TTGCA	0.107	0.071	0.120	0.230	**0.0012**	**0.0059**	0.201
Haplotype 4	CGACG	0.074	0.092	0.088	0.046	>1	>1	>1

Abbreviations: AR, allergic rhinitis; SNP, single-nucleotide polymorphism.
[a]Raw *P*-values are multiplied by 12 (that is, number of haplotypes×number of diseases).
Significant *Pcor*-values (<0.05) are in boldface.

marker of functional SNP1 and almost the same results were expected. The lowest *P*-value for log(total IgE), 0.043, was observed for SNP5 and was not significant if the value was corrected by the number of SNPs. SNP5 was significantly associated with cedar pollen-specific IgE (*Pcor*=0.022). Although SNP1 had a raw *P*-value of 0.0041, the value was >0.05 after a multiple-test correction. A similar tendency was observed with regard to the association of these SNPs with orchard grass-specific IgE. The raw *P*-values for these SNPs were slightly <0.05 and did not reach the significance level after correction. None of the SNPs showed significant association with mite-specific IgE.

Association of haplotype with AR and serum IgE levels

The estimated frequencies of haplotypes consisting of SNP1 to SNP5 are shown in Table 4. The frequency of the TTCGA haplotype [haplotype 3 (H3)] between cedar (10.7%) and orchard grass (7.1%) of childhood AR was significantly lower than that in child controls (23.0%) (*Pcor*=0.0012 and *Pcor*=0.0059, respectively). This haplotype was not significantly associated with mite-positive perennial AR (*Pcor*=0.201). H3 consists of minor alleles of SNP1, SNP2 and SNP5, whereas other haplotypes [haplotype 1 (H1), haplotype 2 (H2) and haplotype 4 (H4)] correspond to the major allele of SNP1, SNP2 and SNP5 (CGXXG, X: any allele of SNP3/SNP4). Because the test of association was performed with H3 vs H1+H2+H4, the result is equivalent to the association between single SNP of SNP1, SNP2 or SNP5 and disease. The frequency of H1, H2 and H4 was higher in all three types of AR patient groups than in the control group. Association test of these haplotypes with the diseases, however, did not reach the level of significance. CGXXG-type haplotypes were equally divided into H1, H2 and H4 in all AR groups and in the control group by SNP3–SNP4 alleles that were not in LD with SNP1–SNP2–SNP5 and not associated with any diseases. This reduced the power to detect an association between each haplotype (H1, H2 or H4) and the disease type.

Next, we determined whether H3 has an impact on serum total and specific IgE levels (Table 5). Individuals with at least one H3 allele showed significantly lower cedar-specific IgE levels (*Pcor*=0.0041). The *Pcor*-value for the association with orchard grass-specific IgE was slightly >0.05. This suggests that individuals with H3 are less prone to pollen sensitization. No effect of H3 on total IgE level and mite-specific IgE level was evident.

Effect of amino-acid changes on MMP9 activity

Different promoter activities of alleles of SNP1 were reported previously.[17] However, it was unknown whether the amino-acid change of SNP5 has any effect on MMP9 activity or function. To evaluate the effect of SNP5, we constructed four different MMP9 proteins composed of different combination of SNP3, SNP4 and SNP5 alleles because SNP3 and SNP4 may influence the effect of SNP5 on the enzyme activity.

As shown in Figure 2, type 1 enzyme showed significantly higher proteolytic activity than any of the other types of enzyme tested. Because H1 corresponds to type 1 enzyme and did not show significant association with pollinosis, a difference in enzyme activity

Table 5 Association between haplotype 3 and serum IgE levels

		log(total IgE)			log(cedar IgE)			log(orchard grass IgE)			log(mite IgE)		
	Number of subjects	Mean	s.d.	Pcor[a]	Mean	s.d.	Pcor[b]	Mean	s.d.	Pcor[b]	Mean	s.d.	Pcor[b]
Haplotype 3													
H3/H3+H3/others	194	2.03	0.67		0.21	0.89		−0.26	0.49		0.33	0.97	
(H3)													
Others/others	474	2.13	0.69	0.237	0.44	0.95	**0.0041**	−0.15	0.60	0.064	0.48	1.02	0.262

[a]*P*-values of coefficients of haplotype 3 (H3) for log-transformed total IgE value are calculated as a general linear model with age and sex as covariates. *Pcor*-values are calculated by multiplying raw *P*-values by 4 (that is, number of IgE values).
[b]*P*-values of coefficients of H3 for log-transformed specific IgE values are calculated using a tobit regression model with age and sex as covariates. *Pcor*-values are calculated by multiplying raw *P*-values by 4 (that is, number of IgE values).
Significant *Pcor*-values (<0.05) are in boldface.

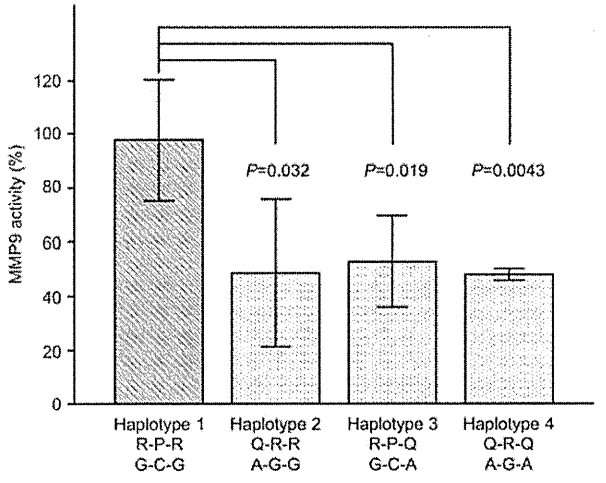

Figure 2 Comparison of peptide cleavage activity of different sequences of MMP9. MMP9 activity for peptide cleavage was monitored by 5-carboxyfluorescein fluorescence. In each experiment, duplicate samples of cell culture medium were assayed. The activity of wild-type enzyme was set to 100% in each experiment. Values are expressed as mean and standard deviation of three independent experiments. Significance was evaluated using analysis of variance test.

was not associated with risk for pollinosis development in a straightforward manner. Compared with the type 1 (wild-type) enzyme, the amino-acid changes from R to Q at SNP5 (type 3) decreased enzyme activity by half (P=0.019). The amino-acid change of 279R (SNP3) + 574R (SNP4) (type 2) also decreased the activity (P=0.032) to a similar extent. The activity of type 4 was similar to that of type 2 and type 3, suggesting no additive effect by type 2 and type 3 amino-acid changes. SNP5 showed an association with cedar pollen sensitization and pollinosis and had an impact on MMP9 enzyme activity. However, a reduction in enzyme activity was also observed with SNP3 and SNP4, neither of which showed an association with disease. Thus, reduced activity was not enough to show an association with clinical phenotypes.

DISCUSSION

In this study, we investigated the association between (potentially) functional sequence variations of the *MMP9* gene and AR in Japanese children. The SNPs −1590C/T (SNP1) and R668Q (SNP5) were in strong LD and were significantly associated with cedar and orchard grass pollinosis. SNP5 was also significantly associated with cedar pollen sensitization. To evaluate the pathological importance of SNP5, we measured the proteolytic activity of different types of MMP9 due to SNP3, SNP4 and SNP5. To our knowledge, the present study is the first to experimentally evaluate the effects of amino-acid changes in MMP9 on its proteolytic activity. Compared with the wild-type enzyme, enzymes with any tested combination of amino-acid substitution showed lower enzyme activity. However, lower enzyme activity and disease risk were not exactly correlated. As seen in Table 3, frequencies of H1 that correspond to the wild-type enzyme in the disease groups were higher than in the control group. It is possible that H1 is associated with higher risk for pollinosis, but this was not statistically significant. H3 was significantly associated with lower risk for pollinosis and is the only haplotype that contains the T allele of SNP1, which was shown previously to have higher promoter activity.[17,24] From these observations, we speculate that different promoter activity associated with SNP1 may be more necessary or important for change in pollinosis risk than different enzyme activity associated with SNP5.

Although we observed significant associations between orchard grass pollinosis and both SNP1 and SNP5, this finding should be interpreted with caution because 89% (48/54) of orchard grass pollinosis patients also had cedar pollinosis. We could not test an association between the SNPs and genuine orchard grass pollinosis because only six children showed orchard grass-only pollinosis. In this test, we found an association between SNPs and pollinosis in patients with sensitization for two different pollens and longer duration of symptoms. Lower OR values and more significant *P*-values found in these patients compared with cedar pollinosis-only patients suggest that children with MMP9 susceptible allele tend to show a more severe phenotype: sensitization for more pollen types and longer duration of symptoms.

Patients with perennial AR with positive mite IgE did not show significant association with *MMP9* SNPs. ORs of SNP1 and SNP5 of those children who also had pollinosis were a little lower than in the entire group, but were higher than in patients with both cedar and orchard grass pollinosis. Further, ORs in mite-positive perennial AR-only patients were similar to those in control children. The relationship between cedar pollinosis and mite-positive perennial AR was not significant. These results suggest a different pathogenesis for pollinosis and mite-positive perennial AR; in addition, MMP9 may have a more important role in the pathogenesis of cedar sensitization and pollinosis than in that of mite sensitization and mite-positive perennial AR.

A strong association between *MMP9* gene variation and serum pollen-specific IgE levels suggests that MMP9 may be involved in the

sensitization process for pollen in the upper airways. Several studies have investigated the role of MMP9 in the immune system. Ichiyasu *et al.*[25] reported that DCs of the bone marrow from MMP9-deficient mice may have impaired migration through the tight junctions. Yen *et al.*[26] reported that DCs matured within inflammatory sites require both chemokine receptor type 7 and prostaglandin E2-induced MMP9 for directional migration to draining lymph nodes. Hintzen *et al.*[27] reported that continuous DC-mediated transport of inhaled antigen to the bronchial lymph node is critical for the induction of tolerance to innocuous antigens. The roles of MMP9 are not restricted to DC migration. MMP9 was also reported to be involved in transmigration of lymphocyte,[28] neutrophils[28] and eosinophils.[29] Recent results demonstrated that tissue-type plasminogen activator promoted several types of bone marrow cells to move to tissue remodeling sites and that MMP9 had a key role on this process by promoting Kit-ligand secretion and vascular endothelial growth factor A (VEGF-A) tissue store release.[30] Furthermore, MMP9 is also known to be expressed in airway epithelial and subepithelial cells.[31,32] These results suggest multipotent effects of MMP9 in the immune system and on tissue remodeling. The results of our association studies, and of many other studies, suggest that MMP9 is involved in the sensitization processes, in particular through migration of DCs and other cell types.

The T allele of SNP1 has been shown to have higher promoter activity,[17] whereas enzyme activity associated with H3 was lower than H1 enzyme. Thus, the association between MMP9 expression/activity and risk for disease development appears to be complex. Because SNP1 and SNP5 are in strong LD and associated with cedar and orchard grass pollinosis to a similar extent, we cannot determine conclusively from the present results which of the SNPs is more important for disease development. As noted above, MMP9 is essential for the action of a variety of cell types, and some of these actions may antagonize *in vivo* biologic an immunologic processes such as IgE production, inflammation and tissue remodeling. Even in the standard allergic asthma model of *Mmp9* knockout mice, totally discrepant results have been reported.[4,5] It is therefore possible that subtle differences in environmental or genetic backgrounds are responsible for the changes in MMP9 expression/activity. Dissection of the mechanistic link between *MMP9* variations and predisposition to sensitization or disease risk is very difficult at present.

A recent large cohort study in Germany investigating *MMP9* SNPs and asthma development showed an association between Q279R and "non-atopic" asthma.[33] This result is somewhat different from our observation that *MMP9* SNPs were involved in pollen sensitization, suggesting that *MMP9* may be involved in chronic airway inflammation processes through a mechanism other than sensitization. The different results may be due to the fact that cedar pollen is not a prevalent allergen in Germany and the larger statistical power of the study may reveal different roles of *MMP9*. Different patterns of association between SNPs and phenotypes may be reflected by the multifaceted nature of *MMP9* and its complex interactions with other genes and factors.[34] Genotype–phonotype association studies under different environmental conditions may shed light on different functions of the *MMP9* gene.

Currently, data on the relevance of MMP9 to nasal tissue pathogenesis in humans are limited. Lee *et al.*[35] examined nasal polyp tissues from asthma patients and showed that the MMP9 level was correlated with the level of inflammatory cell markers such as eosinophilic cationic protein and tryptase. The expression of MMP9 mRNA was higher in nasal polyps when compared with inferior turbinate mucosa in patients with chronic rhinosinusitis.[36] Wang *et al.*[37] reported that −1590C/T and R668Q were associated with chronic rhinosinusitis with nasal polyposis. The haplotype corresponding to H3 in our study was also associated with the disease (P=0.0045). However, negative results have also been reported.[38] Biopsy specimens of nasal mucosa were taken from patients with perennial AR and non-rhinitic control subjects. MMP1, 2, 3 and 9 mRNA were measured and no upregulation of MMPs was found in the tissue from patients. MMP9 in nasal tissue in animals has also been studied. Lim *et al.*[12] investigated upper- and lower-airway remodeling in murine model. OVA (ovalbumin)-sensitized mice were repeatedly exposed to inhalation of OVA for 1 month or 3 months. Repetitive OVA challenge for 3 months induced circumferential peribronchial fibrosis in the lung. Subepithelial fibrosis, increased MMP9 and Timp-1 expression, goblet cell hyperplasia and submucosal grand hypertrophy were observed in the nose. These findings are important because nasal mucosa may show similar pathologic changes to lung tissue and the *MMP9* gene may have a significant role in the pathogenesis of nasal mucosa.

The present results support an important role of the *MMP9* gene in pollen sensitization and pollinosis in Japanese children. Identifying the role of the *MMP9* gene in the sensitization process in upper-airway tissues is of importance to understand the development of pollinosis. In addition, whether stimulation or inhibition of MMP9 activity may benefit treatment of AR is of interest.

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Short Communication

Relationship between *RANTES* Polymorphisms and Respiratory Syncytial Virus Bronchiolitis in a Japanese Infant Population

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SUMMARY: Respiratory syncytial virus (RSV) is the most important virus associated with bronchiolitis in infants and young children. The regulated upon activation, normal T-cell expressed and secreted protein (RANTES, also known as CCL5) appears to be a key player in the etiology of RSV-infected airway inflammation. In this study, we genotyped three single-nucleotide polymorphisms in the *RANTES* gene: −403G/A, −28C/G, and In1.1T/C in 59 infants with severe RSV bronchiolitis and 201 control subjects. The frequencies of the −403G/A + A/A, −28C/G + G/G, and In1.1T/C + C/C genotypes were significantly lower in patients with severe RSV bronchiolitis than in control subjects, and the frequencies of the −403A, −28G, and In1.1C alleles were significantly lower in RSV patients than in control subjects. The present results suggest that *RANTES* polymorphisms may confer risk for severe RSV bronchiolitis.

Respiratory syncytial virus (RSV) is the most important pathogen causing lower respiratory tract infection in infants and young children (1-3). Bronchiolitis is an important disease in infancy and early childhood, and the development of severe bronchiolitis is closely related to RSV infection. Previous studies have implicated cellular immunity in airway inflammation after RSV infection (4,5). Multiple proinflammatory cytokines and chemokines released by alveolar macrophages and epithelial cells are involved in the activation of cellular immunity after RSV infection (6). Regulated upon activation, normal T-cell expressed and secreted protein (RANTES, also known as CCL5) is a chemokine that attracts monocytes, eosinophils, basophils, and memory T lymphocytes (7-11). RANTES is generated by macrophages, $CD8^+$ T lymphocytes, and epithelial cells (12-15).

The human *RANTES* gene is composed of three exons and two introns (16), and in the *RANTES* gene, three single-nucleotide polymorphisms (SNPs) have been characterized: −403G/A (rs2107538), −28C/G (rs2280788), and In1.1T/C (rs2280789) (17-19). SNPs −403G/A and −28C/G are located in the promoter region of the human *RANTES* gene, and In1.1T/C is located in intron 1. Thus far, a few studies have reported on the association between these *RANTES* SNPs and RSV bronchiolitis (12,20,21), with conflicting results. Tian et al. compared the allele frequency and genotype of *RANTES* −403G/A in an RSV bronchiolitis group to a control group and failed to find a significant difference (20). Zhao et al. reported a significant association between *RANTES* −28C/G and RSV bronchiolitis (21). Amanatidou et al. examined −403G/A, −28C/G, and In1.1T/C and found no significant association between these SNPs and RSV bronciolitis when tested separately; however, there was a significant difference in the frequency of the genotype combination −28C/C + −403G/A + In1.1T/T between patients and control subjects (12).

The purpose of the present study was to survey the association between genetic variation in the *RANTES* gene and RSV bronchiolitis in a Japanese infant population.

A total of 59 infants who had been hospitalized with severe RSV bronchiolitis at Chiba University Hospital, Asahi Central Hospital, Chiba Children's Hospital, Shimosizu Hospital (Chiba, Japan), and Ono Pediatric Clinic (Nagasaki, Japan) were recruited. The diagnosis of RSV bronchiolitis was established on the basis of wheezing and the presence of RSV antigen in nasopharyngeal secretion specimens. Exclusion criteria included prematurity, chronic respiratory disease, previous wheezing episodes, cardiac disease, and age >24 months. The mean age of the patients (± standard error of the mean [SEM]) was 5.32 ± 0.81 months (range, 1-19 months).

The control subjects were 201 children who had never had a wheezing episode and were selected from 411 children recruited at an elementary school affiliated with Chiba University. This study was approved by the Ethics Committee on Human Research at Chiba University. Informed consent was obtained from the parents or guardians of all subjects.

Peripheral blood was collected in tubes containing ethylenediaminetetraacetic acid. DNA was extracted from blood with a QIAamp DNA Blood Kit (Qiagen, Valencia, Calif., USA) or from buccal cells with a Buc-

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calAmp DNA Extraction Kit (Epicentre Biotechnologies, Madison, Wis., USA), according to the manufacturers' instructions. Genomic DNA was amplified with an Illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare UK, Little Chalfont, Buckinghamshire, UK) according to the manufacturer's instructions. *RANTES* gene polymorphisms in In1.1T/C (rs2280789) were genotyped with a Taqman system (Applied Biosystems, Foster City, Calif., USA), while genotyping of −403G/A (rs2107538) and −28C/G (rs2280788) was performed using the SNaPshot method (Applied Biosystems).

Statistical comparisons between patients and control subjects were performed using the contingency χ^2 test or unpaired Student's t test. Hardy-Weinberg equilibrium was assessed with a χ^2 goodness of fit test. Strength of linkage disequilibrium (LD) and haplotype frequencies were estimated with SNPAlyze Pro software (version 7.0; Dynacom, Mobara, Japan). All statistical analyses were performed with SPSS Statistics software (version 17.0; SPSS Japan, Tokyo, Japan) unless otherwise stated. P values of less than 0.05 were considered significant unless otherwise stated.

We investigated the characteristics of the 59 patients and 201 control subjects. The following were considered risk factors for the development of RSV bronchiolitis: sex, birth weight, the presence of older siblings, breastfeeding, day care attendance during infancy, and parental smoking during infancy. None of these factors differed significantly between the patients and control subjects (data not shown).

The genotypes and allele frequencies of the three SNPs in the patients and control subjects are shown in Table 1. In the control subjects, all the genotypes were in Hardy-Weinberg equilibrium. The −403A, −28G, and In1.1C alleles were minor alleles. In a dominant model, the frequencies of −403G/A + A/A, −28C/G + G/G, and In1.1T/C + C/C were significantly lower in patients than in control subjects.

The LD status was evaluated for the 201 control subjects; the r^2 values between −403G/A and −28C/G, −403G/A and In1.1T/C, and −28C/G and In1.1T/C were 0.297, 0.948, and 0.313, respectively. Because the LD between −403G/A and In1.1T/C was strong, the genotype results for these two SNPs were nearly identical (Table 1).

We then analyzed the association between the three SNP haplotypes and RSV (Table 2). The estimated frequencies of the H4 haplotype (−403A, −28C, and In1.1T), H5 haplotype (−403G, −28C, and In1.1C), and H6 haplotype (−403G, −28G, and In1.1T) were very low (H4 haplotype [patients, 0.026; control subjects, 0.012]; H5 haplotype [patients, 0.017; control subjects, 6.24×10^{-22}]; H6 haplotype [patients, 9.24×10^{-3}; control subjects, 4.79×10^{-9}]) and were nearly undetectable in the actual samples. We therefore excluded the H4, H5, and H6 haplotypes from the association analysis. Among the three remaining haplotypes, the frequency of H3 (−403A, −28G, and In1.1C) was significantly lower in patients than in control subjects ($P = 0.0156$). The frequency of H1 (−403G, −28C, and In1.1T) was higher ($P = 0.0443$) by 0.100 in patients than in control subjects. However, this difference was not significant after Bonferroni correction. The frequency of the H2 haplotype was similar between the two groups ($P = 0.2081$).

In this study, we examined the association of three SNPs in the *RANTES* gene with RSV bronchiolitis in a population of Japanese infants. The frequencies of genotypes containing −403A, −28G, and In1.1C were

Table 1. Distribution of genotype in infants with severe respiratory syncytial virus (RSV) bronchiolitis and control subjects

	Infants with severe RSV bronchiolitis ($n = 59$)	Control subject ($n = 201$)	P	OR (95% CI)
RANTES −403 G/A				
G/G	34 (57.6)	80 (39.8)		
G/A + A/A	25 (42.4)	121 (60.2)	0.017*	0.49 (0.27–0.88)
Allele				
G	88 (74.6)	249 (61.9)		
A	30 (25.4)	153 (38.1)	0.012*	0.56 (0.35–0.88)
RANTES −28 C/G				
C/C	52 (88.1)	143 (71.1)		
C/G + G/G	7 (11.9)	58 (28.9)	0.010*	0.33 (0.14–0.77)
Allele				
C	109 (92.4)	340 (84.6)		
G	9 (7.6)	62 (15.4)	0.032*	0.45 (0.22–0.94)
RANTES In1.1 T/C				
T/T	34 (57.6)	83 (41.3)		
T/C+C/C	25 (42.4)	118 (58.7)	0.037*	0.52 (0.29–0.93)
Allele				
T	89 (75.4)	254 (63.2)		
C	29 (24.6)	148 (36.8)	0.015*	0.56 (0.35–0.89)

Data are presented as number (%) of subjects unless otherwise indicated.
CI, confidence interval; OR, odds ratio. *Statistically significant.