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1. 特許取得
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玉利真由美、広田朝光、久保充明 理化学研究所 特願2012-192247

2. 実用新案登録
なし

3.その他
なし

アトピー性皮膚炎の発症・症状の制御および治療法の確立普及に関する研究

（副題）痒み・掻破行動と皮膚炎増悪メカニズムに関するアトピーモデルを用いた研究

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研究協力者　古江増隆（同教授）、江﨑仁一（同大学院生）

A. 研究目的

アトピー性皮膚炎の慢性皮膚病変部においては、表皮内知覚神経侵入・伸長とその執拗な痒みとの関連が指摘されている。マウスの Th2 型慢性皮膚炎モデルにおいて、アトピー性皮膚炎治療外用薬であるタクロリムス(FK506 ; FK) は表皮内神経伸長を抑制し、掻破行動も抑制すると報告され、これらの関連が指摘されている。我々はこれまでに、ハプテンおよびダニ抗原誘発性のアトピー性皮膚炎モデルにおいて、MAPK /ERK kinase1/2 (MEK1/2)阻害薬のひとつであるCX659S（CX）が、FKと同程度に表皮内神経伸長は抑制する一方、FKとは異なり掻破行動の抑制はしないことを確認した。

その中で掻破行動を抑制したステロイド外用(betamethasone valerate: BV)、FKと掻破行動を抑制しなかったCXとの検討では、血清 IgE 値や局所でのIL-17A発現が掻破行動と何らかの関連を持つ可能性が考えられた。今回、抗 IgE 抗体を投与することで掻破行動を抑制するか検証したい。

B. 方法

NC/Ngaマウスの剃毛した背部と耳に、精製ダニ蛋白抗原（ビオスタ AD®）を週に 2 回、 3 週間塗布して慢性皮膚炎を発症させた。同時に外用薬として BV 、FK、CXを用いて連日外用し、CX外用群に対し週に 1 度抗 IgE 抗体を皮下注射により投与し、掻破行動、皮膚炎の状態、組織像、血清、mRNA等を比較検討した。

C. 結果

「耳介腫脹反応」および「皮膚炎スコア」については、BV、FK外用群では陽性対照群と比較して有意に抑制されたものの、CX外用群では抑制されず、CX＋抗 IgE 抗体投与群においても陽性対照群と同等の「耳介腫脹反応」が見られた。第 3 週目における「掻破行動」においても、BV、FK外用群で陽性対照群と比較して有意に抑制されたものの、CX外用、 CX+ 抗IgE 抗体投与群では抑制される傾向がみられなかった。第 3 週後の血清総 IgE 値は BV 、FK外用群でのみ有意に減少し、CX+ 抗 IgE 抗体投与群では低下する傾向にあった。血清TARC値は BV 外用群でのみ有意に減少していた。第 3 週後の背部皮膚炎惹起部の組織学的解析では、BV、FK、CX外用群ともに陽性対照群と比較して、浸潤「炎症細胞数」、「肥満細胞数」、および「IL-4陽性細胞数」は有意に少なかった。また「 IL-17 陽性細胞数」および「 IgE 陽性細胞数」は BV 、FK外用群でのみ有意に減少していた。「PGP9.5陽性神経線維」による表皮内神経伸長に関しては、BV、FK、CX外用、CX＋抗 IgE 抗体投与群ともに陽性対照群と比較して、有意に神経伸長の程度が抑制されていた。病変部皮膚から抽出したmRNAレベルでの発現量では、BV、FK外用群でのみIL-17a、TSLP、IL-6の有意な発現量低下を認めた。

D. 考察

今回、精製ダニ蛋白抗原塗布を用いてアトピー性皮膚炎モデルのNC/Ngaマウスに皮膚炎を発症させ、CX外用＋抗 IgE 抗体投与による掻破行動に対する効果を検討した。CX外用群と同様に組織学的な皮膚炎、神経伸長は抑制するものの、掻破行動に関しては抗 IgE 抗体投与郡でも抑制がみられなかった。皮膚炎発症後の血清総 IgE 値が陽性対照群と比較して有意に低下していないことから、完全に IgE 抗体のブロッキングができていなかったことが示唆され、投与量、投与法、ブロッキング法の検討などが必要と思われた。

E. 結論

抗 IgE 抗体の皮下投与では掻破行動の抑制は見られなかった。 IgE 抗体の完全なブロッキングができていなかったため、今後投与方法や投与量の検討が必要である。（平成25年 1 月30日）

アトピー性皮膚炎の発症・症状の制御および治療法の確立普及に関する研究

（副題）1.脊髄内痒み神経の同定に関する研究　2.石垣島コホート研究

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A. 研究目的

1. 痒みという感覚は「掻きたいとう欲求を引き起こす不快な感覚」であり、皮膚における痒みを伝達する一次求心性神経線維に関してはC線維がその役割を担っていることが知られている。一方で、C線維は痛みや冷温覚をはじめとした痒みとは異なる感覚を伝達することも知られている。我々はこれまでに、痒み特異的な一次求心性神経線維の同定のため、ラットにセロトニン（5-HT）塗布による痒み知覚モデルを作製して脊髄後根神経節における電気生理学的記録を行い、5-HTによる痒み刺激に特異的に反応するC線維を同定した。本研究では後根神経節で記録した一次求心性神経線維が脊髄後角ではどの部分に入力し、その後どの神経伝導路を通り伝達されていくのかを検討したい.

2. アトピー性皮膚炎の発症に寄与する諸因子の解明は重要な課題であるが、我が国におけるコホート研究は数少ない。そのような現状の中、我々はこれまでに平成13年度から沖縄県石垣島の乳幼児（保育園児）の集団検診と保護者への質問票によるアンケート調査、採血による血液データの解析を開始し、石垣島の乳幼児コホートの前向き調査を可能としている。これまでに、発症頻度・IgE値・感染症の合併・本症発症の寄与因子などを報告してきた。今後、CCL17/TARCやCCL22/MDCなどのTh2ケモカインの推移とアトピー性皮膚炎の発症や予後を検証したい。

B. 方法

1. SDラットの胸髄を麻酔下に露出し、末梢に5-HTを塗布してパッチクランプ法にて脊髄後角部の膜電流を測定することで、脊髄後角における5-HTによる痒み刺激の入力部位を検討した。

2. 患者アンケート結果、病歴、血清CCL17/TARC、CCL22/MDCの結果を元にデータ解析を行った。

C. 結果

1. 5-HTによる痒み刺激に応答するC線維は、応答しないC線維と比較すると有意に脊髄後角の浅層に入力していた。また、5-HTによる痒み刺激に反応する脊髄後角ニューロンは、後根神経節ニューロンと同じく機械的刺激に反応する性質を持っていた。

2. アンケート調査の多変量解析の結果、患児のアトピー性皮膚炎罹患の危険因子として、本人の気管支喘息または卵アレルギーの既往、父親や同胞のアトピー性皮膚炎の既往が挙がった。採血の結果では、アトピー性皮膚炎や卵アレルギーのある園児は有意に血清TARC値が高く、またアトピー性皮膚炎でかつ卵白特異的IgE値高値群（クラス２以上）で有意に血清TARC値が高かった。一方、血清MDC値はアトピー性皮膚炎有病児で有意に高値であったが、卵アレルギーとの相関は見られなかった。

D. 考察

1. 5-HTによる痒み刺激に反応する１次求心性神経線維は、後根神経節においてC線維の中で機械的刺激にも反応する多様式なニューロンであり、脊髄後角においても同様の性質を持っていた。脊髄後角での入力部位に関しては浅層であることから、侵害刺激や温熱刺激の伝導路と同一部位であることが確認された。

2. 卵アレルギーの既往がアトピー性皮膚炎の危険因子のひとつであり、乳幼児期のアトピー性皮膚炎には卵アレルギーが深く関わっていることが示唆された。また、卵アレルギーの既往があることで血清TARC値（アトピーの疾患重症度マーカー）が高値であったが、血清MDC値には有意差がみられなかった。卵白特異的IgE高値群においても同様の結果であり、TARCとMDCは同じTh2ケモカインでありながら卵アレルギーに関しては何らかの理由によりTARCがより関連性を持つと考えられた。

E. 結論

1. より中枢における痒み特異的神経の伝導路に関する検討を行う必要がある。

2. 経年的なアトピー性皮膚炎の有無とTh2ケモカインの推移などの疫学調査が必要である。

（平成25年１月30日）

アトピー性皮膚炎の発症・症状の制御および治療法の確立普及に関する研究

（副題）本土・琉球クラスターにおけるアトピー関連遺伝子の探索に関する研究

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研究協力者　竹内　聡（九州大学大学院医学研究院皮膚科学共同研究員）、林　純、古庄憲浩（九州大学大学院感染環境医学分野）、玉利真由美、広田　朝光（理化学研究所ゲノム医科学研究センター）、天谷雅行、海老原　全（慶應義塾大学医学部皮膚科学）、工藤　純（慶應義塾大学生命情報学センター）、佐伯秀久（東京慈恵会医科大学皮膚科）

A. 研究目的

アトピー性皮膚炎の有病率は上昇しており、現在、社会問題化している。本疾患の解明は未だ進んでおらず、分子生物学的アプローチを含めた、幅広い視点に立った病因の解明と新規治療薬の開発が切望されている。わが国でも IgE 産生能や気道過敏性に関する候補遺伝子や、最近ではフィラグリン遺伝子の転突然変異が高率に発見されるなどの報告がされつつあるが、未だ疾患を包括的に説明する決定的なものはない。その原因として、本疾患が環境因子に強く左右される面をもち、従来の解析方法がこの環境因子を排除できない方法論であり、候補遺伝子としては正確性を欠く結果になっているためである。より正確な候補遺伝子の探索のためには、ある地域に生活する集団を全体として解析するコホート解析を用いる必要がある。

我々はこれまでに、平成13年度から沖縄県石垣島の乳幼児の集団検診と採血による血液データの解析を開始しているが、初年度は0－6歳（平均 3.1 歳）の乳幼児 565 例のアトピー性皮膚炎の頻度と採血による IgE 値を測定し、さらに種々のアンケートを行ってきた。この検診は毎年継続されており、石垣島の乳幼児コホートの前向き調査を可能としている。H15-16 年にかけては最近保険適応となったアトピー性皮膚炎の重症度マーカー、TARC の血清値がアトピー患児での発症、持続、消退などの自然経過と強く関連することを見いだした。さらにアトピー疾患関連遺伝子の解明を進めるべくこのコホート群における遺伝子解析を行うため、血液サンプルの採取を行っている。

最近、日本人が SNP タイピングにより大きく Ryukyu-と Hondo クラスターの2つに分けられることが判明した。石垣島のコホート群における追跡調査、血液検査、遺伝子研究などで得られた成果 すなわち様々な臨床的アトピー関連因子や数々の候補遺伝子群やまた将来的にそれらを元にした病因の解明・新規治療などが日本人に広く応用可能であるかどうか、またはある特定の疾患サブグループや地域特異性（石垣島など）に認められる傾向にあるのかなどをより正確に検討するには、次なるステップとして同様の採血検査、遺伝子調査を本邦の他地域においても実施し、石垣島で得られた結果の有意性を確認する必要がある。そこで九州・山口地域一円からの人口流入地域である福岡と、全国からの人口流入地域である東京地域において得られる結果を、石垣スタディの結果と比較・検討したい。（本研究は平成 20 年 6 月 23 日付けで九州大学ヒトゲノム・遺伝子解析倫理審査専門委員会の承認を得ている。）

B. 方法

九州大学病院皮膚科、同総合診療部、慶応義塾大学病院皮膚科、東京大学にてアトピー性皮膚炎患者、健常者各 1000 人を目標に血液検体、アンケート調査票、ゲノムタイピングの結果を用いて解析する。

C. 結果

本研究用に「医師調査票」と ISSAC13-14 をと総合診療部調査票を元にした「患者アンケート票」を作成した。上記施設にて倫理委員会での承認申請後、調査・血液サンプル取得終了した。GWAS の結果、過去に報告されたヨーロッパあるいは中国から報告された7領域に加え、新たに 8 領域が同定された。（Hirota T, et al. Genome-wide association study identifies eight new susceptibility loci for atopic dermatitis in the Japanese population. Nat Genet 2012;44(11):1222-6.）

D. 考察

新たに同定された 8 領域は、気管支喘息における GWAS と同様に好塩基球活性化因子を含んでおり、また免疫調節因子であるビタミン D 代謝経路に関わる因子が含まれていた。

E. 結論

今後、GWAS で同定されたそれぞれの因子についてさらなる検討が必要である。GWAS の結果は、アトピー性皮膚炎のメカニズム及び治療を考える上で重要な知見と考える。（平成 25 年 1 月 30 日）

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Variants of C-C Motif Chemokine 22 (*CCL22*) Are Associated with Susceptibility to Atopic Dermatitis: Case-Control Studies

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Abstract

Atopic dermatitis (AD) is a common inflammatory skin disease caused by multiple genetic and environmental factors. AD is characterized by the local infiltration of T helper type 2 (Th2) cells. Recent clinical studies have shown important roles of the Th2 chemokines, CCL22 and CCL17 in the pathogenesis of AD. To investigate whether polymorphisms of the *CCL22* gene affect the susceptibility to AD, we conducted association studies and functional studies of the related variants. We first resequenced the *CCL22* gene and found a total of 39 SNPs. We selected seven tag SNPs in the *CCL22* gene, and conducted association studies using two independent Japanese populations (1st population, 916 cases and 1,032 controls; 2nd population 1,034 cases and 1,004 controls). After the association results were combined by inverse variance method, we observed a significant association at rs4359426 (meta-analysis, combined $P = 9.6 \times 10^{-6}$; OR, 0.74; 95% CI, 0.65–0.85). Functional analysis revealed that the risk allele of rs4359426 contributed to higher expression levels of *CCL22* mRNA. We further examined the allelic differences in the binding of nuclear proteins by electrophoretic mobility shift assay. The signal intensity of the DNA-protein complex derived from the G allele of rs223821, which was in absolute LD with rs4359426, was higher than that from the A allele. Although further functional analyses are needed, it is likely that related variants play a role in susceptibility to AD in a gain-of-function manner. Our findings provide a new insight into the etiology and pathogenesis of AD.

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Introduction

Atopic dermatitis (AD) is a pruritic and chronically relapsing inflammatory skin disease involving disturbed skin barrier functions, cutaneous inflammatory hypersensitivity and defects in the antimicrobial immune defense with a strong genetic background [1]. Predominant infiltration of Th2 cells is a hallmark of acute atopic AD skin lesions [2]. Most patients with AD have peripheral blood eosinophilia and increased serum IgE levels, which are reflected in an increased frequency of peripheral blood skin-homing Th2 cells producing IL-4, IL-5 and IL-13 [1]. C-C motif chemokine 22 (CCL22) and CCL17 are high-affinity ligands for CC-chemokine receptor 4 (CCR4) and induce selective migration of Th2 cells [3]. CCL22 plays a crucial role in controlling the trafficking of Th2 cells into sites of allergic inflammation and is considered to be involved in the pathology of

AD [4]. Keratinocytes from patients with AD highly express thymic stromal lymphopoietin (TSLP), and CCL22 is produced by TSLP-treated dendritic cells [5]. CCL22 is upregulated in lesional atopic dermatitis skin compared with healthy skin [6], and keratinocytes in the epidermal layer of AD skin express CCL17 and CCL22 [7]. Serum levels of CCL22 in AD patients are significantly higher than those found in normal controls [8], and the levels correlate positively with disease severity in AD patients [9]. Strong positive correlations between the levels of CCL17, CCL22, and total IgE in serum of patients with AD and SCORing Atopic Dermatitis (SCORAD) have also been reported [10]. Another study reported that overproduction of IgE induced CCL22 secretion from basophils, which are essential for IgE-mediated chronic allergic dermatitis [11]. These findings prompt-

Table 1. Frequencies of polymorphisms of the *CCL22* gene.

	SNP*	Location	Amino acid	MAF‡	NCBI†	
1	-3075G/A	5′-flanking region	-	0.125	rs223884	
2	-2938G/A	5′-flanking region	-	0.208	rs223885	
3	-2903T/A	5′-flanking region	-	0.333	rs223886	
4	-2668G/T	5′-flanking region	-	0.458	rs34569362	
5	-2550G/C	5′-flanking region	-	0.458	rs76295899	
6	-2511G/T	5′-flanking region	-	0.458	rs4784799	
7	-2191G/C	5′-flanking region	-	0.042	rs76720124	
8	-1795G/A	5′-flanking region	-	0.458	rs34885482	
9	-1775G/T	5′-flanking region	-	0.083	rs72784894	
10	-1618C/T	5′-flanking region	-	0.458	rs77239447	
11	-1515G/T	5′-flanking region	-	0.333	rs223887	
12	-1338A/G	5′-flanking region	-	0.208	rs182668	
13	-961G/A	5′-flanking region	-	0.208	rs223888	
14	-740A/G	5′-flanking region	-	0.083	rs3760071	
15	-488T/C	5′-flanking region	-	0.333	rs223889	§
16	-215WT/DelG	5′-flanking region	-	0.333	rs3214179	
17	5C/A	exon 1	Ala2Asp	0.125	rs4359426	§
18	88C/A	intron 1	-	0.458	rs2074543	§
19	493T/C	intron 1	-	0.458	rs72784897	
20	559G/A	intron 1	-	0.333	rs223816	
21	902C/T	intron 1	-	0.333	rs223817	
22	2030G/C	intron 2	-	0.208	rs223818	§
23	2134T/C	intron 2	-	0.208	rs223819	
24	2198T/C	intron 2	-	0.208	rs223820	
25	2314G/A	intron 2	-	0.292	rs598366	
26	2936A/G	intron 2	-	0.125	rs170359	
27	3062A/G	intron 2	-	0.458	rs73557194	
28	3766T/A	intron 2	-	0.042		
29	3970G/A	intron 2	-	0.125	rs223821	
30	4064WT/InsAAAAC	intron 2	-	0.125	rs72030112	
31	5222T/C	3′ UTR	-	0.125	rs170360	
32	5978WT/DelT	3′ UTR	-	0.125	rs57450696	
33	5979C/G	3′ UTR	-	0.375	rs57186204	
34	6089T/C	3′ UTR	-	0.125	rs223823	
35	6621A/G	3′ UTR	-	0.458	rs121565	§
36	6910G/A	3′ UTR	-	0.417	rs658559	§
37	7858C/T	3′-flanking region	-	0.458	rs3859048	§
38	7883G/A	3′-flanking region	-	0.458	rs72301	
39	8021G/A	3′-flanking region	-	0.042	rs11865093	

*Numbering according to the genomic sequence of *CCL22* (AC003665). Position 1 is the A of the initiation codon.
‡Minor allele frequencies (MAF) in the screening population (N = 12).
†NCBI, number from the dbSNP of NCBI (http://www.ncbi.nlm.nih.gov/SNP/).
§SNPs were genotyped in this study.
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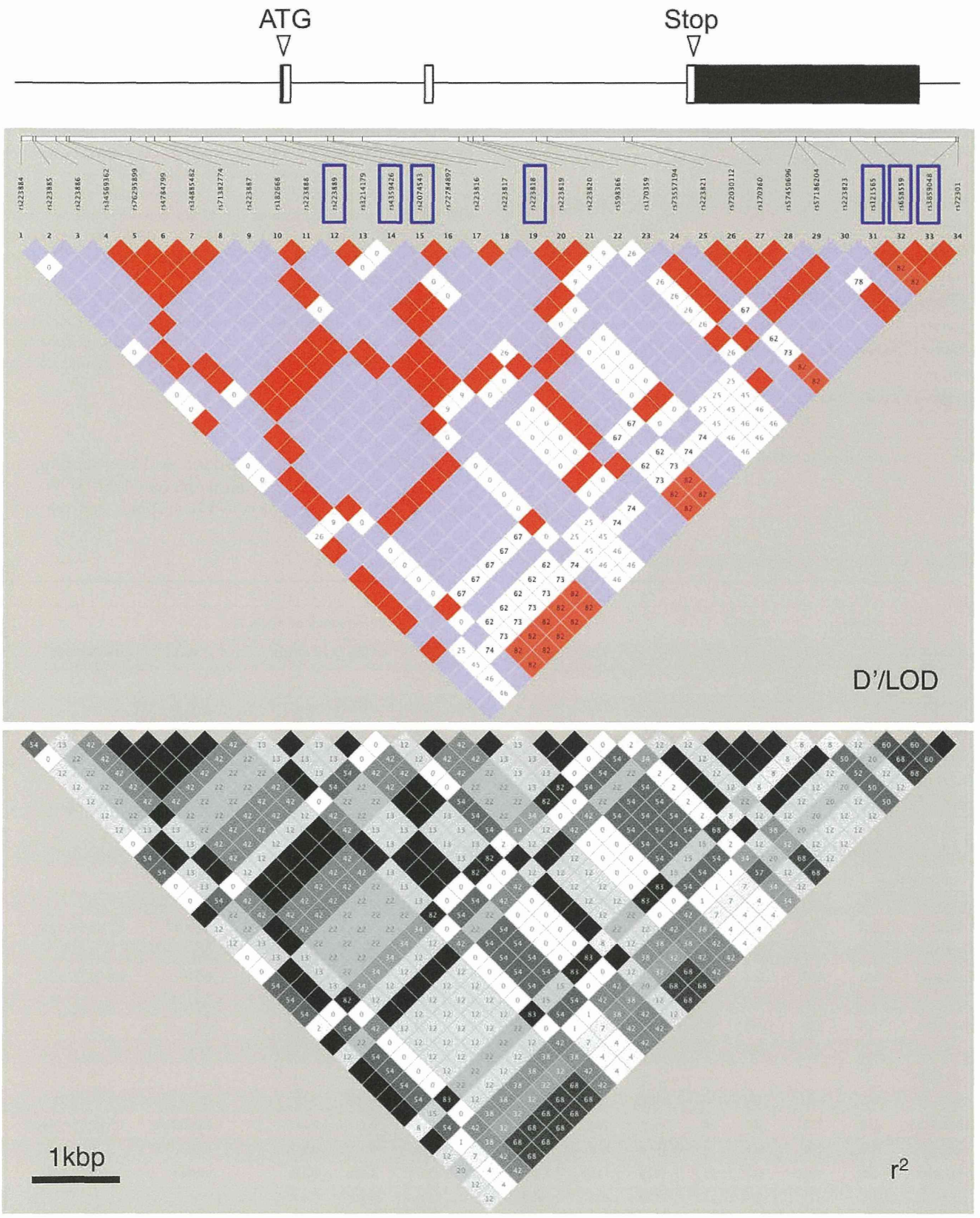

Figure 1. Pairwise linkage disequilibrium between 34 SNPs. LD was measured by D'/LOD (upper) and r^2 (lower) estimated using the Haploview 4.2 program (http://www.broad.mit.edu/mpg/haploview/). Boxed variants were genotyped in this study.
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Table 2. Clinical characteristics of the subjects.

	Case	Control
1st population		
Source	The University of Tokyo Keio University Kyushu University Takao Hospital	Control volunteers
Number of samples	916	1,032
Ethnicity	Japanese	Japanese
Female	43.6%	33.0%
Age (mean ± sd)	30.1±9.5	48.5±13.7
2nd population		
Source	BioBank Japan	University of Tsukuba
Number of samples	1,034	1,004
Ethnicity	Japanese	Japanese
Female	43.8%	54.4%
Age (mean ± sd)	30.8±12.7	50.0±9.2

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ed us to conduct an association and functional study to test whether genetic variations of *CCL22* contribute to AD susceptibility.

Several association studies using genetic variants of genes *CCL17* and *CCR4* in the CCR4 pathway have been conducted to discover genetic components in the pathogenesis of atopic dermatitis [12,13]. A promoter polymorphism of *CCL17*, -431C>T, increases the promoter activity and the 431T allele influences higher serum levels of CCL17 [12], but genetic variants in the *CCL17* gene are not associated with susceptibility to AD. A recent study also reported that C1014T polymorphism in the *CCR4* gene was not associated with AD [13]. However, those studies were performed with small sample sizes and without replication studies. Genetic study of the *CCL22* gene has not been conducted.

In this study, we focused on the *CCL22* gene, resequenced the gene regions including all exons and introns, and carried out linkage disequilibrium mapping. We performed an association study using two independent populations and functional analyses of the related variants.

Results

Polymorphisms of the *CCL22* gene and LD mapping

We identified a total of 39 polymorphisms (Table 1). We next performed linkage disequilibrium (LD) mapping and calculated

Table 3. Genotype counts and case-control association test results of seven tag SNPs.

	Allele		Case			Control				Frequency of allele 2			
db SNP ID	**1/2**	**1/1**	**1/2**	**2/2**	**N**	**1/1**	**1/2**	**2/2**	**N**	**Case**	**Control**	***P* value**	**OR (95%CI)**
1st population													
rs223889	T/C	321	435	151	907	360	502	161	1023	0.406	0.403	0.82	-
rs4359426	C/A	706	191	12	909	736	269	16	1021	0.118	0.147	0.0072	0.77(0.64–0.93)
rs2074543	G/C	386	404	113	903	447	469	110	1026	0.349	0.336	0.39	-
rs223818	A/G	563	311	39	913	596	369	56	1021	0.213	0.236	0.093	-
rs121565	A/G	294	439	173	906	325	509	195	1029	0.433	0.437	0.82	-
rs658559	G/A	333	434	134	901	374	491	162	1027	0.390	0.397	0.65	-
rs3859048	C/T	399	410	103	912	466	448	108	1022	0.338	0.325	0.40	-
2nd population													
rs223889	T/C	369	497	163	1029	364	485	150	999	0.400	0.393	0.65	-
rs4359426	C/A	815	202	12	1029	722	249	22	993	0.110	0.148	0.00037	0.71(0.59–0.86)
rs2074543	G/C	404	484	133	1021	418	459	120	997	0.367	0.351	0.26	-
rs223818	A/G	647	331	42	1020	585	351	57	993	0.203	0.234	0.019	0.84(0.72–0.97)
rs121565	A/G	317	530	179	1026	317	500	180	997	0.433	0.431	0.92	-
rs658559	G/A	389	486	154	1029	363	479	148	990	0.386	0.391	0.71	-
rs3859048	C/T	425	484	117	1026	441	446	113	1000	0.350	0.336	0.35	-
Combined													
rs223889	T/C	690	932	314	1936	724	987	311	2022	0.403	0.398	0.63	-
rs4359426	C/A	1521	393	24	1938	1458	518	38	2014	0.114	0.147	0.0000096	0.74(0.65–0.85)
rs2074543	G/C	790	888	246	1924	865	928	230	2023	0.359	0.343	0.16	-
rs223818	A/G	1210	642	81	1933	1181	720	113	2014	0.208	0.235	0.0044	0.86(0.77–0.95)
rs121565	A/G	611	969	352	1932	642	1009	375	2026	0.433	0.434	0.93	-
rs658559	G/A	722	920	288	1930	737	970	310	2017	0.388	0.394	0.56	-
rs3859048	C/T	824	894	220	1938	907	894	221	2022	0.344	0.330	0.21	-

P values of the two populations were calculated by logistic regression analysis under an additive model. The combined *P* values were calculated using the inverse variance method. OR, odds ratio; CI, confidence interval; -, not significant.

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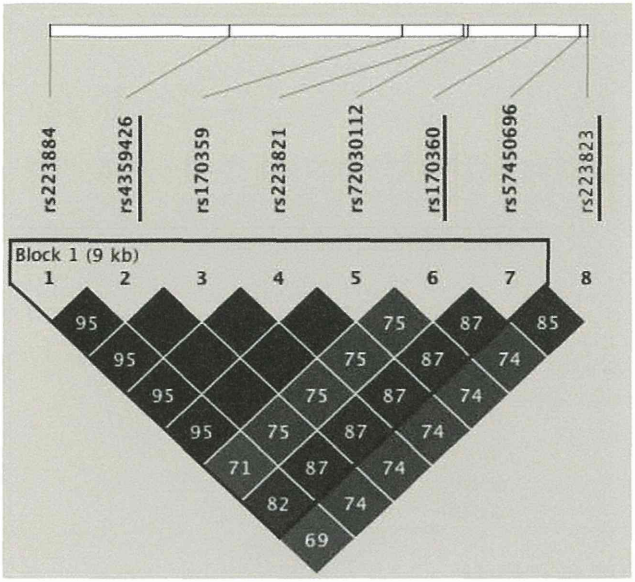

Figure 2. Pairwise linkage disequilibrium (r^2) among eight SNPs in strong LD with rs4359426 in 94 control subjects. Two tag SNPs, rs170360 and rs223823, were selected for further association study. Underlined SNPs were examined.
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pairwise LD coefficients D′ and r^2 among the 34 polymorphisms with MAF>10% using the Haploview 4.2 program (Figure 1). Seven tag SNPs were selected for association studies using tagger in Haploview 4.2, and these polymorphisms captured 34 of the 34 alleles with a mean r^2 of 0.990 (r^2>0.82). The HapMap JPT database contains genotype data for six SNPs with MAF>10% in the region (data not shown). The SNPs examined in this study covered all six SNPs shown in the HapMap JPT database.

Association of *CCL22* SNPs with susceptibility to atopic dermatitis

We recruited 916 cases and 1,032 control subjects for the 1st population and 1,034 cases and 1,004 control subjects for the 2nd population, respectively (Table 2). We genotyped seven tag SNPs and all genotype frequencies are shown in Table 3. The rs4359426 (A2D) SNP was associated with AD under an additive genotype model by logistic regression analysis in the first population (P = 0.0072; OR, 0.77; 95% CI, 0.64–0.93) (Table 3). In a replication study, rs4359426 was also associated with AD in the second population (P = 0.00037; OR, 0.71; 95% CI, 0.59–0.86) (Table 3). The direction of association of the SNP was similar in both of the populations. We combined the results using inverse variance method, and observed a significant association at rs4359426 (meta-analysis, P = 0.0000096; OR, 0.74; 95% CI, 0.65–0.85) (Table 3). We next performed further mapping analyses using two genetic variants, rs170360 and rs223823. The two SNPs were selected from among SNPs that were in strong LD (r^2>0.87) with rs4359426 (Figure 2). Among the three variants, the strongest association was observed at rs4359426 (Table 4).

Contribution of 5′UTR rs4359426 SNP to mRNA expression levels of *CCL22*

Next, using allele-specific transcript quantification (ASTQ), we evaluated whether the related variants could affect the mRNA expression level in EBV-transformed lymphoblastoid cells. As rs4359426 was located at the 16th nucleotide from the 5′ end of the *CCL22* gene (NM_002990.3), we were not able to design primers of the SNP for ASTQ analysis. We therefore designed PCR primers to encompass a SNP in the 3′-UTR of *CCL22* (rs170360) that was in strong LD with rs4359426 (Figure 3A). We isolated total RNA from 24 cell lines that were heterozygous with rs170360, and genomic DNA was used as a control for equal biallelic representation. Predicted haplotype frequencies are shown in Figure 3B. The ratio of PCR products was approximately 1.6 for cDNAs and 1.0 for genomic DNA from 21 subjects who were heterozygous for rs4359426 (Figure 3C, left panel); however, such

Table 4. Genotype counts and case-control association test results for SNPs rs4359426, rs170360 and rs223823.

	Allele		Case			Control				Frequency of allele 2			
db SNP ID	1/2	1/1	1/2	2/2	N	1/1	1/2	2/2	N	Case	Control	*P* value	OR (95%CI)
1st population													
rs4359426	C/A	706	191	12	909	736	269	16	1021	0.118	0.147	0.0072	0.77(0.64–0.93)
rs170360	T/C	695	199	12	906	734	269	20	1023	0.123	0.151	0.011	0.78(0.65–0.95)
rs223823	T/C	728	170	11	909	765	252	10	1027	0.106	0.132	0.0093	0.77(0.63–0.94)
2nd population													
rs4359426	C/A	815	202	12	1029	722	249	22	993	0.110	0.148	0.00037	0.71(0.59–0.86)
rs170360	T/C	792	220	19	1031	728	238	26	992	0.125	0.146	0.055	0.84(0.70–1.00)
rs223823	T/C	823	189	8	1020	780	193	19	992	0.100	0.116	0.11	0.85(0.70–1.04)
Combined													
rs4359426	C/A	1521	393	24	1938	1458	518	38	2014	0.118	0.147	0.0000096	0.74(0.65–0.85)
rs170360	T/C	1487	419	31	1937	1462	507	46	2015	0.123	0.151	0.0017	0.81(0.72–0.93)
rs223823	T/C	1551	359	19	1929	1545	445	29	2019	0.106	0.132	0.0030	0.81(0.70–0.93)

P values of the two populations were calculated by logistic regression analysis under an additive model.
The combined P values were calculated using the inverse variance method. OR, odds ratio; CI, confidence interval.
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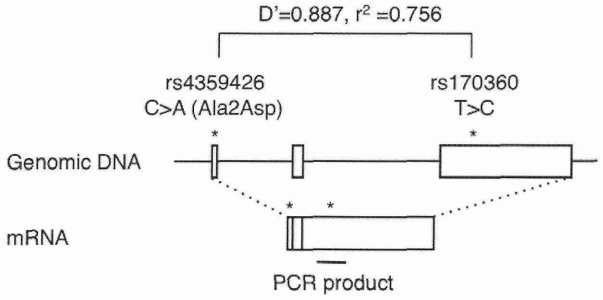
A



B

Haplotype	rs4359426 C>A	rs170360 T>C	Frequency
I	C	T	0.849
II	A	C	0.120
III	C	C	0.018
IV	A	T	0.013

C

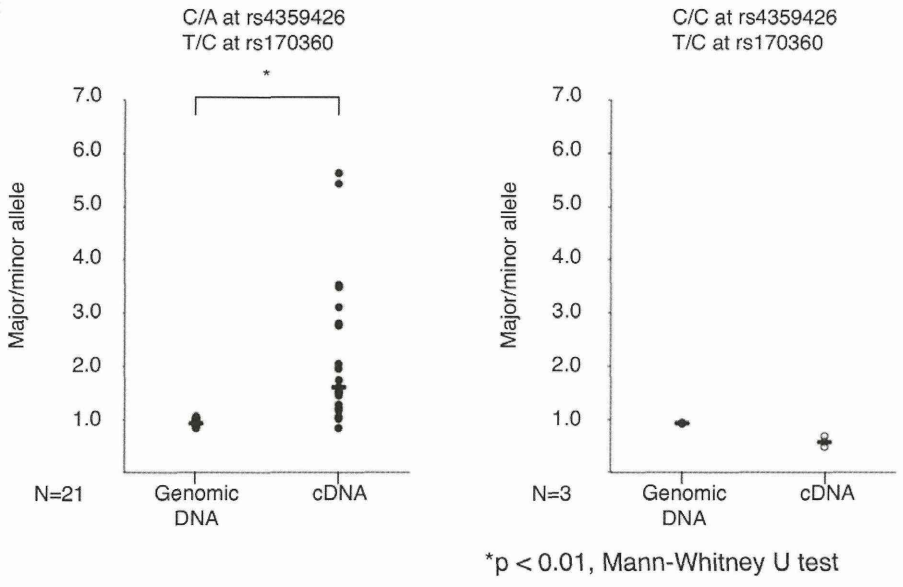


Figure 3. Allelic imbalance of gene expression of *CCL22* in EBV-transformed cells with heterozygous genotypes. (**A**) Genomic structures, locations and LD of the two SNPs. (**B**) Haplotypes for the two SNPs in the 1st population. (**C**) The allelic ratio of PCR products from individuals. Heterozygous (left) and homozygous (right) at rs4359426. *Two-tailed $P = 0.0000006$ by the Mann-Whitney U test.
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differences were not observed in cells from three subjects who were homozygous for the C allele at rs4359426 (Figure 3C, right panel). These results implied an effect of rs4359426 and/or variants in strong LD with rs4359426 on mRNA expression levels of *CCL22*. rs4359426 and rs170360 are in absolute LD in the HapMap Caucasian populations. We further examined the expression patterns of rs4359426 and rs170360 using Genevar 3.0.2 dataset, and confirmed that the expression patterns were similar to our findings (data not shown).

Transcription factor binding to the rs223821 SNP

As rs4359426 was in absolute LD with rs170359, rs223821 and rs72030112 (n = 94) (Figure 2), we further examined the allelic differences of these three SNPs in the binding of nuclear proteins by electrophoretic mobility shift assay (EMSA). We could not find any specific binding of nuclear factor(s) to oligonucleotides containing rs170359 and rs72030112. However, we observed that the signal intensity of the DNA-protein complex derived from the G allele of rs223821 was higher than that from the A allele in the presence of THP-1 nuclear extract stimulated with LPS (1 μg/ml) (Figure 4). We confirmed that the complex was diminished by an excess amount of a non-labeled allele-specific competitor probe (Figure 4). This result suggested that an unidentified nuclear factor(s) interacted with the genomic region at intron 2 of *CCL22* and the SNP might have an allele-specific effect on expression through varying affinity for a transcription factor.

Discussion

CCL22 plays an important role in the recruitment of Th2 cells into the inflammatory lesions of Th2-related diseases such as AD [14]. A recent study reported upregulation of *CCL17*, *CCL18* and *CCL22* expression in patients with AD, and suggested that the disease-specific chemokines might recruit specific memory T-cell subsets into the skin [15]. The plasma levels of CCL22 are

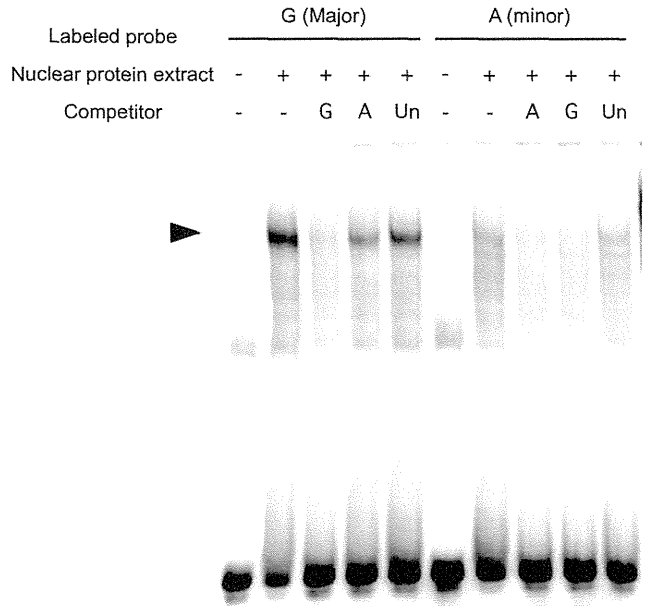

Figure 4. Electrophoretic mobility shift assays of rs223821. EMSA was performed using nuclear extracts from THP-1 cells stimulated with LPS (1.0 μg/ml) for 1 hour. DIG-labeled oligonucleotides corresponding to the G allele (lanes 1–5) and A allele (lanes 6–10) were used as probes. Three independent experiments were performed with similar results.
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significantly elevated in AD patients, and the values strongly correlate with disease severity [7,10]. We identified and replicated the rs4359426 (A2D) variant of *CCL22*, which was significantly associated with AD. rs4359426 is a non-synonymous SNP and causes an amino acid substitution in the signal peptide-encoding region. We examined the influence of the amino acid substitution on the structure using SIFT (Sorting Intolerant From Tolerant) software, and the substitution at position 2 from Ala to Asp was predicted to be tolerated. In addition, no possible impacts of the amino acid substitution on the structure and function of CCL22 were predicted by PolyPhen-2 (polymorphism phenotyping v2).

Functional analyses of the related variants of *CCL22* polymorphisms showed that the susceptible allele of rs4359426 might be involved in higher mRNA expression in ASTQ analysis. We confirmed that the expression patterns from Genevar 3.0.2 dataset were similar to our findings. We also demonstrated that the genomic fragment including the risk allele of rs223821 had much higher binding affinity to the nuclear factor(s). Although it is unclear whether higher mRNA expression is influenced by altering expression enhancer activity or mRNA stability, polymorphisms in the *CCL22* gene appear to be a genetic component of the pathologic mechanisms leading to atopic dermatitis, putatively via increased *CCL22* mRNA expression.

Genetic studies reveal underlying cellular pathways, and in some cases, point to new therapeutic approaches. A recent study using a humanized model of asthma showed a critical role for DC-derived CCL17 and CCL22 in attracting Th2 cells and inducing airway inflammation [16]. In the study, administration of a CCR4-blocking antibody abolished airway eosinophilia, goblet cell hyperplasia, IgE synthesis and bronchial hyperreactivity [16]. IL-13 is an important mediator of Th2 immune responses, and there many IL-13-positive cells in AD skin lesions [17]. A recent study has shown that IL-13 induces a significant increase in the expression of CCL22 in human keratinocytes, and blocking of CCL22 in IL-13-stimulated cells results in 70–90% inhibition in migration of CD4+CCR4+ T cells [18]. These findings suggest that targeting the CCL22/CCR4 pathway might be therapeutically efficacious as a new treatment for atopic dermatitis.

The involvement of CCL22 has been reported in several immune-mediated diseases. A recent study has shown by immunohistochemistry that CCL22 is not expressed in normal skin and is markedly expressed in the lesions of atopic dermatitis, allergic contact dermatitis, and psoriasis vulgaris [19]. Another report has shown that CCL22 is present within the synovial membrane in rheumatoid arthritis and osteoarthritis patients and in high amounts in the synovial fluid of patients with rheumatoid arthritis and psoriatic arthritis [20]. To examine whether the functional SNPs found in this study are associated with those diseases will be needed for understanding of the interconnectivity of the molecular mechanisms underlying distinct diseases.

In summary, we found a significant association between susceptibility to AD and polymorphisms affecting *CCL22* expression in Japanese populations. Our findings strongly support the important role of CCL22 in AD. Although the effect of the non-synonymous SNP on protein function remains unclear, it is likely that related variants play a role in susceptibility to AD in a gain-of-function manner. Further functional analyses and replication studies in other populations are needed; however, our findings provide insights into the pathophysiology of AD.

Materials and Methods

Subjects

A total of 1,950 case subjects with AD were recruited from several hospitals as described [21]. Case subjects in a second population were obtained from the BioBank Japan [22]. All case subjects were diagnosed according to the criteria of Hanifin and Rajka [23]. A total of 1,032 control volunteers in the first set who had no history of AD were recruited by detailed physicians' interviews. For the second set, a total of 1,004 controls who had never been diagnosed with AD were recruited during their annual health checkup in the University of Tsukuba (Table 2). All individuals were Japanese and gave written informed consent to participate in the study. This research project was approved by the ethics committees at the Institute of Medical Science, the University of Tokyo and the RIKEN Yokohama Institute.

Resequencing of the *CCL22* gene and genotyping

We first resequenced the *CCL22* region to identify genetic variations using DNA from 12 subjects with AD. We surveyed the gene from 3 kb of the 5′ flanking region to a 1 kb continuous 3′ flanking region of the last exon on the basis of genomic sequences from the NCBI database (NC_000016.9). The PCR product was reacted with BigDye Terminator v3.1 (Applied Biosystems), and sequences were assembled and polymorphisms identified using the SEQUENCHER program (Gene Codes Corporation, Ann Arbor, MI).

Genotyping of the seven SNPs in *CCL22* was performed by the TaqMan^{TM} allele-specific amplification (TaqMan-ASA) method (Applied Biosystems) and multiplex-PCR based Invader assay (Third Wave Technologies).

Allele-specific transcript quantification (ASTQ)

We conducted allelic expression analyses by TaqMan assay using SNP genotyping probes as described [24]. EBV-transformed lymphoblastoid cells were obtained from the Health Science Research Resources Bank of Japan. Genomic DNA was used as a

control for equal biallelic representation. The allelic ratio for each cDNA and genomic DNA was measured.

Electrophoresis Mobility Shift Assay

EMSA was performed using nuclear extracts from THP-1 cells stimulated with LPS (1.0 μg/ml) for 1 hour. DIG-labeled oligonucleotides corresponding to the G allele (lanes 1–5) and A allele (lanes 6–10) were used as probes. The oligonucleotide sequences were 5′-ATCGCCTGAACCCGGGAGTTGGAGGTT for the G allele and 5′-ATCGCCTGAACCCAGGAGTTGGAGGTT for the A allele. For competition, a 100-fold excess of unlabeled G or A allele oligonucleotides or unrelated oligonucleotides (Un) (TFIID) was used.

Statistical analysis

We calculated allele frequencies and tested agreement with Hardy-Weinberg equilibrium using a chi-square goodness-of-fit test. We then compared differences in the allele frequencies between case and control subjects by logistic regression analysis under an additive model and calculated odds ratios (ORs) with 95% confidence intervals (CIs). Results for the 1st and 2nd populations were combined by fixed effect inverse-variance method using Genome-Wide Association Meta Analysis (GWAMA, http://www.well.ox.ac.uk/gwama/tutorial.shtml) [25]. We applied Bonferroni corrections, the multiplication of P values by the number of variants investigated. Corrected P values of less than 0.05 were judged to be significant. The expression patterns of SNPs were obtained from Genevar (GENe Expression VARiation) 3.0.2 (Wellcome Trust Sanger Institute). We examined the influence of amino acid substitution on the structure using SIFT software (http://sift.jcvi.org/) and PolyPhen-2 (polymorphism phenotyping v2) (http://genetics.bwh.harvard.edu/pph2/).

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Author Contributions

Conceived and designed the experiments: TH MT. Performed the experiments: TH KT STanaka HM TSasaki STakeuchi. Analyzed the data: TH. Contributed reagents/materials/analysis tools: HS KE MS TY SF AM SD TE NH TSakamoto HM TSasaki TE MA HE STakeuchi MF EN YN MK. Wrote the paper: MT. Supervised the study: NK YN.

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ORIGINAL ARTICLE

A Randomized, Open-Label, Multicenter Trial of Topical Tacrolimus for the Treatment of Pruritis in Patients with Atopic Dermatitis

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Background: Pruritis caused by atopic dermatitis (AD) is not always well controlled by topical corticosteroid therapy, but use of tacrolimus often helps to soothe such intractable pruritis in clinical settings. **Objective:** To determine the anti-pruritic efficacy of topical tacrolimus in treating AD in induction and maintenance therapy. **Methods:** Prior to the study, patients were randomly allocated into two groups, induction therapy followed by tacrolimus monotherapy maintenance, and induction therapy followed by emollient-only maintenance. In the induction therapy, the patients were allowed to use topical tacrolimus and emollients in addition to a low dose (< 10 g/week) of topical steroids. Patients showing relief from pruritis were allowed to proceed to maintenance therapy. Recurrence of pruritis in maintenance therapy was examined as a major endpoint. **Results:** Two-thirds of patients (44/68; 64.7%) showed relief from pruritis after induction therapy. Pruritis recurred in 23.8% (5/21) of the tacrolimus monotherapy group and in 100% (21/21) of the emollient group during maintenance period, a difference that was statistically significant. **Conclusion:** Use of topical tacrolimus is effective in controlling pruritis of AD compared to emollient. **(Ann Dermatol 24(2) 144~150, 2012)**

-Keywords-
Atopic dermatitis, Maintenance therapy, Pruritis, Randomized trial, Tacrolimus

INTRODUCTION

Atopic dermatitis (AD) is a common chronic or chronically relapsing, severely pruritic, and eczematous skin disease whose prevalence appears to have increased significantly in recent decades[1,2]. The control of pruritis, a primary symptom of AD, is very important in its treatment since pruritis itself is an unpleasant sensation that often disturbs patients' sleep. Additionally, incidental scratching exacerbates and sustains skin eruptions, thereby significantly reducing patient quality of life. However, pruritis

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caused by AD is not readily controlled with clinically available oral antihistamines, probably due to the presence of many inflammatory pruritogenic factors other than histamine[3-5]. Thus, one of the simplest and most practical answers is to reduce or eliminate skin inflammation by the use of strong anti-inflammatory agents such as topical corticosteroids. Indeed, this strategy is effective in most cases in treating pruritis as well as skin inflammations caused by AD[6]. However, there are substantial numbers of patients undergoing topical corticosteroid therapy who still suffer from intractable pruritis and whose extensive scratching aggravates their dermatitis. Calcineurin inhibitors are a relatively new treatment for AD, and orally administered cyclosporine has been reported effective in treating refractory pruritis in patients with AD[7]. Similarly, the anti-pruritic effects of topical calcineurin inhibitors have also been reported[8]. Thus, the purpose of this study was to further evaluate the anti-pruritic efficacy of topical tacrolimus, a calcineurin inhibitor, in the treatment of patients with AD in inductive and maintenance treatment.

MATERIALS AND METHODS

Inclusion/Exclusion

Patients with AD who were >10 years old and whose visual analogue scale (VAS)-itch scores (max=100) were 30~80 were recruited after written informed consent was obtained. Patients whose VAS-itch scores were >80 were excluded because of their desperate need for anti-pruritic treatment including antihistamines or more potent systemic anti-inflammatory treatment. Conversely, patients whose VAS-itch scores were <30 were excluded because of their lesser need for additional anti-pruritic therapy and the limited window in assessing pruritis improvement. Patients who had been treated with orally administered corticosteroids, cyclosporine, or antihistamines within two weeks prior to the registration were also excluded because of

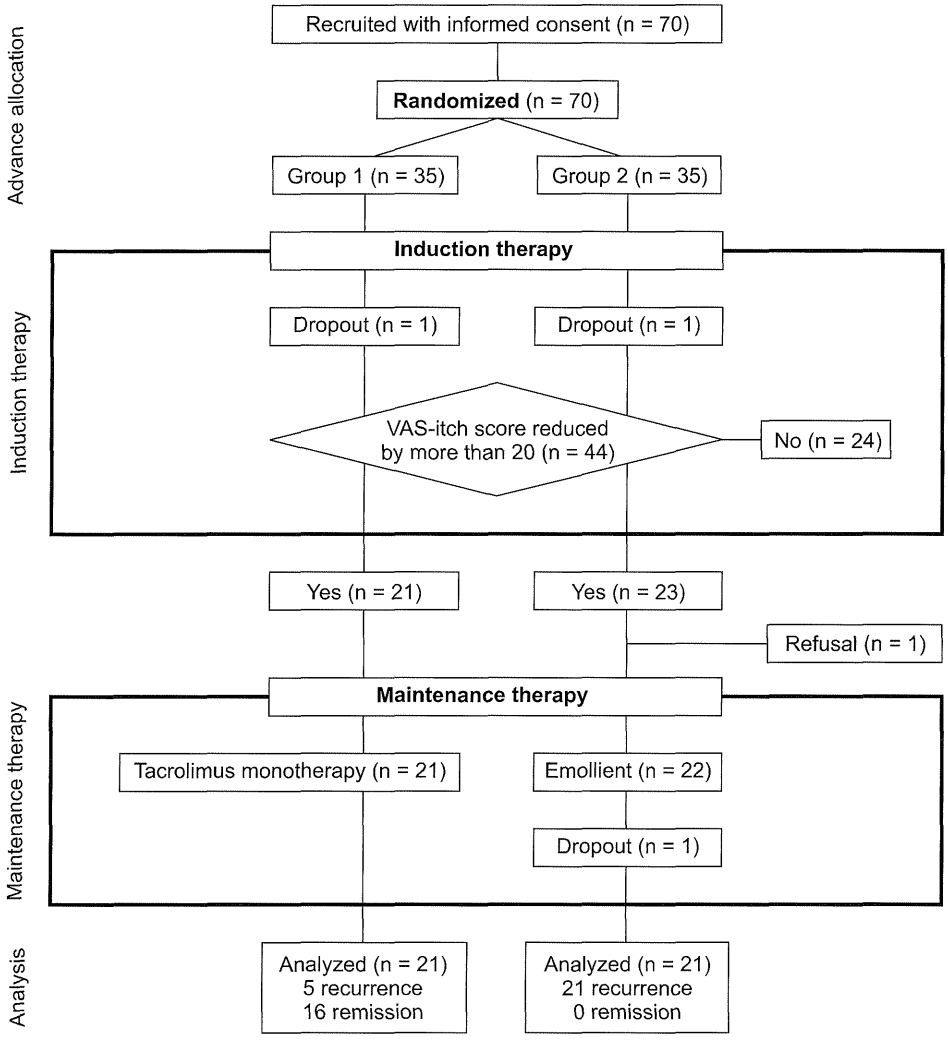

Fig. 1. Flow diagram showing subjects' progress. Patients were advance-allocated after registration, received introduction therapy (add-on tacrolimus therapy), and the responders to the introduction therapy proceeded into maintenance therapy. There were several dropouts and one refusal during the study. VAS: visual analogue scale.

their potential influence on pruritis.

Study design

All of the patients received induction (1~4 weeks) and maintenance (>4 weeks) therapy. Prior to the study, patients were randomly allocated in advance into two groups: patients who received topical tacrolimus monotherapy as maintenance therapy after induction therapy and patients who received emollient only for maintenance therapy after induction therapy. In the induction therapy,

Table 1. Baseline values of patients

	Total (n=70)			Completion of study (n=42)		
	Tacrolimus monotherapy-allocated (n=35)	Emollient-allocated (n=35)	Total	Tacrolimus monotherapy (n=21)	Emollient (n=21)	Total
Gender, numbers (%)						
Male	17 (48.6)	20 (57.1)	37 (52.9)	12 (57.1)	9 (42.9)	21 (50.0)
Female	18 (51.4)	15 (42.9)	33 (47.1)	9 (42.9)	12 (57.1)	21 (50.0)
Age (yr)						
Mean (SD)	30.5 (13.2)	30.8 (11.9)	30.7 (12.5)	31.3 (13.6)	31.3 (13.3)	31.3 (13.3)
10~24, numbers (%)	12 (34.3)	14 (40.0)	26 (37.1)	9 (42.9)	6 (28.6)	15 (35.7)
25~35, numbers (%)	13 (37.1)	10 (28.6)	23 (32.9)	5 (23.8)	8 (38.1)	13 (31.0)
36~64, numbers (%)	10 (28.6)	11 (31.4)	21 (30.0)	7 (33.3)	7 (33.3)	14 (33.3)
Institution, numbers (%)						
Kyushu University	14 (40.0)	14 (40.0)	28 (40.0)	12 (57.1)	9 (42.9)	21 (50.0)
University of Tokyo	12 (34.3)	13 (37.1)	25 (35.7)	4 (19.0)	8 (38.1)	12 (28.6)
National Center for Child Health and Development (Allergy Division)	3 (8.6)	4 (11.4)	7 (10.0)	2 (9.5)	2 (9.5)	4 (9.5)
Social Insurance Chuo General Hospital	1 (2.9)	2 (5.7)	3 (4.3)	0 (0.0)	2 (9.5)	2 (4.8)
Saitama Medical University	2 (5.7)	0 (0.0)	2 (2.9)	2 (9.5)	0 (0.0)	2 (4.8)
National Center for Child Health and Development (Dermatology Division)	1 (2.9)	0 (0.0)	1 (1.4)	1 (4.8)	0 (0.0)	1 (2.4)
St. Marianna University School of Medical Hospital	1 (2.9)	1 (2.9)	2 (2.9)	0	0	0
Hiroshima University	1 (2.9)	1 (2.9)	2 (2.9)	0	0	0
Numbers (%)						
Complications -yes-	19 (54.3)	20 (57.1)	39 (55.7)	11 (52.4)	11 (52.4)	22 (52.4)
Past history -yes-	21 (60.0)	26 (74.3)	47 (67.1)	13 (61.9)	16 (76.2)	29 (69.0)
Family history* -yes-	15 (46.9)	24 (72.7)	39 (60.0)	8 (42.1)	14 (70.0)	22 (56.4)
Complications (details)						
Asthma	10 (31.3)	10 (30.3)	20 (30.8)	6 (28.6)	6 (28.6)	12 (28.6)
Allergic rhinitis	10 (28.6)	18 (51.4)	28 (40.0)	6 (28.6)	10 (47.6)	16 (38.1)
Allergic conjunctivitis	1 (2.9)	0 (0.0)	1 (1.4)	1 (4.8)	0 (0.0)	1 (2.4)
Scoliosis	1 (2.9)	0 (0.0)	1 (1.4)	0	0	0
Autistic tendency	1 (2.9)	0 (0.0)	1 (1.4)	0	0	0
Medical history						
Asthma	14 (40.0)	18 (51.4)	32 (45.7)	9 (42.9)	11 (52.4)	20 (47.6)
Allergic rhinitis	10 (28.6)	17 (48.6)	27 (38.6)	6 (28.6)	10 (47.6)	16 (38.1)
Pollen allergy	0 (0.0)	1 (2.9)	1 (1.4)	0 (0.0)	1 (4.8)	1 (2.4)
Family history						
Atopic dermatitis	12 (37.5)	17 (51.5)	29 (44.6)	6 (31.6)	10 (50.0)	16 (41.0)
Asthma	4 (12.5)	5 (15.2)	9 (13.8)	2 (10.5)	2 (10.0)	4 (10.3)
Allergic rhinitis	2 (6.3)	8 (24.2)	10 (15.4)	1 (5.3)	6 (30.0)	7 (17.9)
Pollen allergy	0 (0.0)	2 (6.1)	2 (3.1)	0 (0.0)	1 (5.0)	1 (2.6)
Treatment within the last month						
Topical corticosteroids	30 (85.7)	26 (74.3)	56 (80.0)	18 (85.7)	14 (66.7)	32 (76.2)
Topical tacrolimus	19 (54.3)	19 (54.3)	38 (54.3)	13 (61.9)	11 (52.4)	24 (57.1)
Oral antihistamines	10 (28.6)	12 (34.3)	22 (31.4)	5 (23.8)	6 (28.6)	11 (26.2)
Emollients (heparin)	3 (8.6)	3 (8.6)	6 (8.6)	2 (9.5)	2 (9.5)	4 (9.5)

SD: standard deviation. *65 of 70 in Test 1 and all subjects in Test 2 answered this question.

all of the patients were treated with topical tacrolimus (of 0.03% for patients <16 years old and of 0.1% otherwise) and emollients twice daily in addition to their usual topical corticosteroid treatment (maximum use, 10 g/week), and change of VAS-itch score was examined. Patients who showed a reduced VAS-itch score by >20 points were considered to show relief from pruritis, while only such induction therapy responders proceeded into maintenance treatment. In maintenance therapy, recurrence of pruritis, mean change of VAS-itch scores, and the percentage of patients with pruritis recurrence were measured. Patients who showed increased VAS-itch scores of >20 points were categorized as suffering from pruritis recurrence in maintenance treatment. Secondarily, skin severity score was monitored using the SCORing Atopic Dermatitis (SCORAD) score[9]. This study was an open label, randomized, multi-center study and was approved by the internal ethical review boards of Kyushu University and other institutions.

Statistical analysis

The confidence interval (CI) for the proportion of subjects who experienced pruritis relief was estimated in the induction therapy using Fisher's exact method assuming a binomial distribution, while changes in VAS-itch score and SCORAD were assessed using the paired t-test. The cumulative proportion of pruritis recurrence was estimated using the Kaplan-Meier method, while the CI was estimated using Greenwood's method in maintenance treatment. The percentage difference in pruritis recurrence between the two groups was assessed using Fisher's exact test. The mean difference between VAS-itch score and its 95% CI were estimated using analysis of covariance (ANCOVA). The time elapsed before pruritis recurrence was assessed using the stratified log-rank test, with institutions divided into "Kyushu University," "University

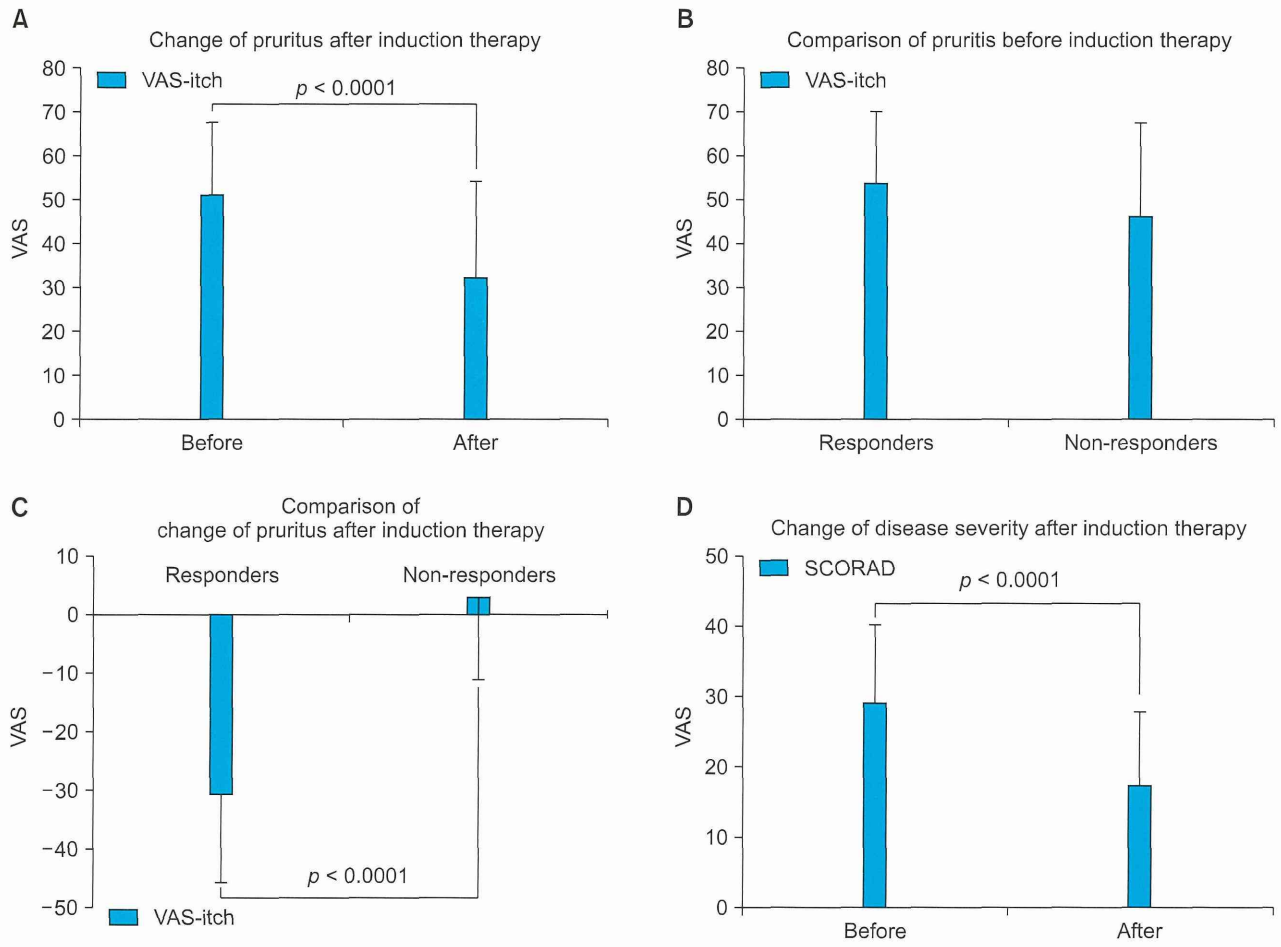

Fig. 2. Change in visual analogue scale (VAS)-itch score and disease severity after add-on tacrolimus therapy. (A) Pruritis (mean VAS-itch score - standard deviation) reduced after add-on topical tacrolimus therapy. (B) There was no statistical difference in mean VAS-itch score between responders and non-responders before the add-on therapy. (C) There was a significant decrease in VAS-itch score in responders after the add-on therapy. (D) SCORing Atopic Dermatitis (SCORAD) score reduced after the add-on topical tacrolimus therapy.

of Tokyo," and "other institutions."

RESULTS

A total of 70 patients with AD were registered, 68 of whom completed induction therapy (Fig. 1). A total of 44 of the 68 patients experienced pruritis relief (64.7%; 95% CI, 53.1~76.4%), while 43 of the 44 responders proceeded to maintenance therapy. The median and mean (standard deviation, SD) of the induction therapy period among the 43 patients were 15 days and 17.9 (7.1) days, respectively. Twenty-one patients each in the tacrolimus monotherapy group and the emollient group completed the maintenance treatment. No marked bias was apparent in the baseline data of the patients who were registered compared to those who completed this whole study (Table 1).

In the induction therapy, mean VAS-itch score (SD) decreased from 51.1 (16.6) to 32.3 (22.1) (Fig. 2A), while the mean difference, 18.8 (95% CI, 13.5~24.1), was statistically significant ($p<0.0001$). There was no statistical difference in mean VAS-itch score between responders and non-responders before the induction therapy (Fig. 2B), but there was a significant decrease of VAS-itch in responders after treatment (Fig. 2C). Data on disease severity (SCORAD) after the induction therapy were obtained from 50 of the 68 subjects who completed treatment (43 of the 44 pruritis-responders and 7 of the 24 non-responders). Mean SCORAD (SD) of the 50 subjects decreased from 29.1 (11.1) to 17.3 (10.6) (Fig. 2D), and the mean difference of 11.8 (95% CI, 9.0~14.7) was statistically significant ($p<0.0001$).

Cumulative itch recurrence in the tacrolimus monotherapy maintenance group and emollient maintenance group at day 28 was 23.8% (95% CI, 10.7~52.9%) and 100%, respectively in maintenance treatment (Fig. 3), and the difference between the two groups was statistically significant ($p<0.0001$). The median time to pruritis recurrence in the tacrolimus monotherapy group and the emollient group was >28 days and 3 days (95% CI, 2~5 days), respectively (Table 2). The mean VAS-itch score in the tacrolimus monotherapy group was well controlled as shown by values of 28.1 (15.4) at the start and 29.6 (20.9) at the end of maintenance treatment, while that in the emollient group significantly increased from 19.3 (16.7) to 50.7 (17.0) (Table 3). The mean change in VAS-itch scores was 1.50 (3.30) in the tacrolimus monotherapy and 31.4 (2.59) in the emollient group, respectively, in maintenance treatment (Fig. 4), and the difference, 28.6 (95% CI, 19.8~37.5), was statistically significant ($p<0.0001$).

A transient burning sensation by topical tacrolimus, the only distinguished side effect, was recorded in 32 of 69

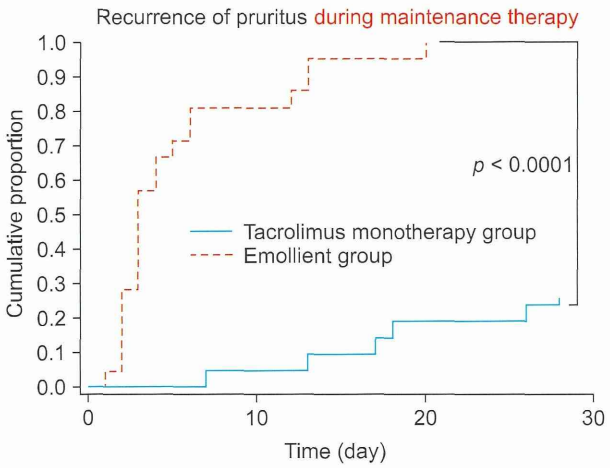

Fig. 3. Cumulative recurrence of pruritis in maintenance therapy. Tacrolimus monotherapy group (solid line) showed significantly much lower recurrence of pruritis compared to that of the emollient group (dotted line).

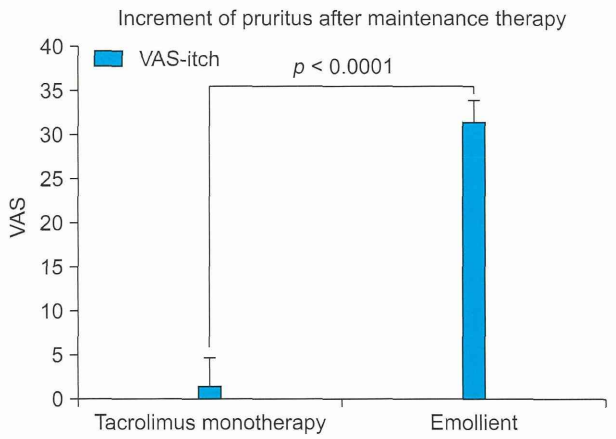

Fig. 4. Efficacy of tacrolimus monotherapy in maintenance therapy. The emollient group showed more pruritis than the tacrolimus monotherapy group at the end of maintenance therapy. VAS: visual analogue scale.

Table 2. Median time to pruritis recurrence in maintenance therapy

	Time to recurrence
Tacrolimus monotherapy	>28 days
Emollient	3 days

Table 3. Change of pruritis (mean [standard deviation]) after maintenance therapy

	Before	After
Tacrolimus monotherapy	28.1 (15.4)	29.6 (20.9)
Emollient	19.3 (16.7)	50.7 (17.0)

patients (46.3%, excluding one dropout patient who never returned after initial registration) in the induction therapy. The other minor side effect was acne/folliculitis (3 cases, 4.3%), herpes simplex (1 case, 1.4%), wart (1 case, 1.4%), and the common cold (2 cases, 2.9%) throughout the study period.

DISCUSSION

In the induction therapy of this study, patients were allowed to use topical tacrolimus and emollients in addition to low-dose application (< 10 g/week) of topical steroids. Almost two-thirds of the patients with AD experienced pruritis relief after the induction therapy. In these responsive patients, sequential maintenance by topical tacrolimus monotherapy was found to be significantly effective in controlling pruritis caused by AD compared with emollient only.

Orally administered cyclosporine appeared to effectively treat intractable pruritis in patients with AD as previously mentioned[7]. However, various adverse effects, such as systemic immune suppression, hypertension, headache and possible renal failure, should be considered and carefully monitored before and during administration. Topical tacrolimus, on the other hand, can basically avoid all of these undesirable adverse effects and is therefore more suitable for use in daily clinics, except for possible local immune suppression of the skin. However, one of our earlier studies and another report showed that topical tacrolimus was not associated with an increase in cutaneous infection[10,11]. Ultraviolet therapy is another option for treating intractable pruritis in patients with AD[12], but it carries the possible risk of developing skin cancer in the long run[13], raising a concern about its use, particularly in infants.

Hon et al.[14] evaluated the clinical efficacy of topical tacrolimus for reducing the sensation of pruritis in children with AD. Three boys and four girls with AD were treated with topical tacrolimus for a consecutive two-week period after a one-week run-in. Nocturnal scratching activity measured using a DigiTrac movement recorder was reduced from 115.0 g/min to 71.5 g/min ($p=0.028$) after two weeks of treatment.

Such anti-pruritic effects of topical tacrolimus are thought to be due to its anti-inflammatory action considering the fact that the efficacy of 0.1% tacrolimus ointment was similar to that of 0.1% hydrocortisone butyrate ointment or 0.12% betamethasone valerate ointment when applied for three weeks in adults[15,16]. However, pruritis is not always readily relieved even after topical application of more potent corticosteroids in clinical settings. Several

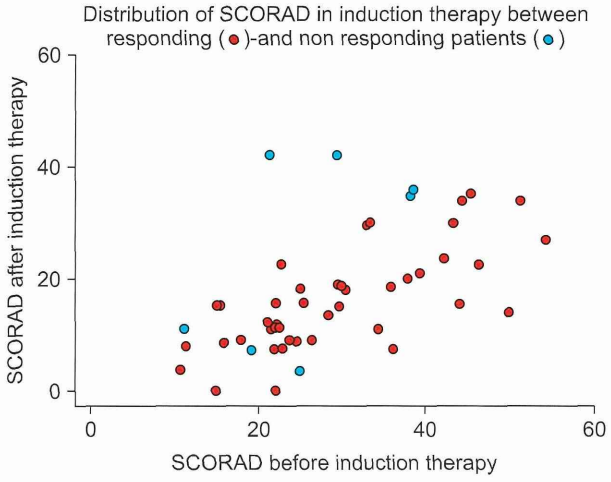

Fig. 5. Change of SCORing Atopic Dermatitis (SCORAD) in induction therapy between treatment-responding (blue circle) and non-responding patients (red circle) in induction therapy. Forty-three treatment-responding patients showed significantly reduced SCORAD compared to that of 7 non-responding patients in induction therapy as assessed by analysis of covariance ($p=0.001$).

unique characteristics of tacrolimus that appear to be related to its anti-pruritic effects, such as inhibition of the epidermal sensory nerve extension[17], transient release of substance P from sensory nerve endings[18], and suppression of mast cell degranulation[19] have been reported. There is currently no conclusive answer as to what governs the mechanism of anti-pruritic action of tacrolimus; however, one important fact is that topical tacrolimus does have an anti-pruritic property that topical corticosteroids lack[17]. We reproduced these results in a very similar experimental setting and extended the findings that the curious anti-pruritic effects of tacrolimus might not be simply due to its anti-inflammatory effects or anti-epidermal nerve extension effects[20]. In this study, the treatment responders (patients with decreased pruritis) showed a better change in SCORAD (disease severity) than non-responders in the induction therapy when data available were analyzed by ANCOVA (Fig. 5). However, we cannot determine from this whether the improved disease severity might come from less itching/scratching by the direct action of topical tacrolimus or that improved disease severity resulted in less production of various inflammatory pruritogens to bring about less pruritis. Anyhow, controlling itching/scratching is important in the formation of allergic skin reaction[21], and further investigations will be needed to precisely identify the mechanism of action of anti-pruritic effects by anti-inflammatory agents.

Finally, this is an open study; therefore the possibility of a placebo effect (no exact vehicle control was used) should