トランスレーショナルリサーチを支援する

遺伝子医学 MOOK 10

Gene & Medicine

DNAチップ/マイクロアレイ臨床応用の実際

基礎、最新技術、臨床・創薬研究応用への実際から今後の展開・問題点まで

別　刷

株式会社 メディカルドゥ

1．疾病の解析 -発現解析各論-
7） 眼科領域におけるアレイ解析の動向

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ここ数ヵ月の間に，ヒト全ゲノムにわたる数十万ヵ所の一塩基多型（single nucleotide polymorphism：SNP）をタイピングし，数千人規模のアソシエーション解析を実施した研究成果が次々に報告され，geneticsの分野では疾患関連遺伝子の同定に沸いている。奇しくも，その先駆けの1つとなった研究例は眼科領域からのものであった。それは，2005年に加齢黄斑変性の原因遺伝子として補体系の調節因子の1つ，H因子遺伝子が同定された研究である。本稿では，DNAチップを用いたSNPをマーカーとするアソシエーション解析がもたらしたブレイクスルーについてこの研究を題材に概説するとともに，もう1つの代表的な眼疾患である緑内障のアソシエーション解析の現状やチップを用いた遺伝子発現解析が眼科領域に与える可能性について考察する。

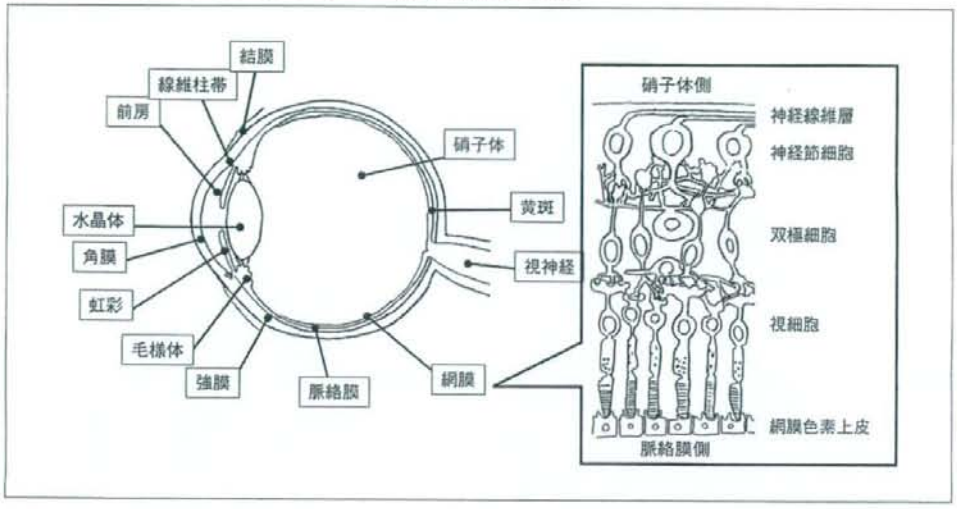
はじめに

近年，数万種類の既知遺伝子の発現解析やヒト全ゲノムにわたる百万ヵ所にも及ぶSNPsのタイピングだけでなく，タイリングアレイを用いたトランスクリプトーム解析や染色体構造変化をコピー数として捉えるcomparative genomic hybridization（CGH），さらにはリシークエンスまでもがチップベースの実験で行えるようになってきた。眼科領域では，歴史の浅いリシークエンスチップの報告例は現在のところゼロで，CGHの報告はretinoblastoma[1]とintraocular uveal melanoma[2]の2例のみであるが，それ以外のアプリケーションは活発に実用されている。これらのチップの技術革新の背景として，①国際HapMapプロジェクト[3]の進展やデータベース上のアノテーション情報の更新によるプローブの質の向上，②チップに搭載できるプローブの高密度化，③チップあたりの解析コストの劇的な低下が挙げられる。個々のチップにはそれぞれ実験上難易度が高い問題点があるものの，今後も質の高いデータをより簡便に得るためのチップ自体の技術開発は着実に進展していくことが予想される。

一方，チップ実験では，定性・定量データにかかわらず，抽出されるデータは膨大かつ無味乾燥である。したがって，解析対象の選択や分類などの事前計画はもちろん，データの処理方法とその解釈，ウェットなサポートデータも含めた研究全般にわたる実験設計に精度の高さが要求される。特に，疾患の発症や進行を制御するメカニズムを解明するためには，圧倒的な情報量により生命現象を包括的に捉える「力づく」だけで達成できるものではなく，ある程度フォーカスした設計を工夫する必要がある。本稿では，個々のチップ研究の設計方法にも着目しながら，眼科領域を取り巻くSNPs解析と遺伝子発現解析の現状を把握し，そ

key words
SNP，疾患関連遺伝子，アソシエーション解析，検出力，眼疾患，加齢黄斑変性，緑内障

図❶　眼球の断面図と各組織の名称および網膜の層構造（拡大）



の将来性について考察を加える。

Ⅰ．SNPをマーカーとするアソシエーション解析

疾患との関連性が示唆されている候補遺伝子上のSNPsをダイレクトシークエンス法やタックマン法で解析する従来のアプローチは眼科領域でも行われている。例えば近年，神経変性疾患としても捉えはじめられている緑内障におけるアルツハイマー病に関与するアポE[4]遺伝子をはじめ，網膜疾患で約50報，Stevens-Johnson症候群とToll-like receptor[5]やIL4R[6]，アトピー性結膜炎とIFNγレセプター[7]といった角結膜疾患でも約10報の興味深い解析例がある。今後，チップを用いた「全ゲノムアソシエーション解析」がこれらの研究をサポートするうえでも重要なアプローチになることは確実である。現在全世界で盛んに実施されはじめている全ゲノムアソシエーション解析は，原因遺伝子群を先入観のバイアスなしに同定できる可能性をおおいに秘めている。

2007年6月に英国のグループから全身性の多因子疾患7疾患についての結果が報告され[8]，各症例2000人と対照3000人のSNPデータを用いたアソシエーション解析のスケールの大きさに震撼させられた。同号のNature誌上に，SNPをマーカーとするアソシエーション解析が満たすべき明確な指針も提示された[9]。これは，これまでのいくつかの解析結果について再現性がとれないとの報告が相次いだためである[10]。この指針では，今後同様の解析を実施する場合，統計学的なパワー（検出力）が十分に高い実験設計に基づいていることと，同程度かそれ以上の規模の別集団で再現性を得ることが推奨されている。改めて大型プロジェクトの強みが浮き彫りになった形である。しかし興味深いことに，全ゲノムアソシエーション解析の先例となった以下に概説する眼疾患研究例では，この指針を必ずしも十分には満たしていなかった。

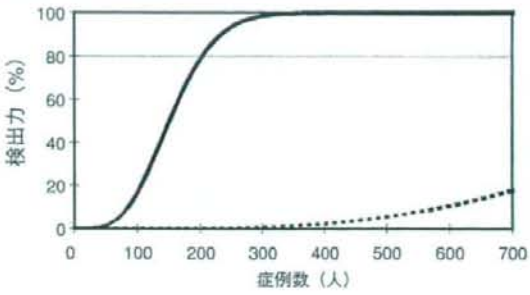
1．加齢黄斑変性

折しも2005年4月は，Affimetrix社やIllumina社が100Kチップを上市してはいたものの，同年8月に更新された国際HapMapプロジェクト[3]の第2相の成果はプローブにほとんど反映されておらず，ごく一部の施設がAffimetrix社500Kチップのベータ版を試用していた過渡期の頃であった。そのような状況下で，Affimetrix社100Kチップを用いた研究成果として，補体系のH因子（complement factor H：*CFH*）遺伝子が加齢黄斑変性[用語1]の原因遺伝子として同定された[11]。

加齢黄斑変性は，光を感じる神経の膜（網膜）の中央に位置し，物を見るために最も重要な視力

や色の判別を司る黄斑が加齢とともに変性する疾患である（図❶）。日本人の罹患率は50歳以上でも1％未満と推計されているが，米国では失明原因の第1位であり，現在は進行を遅延させるための対処療法しかない。本研究では，白人の症例96人と対照50人の全ゲノムアソシエーション解析を実施した結果，「過剰補正」とさえ言われている厳しいBonferroniの補正を突破した有意水準の高いマーカーSNPを2つ見出した。その後の連鎖不平衡ブロックによる領域の規定，領域内のダイレクトシークエンス，候補SNPsのハプロタイプ解析を経て，チロシンからヒスチジンへのアミノ酸変異をもたらす*CFH*遺伝子上のnonsynonymous SNPを同定することに成功した。この研究は，全ゲノム解析によるマーカーSNPから，これが真の生化学的疾患原因SNPであるという完全な証明には至っていないものの，追試されているという意味でかなり本当らしい疾患原因SNPの同定に結びついた最初の典型的な成功例である。

図❷ 統計学的検出力のシミュレーション



加齢黄斑変性のマーカーSNPとして同定された1SNP（rs1329428）を題材に，本アソシエーション解析の検出力をフリーウェアソフト“Power and Sample Size Calculations”（Department of Biostatistics, Vanderbilt University）を用いて概算した。アレル頻度比較に基づき算出するため，オッズ比3.0（実線）とリスクアレル頻度0.52は文献12の情報から仮定した。検体数は，症例96人・対照50人（対照/疾患：0.52），有意水準はBonferroniの補正の閾値に近い$p < 1 \times 10^{-7}$に設定した。オッズ比1.5（点線）の場合についても同様の条件で検討した。

ところが，驚くべきことに本研究の理論上の検出力はかなり低い。症例96人・対照50人のアソシーション解析で，有意水準が$p < 1 \times 10^{-7}$のマーカーSNPを取得するための検出力は約15％に過ぎず（図❷，実線），検出力が80％を超えるためには少なくとも200人規模の症例が必要である。今回マーカーとして取得できた*CFH*遺伝子上のSNPのオッズ比が約3.0と比較的高かった[12]ことが幸運であったと言える。これが仮に多因子疾患で検出されるSNPの平均的なオッズ比1.5程度であった場合（図❷，点線），症例と対照群を合わせて2000人を超える例数を準備する必要があり，理論上，本研究の検体数では同様の結果を得ることは絶望的であった。偶然にもオッズ比が高かったとはいえ，検出力が15％しかない研究設計で高い有意水準のマーカーSNPを取得できたことについて，本研究が幸運に恵まれたことによるという理解は，今後，同様の研究を設計する際には忘れてはならない。

本研究は，同施設からの別集団を用いた報告[12]と他施設からの追随報告で再現性が実証されている。さらに，その後の解析から新たな原因遺伝子として補体系のC3因子上のnonsynonymous SNPも同定されている[13]。危うい実験設計ではあったが，まちがいなく全ゲノムアソシエーション解析が疾患の発症分子メカニズムの一端を解明する糸口になった。しかし残念ながら，*CFH*遺伝子上のSNPは日本人では関連がないことが本邦から報告され，この結果の差異は人種差によると推測されている[14]。今後，欧米人の加齢黄斑変性の発症メカニズムの全容の解明とともに，日本人独自の原因遺伝子の同定に向けた全ゲノムアソシエーション解析の実現が期待される。

2．緑内障

緑内障[用語1]は全人類共通の失明の原因の上位の疾患であることから，根治療法の開発に向けて世界中で原因遺伝子の探索の試みが報告されている。緑内障は，神経線維層が障害され視野が狭くなる疾患で，前房中の液体成分（前房水）の排出障害による眼圧の上昇が最大のリスク因子とされているが，正常眼圧緑内障の概念の確立から神経細胞

死に関わる他因子にも注目が集まっている。古くから緑内障は遺伝性疾患であると考えられており，緑内障家系を用いた連鎖解析によりゲノム上の候補領域が特定されている。それらの領域から，ミオシリン[15]，オプチニューリン[16]，*WDR36*[17]などが同定され，「緑内障遺伝子」と呼称されている。しかし，検出力が十分に高い解析で追試されていないので，これら3つの遺伝子が緑内障の発症メカニズムに関与する可能性はあると考えられるが，どの程度の寄与があるかは今後の研究を待つ必要がある。

日本人でも，ミオシリンなどの遺伝子についてのアソシエーション解析が報告されており[18]，新たな原因遺伝子の候補も挙がっている。しかし，一定水準以上の検出力を有する全ゲノムアソシエーション解析を実施した報告例は皆無である。症例・対照群の設計精度の高い，ある程度規模の大きな集団を準備することができれば，あるいは発症メカニズムの解明や治療法の開発に向けて突破口になるような「真の緑内障遺伝子群」を同定することができるかもしれない。

最近，続発緑内障の一病型である落屑緑内障の原因遺伝子が同定されたとの報告がなされた[19]。本報告を受けて一部では「緑内障の原因遺伝子を同定」とも報じられている。しかし，同定されたSNPは最も一般的な緑内障である開放隅角緑内障では関連が認められず，落屑緑内障のみで強い関連を示した。さらに，緑内障を伴わない落屑症候群に対しても関連が示されていることから，正確には「落屑症候群とそれに伴う続発緑内障の原因遺伝子が同定された」と言うべきであろう。

Ⅱ．遺伝子発現解析

遺伝子の発現の変動を定量的に解析する場合，異なる由来の同一組織（症例と対照など）を準備するか，もしくは特定の組織を異なる条件で処理するか（薬剤の処理の有無など），大別して2通りの検討方法が考えられる。当然のことながら，いずれの場合においても出発材料として解析対象となる組織や細胞のtotal RNAが必要になる。ヒトの組織を入手するには制限があるので，チップデータの意義づけには動物実験も含めて研究の設計が極めて重要である。

1．眼科領域におけるヒト由来の組織・細胞

研究計画が所属施設医学倫理委員会をクリアし，インフォームドコンセントが十分に得られるという条件つきではあるが，眼科領域で入手可能なヒト由来の組織を**表❶**に示した。日本でも米国でも健常者から採取できる組織は角膜と結膜の上皮の一部に限られる。その他の組織については，手術などの治療行為で摘出した組織に限って，当人の同意の下，研究目的での使用が可能である。一方，米国では本邦とは異なり，アイバンクへ提供された眼球であっても，適正な手続きを経ることで眼の各組織を研究目的で使用することができる。実質上この制度が研究社会へ多大な貢献をしている。

2．眼疾患の遺伝子発現変動解析

特定の眼組織での遺伝子発現プロファイルを解析することで，眼疾患の発症や進行との関連（**表❶**）を明らかにしようとする試みが多数報告されている。例えば，年齢差のあるヒト眼球由来の網膜を比較して発現が変動している遺伝子を列挙し，加齢に伴い発症する疾患との関連を考察している報告がある[20]。また，網膜色素上皮細胞を用いて，加齢とともに増加する種々の酸化ストレスの有無により発現が変動する遺伝子を解析した報告も散見される。加齢黄斑変性では，SNP解析から原因遺伝子として補体系の因子が同定されたことを契機に，今後免疫系との関わりが盛んに検討されることが予想される。しかし，眼組織はある種の「免疫寛容」の状態にあり，網膜に侵入した外来抗原は免疫反応を誘起しにくいことが知られている[21]。全身性の補体系が網膜における加齢黄斑変性の発症メカニズムにどのように関わっているのか，興味深い課題が残されている。

緑内障では，障害部位である神経線維層のもととなる神経節細胞と眼圧の維持に関与している線維柱帯細胞を用いた報告が圧倒的に多い。神経節細胞はヒト由来の細胞の入手が困難であることから，ラットから樹立した初代培養細胞で代用している。反面，線維柱帯細胞ではヒト眼球由来の初代培養系が確立されており[22]，眼圧の上昇や下降

表❶ 研究材料として入手可能な眼組織と関連する眼疾患

組織	日本			米国			関連疾患
	健常者	患者（手術）	アイバンク	健常者	患者（手術）	アイバンク	
角膜	○*	○	移植治療のみ	○*	○	○	Stevens-Johnson症候群，感染症など
結膜	○*	○	×	○*	○	○	Stevens-Johnson症候群，翼状片など
線維柱帯	×	○	×	×	○	○	緑内障
虹彩	×	○	×	×	○	○	虹彩炎
水晶体	×	○	×	×	○	○	白内障
網膜	×	○	×	×	○	○	緑内障，加齢黄斑変性など

* Epi-LASIKあるいはPRK屈折手術時における角膜上皮，impression cytologyによる結膜上皮に限る。

に関与しているTGF-βファミリーなどのサイトカインの影響を検討した例が多く見受けられる。筆者らは，前房水に含まれるTGF-β3濃度を定量できる微量測定系を構築し，続発緑内障の一病型である偽落屑緑内障の患者から採取した前房水中のTGF-β3濃度が他の病型の患者に比べて有意に高いことを見出した[23]。現在，TGF-βファミリーが相互に眼圧を制御する機構を統合的に理解するため，線維柱帯細胞にTGF-β1，-β2，-β3をそれぞれ添加し，発現が変動する遺伝子群を検討している。その際，二次的な作用の影響をできるだけ回避するために各サイトカインを添加してからごく早期にtotal RNAを抽出してチップ実験に供している。本研究がTGF-βファミリーによる眼圧の制御機構と緑内障の発症や進行のメカニズムとの関わりを解明する一助になることを期待している。

おわりに

10年以上の歴史があるチップを用いる遺伝子発現解析の結果は連続変数であり，グレーのグラデーションとして表現されるが，SNPs解析は白か黒かの結果を出さなくてはならない。白黒を判定できる実験の質を確保する努力が各研究者に求められる。結果が後世の大規模研究で覆されることがおおいにありうるからである。今後，チップの多様なアプリケーションがもたらす無数のデータを，従来の分子生物学的手法で整理・統合しながら生命現象を捉えていく機会がますます増えていくものと思われる。眼科領域では，日本眼科学会の専門別研究会の1つ「眼科DNAチップ研究会」などで先進的な研究の情報交換や議論をさらに活性化することが重要であると考えられる。

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用語解説

1．**加齢黄斑変性**：黄斑が加齢に伴い変性することによって発症する疾患。脈絡膜から発生する異常な血管（新生血管）の有無で滲出型と萎縮型に分類される。特に滲出型は急激な視力低下を生じ，失明原因となる難治性疾患である。日本人では，男性の発病率が女性の約3倍，50歳以上の罹患率は1％未満と推計されている。米国では失明原因の第1位。

2．**緑内障**：網膜の神経線維層が何らかの原因で障害され視野が狭くなる疾患。糖尿病網膜症とともに「目の成人病」と言われ，全人類共通の失明原因の上位の疾患である。前房水の排出障害による眼圧の上昇が最大のリスク因子とされているが，日本人患者の約6割が「正常眼圧」緑内障である。緑内障は，原発緑内障，先天緑内障，続発緑内障に大別され，原発緑内障はさらに開放隅角緑内障（このうち眼圧が正常範囲のものを正常眼圧緑内障と呼ぶ）と閉塞隅角緑内障に分類される。

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Association of Combined IL-13/IL-4R Signaling Pathway Gene Polymorphism with Stevens-Johnson Syndrome Accompanied by Ocular Surface Complications

Mayumi Ueta, Chie Sotozono, Tsutomu Inatomi, Kentaro Kojima, Junji Hamuro, and Shigeru Kinoshita

Purpose. Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) are acute-onset mucocutaneous diseases induced by infectious agents or inciting drugs. The authors previously reported an association between SJS/TEN and *IL-4R* gene polymorphism that is essential for IL-4 and IL-13 signaling. To examine *IL-4* and *IL-13* gene polymorphisms and the combination of these polymorphisms with *IL-4R* polymorphism, the authors performed polymorphism analysis.

Methods. In 76 Japanese SJS/TEN patients with ocular surface complications and 160 healthy controls, the authors analyzed polymorphisms of the promoter −590C/T in the *IL-4* gene and of the promoter −1111C/T and Arg110Gln in the *IL-13* gene and assessed Gln551Arg in the *IL-4R* gene. Because Arg110Gln affects serum IL-13, plasma IL-13 levels were also examined.

Results. In the SJS/TEN patients, the Arg110Gln SNP of *IL-13* was significantly associated with the disease, and the frequency of Arg110 alleles was significantly higher than that in the controls. Plasma IL-13 tended to be lower in SJS/TEN patients than in the controls. Analysis of the genotype pattern of *IL-4R* SNP Gln551Arg and *IL-13* SNP Arg110Gln showed that the Gln551Gln(A/A)-Arg110Arg(G/G) genotype pattern was also associated with SJS/TEN.

Conclusions. *IL-13* gene polymorphisms might be associated with SJS/TEN with ocular surface complications. The present findings suggest that SJS/TEN is different from allergic diseases such as atopy and asthma because the ratio of each allele in the *IL-13* SNP Arg110Gln was the opposite of the ratio in those diseases. They also reveal that combined polymorphisms in the IL-13/IL-4R signaling pathway were associated with SJS/TEN with ocular surface complications. (*Invest Ophthalmol Vis Sci.* 2008;49:1809-1813) DOI:10.1167/iovs.07-1401

Stevens-Johnson syndrome (SJS), an acute inflammatory vesiculobullous reaction of the skin and mucous membranes first described in 1922,[1] is commonly associated with infectious agents and inciting drugs.[2,3] When there is extensive skin detachment and a poor prognosis, the condition is called toxic epidermal necrolysis (TEN).[4] In the acute stage, SJS/TEN patients manifest vesiculobullous skin lesions, severe conjunctivitis, and persistent corneal epithelial defects because of ocular surface inflammation. In the chronic stage, ocular surface complications, such as conjunctival invasion into the cornea caused by corneal epithelial stem cell deficiency, symblepharon, ankyloblepharon, and, in some instances, keratinization of the ocular surface, persist despite healing of the skin lesions.[5] SJS/TEN is one of the most devastating ocular surface diseases, and it leads to corneal damage and loss of vision. The reported incidence of ocular surface complications in SJS/TEN is 50% to 68%.[3,6]

We previously reported that not only environmental but also genetic factors may play important roles in an integrated etiology of SJS/TEN and that in the Japanese, HLA-A*0206 was strongly associated with SJS/TEN with ocular surface complications.[7] We also documented that in Japanese patients with SJS/TEN, there was an association with toll-like receptor 3 (*TLR3*) polymorphisms.[2] Furthermore, we found that in Japanese patients with SJS/TEN, there is an association with polymorphisms in the allergy-related *IL-4R* gene and that the ratio of each allele in the polymorphism was the opposite of the ratio reported in atopy and asthma.[8]

IL-4Rα is a component of not only the IL-4 but also the IL-13 receptor and is essential for both IL-4 and IL-13 signaling. The type 1 IL-4 receptor is composed of 2 subunits, an α subunit (IL-4Rα), which binds IL-4 and transduces its growth-promoting and transcription-activating functions, and a γ c subunit, common to several cytokine receptors, that amplifies signaling of IL-4Rα. The IL-13 receptor (IL-13R) is composed of the IL-4Rα chain (IL-4Rα) and the IL-13Rα1 chain (IL-13Rα1).[9] Given that IL-4 is able to bind to this receptor, it is also called type 2 IL-4R.[9] There exists another IL-13 binding unit, the IL-13Rα2 chain (IL-13Rα2), which acts as a decoy receptor.[9]

Because there is an association between SJS/TEN and *IL-4Rα* polymorphism, we speculated that there might be an association between IL-4 or IL-13 signaling and SJS/TEN. Therefore, we examined IL-4 and IL-13 gene polymorphisms and the combination of these polymorphisms with *IL-4R* polymorphism.

With respect to *IL-4* gene polymorphisms, a variant of the promoter region of the *IL-4* gene, −590C/T, has been shown to be related to asthma.[10-12] Regarding *IL-13* gene polymorphisms, a variant of the promoter region of the *IL-13* gene, −1111C/T,[13,14] and a variant of Arg110Gln were reportedly associated with asthma.[15] Gln551Arg of the *IL-4R* gene was associated with atopy[16,17] and asthma.[18]

Here we examined polymorphisms of the promoter −590C/T (rs.2243250) in the *IL-4* gene, of −1111C/T (rs.1800925) and Arg110Gln (rs.20,541) in the *IL-13* gene, and of Gln551Arg (rs.1801275) in the *IL-4R* gene in Japanese SJS/TEN patients with ocular surface complications and healthy volunteers. We also examined their plasma IL-13 level because

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TABLE 1. Genotype Frequencies for Various SNPs and SJS/TEN Susceptibility

	Control (%) $n = 160$	SJS/TEN (%) $n = 76$	Allele 1 vs. Allele 2 P OR (95% CI)	Genotype 11 vs. 12 + 22 P OR (95% CI)	Genotype 11 + 12 vs. 22 P OR (95% CI)
IL-4 gene					
Promoter-590 (rs. 2243250)					
11 TT	82 (51.3)	39 (51.3)	0.54	0.99	0.08
12 TC	72 (45.0)	30 (39.5)	—	—	—
22 CC	6 (3.8)	7 (9.2)			
IL-13 gene					
Promoter-1111 (rs. 1800925)					
11 CC	101 (63.1)	57 (75.0)	0.049	0.07	0.23
12 CT	52 (32.5)	18 (23.7)	1.7	—	—
22 TT	7 (4.4)	1 (1.3)	(1.0–3.0)		
Arg(G) 110Gln(A) (rs. 20541)					
11 GG	77 (48.1)	47 (61.8)	0.014	0.049	0.035
12 GA	66 (41.2)	27 (35.5)	1.8	1.8	4.4
22 AA	17 (10.6)	2 (2.6)	(1.1–2.8)	(1.0–3.0)	(1.0–19.6)
IL-4R gene					
Gln(A)551Arg(G) (rs.1801275)					
11 AA	115 (71.9)	69 (90.8)	0.0008	0.0011	—
12 AG	41 (25.6)	7 (9.2)	3.7	3.9	—
22 GG	4 (2.5)	0 (0)	(1.7–8.5)	(1.6–9.0)	

P values were determined by χ^2 testing.

Arg110Gln in the *IL-13* gene has an effect on the serum level of IL-13.[19]

METHODS

Patients

This study was approved by the institutional review board of Kyoto Prefectural University of Medicine, Kyoto, Japan. All experimental procedures were conducted in accordance with the principles set forth in the Declaration of Helsinki. The purpose of the research and the experimental protocols were explained to all participants, and their prior written informed consent was obtained.

For single-nucleotide polymorphism (SNP) analysis, we enrolled 76 patients with SJS/TEN in the chronic or subacute phase; all presented with ocular surface complications. The diagnosis of SJS/TEN was based on a confirmed history of the acute onset of high fever, serious mucocutaneous illness with skin eruptions, and involvement of at least two mucosal sites including the ocular surface. The controls were 160 healthy volunteers without allergic diseases such as atopic dermatitis or asthma. All participants and volunteers were Japanese residing in Japan. The average age of the patients and controls was 46.1 ± 17.3 (SD) and 36.2 ± 11.5 (SD) years, respectively. The male/female ratios in the patient and control groups were 33:43 and 57:103, respectively.

SNP Analysis

SNP analysis was performed by direct sequencing. PCR and sequence primers for SNPs of *IL-4* were 5′-CTTGAGCCCAGGAATTTGAG-3′ (sense) and 5′-ACAGGTGGCATCTTGGAAAC-3′ (antisense) for the −590 promoter (rs.2243250). For SNPs of *IL-13*, they were 5′-CCACATCTGTACAGTAGAGG-3′ (sense) and 5′-GGCTGAGGTCTAAGCTAAGG-3′ (antisense) for Arg110Gln (rs.20,541), and 5′-ATGCCTTGTGAGGAGGGTCAC-3′ (sense) and 5′-CCAGTCTCTGCAGGATCAACC-3′ (antisense) for promoter-1111 (rs.1800925). For Gln551Arg (rs.1801275) of *IL-4R* SNPs, they were 5′-AGCTTCAGCAACTCCCTGAG-3′ (sense) and 5′-CCCAAACCCACATTTCTCTG-3′ (antisense). Genomic DNA was isolated from human peripheral blood at SRL Inc. (Tokyo, Japan). PCR amplification was with DNA polymerase (Takara, Shiga, Japan) for 35 cycles at 94°C for 1 minute, annealing at 60°C for 1 minute, and 72°C for 1 minute on a commercial PCR machine (GeneAmp; Applied Biosystems, Foster City, CA) The PCR products were reacted (BigDye Terminator v3.1; Applied Biosystems), and sequence reactions were resolved on a genetic analyzer (ABI PRISM 3100; Applied Biosystems).

Statistical Methods Used for SNP Analysis

Alleles were counted manually. Each allele was assessed as an independent variable, and separate P values were calculated for each polymorphism. $P < 0.05$ was regarded as significant. In addition, P was corrected for the number of alleles tested in each gene (Bonferroni method).

Measurement of Plasma IL-13 Levels

For the measurement of plasma IL-13 levels, we enrolled 29 patients with SJS/TEN in the chronic or subacute phase; all presented with ocular surface complications (Arg110/Arg110, $n = 14$; Arg110/Gln110, $n = 14$; Gln110/Gln110, $n = 1$). Controls were 160 healthy volunteers (Arg110/Arg110, $n = 77$; Arg110/Gln110, $n = 66$; Gln110/Gln110, $n = 17$). Plasma, obtained at the time of genomic DNA isolation from peripheral blood, was used for analysis. Plasma IL-13 levels were immunoassayed with an ELISA kit (Biotrak Easy ELISA; Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions. The minimum detectable level was 1.56 pg/mL.

RESULTS

A summary of our case-control association study with the four genotyped SNPs is shown in Table 1. All four SNPs were in Hardy-Weinberg equilibrium in the SJS/TEN patients and the healthy controls ($P > 0.01$). In the promoter −590C/T SNP of the *IL-4* gene related to higher IgE levels,[10] there was no significant association. In the promoter −1111C/T SNP of the *IL-13* gene related to asthma,[13,14] there was a weak association with allele frequency (C vs. T, raw $P = 0.049$, corrected $P = 0.099$; odds ratio = 1.7); correction of the P value for the number of alleles detected ($n = 2$) rendered the result not significant. Gln110Arg SNPs of *IL-13* exhibited a significant association with allele frequency (G vs. A, raw $P = 0.014$,

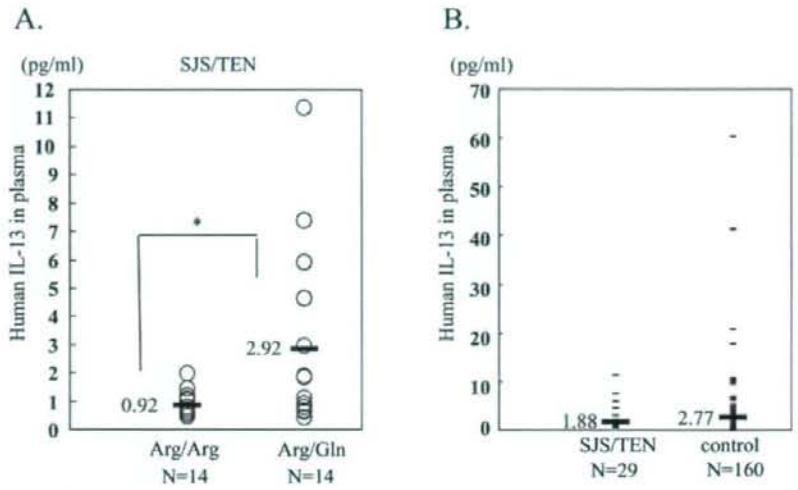

FIGURE 1. Plasma IL-13 levels in SJS/TEN patients. (**A**) Comparison of the plasma IL-13 levels in 28 SJS/TEN patients with Arg110Arg (n = 14) or Arg110Gln (n = 14) showed that levels were significantly higher in patients with Arg110Gln (*P < 0.05). Evaluation was with Student's t-test using a spreadsheet program. (**B**) Comparison of the plasma IL-13 level in 29 SJS/TEN patients (Arg110/Arg110, n = 14; Arg110/Gln110, n = 14; Gln110/Gln110, n = 1) and 160 controls (Arg110/Arg110, n = 77; Arg110/Gln110, n = 66; Gln110/Gln110, n = 17) showed that it tended to be lower in patients than in controls. However, the difference was not statistically significant. Evaluation was with Student's t-test using a spreadsheet program.

corrected P = 0.028; odds ratio = 1.8) even when we corrected P for the number of alleles detected of *IL13* SNPs (n = 2). They also exhibited a weak association with the dominant model (G/G vs. G/A + A/A, raw P = 0.049, corrected P = 0.097; odds ratio = 1.8) and the recessive model (G/G +G/A vs. A/A, raw P = 0.035, corrected P = 0.07; odds ratio = 4.4); correction of the P value for the number of alleles detected (n = 2) rendered the result not significant. These findings contrast with those of Heinzmann et al.,[15] who reported that Gln110 was significantly increased in human asthma. We detected a significant increase in Arg110 in our SJS/TEN patients.

The Gln551Arg SNP of the *IL-4R* gene showed a significant association with allele frequency (A vs. G, raw P = 0.0008; odds ratio = 3.7) and the dominant model (A/A vs. A/G + G/G, raw P = 0.0011; odds ratio = 3.9); these findings coincide with those we reported previously.[8]

We also studied the plasma IL-13 levels in our SJS/TEN patients because these levels were reportedly higher in patients with Gln110.[19] We compared plasma IL-13 levels in Arg110Arg and Arg110Gln genotypes, and, though in the controls it tended to be higher in the Arg110Gln genotype (data not shown), it was significantly higher in SJS/TEN patients with the Arg110Gln than the Arg110Arg genotype (Fig. 1A). Plasma IL-13 levels tended to be lower in SJS/TEN patients than in the controls, but the difference was not statistically significant (Fig. 1B). Our results are in accordance with findings that SJS/TEN with ocular surface complications is associated with Arg110Gln, which affects the plasma IL-13 level; in SJS/TEN there is a significant increase in the Arg110 allele, which might lead to lower serum IL-13 levels than the Gln110 allele.

We also analyzed the genotype pattern of *IL-4R* SNP Arg551Gln and *IL-13* SNP Arg110Gln. We found that the Gln551Gln(A/A)-Arg110Arg(G/G) genotype pattern also associated with SJS/TEN in Japanese patients (χ^2 test; P = 0.0006, OR = 2.6, 95% CI, 1.5–4.6; Table 2). In more detail, 69 of 76 (90.8%) SJS/TEN patients and 115 of 160 (71.9%) controls had IL-4R Gln551Gln (Table 1), and 44 of 76 (57.9%) SJS/TEN patients and 55 of 160 (34.4%) controls had the genotype pattern Gln551Gln(A/A) of the *IL-4R*-Arg110Arg(G/G) of the *IL-13* (type 1 pattern; Table 2). Therefore, 44 of 69 SJS/TEN patients with *IL-4R* Gln551Gln (63.8%) had the type 1 pattern, whereas only 55 of 115 controls with *IL-4R* Gln551Gln (47.8%) had the type 1 pattern. This result shows that SJS/TEN patients with *IL-4R* Gln551Gln have *IL-13* Arg110Arg more frequently than controls with *IL-4R* Gln551Gln; there was a significant difference between SJS/TEN and controls (χ^2 test; P = 0.036, OR = 1.9; 95% CI, 1.0–3.5). Thus, we suggest a combined effect exists between *IL-4R* and *IL-13* polymorphisms.

DISCUSSION

Arg110Gln SNP of *IL-13* was significantly associated with SJS/TEN with ocular surface complications. Arg110Gln affects the

TABLE 2. Pattern Structures and Frequencies of *IL-13* SNP Arg110Gln and *IL-4R* SNP Arg551Gln

Pattern Type	*IL-4R* SNP Arg551 Gln	*IL-13* SNP Arg110 Gln	Control (%) n = 160	SJS/TE (%) n = 76	P	OR (95% CI)
1	A/A	G/G	55/160 (34.4)	44/76 (57.9)	0.0006	2.6 (1.5–4.6)
2	A/A	A/G	48/160 (30.0)	23/76 (30.3)	NS	—
3	A/G	G/G	20/160 (12.5)	3/76 (3.9)	NS	—
4	A/G	A/G	16/160 (10.0)	4/76 (5.3)	NS	—
5	A/A	A/A	12/160 (7.5)	2/76 (2.6)	NS	—
6	G/G	G/G	2/160 (1.3)	0/76 (0.0)	NS	—
7	G/G	A/G	2/160 (1.3)	0/76 (0.0)	NS	—
8	A/G	A/A	2/160 (1.3)	0/76 (0.0)	NS	—

P values were determined by χ^2 testing.

plasma IL-13 level.[19] In SJS/TEN, Arg110 alleles are significantly increased, but in atopy and asthma, Gln110 alleles are significantly increased.[15] Plasma IL-13 tended to be lower in our SJS/TEN patients with ocular surface complications than in the controls because plasma IL-13 was lower in the presence of Arg110Arg than in the presence of Gln110Arg.[19] Although our results suggest that in Japanese SJS/TEN patients with ocular surface complications there might be an association with polymorphisms in the allergy-related *IL-13* genes, SJS/TEN is different from allergic diseases such as atopy and asthma because the ratio of each allele of the *IL-13* SNP Arg110Gln was opposite the ratio in atopy and asthma. In SJS/TEN, Arg110 rather than Gln110 alleles (which are significantly increased in asthma)[15] showed a significant increase. Arima et al.[19] have reported that the Gln110 variant of Gln110Arg decreased the affinity with IL-13Rα2, a decoy receptor, and enhanced stability as a protein, causing upregulation of the IL-13 concentration in vivo. The results we obtained by polymorphism analysis were supported by our findings that the plasma IL-13 level tended to be lower in patients with SJS/TEN.

Given that there is an association with *IL-4R* gene polymorphisms in SJS/TEN,[8] we analyzed the genotype pattern of the *IL-4R* SNP Gln551Arg and the *IL-13* SNP Arg110Gln. We found that Gln551Gln(A/A) of the *IL-4R*-Arg110Arg(G/G) of the *IL-13* genotype pattern associated with SJS/TEN in Japanese patients.

These results reveal not only that *IL-13* and *IL-4R* gene polymorphisms but also that combined polymorphisms in the IL-13/IL-4R signaling pathway are associated with SJS/TEN with ocular surface complications.

We previously reported that SJS/TEN was associated with Gln551Arg of *IL-4R* polymorphisms, which had no effect on IgE synthesis.[8] In addition, in Gln551Arg polymorphisms, Gln551 but not Arg551 alleles were significantly increased in SJS/TEN, whereas Arg551 alleles were significantly increased in atopy and asthma. Our earlier study on the relationship between serum IgE and SJS/TEN also showed that there was no significant difference between SJS/TEN patients and controls with respect to the incidence of high total serum IgE.[8]

In Arg110Gln of *IL-13* polymorphisms and Gln551Arg of *IL-4R* polymorphisms, the ratio of each allele was the inverse of the ratio reported for atopy and asthma; therefore, SJS/TEN appears to be different from those allergic diseases. Moreover, combined polymorphisms in the IL-13/IL-4R signaling pathway are also associated with SJS/TEN patients; we document that Gln551Gln(A/A) of the *IL-4R* and Arg110Arg(G/G) of the *IL-13* genotype pattern were also associated with SJS/TEN with ocular surface complications.

In patients with acute-phase SJS/TEN, dermatologists have examined IL-13 levels in serum or skin lesions and reported that the expression level of IL-13 is upregulated in patients with Steven-Johnson syndrome. They also reported that normalization in serum IL-13 levels was demonstrated in all three patients with SJS/TEN, who were tested after the resolution of the cutaneous disease. In this study, we examined the serum IL-13 levels in SJS/TEN patients in the chronic phase or the subacute phase, in which the cutaneous disease was resolved. Thus, we suggest that the serum IL-13 levels of baseline such as in the chronic phase or the subacute phase, but not in the acute phase, tend to be lower in SJS/TEN patients with ocular surface complications than in the controls because the Arg110Arg genotype, in which the IL-13 level was reportedly lower than in Arg110Gln, was significantly increased in SJS/TEN patients with ocular surface complications.

It has been suggested that the pathogenesis of TEN is immunologically mediated and that it involves cytotoxic $CD8^+$ lymphocytes.[20,21] Given that $CD8^+$ T cells involve Th1 cytokine-driven inflammatory mechanisms, such mechanisms may be involved in the skin inflammation seen in the acute stage of SJS/TEN. In contrast, Th2 cytokine-driven inflammatory mechanisms may play a role in the inflammation seen in allergic diseases such as atopy and asthma.[22] Thus, genetic alterations in the IL-13/IL-4R signaling pathway may regulate Th1 or Th2 cytokine-driven inflammatory mechanisms.

We suggested elsewhere that IL-4R might be linked to innate immunity.[8] The innate immune system may constitute a link between the environment and the adaptive immune system. We are continuing to examine the pathophysiology of SJS/TEN with ocular surface complications.

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HLA-B locus in Japanese patients with anti-epileptics and allopurinol-related Stevens–Johnson syndrome and toxic epidermal necrolysis

Introduction: Stevens–Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) are rare but life-threatening severe cutaneous adverse reactions. Recently, strong associations of *HLA-B*1502* and *HLA-B*5801* with carbamazepine- and allopurinol-induced severe cutaneous adverse reactions were found in Han Chinese patients, respectively, but ethnic differences in the associations have been reported. The objective of this study is to clarify the involvement of *HLA-B*1502* and *HLA-B*5801* in Japanese SJS/TEN patients. **Methods:** *HLA-B* genotyping was performed on 58 Japanese SJS/TEN patients between July 2006 and April 2008 from multicenters in Japan. **Results:** There were no *HLA-B*1502* carriers among 58 SJS/TEN patients. This patient group included seven carbamazepine-related and 11 aromatic anti-epileptic agent-related SJS/TEN patients. In addition, there were five *HLA-B*5801* carriers, which included four allopurinol-related SJS/TEN patients. **Conclusion:** While *HLA-B*1502* is unlikely to be associated with carbamazepine-related or aromatic anti-epileptic agent-related SJS/TEN, *HLA-B*5801* was significantly associated with allopurinol-related SJS/TEN in Japanese.

KEYWORDS: allopurinol, anti-epileptic drugs, carbamazepine, *HLA-B*1502*, *HLA-B*5801*, Japanese patient, Stevens–Johnson syndrome, toxic epidermal necrolysis

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Introduction

Stevens–Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) are life-threatening severe adverse drug reactions with mucosal and cutaneous disorders, and very often accompanied by high fever and systemic complications. Some investigators have proposed that SJS and TEN are variations of the same disease expressed with different severity [1,2], although this is controversial. Although SJS and TEN incidence is very low (0.4–6 per million per year) [3,4], more than 100 different causative drugs have been reported [5]. The diseases are probably T-cell-mediated delayed allergic reactions [6], and typically begin within 1–3 weeks after first exposure to a drug.

Recently, an extremely strong association (odds ratio: ~2504) between human leukocyte antigen (*HLA*)-*B*1502* and carbamazepine-induced SJS/TEN in Han Chinese patients in Taiwan was reported [6]. Another Taiwanese study showed that *HLA-B*5801* was detected in all Han Chinese patients with SJS/TEN or drug-induced hypersensitivity (DIHS) induced by allopurinol [7]. The involvement of *HLA-B* 1502* was also confirmed in SJS/TEN caused by other aromatic epileptic agents such as phenytoin in Han Chinese or Thai population [8,9]. However, such a strong association between *HLA-B*1502* and carbamazepine-induced SJS/TEN was not detected in Caucasian patients [5]. These reports suggested that HLA involvement in severe cutaneous adverse reactions may be drug-specific as well as ethnic group-specific. Thus, we started a retrospective case–control study to explore genetic biomarkers related to SJS and TEN in Japanese patients living in Japan.

Patients & methods

Patients

The ethics committees of each participating institute of the Japan Severe Adverse Reactions (JSAR) research group approved this study. Written informed consent was obtained from each patient. A total of 58 Japanese patients from unrelated families in Japan were recruited from JSAR research group hospitals or through a nationwide blood-sampling network system in Japan for SJS/TEN onset patients, operated by the National Institute of Health Sciences in cooperation with the Ministry of Health, Labour and Welfare in Japan and the Federation of Pharmaceutical Manufacturers' Association of Japan. All patients, two of whom were referred to in a previous report [10], were diagnosed as SJS or TEN by JSAR research group experts based on diagnostic criteria proposed by Bastuji-Garin *et al.* [1], which are currently used in Japan [11,12] using a standardized case report form including medicinal records,

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disease progress and involvement of systemic complication, as well as SJS/TEN treatment [1]. TEN and SJS are defined as mucocutaneous disorders characterized by extensive erythema, blisters, epidermal detachment, erosions, enanthema and high fever. SJS is defined as skin detachment of 10% or less of the body surface area, and TEN is defined as skin detachment of more than 10%, excluding staphylococcal scaled skin syndrome. The severity of ocular complication was scored as follows: 0: no involvement; 1: only hyperemia of bulbar and palpebral conjuctiva; 2: pseudomembrane formation; 3: defect of conjunctival or corneal epithelia.

HLA-B typing

High-resolution *HLA-B* typing was performed by a sequence-based method using SeCore™ B Locus Sequencing Kit (Invitrogen Corp., WI, USA) and an Applied Biosystems (ABI) 3730 DNA sequencer (ABI, CA, USA). Genomic DNA (250 ng) was used for PCR amplification and sequencing exons 2, 3 and 4. *HLA-B* haplotype was estimated with the Assign SBT software (version 3.2.7b, Conexio Genomics, Western Australia, Australia).

Statistical analysis

*HLA-B*5801* allele frequency reported by Tanaka *et al.*, who performed typing of *HLA-A*, and *-B* for 493 Japanese healthy subjects living in Japan was used as the frequency in control subjects [13]. Fisher's exact test was conducted using Prism 4 (GraphPad Software, Inc., CA, USA) to calculate the odds ratio and the 95% confidence interval.

Results

Demographics of patients recruited in this study are summarized in Table 1. A total of 36 and 22 patients were diagnosed with SJS and TEN, respectively. Approximately 80% of SJS/TEN patients complained of ophthalmic disorders, and two patients were coadministered anti-epileptic agents and allopurinol.

*HLA-B*1502* & *HLA-B*0702* in carbamazepine-related SJS/TEN

In our study, carbamazepine was prescribed for seven patients, and other aromatic anti-epileptic agents, such as phenytoin, phenobarbital or zonisamide, were prescribed for 11 patients. By contrast to data on the Han Chinese [6,8] and Thai populations [9], *HLA-B*1502* was neither detected in patients administered carbamazepine, nor in patients administered other aromatic epileptic drugs (Table 2).

Alfirevic *et al.* reported a potential protecting effect of *HLA-B*0702* against carbamazepine-induced severe cutaneous adverse reactions in Caucasian patients [14]. In line with this, no SJS/TEN patients receiving carbamazepine or other aromatic anti-epileptic drugs carried *HLA-B*0702* in this study. However, we found *HLA-B*0702* in two patients who did not receive anti-epileptic drugs, and there was no significant difference in the carrier frequencies between patients (1.72%) and the Japanese population (5.17%) ($p = 0.1113$).

*HLA-B*5801* in allopurinol-related and -unrelated SJS/TEN patients

As shown in Table 3, we found five carriers of *HLA-B*5801*, and four patients (patients 23,

Table 1. Demographics of Japanese patients recruited in the current study.

Factor	Value
Disease (SJS, TEN)	36, 22
Sex (male, female)	35, 23
Age (mean [range])	55 (5–94)
Severity in ophthalmic disorders	
Score 0 (no ophthalmic involvement)	12
Score 1 (only hyperemia of bulbar and palpebral conjunciva)	21
Score 2 (pseudomembrane without epithelial defect)	1
Score 3 (conjunctival and/or corneal epithelial defect)	14
Severity unknown ocular disorders	9
No description on ophthalmic symptom	1
Administered drugs before development of SJS/TEN	
Carbamazepine	7
Other aromatic anti-epileptic drugs	11
Allopurinol	10*

*One patient was treated with both carbamazepine and allopurinol, and another patient was treated with phenytoin and allopurinol.
SJS: Stevens–Johnson syndrome; TEN: Toxic epidermal necrolysis.

Table 2. Characteristics of SJS/TEN patients administered aromatic anti-epileptic drugs.

ID number	Sex	Age (years)	Disease	Aromatic anti-epileptic drugs prescribed	Severity score in ophthalmic disorders	*HLA-B* diplotype
1	M	73	SJS	Carbamazepine	1	*1511/*4801
2	F	42	SJS	Carbamazepine	3	*4001/*5201
3	M	45	SJS	Carbamazepine	3	*4801/*5601
4	M	54	SJS	Carbamazepine	0	*1501/*3501
5*	F	6	SJS	Carbamazepine	Severity unknown	*4006/*5101
6*	F	52	SJS	Carbamazepine/zonisamide	Severity unknown	*4601/*5901
7	M	17	TEN	Carbamazepine/zonisamide	3	*4601/*5601
8	M	67	SJS	Phenytoin	Ocular involvement unknown	*4001/*4601
9	F	5	SJS	Phenytoin	0	*5504/*6701
10	F	64	TEN	Phenytoin	3	*1501/*5101
11	F	56	TEN	Phenytoin	0	*1501/*5401
12	M	6	SJS	Phenobarbital	Severity unknown	*1501/*5101
13	M	69	SJS	Phenobarbital	1	*1501/*5101
14	F	42	TEN	Phenobarbital	0	*5101/*5401
15	M	25	SJS	Zonisamide	2	*1301/*4601
16	F	71	SJS	Zonisamide	1	*4002/*5101
17	M	52	TEN	Zonisamide	Severity unknown	*3501/*4601
18	M	78	TEN	Zonisamide	Severity unknown	*3901/*6701

These patients were reported in the previous report [10]. F: Female; M: Male; SJS: Stevens–Johnson syndrome; TEN: Toxic epidermal necrolysis.

24, 27 and 28) received allopurinol. Since a total of ten patients received allopurinol, *HLA-B*5801* carrier frequency in allopurinol-related patients was 40.0%. Table 4 shows a significant increase in *HLA-B*5801* allele frequency in allopurinol-administered patients when compared with the Japanese population (odds ratio: 40.83, $p < 0.0001$). *HLA-B*5801* was detected in one patient (patient 41) who did not receive allopurinol. This is the first report that *HLA-B*5801* was detected in a SJS/TEN patient unrelated to allopurinol.

Discussion

Recently, involvement of *HLA* loci have been detected in idiosyncratic adverse drug reactions, including cutaneous [6,7,15] or liver [16] injury. Regarding severe cutaneous reactions, some HLA class I antigen genotypes, such as *HLA-B*1502* [6], *HLA-B*5801* [7] and *HLA-B*5701* [15], have been reported to be very promising biomarkers for discriminating patients at high risk of SJS, TEN or DIHS induced by carbamazepine, allopurinol or abacavir, respectively. The very strong association of *HLA-B*1502* with

Table 3. Characteristics of SJS/TEN patients administered allopurinol and an allopurinol-unrelated patient carrying *HLA-B*5801*.

ID number	Sex	Age (years)	Disease	Allopurinol prescribed	Severity score in ophthalmic disorders	*HLA-B* diplotype[§]
1	M	73	SJS	Yes	1	*1511/*4801
8	M	67	SJS	Yes	Severity unknown	*4001/*4601
23	F	53	SJS	Yes	1	*4002/**5801**
24	M	77	TEN	Yes	Severity unknown	*5201/**5801**
25	M	75	SJS	Yes	Severity unknown	*4002/*4006
26	M	67	SJS	Yes	1	*3901/*4001
27	F	81	SJS	Yes	1	*4601/**5801**
28	M	83	SJS	Yes	1	*3901/**5801**
29	M	58	TEN	Yes	1	*1501/*5601
30	M	75	TEN	Yes	0	*3501/*5201
41	F	55	TEN	No[‡]	Severity unknown	*5401/**5801**

[‡]Leflunomid was prescribed for this patient.
[§]5801 is indicated in bold.
F: Female; M: Male; SJS: Stevens–Johnson syndrome; TEN: Toxic epidermal necrolysis.

Table 4. Associations of *HLA-B*5801* with Japanese SJS/TEN patients.

Patient group	Allele frequency (%)		p-value (Fisher's exact test)	Odds ratio	95% confidence interval for odds ratio
	SJS/TEN patients	*Japanese population**			
Allopurinol-related patients	20.0 (4/20)	0.61 (6/986)	<0.0001	40.83	10.50–158.9

*Data of 493 healthy Japanese reported in [13].
SJS: Stevens–Johnson syndrome; TEN: Toxic epidermal necrolysis.

carbamazepine-induced SJS/TEN found in Han Chinese patients in Taiwan [6] was further confirmed by an extended study in Taiwan [17], and studies on Asian patients living in Europe [5], Han Chinese patients in Hong Kong [8] and the Thai population [9]. Man *et al.* and Locharernkul *et al.* reported that *HLA-B*1502* was also detected in patients who suffered from SJS/TEN caused by aromatic anti-epileptic agents such as phenytoin and lamotrigine [8,9]. By contrast, no SJS/TEN patients receiving aromatic anti-epileptic drugs including carbamzepine carried *HLA-B*1502* in our study using Japanese patients. Thus, we could not confirm the association of *HLA-B*1502* with SJS/TEN in Japanese patients. This is reminiscent of the lack of the association in Caucasian carbamazepine-induced SJS/TEN patients [5]. *HLA-B*1502* was not detected in 486 healthy Japanese [13], while its allele frequency in Han Chinese was reported to be 8.6% [6]. The very low allele frequency of *HLA-B*1502* in the Japanese may account for why no association between *HLA-B*1502* and SJS/TEN was detected in our study. To date, useful genetic biomarkers have not been found for carbamazepine-induced SJS/TEN in ethnic groups other than some Asian ethnic groups, including Han Chinese.

Alfirevic *et al.* reported a significant low carrier frequency of *HLA-B*0702* in Caucasian patients with carbamazepine-induced severe cutaneous adverse reactions, and its potential protecting effect against severe cutaneous adverse reactions [14]. Since we detected *HLA-B*0702* in two SJS/TEN patients unrelated to carbamazepine administration, further studies are necessary to clarify the relationship between *HLA-B*0702* and SJS/TEN.

The association of *HLA-B*5801* with allopurinol-induced severe cutaneous adverse reactions detected in Han Chinese in Taiwan [7] has been confirmed in Caucasians [5]. Although the association observed in Han Chinese in Taiwan was extremely strong (odds ratio: ~580), only a moderate association of *HLA-B*5801* with allopurinol-induced SJS/TEN was observed in a European study by Lonjou *et al.* ($p < 10^{-18}$, odds ratio: 80) [5]. In their study, the carrier frequency in European patients was 55.6%, while that in a European population was 1.5%. A moderate but statistically significant association ($p < 0.0001$, odds ratio: ~40) between *HLA-B*5801* with allopurinol-administered SJS/TEN was also detected in the current study using Japanese patients. Although the carrier frequency of *HLA-B*5801* in the Japanese population (1.2%) [13] is comparable to that in the European population (1.5%), the carrier frequency of *HLA-B*5801* in allopurinol-administered Japanese patients (40.0%) was lower than that observed in European patients. The sample size of our study was not sufficient to estimate the accurate carrier frequency in patients. Recently, Ueta *et al.* reported a case–control study on relationships between HLA class I and II genetic polymorphisms with severe ocular

Executive summary

Backgrounds of genetic biomarkers for severe cutaneous adverse reactions

- Recently, strong drug-specific associations of human leukocyte antigen (*HLA*)-*B*1502* and *HLA-B*5801* with carbamazepine- and allopurinol-induced severe cutaneous adverse drug reactions were found in Han Chinese patients, respectively.
- However, a European study suggested that HLA involvement in severe cutaneous adverse reactions may be ethnic-group-specific, as well as drug-specific.

Objective of this study

- We began a retrospective case–control study to explore genetic biomarkers related to Stevens–Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) in Japanese patients living in Japan.

Conclusion

- We could not find any association between *HLA-B*1502* and carbamazepine-aromatic anti-epileptic agent-associated SJS/TEN in Japanese patients.
- We detected a moderate association of *HLA-B*5801* with Japanese allopurinol-related SJS/TEN patients.

complications using 71 Japanese drug-unspecified SJS/TEN patients and 111 Japanese controls, and they did not detect any *HLA-B*5801* carriers both in cases and controls [18]. However, no allopurinol-induced patients were included in their sample [UETA M, TOKUNAGA K, SOTOZONO C *ET AL.*: PREFECTURAL UNIVERSITY OF MEDICINE, KYOTO, JAPAN. PERS. COMMUN.]. On the other hand, Dainichi *et al.* detected three *HLA-B*5801* carriers in all three allopurinol-associated patients diagnosed with SJS, DIHS and TEN, respectively [19]. Their data and the current study lead to a conclusion that *HLA-B*5801* is one of the (surrogate) genetic biomarkers for allopurinol-associated SJS/TEN also in Japanese patients.

Conclusion

While *HLA-B*1502* is unlikely to be associated with carbamazepine-related or aromatic anti-epileptic agent-related SJS/TEN, *HLA-B*5801* was significantly associated with allopurinol-related SJS/TEN in Japanese.

Future perspective

Recently, the US FDA approved the revision of the label of products containing carbamazepine. In the updated label, it is clearly stated that patients with Chinese ancestry should be screened for the *HLA-B*1502* allele before starting treatment with carbamazepine, and that *HLA-B*1502*-positive patients should basically not be given the drug. On July the 24th, 2008, FDA published an 'alert' [101] based on several studies [15,20–22] informing healthcare professionals that the screening for *HLA-B*5701* is necessary before initiating treatment with abacavir ,and that abacavir should not be administered to *HLA-B*5701* carriers. The Committee for Medicinal Products for Human Use (CHMP) is also considering the revision of the Summary of Product Characteristics (SPC) of abacavir-containing products. Thus, personalized medicine based on pharmacogenomics using biomarkers with excellent performance characteristics has started to identify patients at high risk of idiosyncratic adverse reactions. However, biomarkers only for restricted drugs such as carbamazepine (*HLA-B*1502* for some Asian ethnic groups excluding Japanese), abacavir (*HLA-B*5701* for people living in the USA and Europe) or allopurinol (*HLA-B*5801*) among more than 100 causative ones have been detected to date. Therefore, more intensive, nationwide or even international case–control studies are necessary to find corresponding biomarkers identifying patients at high risk for individual ethnic populations or individual causative drugs. The accumulation of such data may uncover pathogenic mechanisms of SJS/TEN, which will be useful for the identification of new molecules that cause severe cutaneous adverse reactions at an early stage of the drug-development process.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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Association of Fas Ligand gene polymorphism with Stevens Johnson syndrome

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Association of Fas Ligand gene polymorphism with Stevens–Johnson syndrome

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ABSTRACT

Background: Stevens–Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) are acute severe blistering diseases of the skin and also two of the most devastating ocular surface diseases leading to corneal damage and loss of vision. The extreme rarity of cutaneous and ocular surface reactions to drug therapies led us to suspect individual susceptibility. SJS/TEN patients in the acute stage were reported to manifest increased serum levels of Fas Ligand (FasL). Thus, we performed SNP association analysis of the FasL gene.

Methods: In 76 Japanese SJS/TEN patients with ocular surface complications and 160 Japanese healthy controls, we examined four SNPs of FasL reported in the Japanese Single Nucleotide Polymorphisms (JSNP) database by sequencing.

Results: The SNP rs.3830150 A/G showed a significant strong inverse association with SJS/TEN. Analysis of the genotype pattern of SNPs rs.3830150 and rs.2639614 (rs.3830150 A/A–rs.2639614 G/G) also manifested a strong inverse association with SJS/TEN.

Conclusion: FasL gene polymorphisms might be associated with SJS/TEN.

Stevens–Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN), considered variants of a single disease, are acute-onset mucocutaneous diseases induced by infectious agents and/or inciting drugs.[1 2] SJS/TEN are acute severe blistering diseases of the skin which carry high mortalities.[3–4] In addition, SJS and TEN are also two of the most devastating ocular surface diseases leading to corneal damage and loss of vision. The reported incidence of ocular complications in SJS/TEN is 50–68%.[2 4] From the ophthalmological standpoint, in the acute stage, SJS/TEN patients manifest severe conjunctivitis, and persistent corneal epithelial defects due to ocular surface inflammation. In the chronic stage, ocular surface complications such as conjunctival invasion into the cornea due to corneal epithelial stem-cell deficiency, symblepharon and, in some instances, keratinisation of the ocular surface persist despite the healing of the skin lesions.[5] We observed that more than 95% of SJS/TEN patients with ocular surface complications had lost their fingernails in the acute or subacute stage and that some continue to have transformed nails even after healing of the skin lesions.[1 6]

Although the pathobiological mechanisms underlying the onset of SJS/TEN have not been fully established, the extreme rarity of cutaneous- and ocular surface reactions to drug therapies led us to suspect individual susceptibility. We have documented the association with TLR3[1] and IL4R polymorphisms in Japanese SJS/TEN patients with ocular complications.[6] We also reported that in the Japanese, HLA-A*0206 was strongly associated with SJS/TEN with ocular surface complications.[7] Thus, genetic and environmental factors may play an important role in an integrated aetiology of SJS/TEN.

It has been reported that the skin lesion of SJS/TEN in the acute stage is histologically characterised by marked keratinocyte apoptosis in the epidermis with dermo-epidermal separation, resulting in bullae.[8] Moreover, SJS/TEN patients in the acute stage manifested increased serum levels of Fas Ligand (FasL),[9 10] and the activation of Fas through FasL was reported to be an initial important step leading to the diffused apoptotic cell death of epidermal cells in SJS/TEN.[9 10] Thus, we performed SNP association analysis of the FasL gene. We examined four SNPs of FasL reported in the Japanese Single Nucleotide Polymorphisms (JSNP) database and found that rs.3830150 A/G (intron) were significantly associated with SJS/TEN. Analysis of the genotype pattern of SNPs rs.3830150 and rs.2639614 (rs.3830150 A/A–rs.2639614 G/G) also manifested a strong inverse association with SJS/TEN in Japanese patients.

MATERIALS AND METHODS

Patients

This study was approved by the institutional review board of Kyoto Prefectural University of Medicine, Kyoto, Japan. All experimental procedures were conducted in accordance with the principles set forth in the Declaration of Helsinki.

For SNPs analysis, we enrolled 76 patients with SJS/TEN in the chronic or subacute phase; all presented the symptom of ocular surface complications. The diagnosis of SJS/TEN was based on a confirmed history of the acute onset of high fever, serious mucocutaneous illness with skin eruptions, and involvement of at least two mucosal sites, including the ocular surface. The controls were 160 healthy volunteers. All participants and volunteers were Japanese residing in Japan. The average age of the patients and controls was 46.1 (SD 17.3) and 36.2 (11.5) years, respectively. The male:female ratio in the patient- and control groups was 33:43 and 57:103, respectively.

SNPs analysis

FasL SNP analysis was performed by sequencing from both sides, forward and reverse, in order to carefully confirm the results. For SNPs of FasL, the PCR- and sequence primers were 5′-TTTGGGAAACCCTCTCAAGC-3′ (sense) and 5′-CGTGGCTGAGTGCCAGATTAG-3′ (antisense)