継続した場合（65.9%：137/208例）の改善率に違いは認められなかった．また，投薬開始月別や年齢別の全般改善度にも違いは認められず，間欠型では67.9%（152/224例），軽症持続型では59.6%（205/344例）の改善率であった．3段階（有効，やや有効，無効）で評価したアトピー性皮膚炎およびアレルギー性鼻炎への有効率（やや有効以上）は，それぞれ65.6%（99/151例），76.8%（129/168例）であり，気管支喘息への効果と同等または，それ以上の改善を示した．

4）血清IgEおよび末梢血好酸球数の推移

血清IgEの推移を図**4**に示す．投与前平均値（Mean±SD）は677.2±1,077 IU/mLであり，投与後は446±436.3 IU/mLであった．

また同様に，末梢血好酸球数の推移を図**5**に示す．投与前平均値は363.8±263.4/μLであり，投与後は338.0±314.9/μLであった．

表4　患児背景別全般改善度

背景		著明改善	中等度改善	軽度改善	不変	悪化	改善率	改善率 95%信頼区間
全体		111	246	136	57	18	62.9%	58.7～66.8
投与方法	LT拮抗薬を中止し切替	69	135	85	33	14	60.7%	55.3～66.0
	LT拮抗薬継続し追加	39	98	46	21	4	65.9%	59.0～72.3
	LT拮抗薬増量し追加	3	11	5	3	0	63.6%	40.7～82.8
投薬開始月	3～5月	33	66	42	17	4	61.1%	53.1～68.7
	6～8月	29	59	38	18	8	57.9%	49.6～65.8
	9～11月	31	77	32	13	5	68.4%	60.5～75.5
	12～2月	18	44	24	9	1	64.6%	54.2～74.1
性別	男	75	156	81	33	11	64.9%	59.7～69.8
	女	36	90	55	24	7	59.4%	52.5～66.1
入院外来区分	入院	0	1	1	1	0	33.3%	0.8～90.6
	外来	110	239	131	53	16	63.6%	59.4～67.6
	入院＋外来	1	6	4	3	2	43.8%	19.8～70.1
年齢	3歳未満	30	49	31	14	1	63.2%	54.1～71.6
	3歳以上5歳未満	35	88	41	26	10	61.5%	54.4～68.3
	5歳以上11歳未満	44	96	57	15	7	63.9%	57.2～70.3
	11歳以上	2	13	7	2	0	62.5%	40.6～81.2
発作型	間欠型	42	110	54	14	4	67.9%	61.3～73.9
	軽症持続型	69	136	82	43	14	59.6%	54.2～64.8
病型	アトピー型	69	178	96	43	14	61.8%	56.8～66.5
	非アトピー型	42	68	39	14	3	66.3%	58.5～73.4
合併症	無	64	117	67	27	9	63.7%	57.8～69.3
	有	47	129	69	30	9	62.0%	56.0～67.6
	アトピー性皮膚炎	22	66	37	20	6	58.3%	50.0～66.2
	アレルギー性鼻炎*	33	77	44	16	2	64.0%	56.3～71.1

*：アレルギー性鼻炎に対する改善度の評価ができなかった症例が4例存在した

3. 安全性の評価

副作用発現症例数は12例，副作用発現件数は13件であり，副作用発現症例率は1.9%（12/631例）であった．副作用一覧を**表5**に示す．

重篤例は喘息症状の悪化およびクループ症候群の合計2例であったが，いずれも治療のための入院あるいは入院期間の延長で，かつトシル酸スプラタスト投与継続のまま軽快しており，問題ないと考えられた．非重篤の副作用の多くは胃腸障害（悪心，嘔吐，腹痛）であり，いずれも回復・軽快していた．

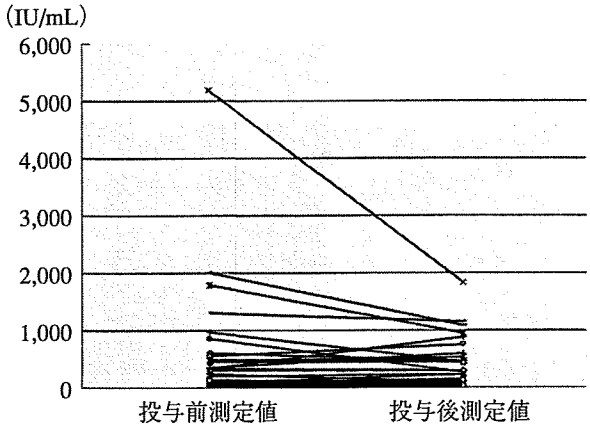

図4　血清IgEの推移

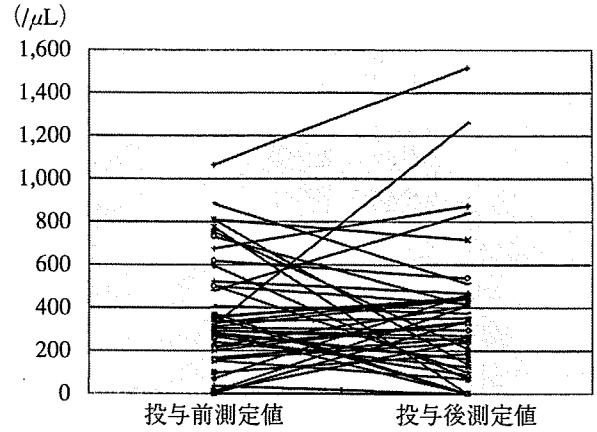

図5　末梢血好酸球数の推移

表5　副作用一覧

	性　別	年　齢	合併症	副作用名*1	発現日数	重篤性	転　帰	本剤以外の要因
1	男	4歳	無	嘔吐	1	非重篤	軽快	無
2	女	10歳	アレルギー性鼻炎	腹痛	1	非重篤	軽快	無
				嘔吐	1	非重篤	軽快	無
3	女	1歳	アトピー性皮膚炎 アレルギー性鼻炎	悪心	3	非重篤	軽快	無
4	女	3歳	アトピー性皮膚炎 アレルギー性鼻炎	悪心	2	非重篤	回復	無
5	男	8歳	無	悪心	46	非重篤	回復	無
6	男	4歳	無	鼻出血	2	非重篤	回復	無
7	男	2歳	無	喘息	13	重篤	軽快	有（ウイルス感染）
8	男	1歳	アトピー性皮膚炎 食物アレルギー	上気道の炎症	29	非重篤	回復	有（感染症）
9	男	4歳	無	クループ症候群	83	重篤	軽快	有（ウイルス感染）
10	男	3歳	食物アレルギー	じんま疹	5	非重篤	回復	有（上気道感染症）
11	男	2歳	アトピー性皮膚炎 食物アレルギー	じんま疹	36	非重篤	回復	有（アトピー素因）
12	男	3歳	アトピー性皮膚炎	好酸球数増加	385*2	非重篤	不明	有（アレルギーの増悪）

*1：MedDRA/J　ver10.1で表記
*2：12週後以降に発現した副作用も表記した

考　察

「小児気管支喘息治療・管理ガイドライン」の普及により吸入ステロイド薬を中心とした長期管理薬が適切に投与されるようになり，喘息症状のコントロールという点ではめざましい進歩を遂げている．抗アレルギー薬の中ではロイコトリエン受容体拮抗薬が気管支喘息に対して多くのエビデンスを有しており，長期管理薬として幅広く用いられている．しかしながら，ロイコトリエン受容体拮抗薬で十分コントロールできない症例も日常診療の中で見受けられ，喘息治療を実施していくうえでは長期管理薬のより多くの選択肢が望まれるところである．

トシル酸スプラタストは抗アレルギー薬のTh2サイトカイン阻害薬に分類され，Th2細胞から産生されるIL-4，5，13などのサイトカインの阻害によりIgE産生ならびに好酸球組織浸潤を抑制することが認められており[1)~6)]，他の抗アレルギー薬とは異った薬理作用よりアレルギー炎症を制御すると考えられている．また，小児に対する有用性の一つとして，アレルギーマーチ症例に対する喘息発症抑制効果[8)]も報告されている．

ロイコトリエン受容体拮抗薬に反応を示さなかった症例に対し，トシル酸スプラタストの有用性が示唆される結果が報告されているが[9)]，少数例での検討であり，一般臨床における大規模な検討が必要と考えられた．そこで今回，ロイコトリエン受容体拮抗薬により効果不十分と考えられる気管支喘息患児を対象として，ロイコトリエン受容体拮抗薬にトシル酸スプラタストを追加投与する，もしくはロイコトリエン受容体拮抗薬を投与中止してトシル酸スプラタストを投与した場合の喘息症状の改善効果，喘息に合併するアトピー性皮膚炎やアレルギー性鼻炎の改善効果，QOL，血清IgE値や末梢血好酸球数の動態および安全性について，多施設共同調査にて検討した．

自覚症状に関して，観察期と比較して気管支喘息症状発現日数のいずれの項目も有意に改善がみられた（$p<0.001$）．同様に，QOLの多くの調査項目で投与後に有意に改善がみられ，とくに4歳未満における身体的領域の改善が著明であった．

全般改善度では，中等度改善以上は357例であり，全般改善率は62.9%（357/568例）であった．また，ロイコトリエン受容体拮抗薬の投与を中止し切替えた場合とそのまま継続した場合の改善率に違いは認められなかった．次に，患児背景別に全般改善度を検討したが，性別，年齢，発作型，病型，合併症の有無のいずれの項目においても大きな違いは認められておらず，今回の調査では有効性に影響を及ぼす因子の検出はできなかった．

また，喘息に合併したアトピー性皮膚炎およびアレルギー性鼻炎の有効率はそれぞれ65.6%（99/151例），76.8%（129/168例）であり，合併するアレルギー症状に対してもトシル酸スプラタストは有用であると考えられた．

血清IgE値ならびに末梢血好酸球数について，トシル酸スプラタスト投与前後で測定できた症例について検討したところ，IgE値および好酸球数ともに投与前より投与後で低下を示した．本結果はIgEおよび好酸球の抑制と効果について関連性は示唆できるものの，症例数が少なく統計学的に有意には至っておらず，今後，さらなる検討が必要と思われる．

有効性の評価について，治験時と同様に5段階の全般改善度で検討したが，全般改善度の各段階の定義は，すべての調査項目で定量化あるいは定性化が困難であること，各調査項目について疾患に対する影響が一律でないことから，定義をすることが困難である．そこで，気管支喘息症状合算発現日数の変化量およびQOL総変化量と全般改善度の分布を検討した（**図6**，**図7**）．

いずれも変化量の増大とともに改善度は高くなっており，とくに定義を定めなかったが，今回得られた全般改善度は気管支喘息症状やQOLの改善結果を反映しているものと考えられる．

一方，安全性に関しては，副作用発現症例率は1.9%（12/631例）であり，高い安全性を示した．副作用の内訳は，悪心，嘔吐，腹痛の消化器症状が5例，喘息，じんま疹などもみられたが，いずれも投与終了後，回復もしくは軽快した．また，問題となる重篤な事象も認められなかった．

これらの結果より，ロイコトリエン受容体拮抗薬を投与していても十分なコントロールが得られない間欠型および軽症持続型の小児気管支

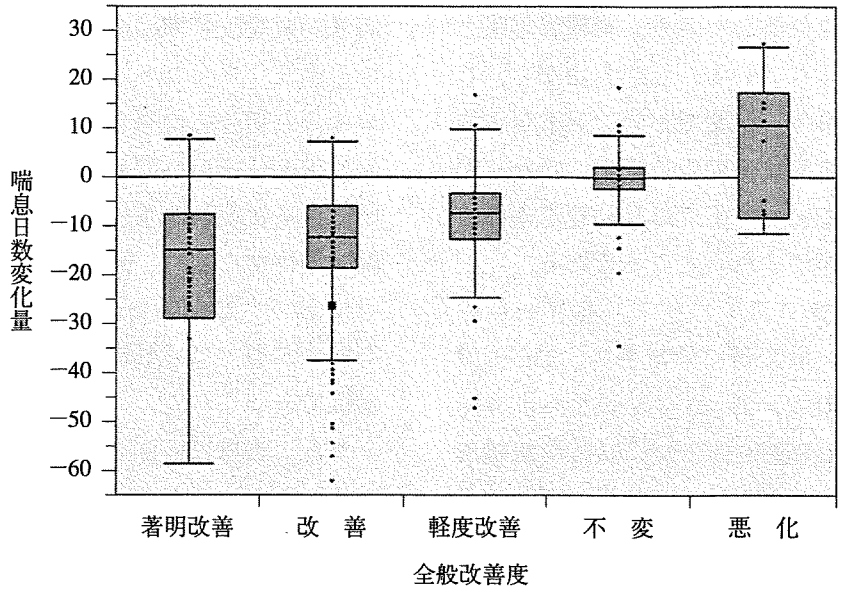

図6　喘息日数変化量と全般改善度の散布

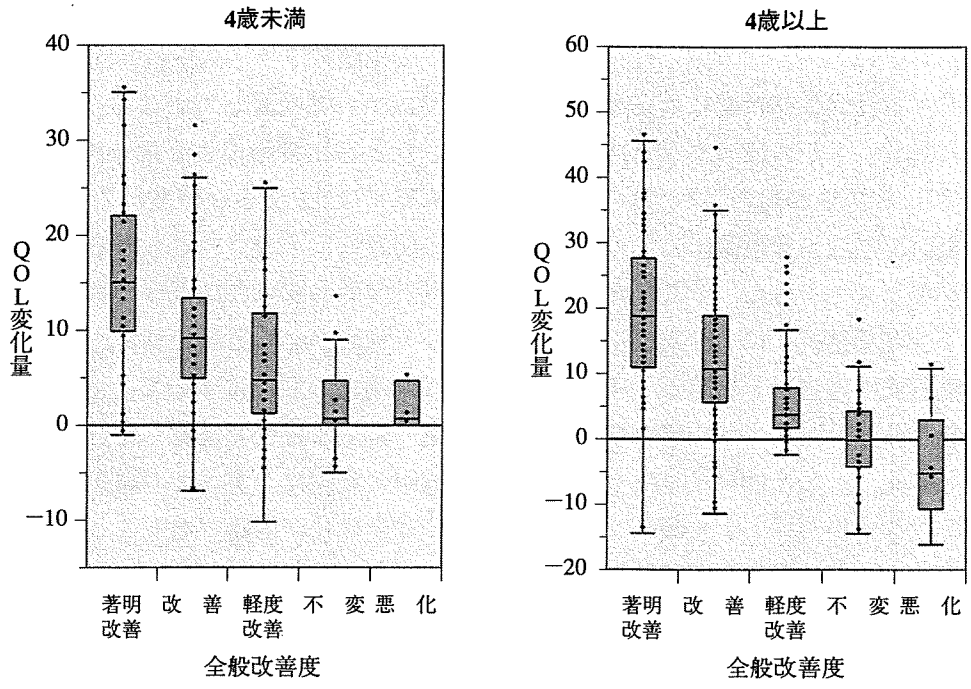

図7　QOL変化量と全般改善度の散布

喘息患児に対して，トシル酸スプラタストドライシロップへ切替え，もしくは追加投与することは，喘息症状ならびに合併症の改善を示し，臨床上有用であると考えられた．また，患児および保護者のQOL向上にもつながることが示唆された．しかしながら，ロイコトリエン受容体拮抗薬で効果が不十分と思われる患者群やトシル酸スプラタストの有効性が認められる患者群の特定については，今回の検討では確認できなかった．

今回の結果より，トシル酸スプラタストを小児気管支喘息治療薬として再認識することができ，今後の臨床研究につながる有意義な結果であったと思われる．

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著者一覧

医療機関名	診療科名	医師名
医療法人社団小児科西町クリニック	小児科	池田和男
岩見沢市立総合病院	小児科	佐藤俊哉
医療法人王子総合病院	小児科	内藤広行，小林徳雄
医療法人社団土田こどもクリニック	小児科	土田　晃
社会福祉法人北海道社会事業協会富良野病院	小児科	角谷不二雄，藤保洋明
JA北海道厚生農業協同組合連合会網走厚生病院	小児科	立花幸晃，松本直也
医療法人社団慈友会望ケ丘医院	小児科	藤崎志保子
医療法人社団のろこどもクリニック	小児科	野呂知世
医療法人佐久間眼科小児科医院	小児科	佐久間実香
医療法人加納こども医院	小児科	加納一毅
どんぐりこどもクリニック	小児科	佐々木正人
医療法人圭尚会金病院	小児科	金由美子
秋田県厚生農業協同組合連合会湖東総合病院	小児科	武田　修
しおたこどもクリニック	小児科	塩田輝和
医療法人明和会中通総合病院	小児科	渡邊　新，安岡健二，平山雅士
医療法人舟山病院	小児科	斎藤誠一
鶴岡市立荘内病院	小児科	伊藤末志
川原田小児科医院	小児科	川原田隆司
福島県厚生農業協同組合連合会白河厚生総合病院	小児科	根本健二，村井弘通，武田良淳，武田いづみ
福島県厚生農業協同組合連合会塙厚生病院	小児科	澁川靖子，鈴木順造
医療法人社団東光会戸田中央総合病院	小児アレルギー科	島貫金男
医療法人社団夢の輪会井上小児科皮フ科	小児科	井上　充
医療法人久恩会下条医院	内科	下条　順
獨協医科大学病院	小児科	吉原重美
医療法人わたなべ会渡辺内科クリニック	内科	渡辺茂男
医療法人湯川会湯川内科クリニック	内科	湯川龍雄

著者一覧　つづき

医療機関名	診療科名	医師名
医療法人聖穂会ふるしま医院	内科	古島吉之
塙医院	小児科	塙　光一
社団法人伊勢崎佐波医師会病院	小児科	中野和俊
川島内科クリニック	小児科	川島　崇
新潟県厚生農業協同組合連合会佐渡総合病院	小児科	岡崎　実
新潟県立吉田病院	小児科	松野正知
新潟県済生会新潟第二病院	小児科	平野泰伸
新潟県立十日町病院	小児科	金山哲也
川端内科消化器科医院	内科	川端英博
深谷赤十字病院	小児科	河野美幸
埼玉医科大学病院	小児科	岡田邦之
山田医院	小児科	山田勝巳
中田小児科クリニック	小児科	中田博一
イルカ耳鼻咽喉科	耳鼻咽喉科	小島好雅
つばきこどもクリニック	小児科	椿　俊和
独立行政法人国立病院機構下志津病院	小児科	渡邊博子，佐藤一樹
たむら小児科クリニック	小児科	田村和善
兼子内科クリニック	内科	兼子裕一
小池医院	小児科	小池洋子
医療法人社団威恵会三岳荘小松崎病院	小児科	榎戸　久
こしぶ小児科	小児科	小渋達郎
井上こどもクリニック	小児科	井上成彰
えんぴつ公園こどもクリニック	小児科	金　孝一
東京慈恵会医科大学附属柏病院	小児科	和田靖之，高畠典子，布山裕一，大島早希子，南波広行，久保政勝
新浦安こどもクリニック	小児科	内藤茂樹
抜弁天クリニック	内科	菊池智津
東京逓信病院	小児科	鈴木淳子，小野正恵，入江　学，北爪　勉
社会福祉法人恩賜財団済生会支部東京都済生会中央病院	小児科	韮澤眞理
東京慈恵会医科大学附属病院	小児科	衛藤義勝，勝沼俊雄
医療法人社団柴田小児科医院	小児科	柴田雄介
こじまこどもクリニック	小児科	小島　明
千葉クリニック	小児科	千葉友幸
みやのこどもクリニック	小児科	宮野孝一
社会福祉法人同愛記念病院	小児科	向山徳子
お花茶屋駅前クリニック	内科	白木康夫
なかにし小児科クリニック	小児科	中西茂則
鶴井内科	内科	鶴井光治
せきぐちクリニック	小児科	関口　茂
星医院	内科・小児科	星　一
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医療法人青空の会小瀬こどもクリニック	小児科	高野美紀子

著者一覧　つづき

医療機関名	診療科名	医師名
バンビーナ小児科	小児科・アレルギー科・内科	真砂野仁
おんだクリニック	小児科・アレルギー科	恩田威文
おばた小児クリニック	小児科・アレルギー科	小幡俊彦
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医療法人社団中野こどもクリニック	小児科	中野康伸
医療法人あいかわこどもクリニック	小児科・アレルギー科	相川博之
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おくやま小児科	小児科	奥山利也
ひろこどもクリニック	小児科	黒住浩子
医療法人慈愛会川辺小児科内科医院	小児科	川邊修二
小児科内科落合医院	小児科	片山文彦
特定医療法人興生会相模台病院	小児科	白井宏幸，敷島康史
特定医療法人社団ジャパンメディカルアライアンス海老名総合病院	小児科	笈浦克則，小川倫史
医療法人社団草柳小児科医院	小児科	草柳哲生
国立大学法人金沢大学附属病院	小児科	谷内江昭宏，東馬智子，和田泰三
金沢医科大学病院	小児科	中村利美
おおのこどもクリニック	小児科	大野高史
中新湊内科クリニック	内科	山崎雅和
前川耳鼻咽喉科	耳鼻咽喉科	前川謙一
医療法人社団和康会河合医院	耳鼻咽喉科	河合晃充，河合康守
まるやま小児科医院	小児科	丸山和男
瀬尾小児科内科医院	小児科	瀬尾　究
国際医療福祉大学熱海病院	小児科	板倉敬乃
医療法人社団わかばファミリークリニックたうち小児科医院	小児科	田内守之
共立湖西総合病院	小児科	西田光宏，田口智英，宮本　健
あいち小児保健医療総合センター	アレルギー科	伊藤浩明，二村昌樹，高岡有里
名古屋第二赤十字病院	小児科	神田康司
愛知医科大学医学部	小児科	緤　裕篤，竹内三奈，川合紀子
山田医院	内科・小児科	山田健司
Kこどもクリニック	小児科	笠井啓子
医療法人おおにしこどもクリニック	小児科・アレルギー科	大西正純
のもと内科小児科	内科	野本　茂
医療法人仁雅会畑川クリニック	小児科	畑川惠子
医療法人宝美会総合青山病院	小児科	牧野健一
小林小児科	小児科・アレルギー科	小林敏美
児玉医院	小児科	児玉　央
独立行政法人国立病院機構長野病院	小児科	森　哲夫
医療法人康友会花岡こどもクリニック	小児科	花岡康彦
医療法人芳生会やまだ小児科クリニック	小児科	山田　節
国立大学法人岐阜大学医学部附属病院	小児科	近藤直実，松井永子，深尾敏幸，金子英雄，折居建治，寺本貴英，川本美奈子，舩戸道徳，近藤　應，小関道夫，徳見哲司，鬼頭勇輔
岐阜県総合医療センター	小児科	今村　淳，伊藤玲子，松尾直樹，菅原勝美
岐阜県立下呂温泉病院	小児科	鹿野博明，森本　航
独立行政法人国立病院機構三重病院	臨床研究部	藤澤隆夫，井口光正，野間雪子，長尾みづほ

著者一覧　つづき

医療機関名	診療科名	医師名
医療法人医仁会武田総合病院	小児科	岸田憲二，神谷一郎
まつおこどもクリニック	小児科	松尾　敏
京都第二赤十字病院	小児科	清澤伸幸，大野邦彦，伊藤陽里，久保田樹里，東道公人
社会福祉法人宇治病院	小児科	松本良文
宇治武田病院	小児科	石野雄一
医療法人和光会おおの小児科	小児科	大野邦彦
医療法人寺井医院	内科	寺井繁勝
鳥越医院	内科	鳥越公造，鳥越公彰
医療法人社団太田医院	小児科	太田志朗
医療法人さたクリニック	産婦人科	佐田博之
社会医療法人真美会中野こども病院	小児科	木野　稔，園府寺美，目黒敬章
大阪府済生会野江病院	小児科	辻美代子，藤原　亨，舟木克枝
社会福祉法人石井記念愛染園附属愛染橋病院	小児科	西川嘉英
大阪府済生会中津病院	小児科，免疫・アレルギーセンター	末廣　豊，藤本雅之
大阪市立大学大学院医学研究科	発達小児医学	山野恒一，新宅治夫
地方独立行政法人大阪府立病院機構呼吸器アレルギー医療センター	小児科	土居　悟，亀田　誠，吉田之範，錦戸知喜
医療法人博洋会宮本医院	小児科・内科	宮本洋二
阿武山こどもクリニック	小児科	岡本良三
ささきアレルギー科クリニック	アレルギー科	佐々木聖
医療法人さもり小児科	小児科	佐守友仁
かめさきこども・アレルギークリニック	小児科	亀崎佐織
医療法人まつお小児科	小児科	松尾泰孝
かなざきこどもクリニック	小児科・アレルギー科	金崎光治
医療法人長田医院	小児科	長田　亮
福原医院	小児科	福原仁雄
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神戸医療生活協同組合いたやどクリニック	小児科	木村彰宏
すぎもとクリニック	内科	杉本智彦
医療法人社団松本小児科医院	小児科	松本秀憲
にのみや小児科	小児科	二宮道人
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医療法人社団平山こども医院	小児科	平山　毅
医療法人社団町塚医院	耳鼻科	町塚博文
医療法人社団とくやまクリニック	小児科・外科	徳山彰俊
宝塚市立病院	小児科	前田　浩，森田直子，田原俊夫，伊藤裕佳子
うえだこどもクリニック	小児科	上田　亨
ひだ耳鼻咽喉科	耳鼻咽喉科	肥田宏一郎
兵庫県立西宮病院	小児科	山本勝輔，安部治郎，村井竜太郎
兵庫医科大学病院	小児科	谷澤隆邦，前　寛，澤木潤子，村尾吉規
医療法人社団賀鳴医院	内科	賀鳴直隆
早野小児科医院	小児科	早野昌毅
医療法人社団ながたこどもクリニック	小児科	永田　忠
たかはし小児科アレルギー科クリニック	小児科	高橋明裕
国立大学法人広島大学病院	小児科	川口浩史，小野　厚，白尾謙一郎
独立行政法人労働者健康福祉機構中国労災病院	小児科	清水浩志
財団法人津山慈風会津山中央病院	小児科	藤本佳夫，梶　俊策，杉本守治，片山　威，松下博亮，栄徳隆裕
医療法人イナバ小児科	小児科	稲葉　淳

著者一覧　つづき

医療機関名	診療科名	医師名
岡山大学医学部・歯学部附属病院	小児科	萬木　章
独立行政法人国立病院機構米子医療センター	小児科	林原　博
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医療法人社団鈴木小児科医院	小児科	鈴木英太郎
松島こどもクリニック	小児科・アレルギー科	松島　寛
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医療法人ごとう小児科	小児科	後藤悟志
愛媛県立三島病院	小児科	長野恵子，吉野修司
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キッズクリニックパパ	小児科	福崎　良
医療法人えもとこどもクリニック	小児科	榎本新也
健生きたじまクリニック	小児科	田中宏実，山田進一
徳島市民病院	小児科	森　一博
医療法人あいだ医院	小児科	間　克麿
公立学校共済組合九州中央病院	小児科	徳川　健
医療法人荒木小児科医院	小児科	荒木速雄
医療法人大山小児科医院	小児科	橋本信男
財団法人福岡県社会保険医療協会社会保険田川病院	小児科	柊山　了，伊藤晋一
北九州市立八幡病院小児救急センター	小児科	市川光太郎，天本正乃，石橋紳作，今村徳夫，山根浩昌
医療法人きしだ小児科クリニック	小児科	岸田邦雄
医療法人あざかみこどもクリニック	小児科	阿座上才紀
(株) 麻生飯塚病院	小児科	岩元二郎，坂口万里江
医療法人つだ小児科	小児科	津田文史朗
医療法人井口こどもクリニック	小児科	井口雄三
医療法人藤本育成会大分こども病院	小児科	藤本　保，石原高信
医療法人博洋会井手小児科医院	小児科	井手　健
長崎県五島中央病院	小児科	田中　摂，里　龍晴
医療法人社団康友会こどもいいん柳丸	小児科	松元康治
医療法人真愛会うちの小児科小児外科	小児科	内野信一郎
医療法人社団秀洋会市川内科クリニック	内科	市川洋一郎
財団法人慈愛会今村病院	小児科	今村直人
医療法人至政会嘉数医院	内科・小児科・呼吸器科	嘉数朝一

＊順不同

著者連絡先
〒501-1194　岐阜市柳戸1-1
岐阜大学大学院医学系研究科小児病態学
松井永子

Associations of functional *NLRP3* polymorphisms with susceptibility to food-induced anaphylaxis and aspirin-induced asthma

Yuki Hitomi, PhD,[a] Motohiro Ebisawa, MD, PhD,[b] Morimitsu Tomikawa, MD,[b] Takanori Imai, MD, PhD,[b] Takatsugu Komata, MD,[b] Tomomitsu Hirota, DDS, PhD,[a] Michishige Harada, PhD,[a] Masafumi Sakashita, MD,[a] Yoichi Suzuki, MD, PhD,[c] Naoki Shimojo, MD, PhD,[d] Yoichi Kohno, MD, PhD,[d] Kimie Fujita, MD, PhD,[e] Akihiko Miyatake, MD, PhD,[f] Satoru Doi, MD, PhD,[g] Tadao Enomoto, MD, PhD,[h] Masami Taniguchi, MD, PhD,[b] Noritaka Higashi, MD, PhD,[b] Yusuke Nakamura, MD, PhD,[i] and Mayumi Tamari, MD, PhD[a] *Kanagawa, Chiba, Shiga, Osaka, Wakayama, and Tokyo, Japan*

Background: NLR family, pyrin domain containing 3 (NLRP3), controls the activity of inflammatory caspase-1 by forming inflammasomes, which leads to cleavage of the procytokines IL-1β and IL-18. Recent studies have shown associations of human *NLRP3* polymorphisms with susceptibility to various inflammatory diseases; however, the association with allergic diseases remains unclear.

Objective: We sought to examine whether *NLRP3* polymorphisms are associated with susceptibility to food allergy, food-induced anaphylaxis, and aspirin-induced asthma (AIA).

Methods: We selected 15 tag single nucleotide polymorphisms (SNPs) of *NLRP3* and conducted association analyses of *NLRP3* using 574 and 1279 samples for food allergy and AIA, respectively. We further performed functional analyses of the susceptible SNPs.

Results: Two *NLRP3* SNPs (rs4612666 and rs10754558) were significantly associated with susceptibility to food-induced anaphylaxis ($P = .00086$ and $P = .00068$, respectively). The *NLRP3* haplotype of the 2 SNPs also showed a significant association ($P = .000098$). We could confirm the association with susceptibility to another hypersensitivity phenotype, AIA (rs4612666, $P = .0096$). Functional analysis revealed that the risk alleles of rs4612666 and rs10754558 increased the enhancer activity of *NLRP3* expression and *NLRP3* mRNA stability, respectively.

Conclusion: Our results indicate that the *NLRP3* SNPs might play an important role in the development of food-induced anaphylaxis and AIA in a gain-of-function manner. Further research on the NLRP3 inflammasome will contribute to the development of novel diagnostic and therapeutic methods for food-induced anaphylaxis and AIA. (J Allergy Clin Immunol 2009;124:779-85.)

Key words: NLR family, pyrin domain containing 3, gene polymorphism, association study, food allergy, hypersensitivity, anaphylaxis, aspirin-induced asthma, enhancer activity, mRNA stability

Food allergy is defined as an adverse immune response to food proteins, and food-induced allergic reactions are responsible for a variety of symptoms involving the skin, gastrointestinal tract, and respiratory tract.[1] Food allergy has increased in the past 10 to 15 years, particularly in industrialized countries.[2] In Japan large-scale morbidity surveys demonstrated that food allergies occur in 5% to 10% of infants and preschool children, which is similar to the rate seen in other industrialized countries.[3-5] Although environmental factors could contribute to the recent increase in food allergies, several family studies have indicated that genetic factors also influence the risk of food allergies.[6,7] Little is known about the specific genes associated with susceptibility to food allergies, and recent studies have shown that polymorphisms of *CD14*[8]; signal transducer and activator of transcription 6 *(STAT6)*[9]; serine peptidase inhibitor, kazal type 5 *(SPINK5)*[10]; and *IL10*[11] are significantly associated with such susceptibility. Anaphylaxis is a life-threatening allergic reaction, and food is one of the most common responsible allergens.[12] In addition to the cutaneous, respiratory, and gastrointestinal symptoms, patients with anaphylaxis can experience cardiovascular symptoms, including hypotension, vascular collapse, and cardiac dysrhythmia, presumably because of massive mast cell mediator release.[13] Double-blind, placebo-controlled food challenge is the gold standard for the diagnosis of food allergy, and strict elimination of the allergenic food is the basic therapy for it.[13,14] Oral desensitization therapy is conducted in some cases to reduce the risk of a critical allergic reaction with accidental ingestion of allergenic food.[15] Although anaphylaxis is a severe side effect of both the food challenge test and oral desensitization therapy,[16] there are no completely reliable methods to estimate the risk for food-induced anaphylaxis.

From [a]the Laboratory for Respiratory Diseases, Center for Genomic Medicine, RIKEN, Kanagawa; [b]the Clinical Research Center for Allergy and Rheumatology, Sagamihara National Hospital, National Hospital Organization; the Departments of [c]Public Health and [d]Pediatrics, Graduate School of Medicine, Chiba University; [e]the School of Human Nursing, the University of Shiga Prefecture; [f]Miyatake Asthma Clinic, Osaka; [g]the Department of Pediatric Allergy, Osaka Prefectural Medical Center for Respiratory and Allergic Diseases; [h]NPO Japan Health Promotion Supporting Network, Wakayama; and [i]the Laboratory of Molecular Medicine, Human Genome Center, Institute of Medical Science, University of Tokyo.

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Reprint requests: Mayumi Tamari, MD, PhD, Laboratory for Respiratory Diseases, Center for Genomic Medicine, the Institute of Physical and Chemical Research (RIKEN), 1-7-22 Suehiro, Tsurumi-ku, Yokohama 230-0045 Kanagawa, Japan. E-mail: tamari@src.riken.jp.

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Abbreviations used
AIA: Aspirin-induced asthma
LD: Linkage disequilibrium
NLR: Nucleotide-binding domain, leucine-rich repeat–containing
NLRP3: NLR family, pyrin domain containing 3
OR: Odds ratio
SNP: Single nucleotide polymorphism

Aspirin-induced asthma (AIA) is a common clinical presentation of aspirin hypersensitivity, and this acute reaction is elicited through COX inhibition by nonsteroidal anti-inflammatory drugs.[17] AIA and autoimmune diseases partly share some clinical features and laboratory markers.[18] The natural course of AIA is similar to that of persistent viral infection of the respiratory system, and infectious factors have been shown to play a role in AIA.[17] However, the causative factors for the disease remain elusive.

NLR family, pyrin domain containing 3 (NLRP3; also known as NALP3, CIAS1, PYPAF1, or cryopyrin), is a member of the nucleotide-binding domain, leucine-rich repeat–containing (NLR) family of genes encoding proteins that comprise a nucleotide-binding domain and a leucine-rich repeat domain, and it controls the activity of inflammatory caspase-1 by forming complexes called inflammasomes.[19] Recent studies have shown that tight collaboration between pathogen-associated molecular patterns and damage-associated molecular patterns is needed to start an innate immune response to allergens,[20] and NLRP3 inflammasomes are activated by pathogen-associated molecular patterns, microbial toxins, live bacteria, viruses, and damage-associated molecular patterns.[21] However, the mechanism of activation of the NLRP3 inflammasome is not fully understood. After being activated, NALP3 recruits apoptosis-associated speck-like protein containing a card and procaspase-1, leading to autocatalytic processing and activation of caspase-1. Active caspase-1 catalyzes cleavage of the procytokines IL-1β and IL-18, which are both proinflammatory cytokines involved in the host response to infection and injury.[22] Excessive production of IL-1β and IL-18 is associated with septic shock and autoimmune disorders.[22]

The human *NLRP3* gene is located in 1q44. Previous studies have determined that the nonsynonymous coding substitution of *NLRP3* causes autoinflammatory diseases: V198M, L353P, A439V, and E627G are associated with familial cold autoinflammatory syndrome; R260W, A352V and G569R are associated with Muckle-Wells syndrome; and D303N, F309S and F537S are associated with chronic infantile neurological, cutaneous, and articular syndrome.[19] Furthermore, recent studies have shown that *NLRP3* polymorphisms are significantly associated with susceptibility to common inflammatory diseases, such as Crohn disease,[23] psoriatic juvenile idiopathic arthritis,[24] and essential hypertension.[25] However, the association of *NLRP3* polymorphisms with susceptibility to allergic diseases has not been reported.

To clarify the genetic factors that increase the risk of the hypersensitive phenotype of allergy, we conducted an association study of *NLRP3* polymorphisms with susceptibility to food allergy, food-induced anaphylaxis, and AIA in a Japanese population. Functional effects of the related *NLRP3* variants were also examined.

METHODS

Subjects

Three hundred twenty pediatric patients with food allergies and positive antigen-specific IgE results (CAP-RAST) were recruited at National Sagamihara Hospital, Kanagawa, Japan. We performed food challenge tests for 178 patients with food allergies. The diagnosis of food allergy was made either based on challenge tests or a definitive episode plus confirmation of antigen-specific IgE levels. Two hundred fifty-four nonatopic, nonasthmatic healthy unrelated control children were recruited from an elementary school affiliated with the Education Department of Chiba University, Chiba, Japan. Healthy subjects included in this group had no history of food allergies, asthma, or atopic dermatitis. Detailed information for patients with food allergy and healthy children is shown in Table I. Food-induced anaphylaxis was defined as symptoms evoked in multiple organs. Five hundred forty-nine adult asthmatic patients were recruited from Miyatake Asthma Clinic and the National Sagamihara Hospital.[26,27] Among the 549 patients, 79 were aspirin intolerant, and 470 were aspirin tolerant. All subjects with asthma were given diagnoses according to the criteria of the American Thoracic Society.[28] Detailed information on adult asthmatic patients is provided in Table E1 (available in this article's Online Repository at www.jacionline.org). All were unrelated Japanese subjects and provided written informed consent to participate in the study according to the rules of the Process Committee at the Center for Genomic Medicine, The Institute of Physical and Chemical Research (RIKEN).

Variation screening of *NLRP3*

We carried out screening of single nucleotide polymorphisms (SNPs) with genomic DNA from 12 healthy subjects. A total of 17 overlapping primer sets were designed on the basis of the *NLRP3* genomic sequence available from the National Center for Biotechnology Information (accession no. NM_001079821.1). The complete coding region of *NLRP3*, intron/exon boundaries, and 100 bases of the surrounding intronic sequence and approximately 3 kb of 5′ genomic DNA were resequenced. The PCR product was reacted with BigDye Terminator v3.1 (Applied Biosystems, Foster City, Calif). Sequences were assembled and polymorphisms were identified with the SEQUENCHER program (Gene Codes Corp, Ann Arbor, Mich).

Selection of *NLRP3* polymorphisms for genotyping

Genomic DNA was prepared from peripheral blood samples by using standard protocols. We selected tag SNPs of the exons and introns of *NLRP3* with a minor allele frequency of greater than 10% in the HapMap Japanese data set (http://www.hapmap.org/). Pairwise linkage disequilibrium (LD) was calculated as r^2 values by using the Haploview 4.1 program (http://www.broad.mit.edu/mpg/haploview/). Haplotype frequencies for the 2 most susceptible SNPs were also estimated with the Haploview 4.1 program. Genotyping of SNPs was performed by using the TaqMan allele-specific amplification method (Applied Biosystems).

Real-time quantitative RT-PCR

Total RNA from normal human tissues was purchased from Clontech (Mountain View, Calif), and total RNA from cell lines was extracted with a NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany). Each RNA was reverse transcribed with Superscript III reverse transcriptase and oligo dT primers (Invitrogen, Carlsbad, Calif). The expression of *NLRP3* transcripts was determined by using real-time quantitative RT-PCR with SYBR Premix Ex Taq (Takara, Shiga, Japan) and specific primers (5′-GGGGTCAT GATGTTCTGTGA-3′ and 5′-CAGGCTTTTCTTCTTGAAGTGTT-3′). In all experiments the amounts of cDNA were standardized by means of quantification of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)*.

Luciferase assay

Intron 7 and the last exon fragments of *NLRP3* from human genomic DNA were amplified by means of PCR by using specific primers (5′-GCCACTATG

TABLE I. Clinical information about patients with food allergy and control subjects

Characteristics	Patients with food allergy			Control subjects, n = 254 (%)
	Total, n = 320 (%)	Anaphylaxis (+), n = 98 (%)	Anaphylaxis (−), n = 222 (%)	
Age (y)	4.2 ± 3.3	5.4 ± 3.1	3.7 ± 3.3	9.0 ± 1.7
Sex				
Male	233 (72.8)	67 (68.4)	166 (74.8)	124 (48.8)
Female	87 (27.2)	31 (31.6)	56 (25.2)	130 (51.2)
Log serum total IgE (IU/mL)	2.6 ± 0.7	2.8 ± 0.4	2.6 ± 0.7	2.4 ± 1.3
Food allergy phenotype				
Infantile atopic dermatitis	268 (83.8)	76 (77.6)	192 (86.5)	—
Immediate type	294 (91.9)	96 (98.0)	198 (89.2)	—
OAS	12 (3.8)	4 (4.1)	8 (3.6)	—
FEIAn	6 (1.9)	5 (5.1)	1 (0.5)	—
Complications				
Atopic dermatitis	223 (69.7)	64 (65.3)	159 (71.6)	—
Bronchial asthma	108 (33.8)	42 (42.9)	66 (29.7)	—
Allergy rhinitis	47 (14.7)	17 (17.3)	30 (13.5)	—
Allergic conjunctivitis	39 (12.2)	15 (15.3)	24 (10.8)	—

OAS, Oral allergy syndrome; *FEIAn*, food-dependent exercise-induced anaphylaxis.

GAAAACAGCAC-3′ and 5′-AAGGAAGCACCCGTACCTGC-3′ and 5′-GTTGTCTGAAATGTATTTCAATT-3′ and 5′-TTTGAAAAATTTCTAGG TACTCT-3′, respectively). PCR products were subcloned into the reporter gene pGL3-promoter vector (Promega, Madison, Wis). Vector pRL-TK was used to normalize for variations in transfection efficiency. These plasmids were transfected into THP-1 cells by using FuGENE 6 (Roche, Basel, Switzerland). The luciferase activities were determined by using the Dual-Luciferase Reporter Assay system (Promega).

Allele-specific transcript quantification

PBMCs were isolated from 3 healthy donors with heterozygous *NLRP3* rs10754558 by means of density gradient centrifugation. Primary monocytes were sorted with human CD14 microbeads and an autoMACS (Miltenyi Biotec, Bergisch Gladbach, Germany), and genomic DNA and total RNA were isolated. We performed allelic expression analyses using the TaqMan assay with SNP genotyping probes. Genomic DNA was used as a control for equal biallelic representation. The ratio of 5-carboxyfluorescein (FAM) intensity to VIC intensity for *NLRP3* was plotted for mixtures of homozygous DNAs at 6 different ratios (3:1, 2:1, 3:2, 2:3, 1:2, and 1:3), with correction based on the signal intensities of heterozygote controls for a standard line. We then measured the allelic ratio for each cDNA and genomic DNA from each subject and calculated the allelic ratio of cDNA and genomic DNA based on the standard line.

Prediction of transcription factor binding sites

The TRANSFAC Professional 10.3 (http://www.biobase.de/pages/) and TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html) Web sites were used to predict putative transcription factor binding sites. RNA secondary structures were predicted by using the mfold Web server (http://mfold.bioinfo.rpi.edu/) setting default parameters and a folding temperature of 37°C.

Electrophoretic mobility shift assays

Nuclear extracts were prepared from THP-1 cells. Extracts were quickly frozen and stored in aliquots at −80°C. Electrophoretic mobility shift assays was performed by using a 2nd Generation DIG Oligonucleotide 3′-end Labeling Kit and 2nd Generation DIG Gel Shift Kit (Roche), according to the manufacturer's instructions. We incubated the nuclear extract with 28-bp double-strand oligonucleotide probes for rs4612666 (C and T) for 30 minutes at room temperature. The oligonucleotide sequences were 5′-GGAGCTGGGAAGACGTAGTATTGGTGGG-3′ for the C allele and 5′-GGAGCTGGGAAGATGTAGTATTGGTGGG-3′ for the T allele, respectively. For the supershift experiments, a rabbit anti-human GATA-2 antibody (Santa Cruz Biotechnology, Santa Cruz, Calif) and GATA-2 consensus probe (5′-CACTTGATAACAGAAAGTGATAACTCT-3′) were used.

Statistical analysis

We calculated allele frequencies and tested agreement with Hardy-Weinberg Equilibrium using a χ^2 goodness-of-fit test at each locus. We then compared differences in allelic frequencies of the polymorphisms between cases and control subjects by using a 2×2 contingency χ^2 test with 1 *df* and calculated odds ratios (ORs) with 95% CIs. Logistic regression analysis was implemented for the susceptibility to anaphylaxis or AIA and genotype to assess the effects of sex (SPSS 14.0 J; SPSS, Inc, Chicago, Ill). The small sample size of this study decreased the ability to detect associations. Power in this study was estimated with the aid of Sample Power 2.0 (SPSS, Inc). If ORs of risk alleles with control group frequencies of 0.05, 0.1, 0.2, and 0.4 were greater than 3.41, 2.61, 2.16, and 1.98, respectively, power exceeded 80% (at $P = .05$) in allelic association tests of food allergy (222 subjects with food allergy without anaphylaxis [control] and 98 subjects with food-induced anaphylaxis). Similarly, in allelic association tests in patients with adult asthma (470 asthmatic patients without AIA [control] and 79 patients with AIA), a power of 80% was ensured if alleles with frequencies of 0.05, 0.1, 0.2, and 0.4 had ORs of greater than 3.41, 2.61, 2.16, and 1.98, respectively. A P value of less than .05 was considered statistically significant. Expression differences between genotypic groups were tested with the Student *t* test and Mann-Whitney *U* test.

RESULTS

Association of *NLRP3* SNPs with susceptibility to food-induced anaphylaxis

Thirty-nine polymorphisms with a frequency of greater than 10% in the *NLRP3* region were contained in the public databases at the National Center for Biotechnology Information dbSNP Web site (http://www.ncbi.nlm.nih.gov/SNP/). We selected 15 polymorphisms for association studies using tagger in the Haploview 4.1 program, and these 15 SNPs captured 39 of the 39 alleles with a mean r^2 value of 0.98 ($r^2 > 0.80$; Fig 1, *A*). We next carried out case-control association studies of the 15 SNPs. The control genotypes did not deviate from Hardy-Weinberg equilibrium. Although no significant association was observed between any SNP and food allergy, 7 SNPs showed significant associations with susceptibility to food-induced anaphylaxis (Table II and see Table E2 in this article's Online Repository at www.jacionline.org). Among

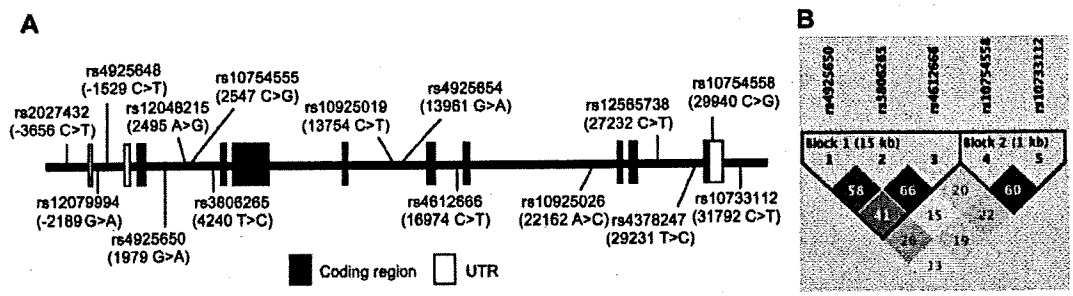

FIG 1. A, Exon–intron structure of the *NLRP3* gene and locations of genotyped tag SNPs. *UTR*, Untranslated region. B, LD structures of the 5 significantly associated *NLRP3* SNPs with susceptibility to food-induced anaphylaxis ($P < .005$). LD was calculated by using genotyping data. Pairwise r^2 values for all combinations of SNPs are shown in gray scale.

TABLE II. Association between *NLRP3* polymorphisms and susceptibility to food-induced anaphylaxis

			Anaphylaxis (+), n = 98 (%)			Anaphylaxis (−), n = 222 (%)			Frequency (allele 1)				
Allele 1/2	dbSNP ID	Location	1/1	1/2	2/2	1/1	1/2	2/2	(+)	(−)	*P* value	OR	95%CI
−3656 C/T	rs2027432	5′ Flanking	78 (80)	19 (19)	1 (1)	169 (76)	50 (23)	3 (1)	0.89	0.87	NS		
−2189 G/A	rs12079994	Intron 1	78 (80)	18 (18)	2 (2)	147 (67)	61 (28)	10 (5)	0.89	0.81	.021	1.81	1.09–2.99
−1529 C/T	rs4925648	Intron 1	81 (83)	17 (17)	0 (0)	166 (75)	54 (24)	2 (1)	0.91	0.87	NS		
1979 G/A	rs4925650	Intron 3	18 (18)	51 (52)	29 (30)	71 (32)	118 (53)	33 (15)	0.44	0.59	.00091	1.77	1.26–2.49
2495 A/G	rs12048215	Intron 3	58 (59)	39 (40)	1 (1)	123 (55)	85 (38)	14 (6)	0.79	0.75	NS		
2547 C/G	rs10754555	Intron 3	42 (43)	47 (49)	8 (8)	77 (35)	115 (52)	29 (13)	0.68	0.61	NS		
4240 T/C	rs3806265	Intron 3	43 (44)	44 (45)	10 (10)	62 (28)	114 (52)	43 (20)	0.67	0.54	.0029	1.71	1.20–2.43
13754 C/T	rs10925019	Intron 6	54 (55)	39 (40)	5 (5)	109 (49)	92 (41)	21 (10)	0.75	0.70	NS		
13961 G/A	rs4925654	Intron 6	67 (68)	25 (26)	6 (6)	144 (65)	71 (32)	7 (3)	0.81	0.81	NS		
16974 C/T	rs4612666	Intron 7	44 (45)	41 (42)	13 (13)	56 (26)	114 (52)	49 (22)	0.66	0.52	.00086	1.81	1.27–2.56
22162 A/C	rs10925026	Intron 8	27 (28)	49 (50)	22 (22)	83 (38)	111 (51)	26 (12)	0.53	0.63	.013	1.53	1.09–2.16
27232 C/T	rs12565738	Intron 10	81 (83)	16 (16)	1 (1)	176 (79)	43 (19)	3 (1)	0.91	0.89	NS		
29231 T/C	rs4378247	Intron 10	84 (86)	13 (13)	1 (1)	181 (82)	40 (18)	1 (1)	0.92	0.91	NS		
29940 C/G	rs10754558	Exon 11 (3′UTR)	25 (26)	46 (47)	27 (28)	83 (38)	109 (50)	25 (12)	0.49	0.63	.00068	1.80	1.28–2.54
31792 C/T	rs10733112	3′ Flanking	35 (36)	49 (50)	14 (14)	46 (21)	119 (54)	57 (26)	0.61	0.48	.0021	1.71	1.21–2.40

NS, Not significant; *UTR*, untranslated region.

5 food-induced anaphylaxis–susceptible SNPs for which the *P* value was less than .005, 3 (rs4925650, rs3806265, and rs4612666; $r^2 \geq 0.41$) and 2 (rs10754558 and rs10733112, $r^2 \geq 0.60$) were in moderate LD with each other (Fig 1, *B*). Because we could not find any SNP that was in strong LD with the 5 variants by resequencing and searching the dbSNP database, we considered the rs4612666 ($P = .00086$; OR, 1.81) and rs10754558 ($P = .00068$; OR, 1.80) variants that were the most susceptible SNPs in each LD block to be associated with the susceptibility to food-induced anaphylaxis. The results of stepwise logistic regression analysis for the susceptibility to anaphylaxis showed significant effects of the genotypes rs4612666 (Wald statistic = 13.38, $df = 1$, $P = .00025$) and rs10754558 (Wald statistic = 6.23, $df = 1$, $P = .013$). There was no significant effect of sex by means of logistic regression analysis. We further divided the subjects with food allergies into 2 groups, those with and without food challenge tests. We found significant associations between the 2 SNPs rs4612666 and rs10754558 and food-induced anaphylaxis in both groups, but a marked effect of food challenges was not observed (data not shown).

We next investigated the effects of *NLRP3* haplotypes with susceptibility to food-induced anaphylaxis. As shown in Table III, the frequency of combination of the most susceptible alleles between LD blocks (rs4612666-C and rs10754558-G) was significantly increased in patients with food-induced anaphylaxis (haplotype_1 vs haplotype_4; $P = .000098$; OR, 2.21).

An association study of *NLRP3* SNPs with susceptibility to AIA

Approximately 10% of adult asthmatic patients are affected by AIA. As well as food-induced anaphylaxis, patients with AIA show acute life-threatening hypersensitivity symptoms.[29] Therefore we performed an association study between the 5 food-induced anaphylaxis–susceptible *NLRP3* SNPs and susceptibility to AIA. rs4612666 showed a significant association with susceptibility to AIA, and the direction of association was similar to that of food-induced anaphylaxis (see Table E3 in this article's Online Repository at www.jacionline.org). The results of stepwise logistic regression analysis for the susceptibility to AIA showed significant effects of genotype rs4612666 (Wald statistic = 4.34, $df = 1$, $P = .037$). No significant effect of sex was found by means of logistic regression analysis. These results indicated that *NLRP3* SNPs were significantly associated with hypersensitivity, such as food-induced anaphylaxis and AIA.

TABLE III. Haplotype frequency in *NLRP3*

	rs4612666	rs10754558	No. Case	No. Control	Frequency Case	Frequency Control
Haplotype_1	C (susceptible)	G (susceptible)	89	130	0.45	0.30
Haplotype_2	T (nonsusceptible)	G (susceptible)	11	27	0.06	0.06
Haplotype_3	C (susceptible)	C (nonsusceptible)	39	92	0.20	0.21
Haplotype_4	T (nonsusceptible)	C (nonsusceptible)	56	181	0.29	0.42
	***P* value**	**OR**	**95% CI**			
Haplotype_1 vs haplotype_4	.000098	2.21	1.48-3.31			

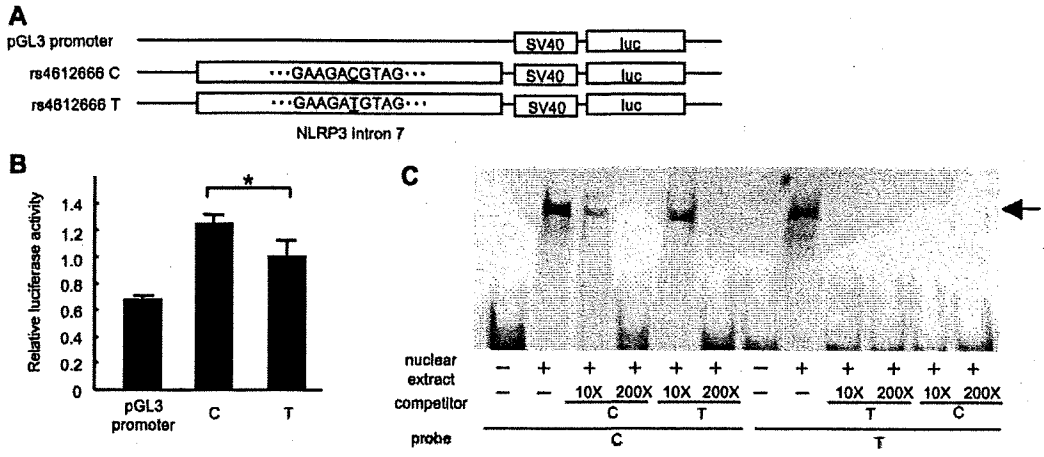

FIG 2. Functional analyses of *NLRP3* rs4612666 using THP-1 cells. **A**, Plasmid constructs used for transfection. **B**, Transcriptional enhancer activities of rs4612666 measured by luciferase *(luc)* activity 24 hours after transfection. Values of relative luciferase activity are shown as means±SDs. **P* < .05, Student *t* test. **C**, Electrophoretic mobility shift assays of rs4612666. An unlabeled probe was used as a competitor. C, C allele; T, T allele.

Influence of rs4612666 on differential expression of *NLRP3* mRNA

We next conducted functional analysis of the rs4612666 SNP. Because rs4612666 was located in intron 7, we examined whether the genomic region around rs4612666 had enhancer activity. At first, to survey the *NLRP3* mRNA expression levels in cells and tissues, we conducted real-time quantitative RT-PCR. *NLRP3* mRNA was dominantly expressed in peripheral leukocytes (see Fig E1*A*, in this article's Online Repository at www.jacionline.org), and among the cell lines of leukocytes, higher expression of *NLRP3* mRNA was detected in the monocyte cell line THP-1 (see Fig E1*B*). We subsequently performed a luciferase assay to test whether the allelic difference contributed to the efficiency of expression of *NLRP3* mRNA using THP-1 cells. The allele-specific constructs containing the food-induced anaphylaxis risk allele rs4612666 showed 1.2-fold higher transcriptional enhancer activity than the other constructs containing the T allele of rs4612666 (Fig 2, *A* and *B*). These experiments were performed 4 times with similar results. We next searched for nuclear transcription factors that might bind to oligonucleotide sequences containing rs4612666 by using TRANSFAC and TFSEARCH, and the genomic region containing the C allele of rs4612666 was found to create a novel consensus sequence corresponding to the putative binding element of GATA-2. Therefore we examined the allelic differences in the binding of nuclear proteins by using the electrophoretic mobility shift assay. The signal intensity of the DNA-protein complex derived from the C allele was higher than that from the T allele in the presence of THP-1 nuclear extract, and the complex was diminished by excess amounts of a nonlabeled allele-specific competitor probe (Fig 2, *C*). Four independent experiments were performed with similar results. However, the band was not supershifted by the addition of antibodies to GATA-2 in the present study (see Fig E2 in this article's Online Repository at www.jacionline.org).

Contribution of the 3′ untranslated region rs10754558 SNP to *NLRP3* mRNA stability

Like the *NLRP3* rs4612666 in intron 7, rs10754558 was significantly associated with susceptibility to food-induced anaphylaxis. Because of the location of rs10754558 in the 3′ untranslated region, we further examined whether the risk allele of rs10754558 affected the stability of the *NLRP3* mRNA. RNA secondary structure prediction with the mfold Web server showed that the risk allele contributed to the mRNA stability more than the other allele of rs10754558 (see Fig E3 in this article's Online Repository at www.jacionline.org). First, we performed a luciferase assay using THP-1 cells. The allele-specific construct containing the G allele of rs10754558 showed 1.3-fold higher activity than the other constructs containing the C allele of rs10754558 (Fig 3, *A* and *B*). These experiments were performed 4 times

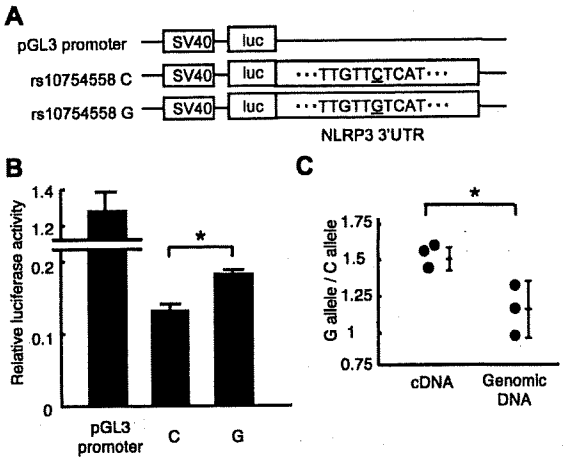

FIG 3. Functional analyses of *NLRP3* rs10754558 using THP-1 cells. **A**, Plasmid constructs used for transfection. *UTR*, Untranslated region. **B**, Effect of the SNP on mRNA stability as measured by luciferase activity. Values of relative luciferase activity are shown as means±SDs. $*P < .0005$, Student *t* test). *C*, C allele; *G*, G allele. **C**, Allele-specific transcript quantification of rs10754558 is shown as means ± SDs. $*P < .05$, Mann-Whitney *U* test.

with similar results. To further investigate the effect of rs10754558 on transcription of mRNA, we performed allele-specific quantitative PCR with a TaqMan probe on human primary monocytes from healthy donors with heterozygous genotypes of rs10754558. In these cells the mean ratio (susceptible vs nonsusceptible allele) was 1.50, which is significantly higher than that of DNA amplicons (ratio = 1.15; $P = .0495$, Mann-Whitney *U* test; Fig 3, *C*). Three independent experiments were performed with similar results. These results indicated that the higher expression of *NLRP3* mRNA was a component of the pathologic mechanisms leading to food-induced anaphylaxis.

DISCUSSION

The common feature of food-induced anaphylaxis and AIA is the immediate hypersensitivity reaction. In the present study we identified significant associations between human *NLRP3* polymorphisms and susceptibility to food-induced anaphylaxis and AIA. Because the 2 *NLRP3* SNPs rs4612666 and rs10754558 were not in strong LD ($r^2<0.20$), it is possible that the SNPs could contribute susceptibility to food-induced anaphylaxis independently. We further found a significant association between AIA and the rs4612666 variant, and the direction of association was similar to the finding in food-induced anaphylaxis. Functional analyses of the 2 related *NLRP3* polymorphisms showed that both variants influenced higher mRNA expression by altering expression enhancer activity or mRNA stability. These observations suggest that human NLRP3 appears to be involved in the hypersensitive immune reaction in allergy through gain-of-function variants.

Several recent studies have shown that nonsynonymous substitutions of *NLRP3* are associated with rare, severe autoinflammatory diseases, such as familial cold autoinflammatory syndrome, Muckle-Wells syndrome, and chronic infantile neurological, cutaneous, and articular syndrome.[19] However, these nonsynonymous substitutions and polymorphisms were not in LD with either of the SNPs associated with susceptibility to food-induced anaphylaxis and AIA in the present study ($r^2 < 0.20$).

In this study the 2 *NLRP3* SNPs associated with food-induced anaphylaxis did not show any association with susceptibility to food allergy. Food-induced allergic reactions exhibit various symptoms, ranging from localized urticaria to severe life-threatening anaphylaxis. In subjects with anaphylaxis caused by insect stings or food, many recent studies have shown no clear relationship between the levels of allergen-specific IgE and the presence, absence, or severity of the clinical response to the allergen.[16] Different genetic factors might be involved in the diverse immunologic responses to foods, and innate immune activation through NLRP3 inflammasomes sensing food components might be one of the immunologic mechanisms in anaphylaxis.

In the present study we found *NLRP3* polymorphisms that increased the risk of the hypersensitive phenotype of allergy. Murine studies have shown that the NLRP3 inflammasome is essential for the establishment of contact hypersensitivity, in which chemical damage to cells or tissues by a hapten is required for establishment of the allergy.[22,30] Recent reports have demonstrated that uric acid, calcium pyrophosphate dehydrate, silica, and asbestos particles activate the NLRP3 inflammasome.[31] Some components contained in food or food ingredients might become activators of the NLRP3 inflammasome and lead to caspase-1 activation to promote the processing and secretion of proinflammatory inflammatory cytokines. However, further analyses to clarify the relationship between NLRP3 inflammasomes and food components are needed. A recent study has reported that aspirin enhances allergic symptoms in patients with food-dependent exercise-induced anaphylaxis, the symptoms of which are severe allergic reactions, such as shock or hypotension.[32,33] The roles of aspirin and nonsteroidal anti-inflammatory drugs in the NLRP3 inflammasome response also remain unexplored.

NLRP3 is in the NLR family of proteins, and other NLR family genes have been shown to be associated with susceptibility to various inflammatory diseases: polymorphisms of nucleotide-binding oligomerization domain containing 1 *(NOD1)* with asthma[34] and inflammatory bowel disease,[35] nucleotide-binding oligomerization domain containing 2 *(NOD2)* with Crohn disease,[36] and *NLRP1* with vitiligo-associated multiple autoimmune disease.[37] Genetic studies on whether the polymorphisms of other NLR family genes are associated with food allergy, food-induced anaphylaxis, and AIA susceptibility remain to be conducted.

Further investigation of the roles of NALP3 inflammasomes in food-induced anaphylaxis and AIA might contribute to our understanding of the pathophysiology of these severe and potentially life-threatening systemic allergic reactions and to the development of novel diagnostic methods for risk assessment of patients with anaphylaxis or AIA.

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Key messages

- Gain-of-function variants of the *NLRP3* gene are associated with food-induced anaphylaxis and AIA.
- The NLRP3 inflammasome might play an important role in the hypersensitivity phenotype of allergy.

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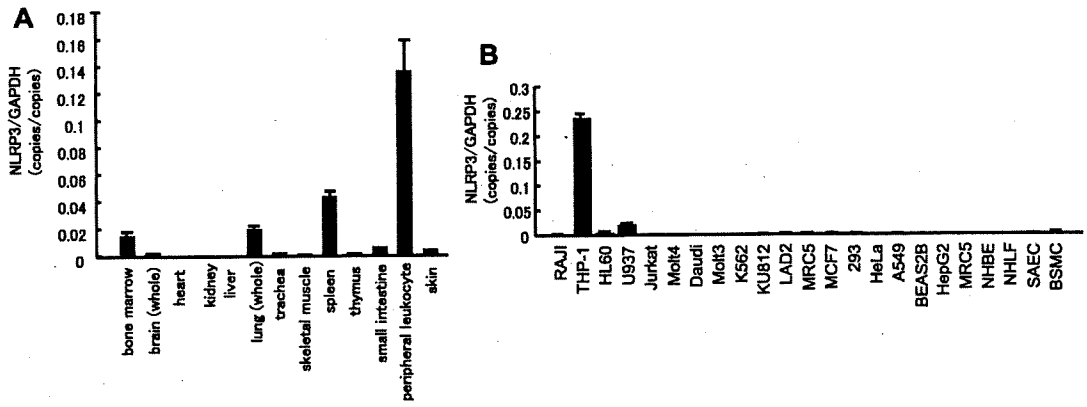

FIG E1. Comparison of relative mRNA expression of *NLRP3* in different tissues **(A)** and cell lines **(B)**. The results were normalized to glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)* transcripts. *NHBE*, Normal human bronchial epithelial cells; *NHLF*, normal human lung fibroblasts; *SAEC*, normal human small airway epithelial cells; *BSMC*, bronchial smooth muscle cells. Results are means ± SDs of triplicate assays.

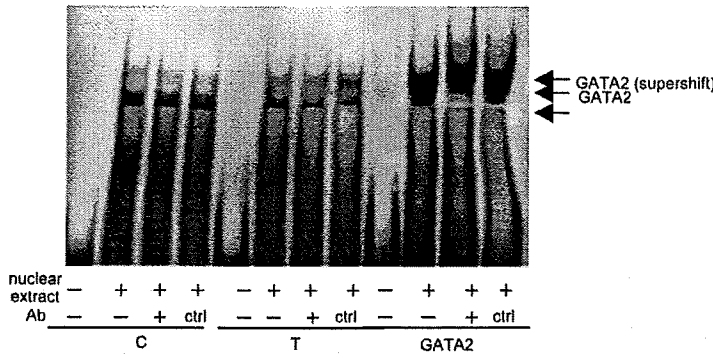

FIG E2. Electrophoretic mobility shift assay of rs4612666. Normal rabbit IgG and a GATA-2 consensus probe were used for control. Two independent experiments were performed with similar results. *Ab*, Antibody; *C*, C allele; *T*, T allele.

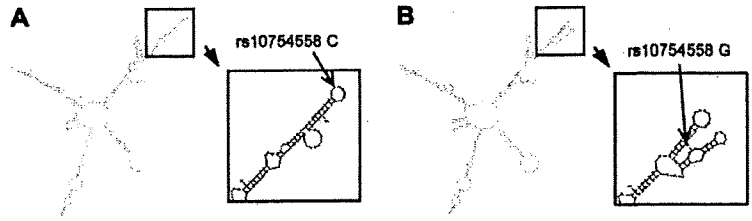

FIG E3. Prediction of the RNA secondary structure. Possible conformations of rs10754558-C **(A)** and rs10754558-G **(B)** and the most stable predicted structure show a dG (Gibbs free energy), which is a free energy increment related to the permissible structural transitions from the unstructured to structured state of −130.3 and −133.1 kcal/mol, respectively. *Arrows* indicate the region in which the rs10754558 SNP is located.

TABLE E1. Clinical information about patients with AIA and control subjects

Characteristics	Patients with bronchial asthma		Control subjects, n = 730 (%)
	AIA (+), n = 79 (%)	AIA (−), n = 470 (%)	
Age (y)	37.7 ± 14.2	37.0 ± 19.8	49.4 ± 14.5
Sex			
Male	25 (31.6)	205 (43.6)	532 (72.9)
Female	54 (68.4)	265 (56.4)	198 (27.1)
Log serum total IgE (IU/mL)	2.3 ± 0.5	2.3 ± 0.6	—