Ⅳ. 主な成果物

自己管理法を含む喘息死ゼロ作戦の実行に関する指針

平成２４～２６年厚生労働科学研究費補助金
免疫アレルギー疾患予防・治療研究事業

「気管支喘息に対する喘息死の予防や自己管理法の
普及に関する研究」研究班作成

目　次

平 成２４～２６年 厚 生 労 働 科 学 研 究 費 補 助 金

免疫アレルギー疾患予防・治療研究事業

「気管支喘息に対する喘息死の予防や自己管理法の
普及に関する研究」研究班作成

研究代表者　　大田　健
独立行政法人国立病院機構東京病院　院長

研究分担者

棟方　充　　福島県立医科大学医学部呼吸器内科学講座　教授
東田有智　　近畿大学医学部内科学講座呼吸器アレルギー内科部門　教授
檜澤伸之　　筑波大学大学院人間総合科学研究科疾患制御医学専攻
呼吸器病態医学分野　教授
近藤直実　　岐阜大学　名誉教授 ／ 平成医療短期大学　学長
下条直樹　　千葉大学大学院医学研究院小児病態学　准教授
田中明彦　　昭和大学医学部内科学講座呼吸器・アレルギー内科　講師
長瀬洋之　　帝京大学医学部内科学講座呼吸器・アレルギー学　准教授

平成 26 年 11 月 3 日まで
秋山一男　　独立行政法人国立病院機構相模原病院臨床研究センター
センター長

平成 26 年 11 月 4 日から
釣木澤尚実　独立行政法人国立病院機構相模原病院臨床研究センター　医師

研究協力者

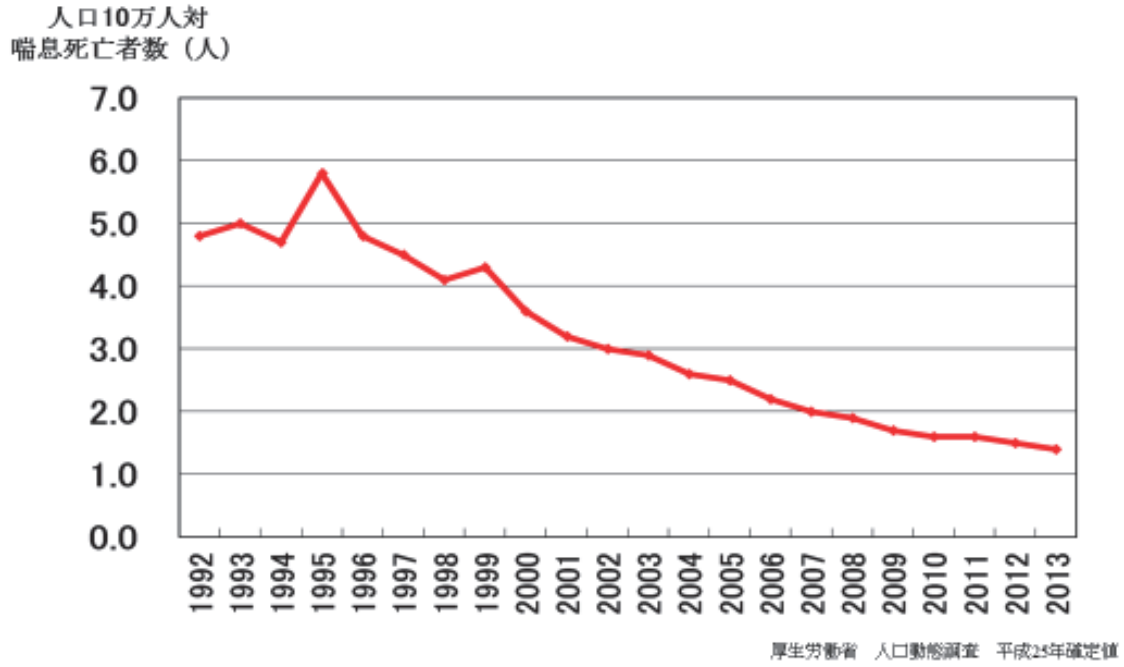
井上博雅　　鹿児島大学大学院医歯学総合研究科呼吸器内科学　教授
大林浩幸　　東濃中央クリニック　院長 ／ 東濃喘息対策委員会　委員長
森川昭廣　　社会福祉法人希望の家附属北関東アレルギー研究所　所長
中村浩之　　金沢大学医薬保健研究域医学系環境生態医学･公衆衛生学　教授

１．現状と課題

厚生労働省人口動態調査によると、５～34歳の年齢階級別喘息死亡率は、1995年には10万人当たり0.7人であったが、1996年以降減少し始め2001年には0.3人にまで減少し、2007年からは総数0.1、男0.1、女0.1以下となり、さらに低下して、わが国の５～34歳の年齢階級喘息死亡率は国際的には最も低い群に属するに至っている。

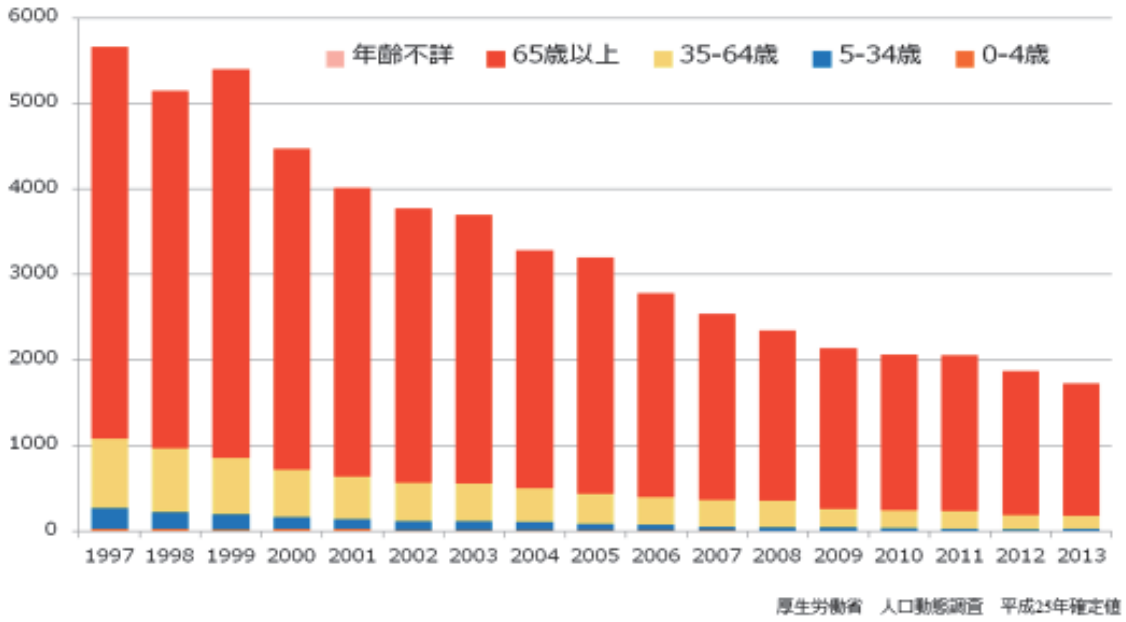
また、全年齢における喘息の死亡数は、1995年7,253人とピークを示した後1996年 5,926人と減少し、2000年 4,427人、2004年 3,283人と順調に減少してきた。そして、「喘息死ゼロ作戦」の取り組みが開始された2006年には前年の3,198人から2,778人へと減少し、2013年には1,728人（10万人当たり1.4人）まで減少して7年間で1,050人の減少をみている（図１）。

図1 喘息死亡率（人口10万人対） 全国



しかし、さらに喘息死をゼロに近づけ喘息の予後を改善するためには、より有効な対策が必要である。なお、死亡患者の年齢は、小児の喘息死亡率は減少して低率となり、2011年の19歳以下の喘息死亡数（総数）は５人であるが、成人では65歳以上の高齢者が毎年80~90%を占めており、高齢者喘息への対応が今後の課題である（図２）。

図2 年齢別 喘息死

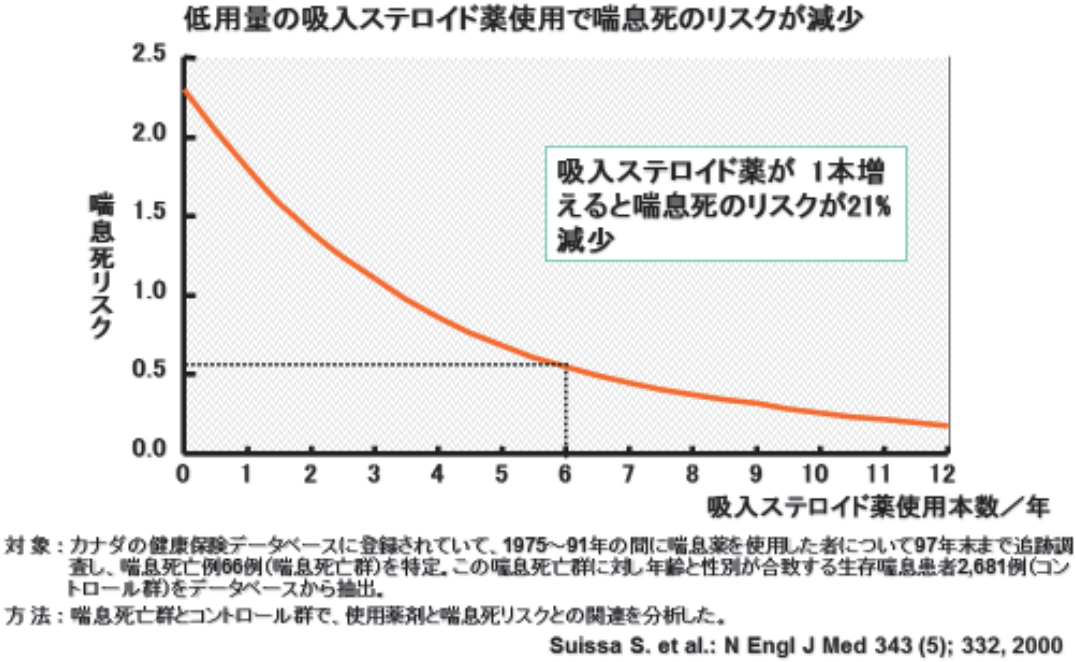


死亡に至る原因は、重篤な発作による窒息死である。そして、重篤な発作の誘因としては、気道感染が最も多く、過労、ストレスがこれに続き三大誘因をなしている。その他には、治療薬の中止、短時間作用性吸入β2刺激薬の過剰使用、副腎皮質ステロイド(ステロイド)の中止・減量、非ステロイド性抗炎症薬(NSAIDs)の投与によるアスピリン喘息の誘発、β2遮断薬の使用(降圧薬、点眼薬)などが挙げられている。成人喘息死では、発作開始後1時間以内が 13.6%、3時間以内と合わせると 29.7%となり、急死が多い。発作から死亡までの状況は、突然の発作で急死が 29.8%、不安定な発作の持続後の急死が 16.2%、不連続な発作後の急死が 17.2%で、重い発作で苦しみながら悪化して亡くなる(21.2%前後)よりも圧倒的に急死が多い。しかし、喘息死の解剖による検討では、臨床的には急死でも気道では慢性に炎症が存在し、悪化した結果であることが示されている。すなわち日常の喘息の管理が不十分な状態で生活していることが、喘息死を来すような重篤な発作を誘発する原因であるとされている。

したがって、喘息死の予防には、炎症を鎮静し維持するための治療、すなわち炎症を標的にした長期管理の治療の実行が有効と考えられる。そして、吸入ステロイドは、炎症を抑制する効果が最も強力で確実な薬剤として位置付けられている。すなわち、吸入ステロイドをベースにした長期管理を実行することにより、気道の炎症は抑えられ、良好な喘息コントロールがもたらされる。吸入ステロイドの使用が喘息死を予防することは、吸入ステロイドの普及率と喘息死とが反比

例することから広く認められている。ある報告では、吸入ステロイドの使用本数が年に１本増える毎に喘息死のリスクが 21%減少すると概算している（図３, Suissa et al: N Engl J Med 343: 332, 2000）。

図３　吸入ステロイド薬使用量と喘息死リスク

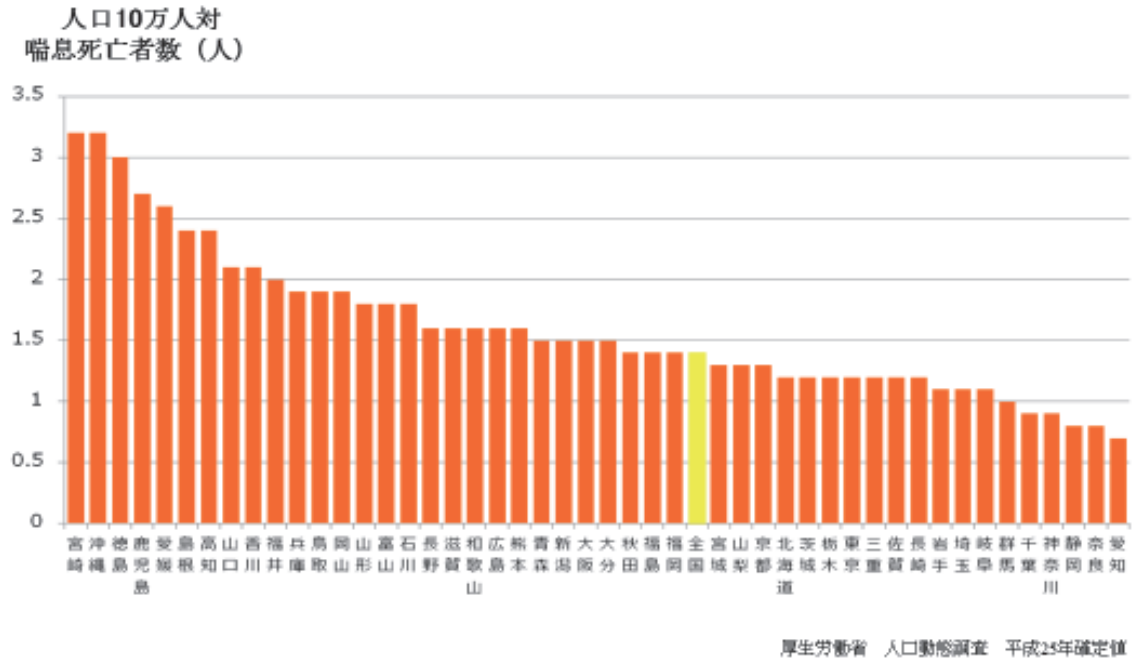


このような背景から、喘息死は、吸入ステロイドをベースに患者の重症度により治療を組み立てる喘息予防・管理ガイドライン 2012（JGL2012)、さらに最新の情報を加えたアレルギー総合ガイドライン 2013 （JAGL2013）等に示された標準的な長期管理を普及し実行することにより予防できると考えられる。また重篤な発作に至っても、発作への対応が十分かつ適切に施行できれば、喘息死を減少させることは可能であると考えられる。

そこで、各都道府県は、全国で喘息死ゼロを実現するために、本作戦に参加することを強く望まれている。

これまでの調査で、都道府県別の喘息の死亡率には、大きなばらつきがみられている（図４)。2013 年の調査で、10 万人あたりの死亡率の全国平均 1.4 人に対して、2.5 人以上の所は、多い順に宮崎県、沖縄県、徳島県、鹿児島県、愛媛県である。さらにそれに次いで 2.0 人を超えているのは島根県、高知県、山口県、香川県である。このような疫学調査の結果は、毎年多少は変化しているが、以上のような自治体あるいは全国平均を上回る自治体では、より積極的に喘息死ゼロ作戦に参加し、本作戦を実行することが望まれる。

図4　喘息死亡率（人口10万人対）2013年



２．喘息死ゼロ作戦とは

喘息死ゼロ作戦とは、予防できる死亡である「喘息死」をゼロにすることを目標として、地域の関係者が連携して病診連携の構築や普及啓発、患者の自己管理の徹底等を図り、医療の質の向上を図るための取り組みである。

そして喘息死をゼロ作戦における最も重要な取り組みは、EBM（evidence based medicine）をベースに作成されたガイドラインに沿った喘息治療の普及である。わが国のガイドライン JGL2009 においては、吸入ステロイド（ICS）を第一選択薬とする長期管理をかかりつけ医において実施すること、患者が普段から自己管理を行うこと、さらに適切な急性増悪（発作）への対応を行うことが標準的な治療とされている。

喘息死を予防するためには、現実の喘息の診療において次のような課題がある。

・喘息の病態には、慢性の気道炎症が重要であることの認識が不十分
・慢性の気道炎症を標的とする長期管理の実行が不十分
・長期管理の第一選択薬である吸入ステロイド（ICS）についての理解と使用が不十分
・喘息の状態の客観的な評価が不十分
・喘息死の約 90%を占める高齢者喘息についての認識が不十分
・喘息発作に対する救急体制の整備が不十分
・専門医と非専門医との連携、医師とコメディカル（看護師、薬剤師、救命救急士など）との連携、病診連携などの医療現場での協力体制の整備が不十分

３．実行のための組織

都道府県に診療所、病院、保健所、その他の医療関係者、関係市区町村、患者会等からなる地域医療連絡協議会を設置し、自治体の現状に即した喘息死ゼロ作戦を実行する。

なお、本研究班においては、日本アレルギー学会および日本アレルギー協会の各支部会の協力のもと、喘息診療におけるオピニオンリーダーの医師から構成される「喘息死ゼロ作戦推進委員会」を設置しており、作戦が効果的に遂行されるよう各地の地域医療連絡協議会を支援する。「喘息死ゼロ作戦推進委員会」の委員に、地域における専門家として、各自治体の地域医療連携協議会の委員として参画を求めることも考えられる。

喘息死ゼロ作戦推進委員会　委員

北海道：西村正治（北海道大学）
東北：田村　弦（仙台気道研究所）
関東・甲信越：土橋邦夫（群馬大学）
北陸：藤村政樹（国立病院機構七尾病院）
中部・東海：新美彰男（名古屋市立大学）
近畿：東田有智（近畿大学）
中国：宗田　良（国立病院機構南岡山医療センター）
四国：横山彰仁（高知大学）
九州：興梠博次（熊本大学）

４．実施内容の実際

（１）モデル医療圏における診療体制の確保及び医療連携事例集の作成

都道府県と地域医療連絡協議会は、地域医師会、救急医療機関等、専門医療機関等の協力を得て、地域の状況に応じた病診連携システムを構築する。

診療体制の構築に当たっては、喘息患者が発作時に受診するだけではなく、ガイドラインに従った長期管理を適切に実施できる体制が求められる。

このため、かかりつけ医、救急医療機関、専門医療機関が連携したうえ、

①かかりつけ医において、ガイドラインに基づく標準治療を広く実施する
②かかりつけ医において、喘息の診断未確定で診断に難渋する例や、難治例を専門医療機関に紹介する
③救急医療機関における発作（急性増悪）時の診療後に、かかりつけ医等における長期管理に結びつける
④診断確定後、症状が安定している例については、長期管理が適切に行われるよう、専門医療機関からかかりつけ医への、いわゆる「逆紹介」を行う

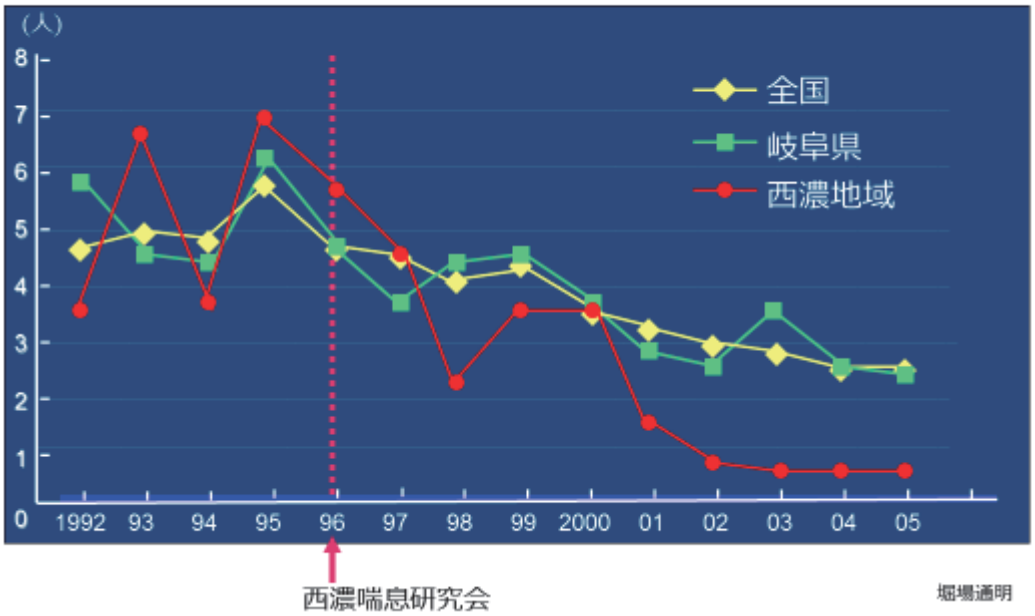
などの取り組みを推進する。それぞれの医療機関において、喘息の長期管理の重要性と、ガイドラインに基づく治療方法を理解し実施するとともに、患者カード（後述）の使用等、治療内容に関して相互の情報提供をスムーズに実施するとともに、患者に対し適切な指導を行い、アドヒアランスの向上を図ることが重要である。

また、喘息死ゼロ作戦においては、このような連携をモデル的な地域で推進し、事例を集積したうえ、他の地域での普及につなげることが期待される。

■事例１：これまでに、喘息の医療を改善するプログラムに関して、国全体として高く評価されている例は、フィンランドである。その内容はヘルシンキ大学のHaahtela 教授を筆頭者に昨年英国の呼吸器学会誌である Thorax に報告されている（Thorax 2006; 61:663-670）。1994 年から 2004 年の 10 年間に国全体で取り組んだ喘息診療の改善プログラムの成果から、プログラムの自己評価を行なっている。そして今後の目標を喘息死ゼロの達成に置いており、これまでに構築したシステムを基盤として、2008 年を比較の対象にその後の 3 年間（2009-2011 年）で目標達成を目指す計画を立てている（個人的情報）。実施されたプログラムの骨子は、喘息が炎症性疾患であることの理解を広める教育、専門医と非専門医（GPs）との連携の構築によるガイドラインの実施であり、個人および国家の喘息による負担の軽減を目標に設定したものである。これまでの成果は、入院日数、喘息死および救急受診の減少、薬剤への経費と定期受診の増加、そして結果として得られる喘息に対する医療費の減少である。国の規模、医療体制、社会の構造、文化や習慣などの違いは無視できないが、その内容はわが国がこれから実行しようとしていることに類似しており、参考となるものである。まず成功の鍵となるのは、実現を目指すための強い意欲と機能的な組織作りであることを強調している。そして国家のサポートを必須とし、共通の治療方針を理解し実行するための組織作りの重要性を示している。

■事例２：わが国における成功例としてよく知られているのが、岐阜県大垣市における取り組みである。大垣市民病院の呼吸器科部長であった堀場通明医師により推進された当地での医療体制の整備では、基幹病院を中心とする病診連携の強化、非専門医への徹底した啓発活動、 薬剤師や看護師などのコメディカルへの啓発活動、患者の教育と診療カードの携行励行、救急医療体制における喘息発作への適切な対処の啓発などが実行された。その結果、該当する西濃地域における喘息死は、活動開始時の 1996 年には 10 万人当たり 5.7 人であったのが、2001 年には 1.6 人、2003 年には 0.62 人程度にまで減少した（図５）。このほか、ガイドラインの普及率の改善などの成果がみられた。この方法は、示唆に富むものであり、本作戦の参考になるものである。

図５ 全国、岐阜県および西濃地域の喘息死者数 (対１０万人口)



（２）病院や診療所等の医療関係者を対象とした研修の実施

都道府県は、地域医療連絡協議会を中心に、診療ガイドラインの実践に向けた啓発活動として、地域ごとの状況に応じた講演会等を積極的に展開する。

研修の重要性が高いテーマとして次のようなテーマが考えられ、地域における普及状況を勘案して必要な研修を実施する。

○ 喘息および小児喘息のガイドラインに基づいた標準的治療方法

喘息予防・管理ガイドライン 2012 (JGL2012) 及び小児気管支喘息治療・管理ガイドライン 2012 (JPGL2012)、さらに最新の情報を含むアレルギー総合ガイドライン 2013 (JAGL2013)、そしてガイドラインに基づいて成人喘息治療のポイントをまとめた本研究班の成果物である「成人気管支喘息治療のミニマムエッセンス」等に示された標準的治療を医療関係者が正しく理解し実行することが重要である。

○ 患者教育の方法

患者が自己管理を継続的に行うためには、発作治療薬と長期管理薬の相違、ステロイド等の吸入薬の正しい吸入法、喘息の悪化の兆候把握や発作の予防法、ピークフローモニタリングなどを患者が正しく理解することが必要であり、医療関係者にとっても患者教育のスキルが求められる。医師だけでなく、看護師、保健師、薬剤師等の連携も重要である。

また、喘息の重症度や改善の可能性を、ピークフローを測定するよりも簡便に

把握するために、イージーアズマプログラム、喘息コントロールテストが開発されており、研修の一環としてこれらの普及をすすめることも有効である。

【イージーアズマプログラムの活用】

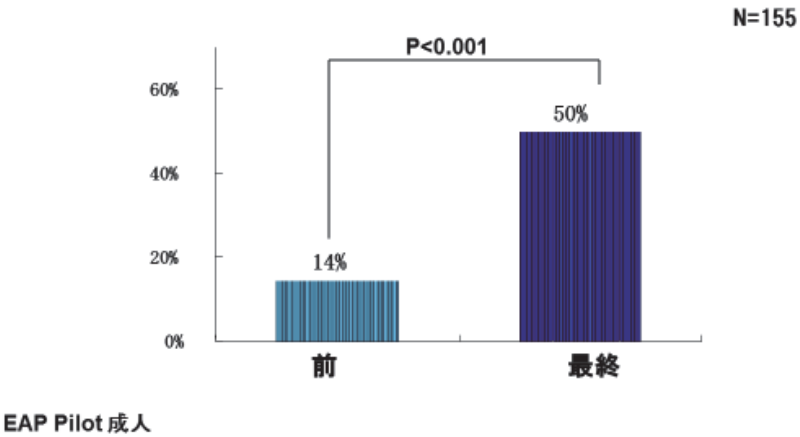
啓発する JGL2012 の実行については、すでにパイロットスタディーで成果を示したイージーアズマプログラム（EAP; Easy Asthma Program）が効果的である。図６に示した基準となる３つの評価項目からなる質問票を用いて患者の状態（重症度）を把握し、JGL2012 に沿った ICS の投与量を含めた治療を施行するものである。その効果は、新潟県と香川県でパイロット研究が実施され、その有効性が実証されている（図７）。

Easy Asthma Program

図６　基準となる評価項目

Easy Asthma Program

図７　導入前後における「3項目全くなし」の患者比率



【喘息コントロールテストの活用】

患者の状態を把握する手段としては、患者自身が実行するピークフロー（PEF; peak expiratory flow）の測定とともに、喘息コントロールテスト（ACT; Asthma Control Test）が有用である。ACT は患者への５つの質問に対する解答から点数をつけ（表１）、その点数によって患者の喘息コントロールを評価するもので（図８）、すでに科学的な検証が行なわれ、その信頼性と有用性が有意であると評価されている。本作戦では両方を用いることを理想とするが、これまでの調査で PEF の測定の普及率が低いことから（図９）、少なくとも ACT により患者の状態を簡便に把握することとする。

表１　ACT －総合点数による評価－
５項目の質問（1）

1．この４週間に、喘息のせいで職場や学校、家庭で思うように仕事や勉強がはかどらなかったことは時間的にどの程度ありましたか？
　いつも：１点　　かなり：２点　　いくぶん：３点　　少し：４点
　全くない：５点
2．この４週間に、どのくらい息切れがしましたか？
　１日に２回以上：１点　　１日に１回：２点　　１週間に３～６回：３点
　１週間に１，２回：４点　　全くない：５点
3．この４週間に、喘息の症状（ゼイゼイする、咳、息切れ、胸が苦しい・痛い）のせいで夜中に目が覚めたり、いつもより朝早く目が覚めてしまうことがどのくらいありましたか？
　１週間に４回以上：１点　　１週間に２，３回：２点　　１週間に１回：３点
　４週間に１、２回：４点　　全くない：５点

表１　ACT －総合点数による評価－
５項目の質問（2）

4．この４週間に、発作止めの吸入薬（サルタノール®やメプチン®など）をどのくらい使いましたか？
　１日に３回以上：１点　　１日に１，２回：２点　　１週間に数回：３点
　１週間に１回以下：４点　　全くない：５点
5．この４週間に、自分自身の喘息をどの程度コントロールできたと思いますか？
　全くできなかった：１点　　あまりできなかった：２点
　まあまあできた：３点　　十分にできた：４点　　完全にできた：５点

図８　ACT点数による喘息コントロール評価

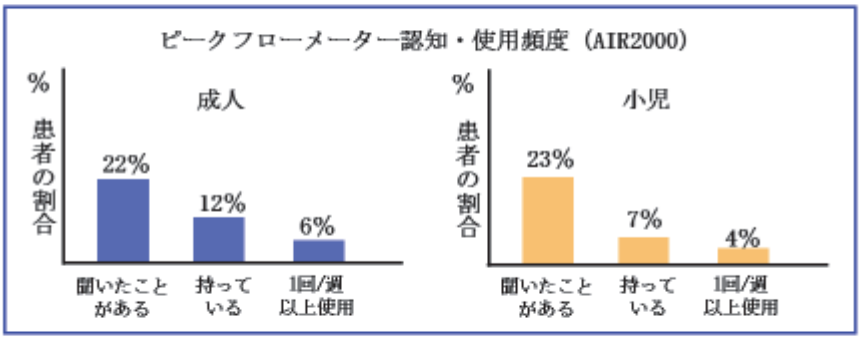
- **25点（満点）**
  完全に喘息がコントロールされている状態
  （TOTAL Control）

- **20 ～24点**
  喘息が良好にコントロールされている状態
  （WELL Control）

- **20点未満**
  喘息がコントロールされていない状態

図９　ピークフローメーターの普及状況

喘息コントロール状態の指標には呼吸機能が最も適している
が特に非専門医におけるピークフローの普及は十分ではない。



喘息コントロールのさらなる改善のためには、
より簡便な評価ツールが必要

喘息コントロールテスト

（3）患者カードの配布の促進並びに患者自己管理の普及

（患者カードの配布）

患者個人の治療内容と急性増悪時の対応、主治医を含めた連絡先などの必要事項を記入した個人の診療情報カード（ぜん息カード）を作成し、各患者が携行することにより、主治医と急性増悪時に受診する医療機関との連携を密にすることができる。喘息カードは、既存のものを参考に全国的に使用できるよう作成した（図 10）。

図10 患者カード(ぜん息カード)

（自己管理の普及）

患者の自己管理を促すためには、医療関係者が適切な指導を行うことが必要である。このため、「（2）病院や診療所等の医療関係者を対象とした研修の実施」に掲げたように、医師、看護師、保健師、薬剤師等が患者教育のスキルを向上し効果的な指導を行うことが重要である。

また、患者が正確な情報にアクセスできるための普及啓発活動を行う。

§日本アレルギー協会

患者様向けの提供および公開資料等

1. 「アレルギー電話相談」の開設。http://www.jaanet.org/
2. 協会で発行している小冊子がダウンロードできるほか、患者会情報等も掲載。
   http://www.jaanet.org/contents/index.html
3. 東北支部のホームページには、「Q&A」等が設けられている。
   http://plaza.umin.ac.jp/thk_jaa/

4. 関東支部欄には「関東支部だより」が掲載されている。
   http://www.jaanet.org/aboutus/4_index_msg.html
5. 九州支部のホームページには、広報欄に「アレルギー・喘息教室」のご案内等が掲載されている。
   http://www.allergy-fk.com/

§ラジオ放送による啓発活動

関西支部では、
ラジオ大阪（OBC）にて「アレルギー診察室」毎週日曜日6：45～7：00
http://www.obc1314.co.jp/timetable_all.html

中国支部では、
山陽放送（RSK）にて「アレルギー談話室」毎週日曜日8：45～9：00
http://www.rsk.co.jp/radio/allergy/index.html

九州支部では、
九州朝日放送（KBC）にて「アレルギー談話室」毎週日曜日6：30～6：45
http://www.allergy-fk.com/keihatu/for-peaple/for-peaple3.htm

§独立行政法人 環境再生保全機構

「成人気管支ぜん息患者の重症度等に応じた健康管理支援、保健指導の実践及び評価手法に関する調査研究」研究班が作成したテキストを入手できる。
喘息の診断がついた時点で喘息の病態と治療の実際について本テキストを用いて説明する。通常は、5分前後で説明できる。
http://www.erca.go.jp

（4）喘息診療担当医師名簿の作成等による医療機関情報の提供

アレルギー疾患の診療を専門的に行う医療機関や医師の名簿としては、

○日本アレルギー学会における認定施設の一覧
http://www.jsaweb.jp/ninteishisetsu/index.html

○日本アレルギー協会における専門医名簿
http://www.jsaweb.jp/ninteilist_general/index.html

が利用可能である。

このほか、医師会の協力のもと、喘息死ゼロ作戦に参加し、専門的な医療

機関と連携して、ガイドラインに基づく喘息治療を行う医師のリストを作成し、利用に供することが望ましい。

これらのリストをもとに、自治体や医療従事者団体等において、適切な喘息診療を行う医療機関の情報を提供することが望ましい。

（５）地域の喘息患者の実態把握を目的とした分析調査の実施

上記の施策を実施するに当たっては、地域の実態を評価することで、地域における問題点を施策の展開に活かすとともに、事業実施の評価にもつなげることができる。

実態把握のための指標としては、人口動態調査による喘息による死亡者数の推移や、年齢別の死亡率等の、既存の統計を活用することができる。また、地域での喘息診療の実態を調査する場合には、中核的な医療機関の協力を得て、喘息発作による救急外来受診患者数・入院患者数の推移、救急外来受診者の普段の治療内容、喘息による死亡例の解析（発症の時期、病型、長期管理、発作時の治療、死亡前の状態、死亡の場所など）等を評価することにより、問題点を具体的に明らかにすることができる。

このほか、喘息の標準的な治療の普及状況を経年的に把握するためのその他の指標としては、製薬企業からの提供が得られる場合には、吸入ステロイドや短時間作用性β2 刺激薬の売り上げ（ガイドラインに基づく治療が普及すれば、吸入ステロイドの処方数が増加し、短時間作用性 β_2 刺激薬の処方数が減少することが予想される)、地域の消防において把握している場合には、若年の呼吸器疾患に対する救急車の出動回数の推移、あるいは、医師や患者の喘息に関する知識、JGL2012の内容の認知と実行などの状況をアンケート調査すること等が考えられる。

これらに基づき、問題点の把握を行い、喘息死ゼロ作戦の実施に活かすことが望ましい。

（６）事業実施の評価

事業実施中に事業のアウトプット、アウトカムや、様々な問題点、改善すべき点に関する評価を行い、創意工夫に結びつけて改善を図ることが重要である。

「（５）地域の喘息患者の実態把握を目的とした分析調査の実施」に掲げた各種の調査は、作戦実施後に再度実施することにより、事業の評価に活用することができる。

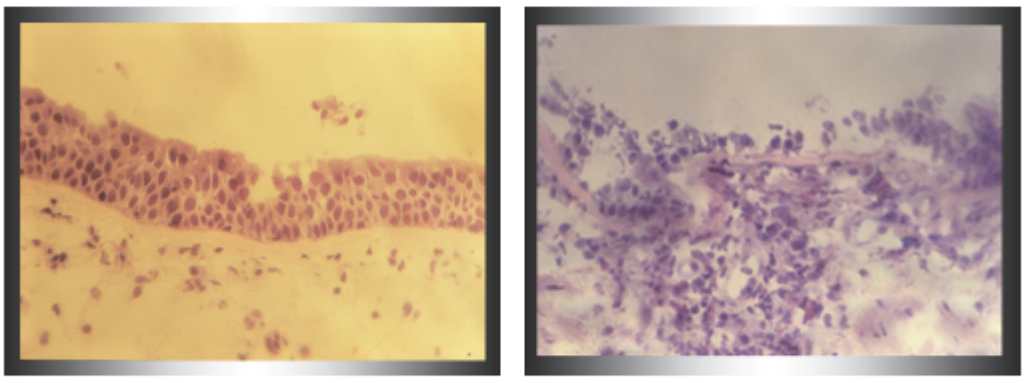
資料　喘息の疾患としての特徴　（医療関係者向け資料）

喘息死ゼロ作戦の実行にあたっては、喘息がどのような特徴をもつ疾患であるかを知っておくことが必要である。そのような観点から、まず喘息の病態、喘息死を含む疫学、喘息の臨床について概説する。

１．喘息の病態

喘息は古代ギリシャ、ヒポクラテスの時代（BC4～5 世紀）からすでに記載のある古い疾患である。発作性の喘鳴（ゼーゼーヒューヒュー）、呼吸困難などの症状が特徴であり、場合によっては死につながる程の重い発作を起こす可能性があるため油断の出来ない疾患として位置付けられる。これまでは、呼吸機能検査の結果から、 薬物治療や自然経過で気道が閉塞している状態が正常化する可逆性の気道閉塞と種々の刺激により気道の閉塞が起こり易い状態である気道過敏性が喘息の特徴とされてきた。しかし、近年気管支鏡により喘息の気道粘膜を生検し検討したところ、喘息の気道には、症状はなくても慢性に炎症という状態が気道に存在することが明らかになった（図 11）。

図11 気管支粘膜生検像



正常　　喘息

炎症には多くの細胞と液性因子が関与して気道粘膜を傷害しており、とくに喘息の炎症では赤く染まる顆粒をもつ好酸球が多くみられるのが特徴とされている。つまり喘息の病態は、可逆性の気道閉塞、気道過敏性と慢性の気道炎症から成り立っている。

では、気道炎症はどのように惹起されるのであろうか。喘息の患者にアレルギ

ーの検査をすると、約 70%で室内塵（ハウスダスト）の主成分であるチリダニに対して陽性の結果が得られる。すなわちチリダニに対する免疫グロブリン E（IgE）抗体が陽性で、チリダニによりアレルギー反応が誘導される状態にある。したがって、喘息になる要素の１つとして、アレルギー体質と生活環境におけるチリダニをはじめとする喘息の原因物質（抗原）への曝露が挙げられる。

次に、喘息を発症する要因として、気道が過敏性になり易い体質も重要である。IgE 抗体があってアレルギー反応が起こっても、気道が閉塞しなければ喘息の症状は出ない。喘息に結びつくアレルギーの発症には、生まれた直後から乳幼児期にかけての生活環境の衛生状態と感染の有無も後天的な要素として関与し、むしろ不衛生な環境への曝露が予防的という仮説も唱えられ、注目されている。喘息は、遺伝子と環境を背景に発症する疾患であると考えられる。

２．喘息の疫学

喘息の気道には慢性の炎症が存在していることが明らかになったが、幸いにも喘息を診断する上で決め手となるのは、これまで通り呼吸生理学的な検査であり、症状としては呼吸困難、喘鳴、咳嗽であることに変わりはない。つまり、喘息の概念に慢性の気道炎症が組み込まれる以前と以後とで、同一の質問表による疫学的なデータの比較が可能である。

1)　喘息の有症率

小児喘息の疫学調査は、同一地域の小学生を対象に定期的な定点観察が可能であり、経年的な変化を反映している。例えば福岡市での検討では、1981-1983 年が 5.7%に対して 1993-1995 年は 7.7%と有意な増加を示している。一方、成人喘息では、異なる研究グループによる各地域での単回調査の結果が報告されている。その結果をみると、喘息の有症率は、1960 年代は 1～1.2%、1970 年代後半から 1980 年代前半は 1～2%となっており、1985 年の静岡県藤枝市の調査結果が 3.14%であることから、有症率の増加が示された。さらに、我々が 1998 年に静岡県藤枝市で行った住民に対するアンケート調査から有症率 4.14%が得られ、全国の罹患者数は 400～500 万人に達している可能性もある。

患者数の増加には、住宅環境の変化（アルミサッシ、絨毯、空調設備）によるチリダニの増加、衛生状態の過剰な改善（無菌化）、食生活、喫煙、大気汚染など種々の因子が関与していると考えられる。そして、疫学調査では、人口密度が有症率と最も密接な相関を示した。また、厚生労働省により報告された有病率をみると、喘息の死亡率とともに都道府県により較差がみられており、有病率に較差を来たす背景因子を検討することは、発症のメカニズムや発症を予防する方法を解明する上で有用と考えられる。当面の課題とは言えないが、今後の課題として

は重要である。

各都道府県で地域の代表的な基幹病院を数カ所選定し、現在受診中の患者を対象にした調査と、その地域全体の調査とを組み合わせることが方法として考えられる。そして、例えば、住宅環境として絨毯の使用、建築歴、家族構成と喫煙者の有無、ペットの有無と種類、掃除の回数、市街地か郊外かなど、家族歴として喘息やアレルギー疾患の有無などを含む統一した質問表を用いた調査を施行し、各都道府県で分析し、全国的に比較検討する。一方で文化的背景として特徴が明らかで、喘息の発症に関係する可能性が考えられる事柄については、仮説を立てて検証するのも別の方法として推奨される。

2) 喘息の臨床像

小児喘息は、乳児期に多く発症する。一方、成人喘息では、1989 年の厚生労働省の実態調査によると、年齢構成のピークは 50 歳代で 24%、次いで 60 歳代 23%、40 歳代 20%となり、発症年齢は 40 歳代 19%、50 歳代 18%、30 歳代 17%の順であった。また、発症時の男女比は、乳児期（生後 1 カ月から 1 年未満）で 2.8、幼児期（満 1 歳から 5 歳）で 1.5、10 歳以後では 1.0 以下で、喘息患者全年齢での男女比はほぼ 1 であった。

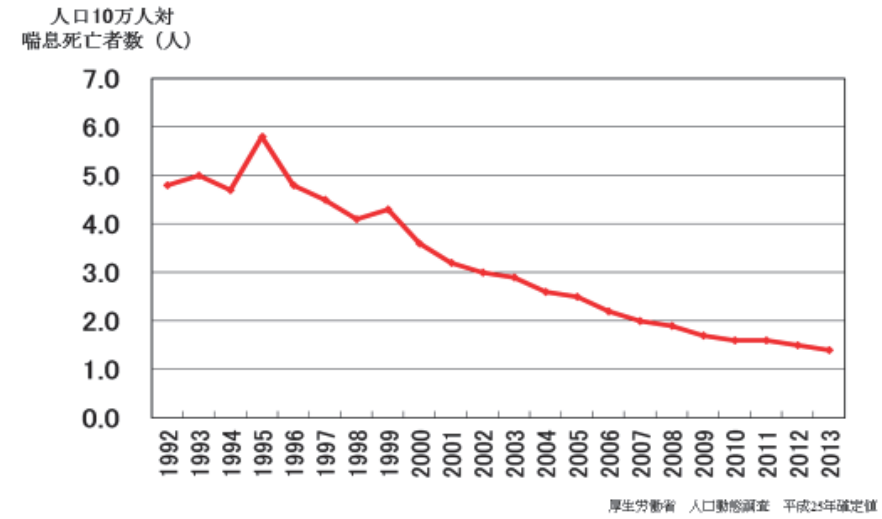
喘息の病型は、環境アレルギーに対する特異的 IgE 抗体が存在するものをアトピー型、IgE 抗体が存在しないものを非アトピー型とすると、アトピー型が成人喘息では約 70%、小児喘息では 90%以上となる。

3) 喘息死

喘息死の動向は、厚生労働省人口動態調査により知ることができる。死亡診断書をもとに喘息死とほぼ正確に判定される 5〜34 歳の年齢階級喘息死亡率は、1995 年には 10 万人当たり 0.7 人であったが、1996 年以降減少し始め 2001 年には 0.3 人にまで減少し、好ましい傾向にある。

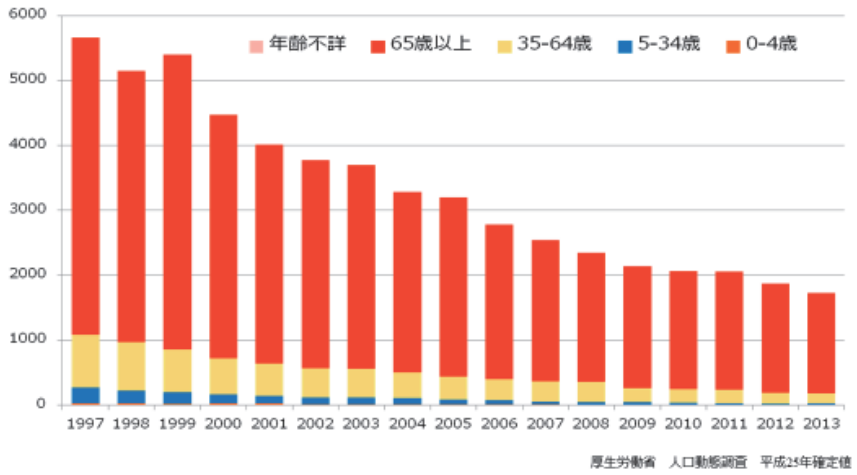
また、成人喘息における死亡数は、1995 年 7,149 人とピークを示した後、1996 年には 5,926 人と減少し、2000 年 4,427 人、2004 年 3,243 人と順調に減少し、とくに「喘息死ゼロ作戦」の取り組みが開始されたと考えられる 2006 年には前年 2005 年の 3,198 人から 2,778 人へと減少し、最新の 2013 年は 1,728 人、10 万人当たり 1.4 人まで減少している（図 1）。

図1　喘息死亡率（人口10万人対）全国



そして、年齢分布では、65 歳以上の高齢者が毎年 90%近くを占めており、高齢者喘息への対応が求められている（図２）。

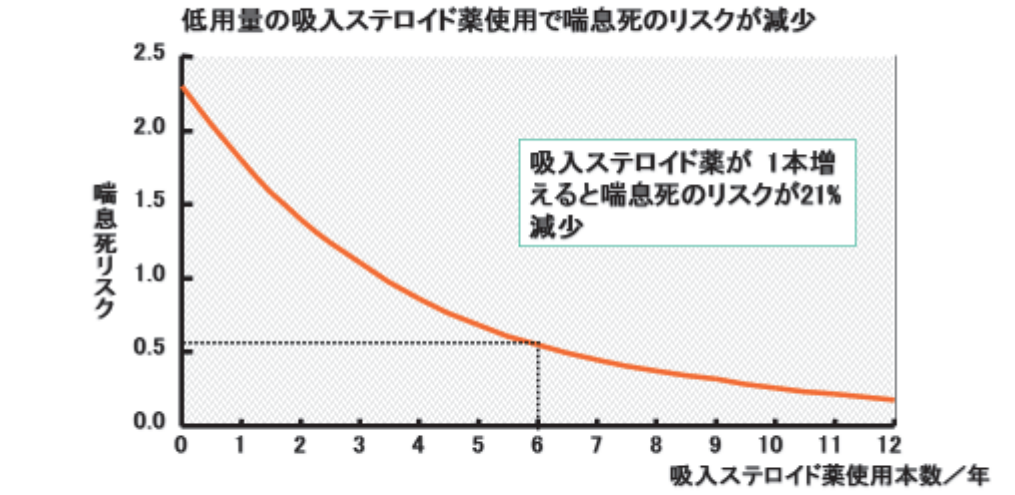
図2　年齢別　喘息死



喘息死は、まだゼロには程遠い数字であるが、上述のように 1995 年のインフルエンザの流行によると思われるピークを境に経年的に減少している。この喘息死の低下には、1992 年に初版が作成され、2006 年以降 3 年ごとに改訂され発刊された「喘息予防・管理ガイドライン」が大いに貢献していると考えられる。したがって、喘息死ゼロ作戦の戦略として現時点で最新のガイドライン、JGL2012 に沿った治療を広めて実行することが、妥当であると考えられる。後述するように、喘息は発作時あるいは症状のある時に治療するだけでなく、慢性の気道炎症を念頭にした長期の管理が重要である。JGL2012 で推奨する吸入ステロイド薬（ICS）を第一選択薬とする長期管理は、多くのエビデンスで支えられている。例えばあ

る報告では、吸入ステロイド薬を使用していない患者では、吸入ステロイド薬の使用が１年に一本増える毎に喘息死のリスクが 21%ずつ減少するという推計結果が示されている（図３）。

図３　吸入ステロイド薬使用量と喘息死リスク

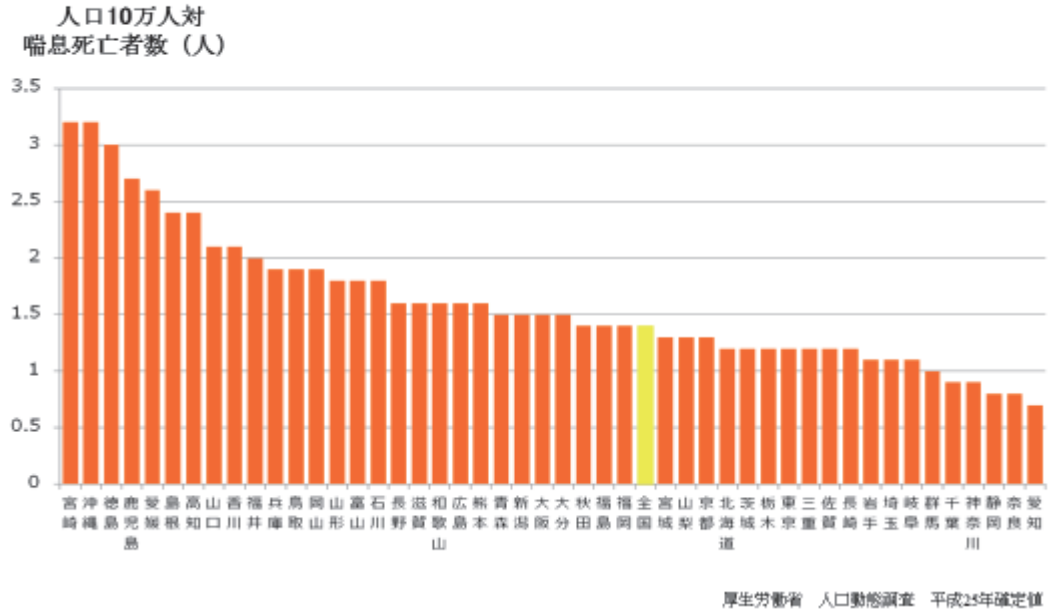


対象：カナダの健康保険データベースに登録されていて、1975～91年の間に喘息薬を使用した者について97年末まで追跡調査し、喘息死亡例66例（喘息死亡群）を特定。この喘息死亡群に対し年齢と性別が合致する生存喘息患者2,681例（コントロール群）をデータベースから抽出。
方法：喘息死亡群とコントロール群で、使用薬剤と喘息死リスクとの関連を分析した。

Suissa S. et al.: N Engl J Med 343 (5); 332, 2000

また、喘息の死亡率を都道府県別に示すと、有病率でもみられたように、都道府県毎に大きなばらつきがみられている（図４）。

図4　喘息死亡率（人口10万人対）2013年



厚生労働省　人口動態調査　平成25年確定値

３．喘息の臨床

喘息の臨床の規範として JGL2012 を用いるにあたり、喘息死ゼロ作戦の理解を深めるために、とくに成人喘息についてその内容の要点を解説する。

1)　診断

発作中に来院すれば、喘息の診断は比較的容易であるが、非発作時や他の呼吸器疾患、とくに慢性閉塞性肺疾患（COPD）を合併する場合には、診断が困難なこともある。

喘息の診断基準は、公式には確立されていないが、JGL2012 の「成人喘息での診断の目安」は、診断への指針として簡便で有用である（表２）。

表２　成人喘息での診断の目安（1）

1. 発作性の呼吸困難、喘鳴、咳（夜間、早朝に出現しやすい）
   の反復

2. 可逆性気道閉塞：自然に、あるいは治療により寛解する。PEF値の日内変動20%以上、β2刺激薬吸入により1秒量が12%以上増加かつ絶対量で200ml以上増加

3. 気道過敏性の亢進：アセチルコリン、ヒスタミン、メサコリンに対する気道収縮反応の亢進

表２　成人喘息での診断の目安（2）

4. アトピー素因：環境アレルゲンに対するIgE抗体の存在

5. 気道炎症の存在：喀痰，末梢血中の好酸球数の増加、ECP高
   値、クレオラ体の証明，呼気中NO濃度上昇

6. 鑑別診断疾患の除外：症状が他の心肺疾患によらない

この表の項目 1、2、5 を満足すれば喘息の診断が強く示唆され、また非発作時の場合で１秒量（FEV_1）やピークフロー（PEF; peak expiratory flow）が正常で可逆性気道閉塞が検出できない時は、1、3、5 を満足しても診断を支持すると考えられる。ただし気道過敏性試験が、喘息で例外なく陽性とは限らないこと、またどこの施設でもできる検査ではない点で、さらに別の指標を考案する余地を残している。

a） 症状（表２の項目１）

臨床症状として、喘鳴、咳、呼吸困難（息切れ）、胸苦しさ（chest tightness）、喀痰などがみられる。また、しばしば鼻炎、副鼻腔炎、鼻茸やアトピー性皮膚炎の合併をみる。喘息の呼吸器症状には発作性の消長がみられ、夜間から早朝にかけて出現することが多い。

b） 呼吸機能検査（表２の項目２と３）

スパイロメトリーによる１秒量、努力性肺活量（FVC; forced vital capacity）、フローボリューム（FV; flow-volume）曲線が有用である。

i） 可逆性気流制限

１秒量は、気道閉塞を評価するゴールドスタンダードであり、FV 曲線は、末梢気道の状態を把握する良い指標となる。また PEF は、１秒量とともに気道閉塞を検出することができ、喘息の日常管理に有用である。

ii） 気道過敏性の亢進

気道の過敏性の評価には、アセチルコリンやその誘導体のメサコリン、あるいはヒスタミンといった気道収縮薬による気道過敏性試験を施行する。方法は、気道収縮薬の吸入により、１秒量の低下を指標とする標準法と、呼吸抵抗の上昇を指標とするアストグラフ法が用いられている。標準法では、１秒量が 20% 以上の低下を示す気道収縮薬の最低濃度（閾値）か、反応曲線から１秒量を 20% 低下させる濃度である PC_{20} を求めて評価する。喘息患者では気道過敏性試験でより低濃度の閾値、あるいは PC_{20} を示すことになる。特に咳のみや胸痛のみを主訴とする cough variant asthma や chest pain variant asthma の診断には、必須の検査である。

c） その他の検査所見（表２の項目４、5、６）

i） アトピー素因

アトピー型では、血清総 IgE 値の上昇がみられ、同時に抗原特異的 IgE 抗体も陽性である。抗原特異的 IgE 抗体は、皮膚反応試験（プリックテスト、皮内テストなど）か、血清反応試験（RAST; radioallergosorbent test や CAP 法、

MAST 法など）により検出される。感度の点では皮膚反応試験が優れているが、最近は、より簡便な血清を用いた検査が好まれる傾向にある。問診においては、アレルギー疾患の家族歴や既往歴、生活環境として住宅環境、本人や同居者の喫煙、室内環境（空調、掃除、カーペット、建築年数、間取りや日当たりなど）、ペットの有無、職業と職場環境などが重要である。最も頻度の高い抗原は、吸入性抗原の室内塵（HD; house dust）やヒョウヒダニ（dermatophagoides）、通称チリダニ（house dust mite）である。また職業性喘息が疑われる場合には、抗原特異性 IgE 抗体の検索を症例毎に疑わしい抗原を用いて（時には研究室で調整して）行なう必要がある。

ii） 気道炎症の存在

気道炎症を臨床的にモニターするための指標は、十分に確立されていない。

血算では、好酸球の増多（500/mm^3 以上）のみられることが多い。喀痰は通常漿液性で気泡に富み、好酸球の増多や剥離した気道上皮からなるクレオラ体を認める。喀痰がでない場合には、高張食塩水による誘発喀痰を採取して検査することも有用である。また、将来的に普及することが予想される呼気中一酸化窒素（NO）の測定では、NO の上昇を認める。

d） 鑑別診断疾患の除外

最初から喘息と決めつけないで、鑑別診断を行なうことが重要である（表３）。

表３ 鑑別すべき疾患（JAGL2013）

1. 上気道疾患：喉頭炎、喉頭蓋炎、vocal cord dysfunction(VCD)
2. 中枢気道疾患：気管内腫瘍、気道異物、気管軟化症、気管支結核、サルコイドーシス
3. 気管支～肺胞領域の疾患：COPD、びまん性汎細気管支炎、肺線維症、過敏性肺炎
4. 循環器疾患：うっ血性心不全、肺血栓塞栓症
5. アンギオテンシン変換酵素阻害薬などの薬物による咳
6. その他の原因：自然気胸、迷走神経刺激症状、過換気症候群、心因性咳嗽
7. アレルギー性呼吸器疾患：アレルギー性気管支肺真菌症、好酸球性肉芽腫性多発血管炎（eosinophilic granulomatosis with polyangiitis, EGPA, Churg-Strauss症候群）、好酸球性肺炎

高齢化社会を迎え、うっ血性心不全による心臓喘息といわれる状態との鑑別、またその原因として、急性心筋梗塞の有無にまで思いを巡らす必要がある。また

中年以降の喫煙者では、慢性閉塞性肺疾患（COPD）との鑑別、あるいは合併の有無を明らかにする。急性発症の呼吸困難と言う点では、緊急な対応を必要とする気胸と肺血栓塞栓症を見逃してはならない。また喘息には気道感染の併発が高率にみられることも考慮することが必要である。喘息を合併するアレルギー性呼吸器疾患では、通常の喘息治療でコントロールされ難い場合が多く、副腎ステロイド薬の全身投与を必要とする難治性喘息では特に、アレルギー性気管支肺アスペルギルス症（アレルギー性気管支肺真菌症）や好酸球性肉芽腫性多発血管炎（eosinophilic granulomatosis with polyangiitis, EGPA, Churg-Strauss 症候群）などとの鑑別が必要である。

４．治療

a） 概要

i） 長期管理

喘息は、発作につながる可逆性の気道閉塞と気道過敏性とともに慢性の気道炎症とその結果引き起こされる気道傷害から成り立つ疾患である。したがって、治療する場合には、発作あるいは喘息症状だけではなく、背景にある気道炎症も標的として考え治療を組み立てることが、発作を起こさないことにつながる。すなわち、JGL2012 を規範として、まず患者毎に喘息の重症度を判定し（表４、表５）、症状に対する治療と炎症を抑え症状を予防する治療（長期管理）の両面から、適切な薬物治療を実行することが基本となる（表６）。

表４　治療前の臨床所見による喘息重症度の分類(成人)

重症度*1		軽症間欠型	軽症持続型	中等症持続型	重症持続型
喘息症状の特徴	頻度	週１回未満	週１回以上だが毎日ではない	毎日	毎日
	強度	症状は軽度で短い	月１回以上日常生活や睡眠が妨げられる	週１回以上日常生活や睡眠が妨げられる	日常生活に制限
				短時間作用性吸入β_2刺激薬頓用がほとんど毎日必要	治療下でもしばしば増悪
	夜間症状	月に２回未満	月２回以上	週１回以上	しばしば
PEF FEV_1*2	%FEV_1, %PEF	80%以上	80%以上	60%以上80%未満	60%未満
	変動	20%未満	20～30%	30%を超える	30%を超える

*1　いずれか１つが認められればその重症度と判断する。

*2　症状からの判断は重症例や長期罹患例で重症度を過小評価する場合がある。呼吸機能は気道閉塞の程度を客観的に示し，その変動は気道過敏性と関連する。%FEV_1＝(FEV_1測定値/FEV_1予測値)×100, %PEF＝(PEF測定値/PEF予測値または自己最良値)×100

表5　現在の治療を考慮した喘息重症度の分類(成人)

現在の治療における患者の症状	現在の治療ステップ			
	ステップ1	ステップ2	ステップ3	ステップ4
コントロールされた状態*1 ●症状を認めない ●夜間症状を認めない	軽症間欠型	軽症持続型	中等症持続型	重症持続型
軽症間欠型相当*2 ●症状が週1回未満 ●症状は軽度で短い ●夜間症状は月に2回未満	軽症間欠型	軽症持続型	中等症持続型	重症持続型
軽症持続型相当*3 ●症状が週1回以上，しかし毎日ではない ●月1回以上日常生活や睡眠が妨げられる ●夜間症状が月2回以上	軽症持続型	中等症持続型	重症持続型	重症持続型
中等症持続型相当*3 ●症状が毎日ある ●短時間作用性吸入β_2刺激薬がほとんど毎日必要 ●週1回以上日常生活や睡眠が妨げられる ●夜間症状が週1回以上	中等症持続型	重症持続型	重症持続型	最重症持続型
重症持続型相当*3 ●治療下でもしばしば増悪 ●症状が毎日ある ●日常生活が制限される ●夜間症状がしばしば	重症持続型	重症持続型	重症持続型	最重症持続型

*1：同一治療継続3～6か月でステップダウンを考慮する。
*2：各治療ステップにおける治療内容を強化する。
*3：治療のアドヒアランスを確認し、必要に応じ是正してステップアップする。

表6　喘息治療ステップ

		治療ステップ 1	治療ステップ 2	治療ステップ 3	治療ステップ 4
長期管理薬	基本治療	吸入ステロイド薬 (低用量)	吸入ステロイド薬 (低～中用量)	吸入ステロイド薬 (中～高用量)	吸入ステロイド薬 (高用量)
		上記が使用できない場合は以下のいずれかを用いる LTRA テオフィリン徐放製剤 ※症状が稀ならば必要なし	上記で不十分な場合に以下のいずれか1剤を併用 LABA (配合剤の使用可[5]) LTRA テオフィリン徐放製剤	上記に下記のいずれか1剤，あるいは複数を併用 LABA (配合剤の使用可[5]) LTRA テオフィリン徐放製剤	上記に下記の複数を併用 LABA (配合剤の使用可) LTRA テオフィリン徐放製剤 上記のすべてでも管理不良の場合は下記のいずれかあるいは両方を追加 抗IgE抗体[2] 経口ステロイド薬[3]
	追加治療	LTRA以外の抗アレルギー薬[1]	LTRA以外の抗アレルギー薬[1]	LTRA以外の抗アレルギー薬[1]	LTRA以外の抗アレルギー薬[1]
発作治療[4]		吸入SABA	吸入SABA[5]	吸入SABA[5]	吸入SABA

そして、あくまでも無症状の状態で健常人と変わらない生活の出来る状態をめざす（表７）。すなわち、症状の治療には即効性の気管支拡張薬、長期管理としては、吸入ステロイド薬を基本薬として継続し、必要に応じて他の薬剤を併用して無症状の状態を維持するのである。喘息の治療を担当する医師側には、JGL2012 に沿った治療を喘息の病態を理解した上で実行することが望まれる。患者側には、薬剤の服用を遵守し（アドヒアランスを堅持し）、喘息の原因への曝露を回避することが要求される。良い生活環境にはフローリング、週３回以上の掃除、寝具の衛生管理が重要とされている。

表７　コントロール状態の評価

	コントロール良好 （すべての項目が該当）	コントロール不十分 （いずれかの項目が該当）	コントロール不良
喘息症状 （日中および夜間）	なし	週１回以上	コントロール不十分の項目が３つ以上当てはまる
発作治療薬の使用	なし	週１回以上	
運動を含む活動制限	なし	あり	
呼吸機能 （FEV_1および PEF）	予測値あるいは 自己最高値の80%以上	予測値あるいは 自己最高値の80%未満	
PEFの日（週）内変動	20%未満	20%以上	
増悪（予定外受診， 救急受診，入院）	なし	年に１回以上	月に１回以上*

*増悪が月に１回以上あれば他の項目が該当しなくともコントロール不良と評価する。

ii）　発作への対応

長期管理を実行していても、発作が出現することもあり、発作に対する適切な対応も長期管理とともに非常に重要である。とくに喘息死をゼロにするためには、長期管理による予防効果だけではなく、死亡の直接の原因である発作に対して、適切に対応することが必須である。

発作は、時と場所を選ばず出現するので、患者自身での対応を指導することが必要である。とくに医療機関を受診しなければならないと判断する基準を明らかにして指導することが重要である。JGL2012 では、表８のように発作強度を分類しており、発作のために横になれない状態（中等度の発作）であれば医療機関を受診することを推奨している。とくに発作が重症化した経験のある患者、アドヒアランスの悪い患者では、担当医が CS の経口薬（例えばプレドニン）を渡しておき、30mg を目安に家庭で内服して受診するよう指導することも推奨されている。

表８　喘息発作の強度と目安となる発作治療ステップ

発作強度[2]	呼吸困難	動作	検査値[1]				選択する発作治療ステップ
			PEF	SpO_2	PaO_2	$PaCO_2$	
喘鳴/胸苦しい	急ぐと苦しい 動くと苦しい	ほぼ普通	80%以上	96%以上	正常	45mmHg未満	発作治療ステップ1
軽度（小発作）	苦しいが横になれる	やや困難					
中等度（中発作）	苦しくて横になれない	かなり困難 かろうじて歩ける	60～80%	91～95%	60mmHg超	45mmHg未満	発作治療ステップ2
高度（大発作）	苦しくて動けない	歩行不能 会話困難	60%未満	90%以下	60mmHg以下	45mmHg以上	発作治療ステップ3
重篤	呼吸減弱 チアノーゼ 呼吸停止	会話不能 体動不能 錯乱 意識障害 失禁	測定不能	90%以下	60mmHg以下	45mmHg以上	発作治療ステップ4

1）気管支拡張薬投与後の値を参考とする。
2）発作強度は主に呼吸困難の程度で判定する（他の項目は参考事項とする）。異なる発作強度の症状が混在する場合は発作強度の思いほうをとる。

基本的には、通常の発作に対する家庭での治療をしても発作が収まらないときは、医療機関を受診し、もっと積極的で有効性の高い治療を施行しなければならないという認識を患者に持たせるよう指導する。

「発作に対する家庭での対応は、まず発作の強さを判定することから始まります。苦しくても横になれれば軽度の発作で、主治医の処方した吸入β_2刺激薬の吸入あるいは経口の発作止めを頓服して下さい。目安として、吸入は１時間で15～20分毎に動悸を感じない限り継続、経口薬は30分後に１回追加可能です。それでも収まらないときや明らかに悪化するときは１時間にこだわらず、受診することをお勧めします。また苦しくて横になれない中等度や話が困難な高度の発作では、ただちに気管支拡張薬を服用して受診して下さい。中等度でも気管内挿管歴や入院歴がある場合、高用量吸入ステロイド薬や経口ステロイド薬を継続投与されている場合には、家庭で経口ステロイド薬を主治医の指示に従い内服し、直ぐに受診して下さい。」という内容の話をして指導することになる。

このような内容を口頭で指導するだけでなく、記載した行動計画表（アクションプラン）を作成し手渡すことも、JGL2012の家庭での対応を実行するうえで必要である。

患者の受診後、その予後を左右する上で重要なのが医療機関での対応で、発作治療ステップとして記述している（表９）。とくに中等度よりも重症の高度（話すのが困難で動けない）や重篤・エマージェンシー（意識障害、呼吸停止）に相当する場合は、救急隊、入院設備のある病院あるいは院内での救命救急部と

の連携が必要となる。そして適切な治療の実行には、各患者の平素の治療内容、発作時に施行する治療内容や治療に当たっての注意点を記した診療カードの作成が有用であると考えられる。カードに含まれる内容としては、処方されている治療薬、推奨される発作時の対応に加えて、喘息の発症時期、治療歴、入院歴、アスピリン喘息の有無、薬剤アレルギーの有無などである。

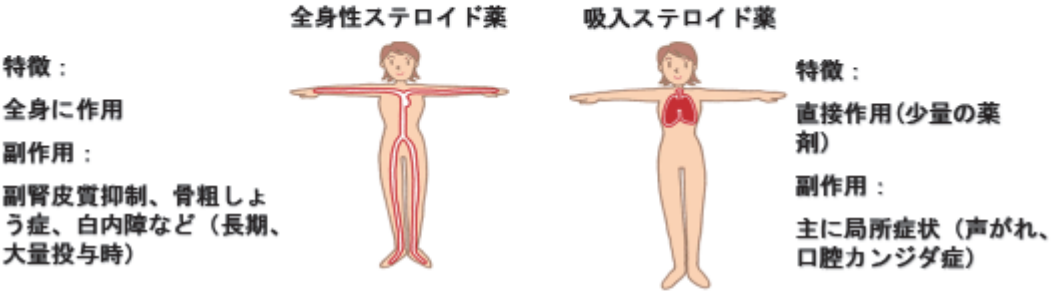
表９　喘息発作の強度に対応した管理法（成人）

	治療	自宅治療可，救急外来入院，ICU管理[1]
発作治療ステップ１	β_2刺激薬吸入，頓用[2] テオフィリン薬頓用	自宅治療可
発作治療ステップ２	β_2刺激薬ネブライザー吸入反復[3] アミノフィリン点滴静注[4] ステロイド薬点滴静注[5] 酸素吸入（鼻カニューレなどで１～２L/分） ボスミン®（0.1％アドレナリン）皮下注[6] 抗コリン薬吸入考慮	救急外来 ・１時間で症状が改善すれば帰宅 ・２～４時間で反応不十分 ┐ ・１～２時間で反応なし ┘ 入院治療 入院治療：高度喘息症状として発作治療ステップ３を施行
発作治療ステップ３	アミノフィリン持続点滴[7] ステロイド薬点滴静注反復[5] 酸素吸入（PaO_2 80mmHg前後を目標に） ボスミン®（0.1％アドレナリン）皮下注[6] β_2刺激薬ネブライザー吸入反復[3]	救急外来 １時間以内に反応なければ入院治療 悪化すれば重篤症状の治療へ
発作治療ステップ４	上記治療継続 症状，呼吸機能悪化で挿管[1] 酸素吸入にもかかわらずPaO_2 50mmHg以下および/または意識障害を伴う急激な$PaCO_2$の上昇 人工呼吸[1]，気管支洗浄 全身麻酔（イソフルラン・セボフルラン・エンフルランなどによる）を考慮	直ちに入院，ICU管理[1]

b)　吸入ステロイド薬の安全性

わが国では、副腎皮質ステロイド薬（CS）は怖い薬として位置付けられ、吸入薬についてもこれまでの恐怖感が、医師と患者の両方に根強く残っている。すなわち、CS は、まず経口薬や注射薬が種々の疾患で使用され、いろいろな副作用が出現することから良く効く反面怖い薬という認識が定着してしまっている。喘息の治療で使用される吸入ステロイド薬（ICS）は、CS の中では最後に登場した剤形であるが、すでに 20 年以上にわたり喘息の治療に用いられ、その効果と安全性から喘息の治療に革命を起こしたと言っても過言ではない（図 12）。喘息は、気道の炎症を特徴とする慢性疾患であり、ICS を長期に投与することが治療の基本となる疾患である。副作用についてもしっかり研究され、ICS の常用量では、CS の全身投与でみられる副腎機能の抑制、骨粗鬆症、糖尿病、消化性潰瘍、免疫不全、異常脂肪沈着などはみられず、また懸念された小児の成長障害、胎児の奇形の発生、気道上皮細胞への悪影響などもみられていない。JGL2012 で推奨されている常用量である限り、咽頭のカンジダ症や嗄声が一般的な副作用で、重篤なものはみられていない。

図12　吸入ステロイド薬について



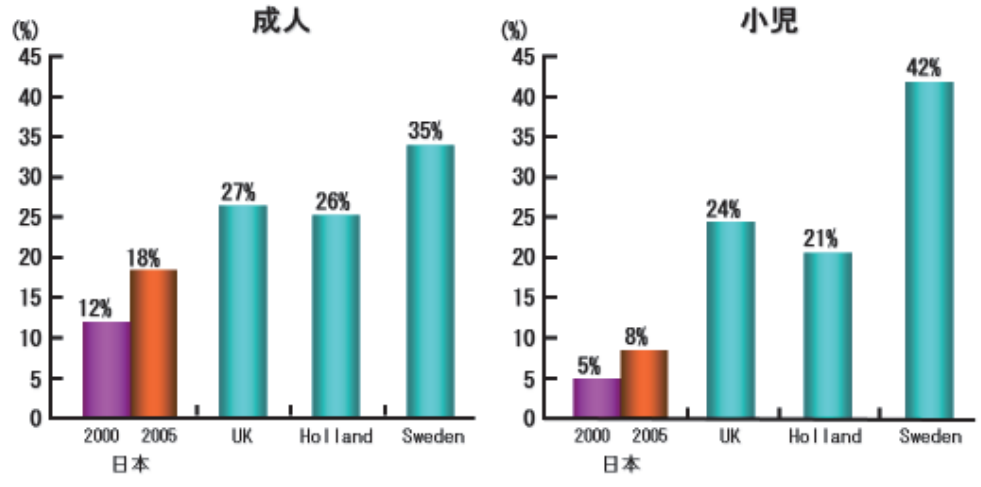
局所抗炎症作用が強力。常用量では副腎機能の副作用はほとんど見られない。吸入薬のステロイド用量は、経口薬に比較して1/1000である。
肝臓において初回通過で90%が代謝される。
局所副作用としては、嗄声、咽頭の異常感、口腔のカンジダ症などがあるがうがいによって多くの場合が防げる。
小児の発育においても、長期の追跡結果では健常な子どもの発育（身長）と差がない。

c)　課題

i)　喘息の臨床に関する実態

わが国での喘息の実態調査として、国際的に共通の質問表を用いた電話による疫学調査が、2000 年（AIRJ2000）と 2005 年（AIRJ2005）に実施された。その結果によると、成人における ICS の使用頻度は、2000 年は 12%、2005 年は 18%で、これは 2000 年の英国 (27%)、オランダ (26%)、スウェーデン (35%) と比べていずれも低値であった（図 13)。

図13 吸入ステロイド薬の使用頻度



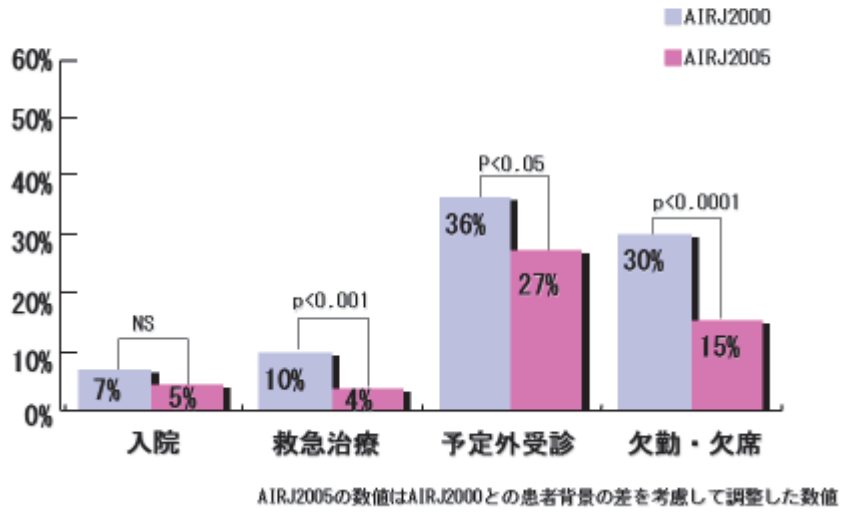
足立　満ほか：アレルギー 2002:51:411-420　(一部改変)

すなわち、ICS を第一選択薬として推奨している喘息治療のガイドラインが十分に実行されていないことを示唆する結果であった。また小児では一層低頻度であることも示された。さらに注目されることは、ICS の使用頻度が 2000 年から 2005 年にかけて 6%増加した結果、救急治療、予定外受診、欠勤・欠席の経験率が有意に減少し（図 14）、吸入ステロイドを用いることの臨床効果を表しており、JGL2012 に沿った ICS による長期管理の有効性を強く支持している。

さらに、AIRJ2000 の結果から、患者の自己管理を評価する上で重視されているピークフローメータの使用が、理想とは程遠く、週 1 回以上使用している成人患者がわずか 6%に過ぎないことが明らかとなった（図８）。すなわち、喘息の状態を客観的に評価するために、より簡便な方法を考えることの必要性が示された。

成人

図14 最近1年間の
入院、救急治療、予定外受診、欠勤・欠席の経験率



AIRJ2005の数値はAIRJ2000との患者背景の差を考慮して調整した数値

ii） 高齢者への対応

喘息死の年齢分布を見ると喘息死の 90%近くが 65 歳以上の高齢者であることが明らかとなっている（図２）。この事実から、JGL2012 を実行するにあたり高齢者への対応をとくに意識することが必要である。

高齢者では、喫煙者であれば COPD を合併していることが稀ではなく、喘息 COPD オーバーラップ症候群（asthma-COPD overlap syndrome, ACOS）と呼ばれている。そして ACOS では、喘息とともに COPD に対する治療も考慮する。禁煙は喘息では ICS の効果が喫煙により抑制されることを阻止するが、COPD では治療においてさらに重要であり、これまでのところ疾患の進行を抑制する唯一の方法といっても過言ではない。したがって禁煙の実行が一層重要となる。

薬物療法では、抗コリン薬を含む複数の気管支拡張薬の使用が COPD の閉塞性換気障害に有効である。発作時には表３の鑑別すべき診断に一層の注意を払い、また喘息に加えてこれらの疾患を併発している可能性もあることを忘れずに対応することが必要となる。さらに、薬剤の代謝も加齢や併用薬により変化するので、その点についても注意が必要である。喘息死ゼロ作戦においては、いかに高齢者の喘息への対応を適切に行なえるようにするかが重要な課題として位置付けられると考えられる。

iii） JGL2012 の普及と実行に関する方策

JGL2012 の普及を妨げることとして、ガイドラインが複雑すぎることが挙げられている。表２～９にまとめられた内容を完全に記憶して日常の臨床で実行するのは、非専門医に限らずたとえ専門医であっても困難であると考えられる。また、ICS を基本薬として用いる長期管理を実行するには、まず喘息の病態に長期管理を必要とする気道の慢性炎症が重要な役割を演じていることの理解を深め、炎症に対して ICS が最も有効で、しかも ICS は長年にわたる臨床的な検討の結果、安全性が極めて高いことの理解を広めることが必須であると考えられる。

したがって、ゼロ作戦の戦略には、喘息の理解を深める教材、ガイドラインの実行を可能にする簡便なプログラム、治療効果として患者の状態を客観的にかつ簡便に評価するための方法などの道具立てが必要と考えられる。

自己管理法を含む喘息死ゼロ作戦の実行に関する指針

平成２７年3月３１日

受託者　　住所　東京都清瀬市竹丘１－１－１
独立行政法人国立病院機構
東京病院
氏名　大田　健

独立行政法人　環境再生保全機構

成人気管支喘息診療のミニマムエッセンス

成人気管支喘息診療のミニマムエッセンス作成ワーキンググループ 編

本書は、平成23年度厚生労働科学研究費補助金で成人気管支喘息診療のミニマムエッセンス作成ワーキンググループ（奥付参照）が作成したものを元に、その後発刊された喘息予防・ガイドライン2012とアレルギー総合ガイドライン2013（一般社団法人日本アレルギー学会作成）の内容を踏まえて改編したミニマムエッセンスです。

1. 診断

診断は、①発作性の呼吸困難、喘鳴、咳、胸苦しさなどの症状の反復、②可逆性の気流制限、③他の心肺疾患などの除外による。過去の救急外来受診歴や、喘息治療薬による症状の改善は診断の参考になる。喘鳴や呼吸困難を認めず、診断に苦慮する場合は、気道過敏性試験を依頼するか、吸入ステロイド薬やβ_2刺激薬による治療的診断を考慮する。

表1. 成人喘息診断の目安

	一般診療	専門診療
1. 発作性の呼吸困難、喘鳴、咳の反復	問診：夜間、早朝に出現しやすい。 聴診：喘鳴は、強制呼気時に頸部で聴取しやすい。	
2. 可逆性の気流制限	問診：無症状期をはさんで、発作が反復	気道可逆性試験
3. 症状が他の心肺疾患によらない（表3）	胸部レントゲン撮影	
4. 気道過敏性の亢進	問診：運動、気道ウイルス感染、アレルゲン曝露、気象変化、精神的ストレス、月経などで症状が惹起される。	気道過敏性試験
5. アトピー素因	血清特異的IgE抗体	即時型皮膚反応
6. 気道炎症の存在	喀痰細胞診や末梢血白血球像における好酸球増多	呼気一酸化窒素濃度測定

1、2、3が臨床診断上重要である。4、5、6は他の所見とともに喘息診断を支持する。

2. 他疾患の鑑別

中高年発症で、喫煙歴を有する場合、COPDの存在を念頭におく。COPDでは、気管支拡張薬吸入後の1秒率が70%未満、高分解能CTで低吸収域、肺拡散能低下などの所見を認める。診断に迷う場合は、専門医へ紹介する。

標準治療に対する反応が十分得られない場合は、**表3**の疾患を念頭に、胸部CT、心機能評価、呼吸器専門医や耳鼻咽喉科医への紹介を考慮する。

表2. COPDとの鑑別のポイント

	COPD	喘息
喫煙歴	ほぼ全例あり	ありうる
40歳未満の場合	稀	多い
呼吸困難	進行性・持続性	発作性・症例により異なる
夜間の咳込み、覚醒	少ない	多い
症状の変動	少ない	多い

COPD診療のエッセンス（日本COPD対策推進会議）より改変

表3. 喘息と鑑別すべき疾患

1. 上気道疾患：喉頭炎、喉頭蓋炎、vocal cord dysfunction（VCD）
2. 中枢気道疾患：気管内腫瘍、気道異物、気管軟化症、気管支結核、サルコイドーシス
3. 気管支～肺胞領域の疾患：COPD、びまん性汎細気管支炎、肺線維症、過敏性肺炎
4. 循環器疾患：うっ血性心不全、肺血栓塞栓症
5. アンジオテンシン変換酵素阻害薬などの薬物による咳
6. その他：自然気胸、迷走神経刺激症状、過換気症候群、心因性咳嗽
7. アレルギー性呼吸器疾患：アレルギー性気管支肺アスペルギルス症、好酸球性多発血管炎性肉芽腫症（Churg-Strauss症候群）、好酸球性肺炎

3. 長期管理における薬物療法プラン

可能な限り呼吸機能を正常化し、QOLを改善し、健常人と変わらない日常生活を送れることが治療の目標である。長期に罹患し、気道リモデリングがある患者では、呼吸機能は正常値まで改善し得ないので、自己最良値に基づいて判定する。コントロール状態は**表4**に基づいて判断するが、短時間作用性β_2刺激薬（SABA）の使用頻度の問診が簡便である。

薬物治療を、**表5**の4つの治療ステップに分ける。未治療患者（**表6**）は、症状が週1回あるかどうかで治療ステップ1と2に分け、連日症状があれば治療ステップ3、さらに治療下でも増悪していれば治療ステップ4とする。治療中の患者は**表4**を参考にコントロール良好を目指し、コントロール不十分であれば、**表5**を参考に治療のステップアップを行う。

表4. コントロール状態の評価

	コントロール良好 （すべての項目が該当）	コントロール不十分 （いずれかの項目が該当）	コントロール不良
喘息症状（日中および夜間）	なし	週1回以上	コントロール不十分の項目が3つ以上当てはまる
発作治療薬の使用	なし	週1回以上	
運動を含む活動制限	なし	あり	
呼吸機能（FEV_1およびPEF）	正常範囲内	予測値あるいは自己最高値の80%未満	
PEFの日（週）内変動	20%未満	20%以上	
増悪	なし	年に1回以上	月に1回以上*

＊増悪が月に1回以上あれば他の項目が該当しなくてもコントロール不良と評価する。

表5. 喘息治療ステップ

		治療ステップ1	治療ステップ2	治療ステップ3	治療ステップ4
長期管理薬	基本治療	吸入ステロイド薬 （低用量）	吸入ステロイド薬 （低～中用量）	吸入ステロイド薬 （中～高用量）	吸入ステロイド薬 （高用量）
		上記が使用できない場合以下のいずれかを用いる LTRA テオフィリン徐放製剤 （症状が稀であれば必要なし）	上記で不十分な場合に以下のいずれか1剤を併用 LABA （配合剤の使用可） LTRA テオフィリン徐放製剤	上記に下記のいずれかを1剤、あるいは複数を併用 LABA （配合剤の使用可） LTRA テオフィリン徐放製剤	上記に下記の複数を併用 LABA （配合剤の使用可） LTRA テオフィリン徐放製剤 上記のすべてでも管理不良の場合は下記のいずれかあるいは両方を追加 抗IgE抗体 経口ステロイド薬
	追加治療	LTRA以外の抗アレルギー薬	LTRA以外の抗アレルギー薬	LTRA以外の抗アレルギー薬	LTRA以外の抗アレルギー薬
発作治療		吸入SABA	吸入SABA	吸入SABA	吸入SABA

LTRA：ロイコトリエン受容体拮抗薬、LABA：長時間作用性β_2刺激薬、SABA：短時間作用性β_2刺激薬

表6. 未治療患者の症状と目安となる治療ステップ

	治療ステップ1 （軽症間欠型相当）	治療ステップ2 （軽症持続型相当）	治療ステップ3 （中等症持続型相当）	治療ステップ4 （重症持続型相当）
喘息症状	週1回未満 軽度で短い	週1回以上だが毎日ではない	毎日	毎日 治療下でもしばしば増悪
夜間症状	月2回未満	月2回以上	週1回以上	しばしば
日常生活の妨げ	なし	月1回以上	週1回以上	持続的

表7. 吸入ステロイド薬、吸入ステロイド薬/長時間作用性β2刺激薬配合剤の治療ステップ別推奨量

商品名	治療ステップ1～2 低用量	治療ステップ3 中用量	治療ステップ4 高用量
キュバール®、フルタイド®エアゾール、オルベスコ®、フルタイド®ロタディスク®、フルタイド®ディスカス®、アズマネックス®ツイストヘラー®	100～200μg/日	200～400μg/日	400～800μg/日
パルミコート®タービュヘイラー®	200～400μg/日	400～800μg/日	800～1600μg/日
パルミコート®吸入液	0.5mg/日	1.0mg/日	2.0mg/日
アドエア®ディスカス®(1吸入2回/日)	100ディスカス	250ディスカス	500ディスカス
シムビコート®タービュヘイラー®	1吸入2回/日	2吸入2回/日	4吸入2回/日
アドエア®エアゾール(2吸入2回/日)	50エアゾール	125エアゾール	250エアゾール
フルティフォーム®	50エアゾール 2吸入2回/日	125エアゾール 2吸入2回/日	125エアゾール 4吸入2回/日
レルベア®エリプタ®(1吸入1回/日)	100エリプタ	100エリプタ または200エリプタ	200エリプタ

●長期管理薬(コントローラー)の使用に関する注意点

①**吸入ステロイド薬(ICS)**：最も効果的な抗炎症薬である。副作用は、口腔･咽頭カンジダ症、嗄声などで全身性の副作用は少ない。妊娠自体に影響しない。喘息患者の呼吸器感染症の頻度を上げる証拠はない。最大呼気位(最大限呼出したところ)から最大吸気位(最大限吸入したところ)まで吸入し、約10秒間息こらえをしてゆっくり吐き出す。デバイス毎に吸入の強さが適切となるように指導する(はやく深く：フルタイド®ロタディスク®･ディスカス®、アドエア®ディスカス®／深く力強く：パルミコート®、シムビコート®、アズマネックス®、レルベア®エリプタ®／ゆっくり深く：キュバール®、オルベスコ®、フルタイド®エアゾール、アドエア®エアゾール、フルティフォーム®)。

②**長時間作用性β2刺激薬(LABA)**：吸入薬、貼付薬、経口薬があり、必ずICSと併用する(単独使用は禁忌)。ICSにLABAを併用すると相乗効果が得られる。

③**吸入ステロイド薬/長時間作用性吸入β2刺激薬配合剤**：ICSとLABAを個別に吸入するよりも有効性が高い。アドヒアランスを向上させてLABAの単独使用を防ぐ。

④**ロイコトリエン受容体拮抗薬(LTRA)**：気管支拡張作用と抗炎症作用を有し、ICSに併用すると有効性が高い。アレルギー性鼻炎合併喘息、運動誘発喘息、アスピリン喘息患者の管理において有用である。

⑤**テオフィリン徐放製剤**：気管支拡張作用を有する。ICSとの併用で相乗効果が得られる。副作用や過剰投与(中毒)を回避するには100mg錠を2～3回/日で開始し、効果が不十分なら保険診療上の常用量である200mgを2回/日まで増量する。重症例では、専門医と相談の上さらに500～600mg/日へと100mg単位(分2～3)で増量できる(レセプト上の詳記を必要とする場合がある)。血中濃度は5～15μg/mLが目標であるが、患者によっては適正な血中濃度でも、それ以下でも中毒症状が生じることがあるので400mg/日の時点で血中濃度のモニタリングをする。

⑥**抗IgE抗体(オマリズマブ)**：高用量ICSと複数の気管支拡張薬の併用下でもコントロール不十分で総血清IgE値が30～1,500 IU/mL、通年性吸入抗原が証明されている場合に投与する。約60%で奏効するとされる。4か月間投与後に効果判定を行う。

4. 急性増悪(発作)時の対応(成人)

発作強度を呼吸困難症状から判定して遅滞なく治療を開始する。前夜横になれていれば小発作、苦しくて横になれないが歩行可能なら中発作、歩行や会話が困難なら大発作、チアノーゼ、意識障害、呼吸停止を認める場合は重篤症状とする。

中等度以上の発作や、吸入β2刺激薬による初期治療に反応が乏しい場合、十分量のICSや、経口ステロイド薬を常用している症例、挿管の既往例、過去1年間に入院、救急受診があった症例では、直ちに全身性ステロイド薬を投与する。入院や集中治療を要すると判断した場合は、遅滞なく専門施設への搬送を手配する。

表8. 喘息発作の強度に対応した管理のポイント

発作強度	呼吸困難	動作	SpO_2	治療	自宅治療可、入院、ICU管理
喘鳴/胸苦しい	急ぐと苦しい 動くと苦しい	ほぼ普通	96%以上	β2刺激薬吸入、頓用[1] テオフィリン薬頓用	自宅治療可
軽度(小発作)	苦しいが横になれる	やや困難		β2刺激薬吸入、頓用[1] テオフィリン薬頓用	自宅治療可
中等度(中発作)	苦しくて横になれない	かなり困難かろうじて歩ける	91〜95%	β2刺激薬ネブライザー吸入反復[2] 0.1%アドレナリン(ボスミン®)皮下注[3] アミノフィリン点滴静注[4] ステロイド薬点滴静注[5] 酸素投与	救急外来 ・1時間で症状が改善すれば帰宅 ・2〜4時間で反応不十分 ・1〜2時間で反応無し 入院治療→高度喘息症状治療へ
高度(大発作)	苦しくて動けない	歩行不能会話困難	90%以下	0.1%アドレナリン(ボスミン®)皮下注[3] アミノフィリン持続点滴[6] ステロイド薬点滴静注反復[5] 酸素投与 β2刺激薬ネブライザー吸入反復[2]	救急外来 1時間以内に反応なければ入院治療 悪化すれば重篤症状の治療へ
重篤	呼吸減弱 チアノーゼ 呼吸停止	会話不能 体動不能 錯乱、失禁 意識障害	90%以下	上記治療継続 症状、呼吸機能悪化で挿管[7] 人工呼吸[7] 気管支洗浄 全身麻酔を考慮	直ちに入院、ICU管理

1)β2刺激薬pMDI 1〜2パフ、20分おき2回反復可。
2)β2刺激薬ネブライザー吸入：20〜30分おきに反復する。脈拍を130/分以下に保つようにモニターする。
3)0.1%アドレナリン(ボスミン®)：0.1〜0.3mL皮下注射20〜30分間隔で反復可。脈拍は130/分以下にとどめる。虚血性心疾患、緑内障[開放隅角(単性)緑内障は可]、甲状腺機能亢進症では禁忌、高血圧の存在下では血圧、心電図モニターが必要。
4)アミノフィリン6mg/kgと等張補液薬200〜250mLを点滴静注、1/2量を15分間程度、残量を45分間程度で投与し、中毒症状(頭痛、吐き気、動悸、期外収縮など)の出現で中止。発作前にテオフィリン薬が十分に投与されている場合は、アミノフィリンを半量もしくはそれ以下に減量する。通常テオフィリン服用患者では可能な限り血中濃度を測定。
5)ステロイド薬静注：ヒドロコルチゾン200〜500mg、メチルプレドニゾロン40〜125mg、デキサメタゾン、あるいはベタメタゾン4〜8mgを点滴静注。以後必要に応じて、ヒドロコルチゾン100〜200mgまたはメチルプレドニゾン40〜80mgを4〜6時間ごとに、あるいはデキサメタゾンあるいはベタメタゾン4〜8mgを6時間ごとに点滴静注、またはプレドニゾロン0.5mg/kg/日、経口。
6)アミノフィリン持続点滴：第1回の点滴に続く持続点滴はアミノフィリン250mg(1筒)を5〜7時間(およそ0.6〜0.8mg/kg/時)で点滴し、血中テオフィリン濃度が10〜20μg/mL(ただし最大限の薬効を得るには15〜20μg/mL)になるように血中濃度をモニターし中毒症状の出現で中止。
7)挿管、人工呼吸装置の装着は時に危険なので、緊急処置としてやむを得ない場合以外は専門施設で行われることが望ましい。

アスピリン喘息の場合、40〜60%相当の症例でコハク酸エステル型(サクシゾン®、ソル･コーテフ®、ソル･メドロール®、水溶性プレドニン®など)で発作誘発の危険があるため、リン酸エステル型(ハイドロコートン®、リンデロン®、デカドロン®など)を使用する。リン酸エステル型であっても、急速静注では添加物による発作誘発の可能性がある。初回投与時や、アスピリン喘息の有無が不明の場合は1時間程度かけて点滴投与する。

5. 専門医への紹介を考慮する条件

1. 治療ステップ3で良好な管理ができず、治療ステップ4に変更する場合。
2. 経口ステロイド薬や高用量の吸入ステロイド薬の長期投与が必要な場合。
3. 経口ステロイド薬高用量短期投与を年に2回以上必要とする場合。
4. 症状が典型的でなく、診断や鑑別が困難で、気道過敏性試験、胸部CTなどが必要な場合。
5. 困難な合併症(例：副鼻腔炎、鼻ポリープ、アレルギー性気管支肺アスペルギルス症、COPD合併、心身医学的問題など)や、特殊な原因(職業喘息、アスピリン喘息、食事アレルギーなど)を有する場合。

平成26年度厚生労働科学研究費補助金　免疫アレルギー疾患等予防･治療研究事業
アレルギー疾患の予後改善を目指した自己管理および生活環境改善に資する治療戦略の確立に関する研究
研究代表者　大田　健(国立病院機構東京病院院長)

成人気管支喘息診療のミニマムエッセンス作成ワーキンググループ(順不同･敬称略)
監修：大田　健　　長瀬　洋之(帝京大学医学部呼吸器･アレルギー内科准教授)
日本医師会　今村　聡(日本医師会副会長)　　大森　千春(大森メディカルクリニック院長)
鈴木　育夫(鈴木医院院長)　　平山　貴度(平山医院院長)
萩原　照久(萩原医院院長)

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「一般住民に対するダニアレルゲン回避のためのパンフレット

作成：研究分担者　釣木澤尚実

ダニアレルゲン対策

- ✓ 普通に生活していてもアレルギーの主たる原因となりうるダニは日本の温暖・湿潤な気候で増殖しやすく、多くの家がダニアレルゲンで汚染されています
- ✓ **「効果的な掃除」**等の環境整備によりダニアレルゲン量を減らすことができます
- ✓ 特に**「寝室」**と**「寝具」**に対する対策が重要です
- ✓ **「除湿」**は**「カビ対策と共通」**しており、カビをエサにするダニの増殖を抑えることができます
- ✓ 掃除の際は**「窓を開けて換気」**し、**「マスクやタオルで口を覆い」**ながら行ってください

→「アレルゲン」に関する参考情報：http://www.jrmunet.jp/allergy/base/allergen/index.html

≪寝具のダニアレルゲンを減らす方法≫

- 週に１回以上、家族全員の寝具カバーをはずして寝具そのものに直接掃除機をかける
- 高密度線維でできた布団・枕カバーを使用する
- ベッドメイキング時に窓を開放する
- 布製のソファを寝室に置かない
- 開放型暖房機器を寝室に置かない
- １日に数回窓を開けて換気する
- 掃除機をかける前に床を水拭きする
- 寝室のカーテンを年２回以上丸洗いする

≪寝室のダニアレルゲンを減らす方法≫

- 室内に植物や水槽、洗濯物、加湿器を置かない
- 週に１回以上寝室を含むすべての部屋に掃除機をかける
- 掃除機をかける前に床の拭き掃除をする
- 床はフローリングである
- 床を化学雑巾やモップで乾拭きする

厚労科研費補助金難治性疾患等克服研究事業「気管支喘息に対する喘息死の予防や自己管理手法の普及に関する研究」
主任研究者：大田　健　分担研究者：　国立病院機構相模原病院　釣木澤尚実　提供資料

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Association of Asthma Education with Asthma Control Evaluated by Asthma Control Test, FEV_1, and Fractional Exhaled Nitric Oxide

JUNPEI SAITO, M.D., PH.D.,* SUGURU SATO, M.D., PH.D., ATSURO FUKUHARA, M.D., YASUKO SATO, M.T., TAKEFUMI NIKAIDO, M.D., YAYOI INOKOSHI, M.D., NAOKO FUKUHARA, M.D., KAZUE SAITO, M.D., TAEKO ISHII, M.D., YOSHINORI TANINO, M.D., PH.D., TAKASHI ISHIDA, M.D., PH.D., AND MITSURU MUNAKATA, M.D., PH.D.

Department of Pulmonary Medicine, School of Medicine, Fukushima Medical University, Fukushima, Japan.

Background. Asthma education is an important adjunct for asthma control although the way asthma education affects asthma outcomes is poorly understood. The asthma control test (ACT), forced expiratory volume in 1 s (FEV_1), and fractional exhaled nitric oxide (FeNO) have all been used as markers of asthma control. However, the use of FeNO as a surrogate marker remains controversial. *Objectives.* (i) To examine whether asthma education is associated with asthma control; (ii) to compare absolute levels and changes of ACT, FEV_1, and FeNO over a year; and (iii) to evaluate whether FeNO can be used as an additional marker of asthma control. *Methods.* Fifty asthmatics with poor adherence (12 mild, 21 moderate, and 17 severe) received asthma education at study entry. Medications were unchanged for the first 3 months, and ACT, FEV_1, and FeNO measurements were recorded at entry, 3, 6, and 12 months. Asthma control was assessed at each visit and patients were categorized as either "stable" or "unstable" asthmatics according to the global initiative for asthma (GINA) guidelines. *Results.* A significant decrease in FeNO and increase in ACT score were noted in the stable asthmatic group at 3 months ($p < .001$), and this persisted over 12 months. Significant correlations were seen between changes (Δ) in FeNO, ACT, and FEV_1 over time. However, significant correlations between the absolute levels were not maintained over 12 months. A decrease of $\geq$18.6% in FeNO and a $\geq$3-point increase in ACT score (sensitivity: 80% and 73.3% and specificity: 83.3% and 87.5%, respectively) were associated with stable asthma control although the absolute levels were not. *Conclusions.* Asthma education may be useful to achieve stable control. In addition, changes rather than absolute levels of FeNO and ACT may be better markers of asthma control.

Keywords asthma control, asthma control test questionnaire, asthma education, fractional exhaled nitric oxide

INTRODUCTION

Interventions that aim to enhance medication adherence have recently been developed (1), although studies of adults and children with asthma show that approximately 30%–50% of those on asthma therapy are nonadherent to therapy at least for part of the time (2–4). Therefore, educating patients on how to manage their asthma more effectively should be used to improve patients' knowledge and influence their decision-making process regarding disease management, resulting in greater treatment adherence. However, the way asthma education affects asthma control remains poorly understood.

Asthma symptom questionnaires, spirometry, sputum eosinophil cell counts, and airway hyperresponsiveness (AHR) are potential ways of monitoring asthma control. Of these, the global initiative for asthma (GINA) guidelines recommend asthma symptom questionnaires and spirometry as conventional approaches for managing asthma. These are easy to perform and are considered to be an indirect marker of underlying airway inflammation (5). However, directly monitoring airway inflammation may be a better and more reliable method. Fractional exhaled nitric oxide (FeNO) is an easy, sensitive, reproducible, and noninvasive marker for directly detecting allergic airway inflammation (6–9). However, the use of FeNO as a surrogate marker of asthma control remains controversial (6, 10).

The aims of this study were to examine whether an intensive asthma educational program is associated with asthma control, to compare changes of predictive markers for asthma control [asthma control test questionnaire (ACT), forced expiratory volume in 1 s (FEV_1), and FeNO] over a 12-month period following asthma education and finally to evaluate whether FeNO measurements could be used as an additional marker of asthma control compared with ACT and FEV_1.

METHODS

Subjects

Asthmatic patients with persistent disease and poor adherence were recruited. All subjects had attended the outpatient clinic at the Department of Pulmonary Medicine, Fukushima Medical University Hospital for more than 6 months. Asthma was diagnosed according to a clinical history of characteristic symptoms (i.e., dry cough, wheezing, chest tightness, and breathlessness) and the presence of either bronchial hyperresponsiveness to methacholine or bronchodilator reversibility $\geq$12% (5). Subjects were excluded if they were current smokers, had other respiratory diseases, or had a respiratory tract infection within 6 weeks of study entry. Poor adherence was defined as the

*Corresponding author: Junpei Saito, M.D., Ph.D., Department of Pulmonary Medicine, School of Medicine, Fukushima Medical University, Hikarigaoka-1, Fukushima 960-1295, Japan; Tel: +81-24-547-1360; Fax: +81-24-548-9366; E-mail: junpei@fmu.ac.jp

presence of at least one of the following in the previous 6 months: (i) failure to follow treatment by missing any prescribed asthma medications more than 1–2 times per week; (ii) a missed follow-up appointment for more than 4 weeks without taking appropriate medications; (iii) difficulty in using prescribed inhalers evaluated by observing and assessing the patient's demonstration of a sample inhaler device, and (iv) the requirement of high-dose inhaled corticosteroid (ICS) due to uncontrolled symptoms. Adherence to therapy and current medications was assessed by respiratory consultants in our department following a patient interview, review of medical records, and asthma diaries. Asthmatic patients attending our hospital's outpatient clinic are seen every 4–8 weeks and medications can only be prescribed at these appointments.

All subjects provided written informed consent, and the Ethics Committee of Fukushima Medical University approved the study.

Study Design

This was a prospective, single-center, observational study. Subjects were reviewed at entry and at intervals of 3, 6, and 12 months (four visits). At study entry, all subjects received an intensive educational program using an illustrated guide. ACT, FeNO measurements, and spirometry [FEV_1 and forced vital capacity (FVC)] were performed, and asthma control, based on the GINA guidelines, was assessed at each subsequent visit in order to evaluate the most appropriate treatment (5). Treatment remained unchanged during the first 3 months to evaluate the association of the intensive asthma educational program with asthma control. Following this 3-month period, the treatment could be changed if the subjects needed to step-up or step-down their treatment in line with the GINA guidelines (5). During the follow-up period, subjects were divided into two groups: (i) stable control group: asthmatic subjects whose treatments had not been changed or had been decreased over the study period and (ii) unstable control group: asthmatic subjects who had to increase their ICS dose or add other antiasthmatic drugs during the study period. These groups categorized as "stable" and "unstable" were used to assess ACT, FeNO, and FEV_1 as outcome predictors in this study.

Intensive Asthma Educational Program

The Ministry of Health and Welfare of Japan has developed a specific educational intervention program for asthma management according to the GINA guidelines. It provides information about asthma pathogenesis, diagnosis, severity, medications (including side effects), differences between reliever and controller agents, importance of asthma treatment, inhaler device instructions, exacerbation management, peak expiratory flow (PEF) monitoring, and a self-management plan. All subjects received a 30-min educational intervention program from a respiratory physician. The program included a detailed explanation and a demonstration of inhaler technique.

The Online FeNO Measurement Methods

FeNO measurements were made prior to spirometry at a constant flow of 50 mL/s according to the American Thoracic Society (ATS)/ European Respiratory Society (ERS) guidelines (11) using a chemiluminescence analyzer (Kimoto, Osaka, Japan). Three measurements were performed and the mean value was documented (coefficient of variation $\leq$10%).

Lung Function Tests

Spirometry was performed using rolling seal spirometers (CHESTAC-11; Chest M.I., Inc., Tokyo, Japan) according to the ATS guidelines (12). Three tests were performed and the highest FEV_1 was recorded. Results of FVC and FEV_1 were presented as percentages of the predicted level for statistical analysis.

Statistical Analysis

Statistical analysis was performed using SPSS for Windows (version 17.0; SPSS, Chicago, IL, USA). Data are shown as means and 95% confidence interval (95% CI). Changes in FeNO, ACT, and FEV_1 are shown as ΔFeNO, ΔACT, and ΔFEV_1, respectively.

The association between intensive asthma education and asthma control was evaluated using a comparison of ACT scores, FeNO levels, and FEV_1 %predicted at study entry and at the 3-month visit using a paired *t* test. The one-way analysis of variance was used to compare changes in FeNO levels, ACT scores, and FEV_1 %predicted over 12 months in the stable and unstable asthmatic groups. Correlations of changes (Δ) and absolute levels between FeNO, ACT, and FEV_1 were evaluated using the Spearman rank correlation coefficient analysis. A receiver operating characteristic (ROC) curve was constructed and the area under the curve (AUC) for each parameter was compared with 0.5 to estimate the optimal cutoff levels for detecting stable control of asthma. Sensitivity, specificity, positive predicted value (PPV), negative predicted value (NPV), positive likelihood ratio (LR(+)), and negative likelihood ratio (LR(−)) were calculated. A two-tailed *p*-value of $<.05$ was considered significant.

RESULTS

Characteristic of Subjects

The study population consisted of 50 asthmatic patients with poor adherence (18 males and 32 females) (Table 1). The mean age was 55.4 years (range: 16–79 years) and the patients were nonsmokers or ex smokers with a <5 pack-year history. Thirty-three patients (66%) were atopic and the cohort consisted of 12 mild, 21 moderate, and 17 severe persistent asthmatic subjects based on symptoms and treatment intensity (13). All the subjects were treated with regular ICS.

TABLE 1.—Subject characteristics.

Characteristics	Data
Patients	50
Sex (male/female)	18/32
Mean age (y.o.)	55.4 (50.4–60.5)
Height (cm)	157.6 (154.8–160.4)
Weight (kg)	61.1 (56.8–65.4)
Atopic status	33 (66%)
Severity of asthma	Mild persistent: 12
	Moderate persistent: 21
	Severe persistent: 17
FEV_1 %predicted (%)	87.8 (81.8–93.9)
FEV_1/FVC (%)	72.4 (69.3–75.5)
FeNO level (ppb)	84.9 (69.6–100.1)
ACT score (point)	19.0 (18–20.1)
ICS dose (μg) BDP equivalent	1106 (957–1255)

Note: Data was expressed as mean (95% CI) or number of patients.
BDP; Beclomethasone dipropionate.

The Effect of Intensive Asthma Educational Program

Asthma treatment remained unchanged during the first 3 months. Following the educational program, a significant decrease in FeNO levels and increase in ACT scores were seen in the stable asthma group ($n = 42$) compared with the unstable asthma group ($n = 8$; $p < .001$; Figure 1A and B). There was no significant difference in FEV_1 %predicted (Figure 1C).

The Changes of FeNO Levels, ACT Scores, and FEV_1 %predicted over 12 Months

In 8 of 50 asthmatic subjects (16%), the ICS dose was increased or other antiasthmatic drugs were added due to poor asthma control. Asthma medications were decreased in 14 of 42 patients (33%) reflecting improved asthma control. Significant decreases in FeNO levels and increases in ACT score following asthma educational program remained consistent in the stable asthmatic group over the course of 12 months (Figure 1A and B).

Correlations between Absolute Levels of FeNO, ACT Scores, and FEV_1 %predicted at Each Visit

There were significant negative correlations between absolute levels of FeNO and ACT scores at the beginning of the study and at 3 months following asthma educational program ($r = -0.47$, $p = .001$; $r = -0.49$, $p = .002$, respectively) (Table 2). Negative weak correlations were found between absolute levels of FeNO and FEV_1 %predicted over the same period ($r = -0.26$, $p = .07$; $r = -0.39$, $p = .02$, respectively). However, these correlations were not present at 6 months (Table 2).

Correlations between Changes (Δ) in FeNO, ACT, and FEV_1 at Each Visit

Highly significant correlations were observed between the ΔFeNO, ΔACT, and ΔFEV_1 which were maintained over the course of 12 months ($p < .05$; Table 3).

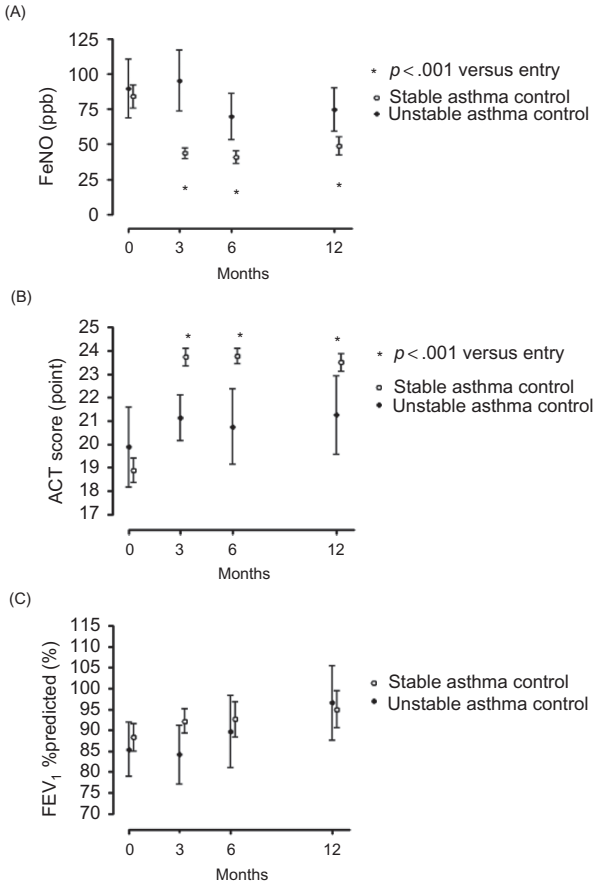

FIGURE 1.—Levels at time points indicated for FeNO levels (A), ACT score (B), and FEV_1 %predicted (C) between the subjects with or without achieving asthma control after the intensive educational interventions. Forty-two asthmatic subjects achieved good control whereas eight asthmatic subjects still had poor control.

Predictive Value of Each Parameter as a Marker to Achieve Stable Asthma Control

ROC curves were constructed to determine the cutoff levels for obtaining stable asthma control using absolute levels or changes in FeNO, ACT, and FEV_1. There were no useful parameters to discriminate subjects with stable asthma from those with unstable asthma using absolute levels (Figure 2A). However, an 18.6% decrease in ΔFeNO [AUC = 0.85, sensitivity of 80.0%, specificity of 83.3%, (LR) (+) of 4.8, LR(−) of 4.17, PPV of 92.3%, and NPV of 40%; $p = .007$] and a 3-point increase in ΔACT (AUC = 0.794, sensitivity of 73.3%, specificity of 87.5%, LR(+) of 5.86, LR(−) of 3.28, PPV of 95.7%, and NPV of 46.7%; $p = .012$) were noted.

DISCUSSION

This is a prospective, observational study to estimate the association of asthma educational program with asthma control. We have compared the absolute levels and changes in FeNO, ACT, and FEV_1 with asthma control. Our results indicate that intensive asthma educational program may be associated with improved asthma control.

TABLE 2.—Correlations between absolute levels of FeNO, ACT scores, and FEV_1 %predicted.

FeNO	Entry	*p*	3 months	*p*	6 months	*p*	12 months	*p*
ACT	−0.47*	.001	−0.49*	.002	−0.20	.249	−0.06	.742
FEV_1 % predicted	−0.26	.07	−0.39*	.019	−0.28	.110	−0.08	.649

Note: Spearman rank correlation coefficients were calculated and the data were expressed as *r* values.
*$p < .05$.

TABLE 3.—Correlations between changes of FeNO levels, ACT scores, and FEV_1.

ΔFeNO	3-entry	*p*	6-entry	*p*	12-entry	*p*
ΔACT	−0.76*	<.001	−0.61*	<.001	−0.53*	.005
ΔFEV_1	−0.53*	.001	−0.54*	.003	−0.42*	.03

Note: Spearman rank correlation coefficients were calculated and the data were expressed as *r* values.
*$p < .05$.

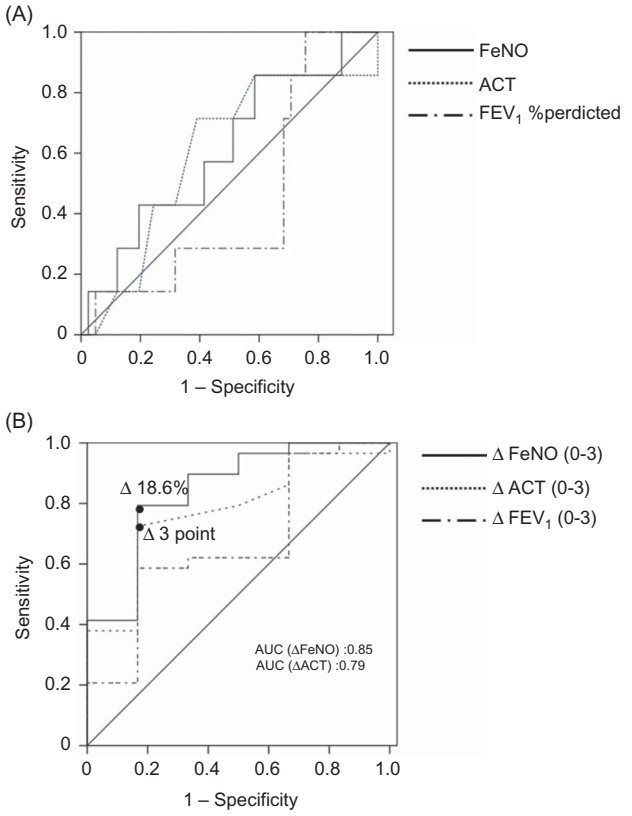

FIGURE 2.—ROC curves of absolute levels (A) and changes (B) of three parameters to determine good asthma control.

FeNO levels and ACT scores, but not FEV_1 %predicted, in the stable asthmatic group indicate improved asthma control over the course of 12 months. Changes (Δ) in FeNO levels, ACT scores, and FEV_1 had a stronger correlation, suggesting that multiple measurements and monitoring these changes may be a better way of predicting future asthma control.

Asthmatic subjects without self-management plans are often less adherent to prescribed medications and are more likely to be poorly controlled (4, 14). Many asthmatic patients do not use their inhaler devices correctly and asthmatic patients who are more adherent to therapy are less likely to suffer exacerbations (15, 16). Therefore, asthma education plays a vital role in improving and obtaining asthma control. The GINA guidelines emphasize the importance of asthma education, highlighting that educational intervention should be an integral part of interactions between patients and physicians (5). This interaction plays an important role by increasing motivation, skills, and confidence with subsequent benefits in asthma control. This results in improved asthma symptoms and lung function, and a decreased economic burden in terms of medical expenses (17).

This study found a significant increase in ACT scores and decrease in FeNO levels at 3 months following asthma educational program in the stable asthmatic group. No significant difference was seen in the unstable asthmatic group. Several previous studies have noted similar findings, reporting that asthma education is associated with improvements in symptoms (4, 18–21), lung function (18–21), and quality of life (QOL) (19, 21). However, while some of these studies noted an association between all three factors (19,21), others found that only symptoms correlated with an educational program (4, 18, 20). This difference may be explained by the content of educational program, study designs, and characteristics of the subjects. In our study, a significant reduction in FeNO levels could be seen in the stable asthma group. There is a paucity of information regarding the relationship between asthma education and FeNO. Dressel et al. noted a significant decrease in FeNO levels and improved asthma symptoms following an educational program in occupational asthmatic patients. In addition, they found that these improvements were not correlated with lung function (22). These findings and results from our study support the view that an intensive asthma educational program may be a valuable adjunct to achieve stable asthma control.

In this study, significant decreases in FeNO levels and increases in ACT scores remained consistent over the course of 12 months in stable asthmatic subjects. Symptom questionnaires and lung function tests are recommended in the GINA guidelines as markers of asthma control (5). Although they are easy to perform on almost all subjects, they are sometimes discordant with airway inflammation (23). Furthermore, symptoms are subjective and results may be influenced by patient's perception (24). Other markers such as sputum eosinophils and AHR have shown good correlations with asthma control (25, 26). However, these tests are time consuming,

invasive, require technical skill to process, and are, therefore, difficult to incorporate into standard clinical practice. Our results suggest that a combination of ACT and FeNO may be a quick and easy way to differentiate between stable and unstable asthmatic subjects.

In this study, we found a significant relationship between ΔFeNO, ΔACT, and ΔFEV_1 over the course of 12 months, whereas significant correlations between absolute levels were only observed at 3 months. FeNO reflects airway inflammation directly and correlates with conventional markers such as FEV_1, AHR, and sputum eosinophil counts (6–9). Several randomized control trials comparing the single or add-on effects of FeNO, with established parameters of asthma control, have only noted a limited benefit (27–30). These findings may reflect differences in study designs, the small number of subjects enrolled, variation in significance levels, and differences in asthma severity. Furthermore, individual patients may have their own personal best and range of FeNO levels, as seen with PEF measurements (31). FeNO levels in some asthmatic patients remain predominantly high regardless of an improvement in lung function and symptoms (29). Therefore, an individual cutoff level or proportional change, from baseline rather than an absolute cutoff level may be more useful.

The results of this study indicate that a 20% ΔFeNO decrease and a 3-point ΔACT increase may predict stable asthma control. To date, few studies have examined the relationship between ΔFeNO and other conventional parameters of asthma control. Jones et al. reported that change in FeNO level from baseline had a higher PPV compared with an absolute cutoff level in terms of asthma control (32). Michils et al. suggested that a single measurement of FeNO was not a good reflection of asthma control, particularly in patients on medium-to-high doses of ICS (33). A study looking at ΔACT found that a minimally important difference (MID) of three points is a significant predictor of asthma control (34). These studies support our observations that using changes in the levels of parameters may be better than using absolute levels for the evaluation of asthma control. We suggest that monitoring changes in FeNO levels and ACT scores in individual patients over time could be used as markers for achieving stable asthma control.

There are several limitations to this study. This is a prospective, observational study with a relatively small number of subjects tested. In addition, the educational intervention may be confounded by the Hawthorne effect, whereby subjects can subconsciously alter their behavior in a specific manner simply because of awareness of being educated and observed. Finally, subjects in this study may not represent the general asthmatic population. Only asthmatic subjects with poor adherence were recruited, and this may generate a positive selection bias. However, this is a group that we believe requires greater asthma education than their well-controlled counterparts.

In conclusion, this study suggests that comprehensive asthma educational program should be viewed as an important treatment option and considered prior to instigating changes in treatment. In addition, the changes (Δ) in FeNO and ACT levels could become useful surrogate markers for long-term asthma management. Further, large randomized studies are warranted to determine the influence of asthma education on disease control and the utility of changes (Δ) of FeNO and ACT.

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DECLARATION OF INTEREST

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REVIEW ARTICLE

Review Series: Non-Invasive Monitoring of Airway Inflammation in Asthma

Exhaled Nitric Oxide (FeNO) as a Non-Invasive Marker of Airway Inflammation

Mitsuru Munakata[1,2]

ABSTRACT
Nitric oxide (NO), previously very famous for being an environmental pollutant in the field of pulmonary medicine, is now known as the smallest, lightest, and most famed molecule to act as a biological messenger. Furthermore, recent basic researches have revealed the production mechanisms and physiological functions of nitric oxide in the lung, and clinical researches have been clarifying its tight relation to airway inflammation in asthma. On the bases of this knowledge, fractional nitric oxide (FeNO) has now been introduced as one of the most practical tools for the diagnosis and management of bronchial asthma.

KEY WORDS
asthma, cut-off, diagnosis, monitor, non-invasive

INTRODUCTION

Previously known as a toxic molecule listed as an environmental pollutant, nitric oxide (NO) is now known to be the smallest, lightest, and most famed molecule to act as a biological messenger in mammals. NO was first recognized as a physiologically important molecule in the manuscript written by Fruchgott and Zawadzki, entitled "The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine", published in Nature in 1980.[1] Initially, the factor released from vascular endothelial cells was named as endothelium derived relaxing factor (EDRF), and large number of scientists had been pursued the true feature of EDRF. In 1987, two groups led by Ignaro and by Moncada, independently discovered and reported that EDRF is a simple gaseous molecule called NO.[2,3] After the discovery, a large amount of studies in field of medicine revealed its many roles in a wide range of pathophysiological status including cardiovascular, immune, metabolic, and neurological diseases. For an ordinary person, it became a very famous physiological mediator of penile erection and opened the door for the treatment of impotence.[4] In 1992, NO was selected as Molecule of the Year for a startlingly simple molecule unites neuroscience, physiology and immunobiology and revised scientists' understanding of how cells communicate and defend themselves.[5] In 1998, the Nobel prize for Physiology or Medicine was awarded to Doctors, Furchgott R, Ignarro L, and Murad F.[6]

In the field of pulmonary medicine, physiological and pathological roles of NO in lung disease have also been investigated. Epithelium dependent inhibition of airway smooth muscle contraction and epithelium dependent relaxation of airway smooth muscle, similar effect of vascular endothelium to vascular smooth muscle, have been reported.[7,8] These phenomena also suggested the existence of epithelium derived relaxing factor (EpDRF).[9] Since NO is also confirmed to be a potent smooth muscle relaxing agent (Fig. 1),[10] several pharmacological studies verifying whether EpDRF is also NO were carried out and confirmed production of NO from airway epithelium.[11] In these processes, measurement systems for NO in exhaled air have been developed.[12] By applying such systems, exhaled NO have been measured in many pulmonary diseases and significant increase

[1]Fukushima Medical University Hospital and [2]Fukushima Medical University, Fukushima, Japan.
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Correspondence: Mitsuru Munakata, MD, PhD, President, Fukushima Medical University Hospital, Director, Fukushima Medical University, Fukushima 960-1295, Japan.
Email: munakata@fmu.ac.jp
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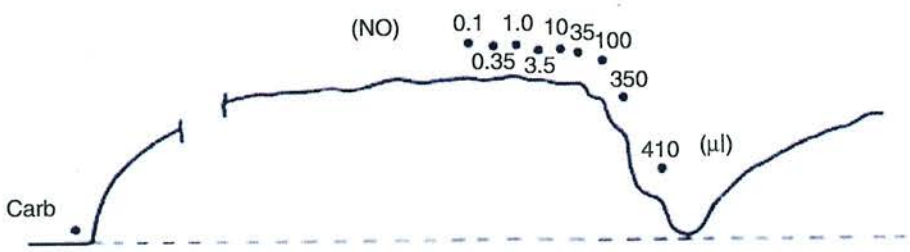

Fig. 1 Airway smooth muscle relaxation induced by nitric oxide (NO). Guinea pig tracheal strip was contracted by stimulated by Carbachol (Carb), then 0.1 to 410 ul of saturated solution of NO (estimated concentration 2 mM) was prepared, and was added to the tissue bath. Clear concentration-dependent relaxation was observed (Adapted from reference 10).

in exhaled NO of the patients with asthma became apparent.[13,14] Since the measurement is noninvasive and effort independent, exhaled NO has been much expected as a new tool for the diagnosis and management of asthma, and large efforts have been devoted on the clinical research.

PRODUCTION AND FUNCTION OF NO IN THE AIRWAYS

In the respiratory tract, NO is produced by a wide variety of cell types and is generated through conversion of L-arginine to L-citrulline by the action of nitric oxide synthase (NOS). Three isoforms of NOS are known: neuronal NOS (NOS I or nNOS), inducible NOS (NOS II or iNOS), and endothelial NOS (NOS III or eNOS).[15] Two isoforms, nNOS and eNOS, are expressed constitutively, but iNOS is not normally expressed in most tissues but is induced in several types of cells by pro-inflammatory cytokines.[16-18] All three types of NOS isoforms are known to be expressed in the lung. Endothelial NOS (eNOS) immunoreactivity is found in endothelial cells of pulmonary vessels. In addition, it is constitutively expressed in human bronchial epithelium and in type II pneumocytes.[19,20] NO produced by eNOS and released from endothelial cells in the pulmonary circulation is speculated to regulate vascular basal tone and counteract hypoxic vasoconstriction.[21] Neuronal NOS (nNOS) is expressed in human airway nerves, including those present in the airway smooth muscle,[22,23] and is estimated to be a major mediator of the inhibitory non-adrenergic non-cholinergic nervous (iNANC) system.[24] Co-localization with vasoactive intestinal polypeptide (VIP) is also observed.[25] Nerves distributed to submucosal glands also contain nNOS and NO regulates secretory function of the glands.[22,26] In the lung, iNOS (or NOS II) is known to be expressed in macrophages,[27] epithelial cells,[28-30] type II pneumocytes,[31,32] endothelial cells,[33] airway and vascular smooth muscle,[34] mast cells,[35] neutrophils,[36] chondrocytes,[23] and fibroblast.[37] Usually, iNOS in these cells is expressed when stimulated by endogenous mediators such as chemokines and cytokines, and by exogenous stimulant such as bacterial toxins, viruses, allergens, etc. Constitutive expression of iNOS in airway epithelial cells and rapid loss of its expression after removal of the epithelial cells from the in vivo airway environment were reported only in humans and suggest that the expression is dependent upon conditions and/or factors present in the airway.[38] iNOS derived NO is also speculated to regulate both airway smooth muscle tone and inflammatory responses.

MEASUREMENT OF EXHALED NO

The presence of NO in the exhaled air of humans was demonstrated by chemiluminescence, diazotization and mass spectrometry in 1991.[12] Thereafter, several measurement systems have been developed. The most commonly used system is chemiluminescence, and in Japan, two types are available; NOA280i (Severs, GE Analytical Instruments, Boulder, USA) and NA623N (Chest MI, Tokyo, Japan). These can be applied for both online and offline measurement of fractional exhaled NO (FeNO) in ppb. With these two types, we can get almost the same FeNO values.[39-42] It is known that FeNO is strongly affected by expiratory flow rate, FeNO levels in dead space air are high, and those in nasal cavities are very high. Therefore, several cautions should be kept in mind to get reasonable FeNO values. These cautions are included in American Thoracic Society (ATS) and European Respiratory Society (ERS) recommendations for the measurement of FeNO,[43,44] and following these recommendations is very important when FeNO is measured with these analyzers.

Another NO measurement system is the electrochemical method. The merits of the system are its compact size and portability. In Japan, two types of analyzers, NIOX MINO (Aerocrine, Stockholm, Sweden) and NObreath (Bedfont Scientific, Kent, UK), are available. There are some differences in FeNO levels measured by these analyzers when compared to a chemiluminescence analyzer. Differences of FeNO levels measured by different analyzers have been examined and conversion equations are also

available.[39-42] Several attempts to separate alveolar NO from airway NO have been performed by measuring FeNO at multiple exhalation flow rates,[45-48] however, it seems very difficult to apply such methods to clinical medicine and they might be useful as research tools.

NO AND AIRWAY INFLAMMATION IN ASTHMA

Asthma is a syndrome characterized by the presence of two physiological characteristics, reversible airflow limitation and airway hyperresponsiveness with respiratory symptoms. However, a recent advance in asthma research revealed the importance of airway inflammation existing behind these physiological characteristics. According to such progress in the concept of asthma, diagnosis and treatment strategies have been changing dramatically. One of the most prominent examples is the introduction of anti-inflammatory therapy with ICS, resulting in the pronounced the improvement in control and quality of life of the patients, and a dramatic reduction in the number of emergency room visit and deaths of the patients in Japan.

The recognition of the important roles of airway inflammation in asthma also promoted the development of new technology to evaluate airway inflammation in asthma. In these processes, a FeNO measurement was recognized as the most anticipated candidate. In early 1990s, significant increase in FeNO of ICS naïve patients with asthma, and decreased FeNO after ICS treatment was revealed,[13,14] suggesting the relationship between airway inflammation and elevation of FeNO. Hamid *et al.* applied immune-histochemical methods for bronchial biopsy specimen to investigate the presence of NOS in asthma. Immunoreactivity to iNOS was observed in the epithelium and some inflammatory cells in 22 of 23 biopsies from asthmatics, but in only 2 of 20 controls.[49] Guo *et al.* also examined iNOS expression by mRNA and protein assay and revealed that human airway epithelium has abundant expression of iNOS due to continuous transcriptional activation of the gene in vivo, and that individuals with asthma have higher than normal NO concentrations and increased iNOS mRNA and protein due to transcriptional regulation through activation of STAT1.[50] In addition, they revealed decreased expression of iNOS mRNA in asthmatics receiving ICS.[50] Redington *et al.* also examined iNOS expression in the airway epithelium and revealed enhanced expression in asthmatic subjects and regulation by corticosteroid treatment.[51]

The regulation mechanisms of iNOS expression are far from full elucidation. Although abundant expression of iNOS is observed in human airway epithelium, it will instantly disappear when these cells are cultured ex vivo,[38] suggesting the existence of in vivo factors or stimuli in the airway. In other types of cells, iNOS expression is only observed after stimulation with cytokines such as IFN-γ, IL-1β, and/or TNF-α.[52] Guo *et al.* revealed that a combination of IFN-γ/IL-4, which occurs naturally in lung epithelial lining fluid, leads to maintenance of iNOS expression in human airway epithelium through production of soluble mediators and stabilization of mRNA.[53] Alving and Malinovschi suggested a possible model of iNOS regulation of human airway from the results of recent studies[54] (Fig. 2). In healthy subjects (Fig. 2a), continuous expression of iNOS is maintained by IFN-γ, which normally exists in the respiratory tract. In this process, induction of unidentified soluble mediators by IFN-γ and the subsequent activation of the JAK/STA pathway are considered to be important.[55] In asthmatic airways (Fig. 2b), different regulation mechanisms are estimated (Fig. 2b). Initially, Th2 cytokines such as IL-4 and IL-13 were recognized to down-regulate iNOS expression.[56] However, several recent studies revealed that IL-4 and IL-13 actually induce iNOS expression in human airway epithelial cells in reasonable medium conditions through the STAT-6 pathway.[50,53,57,58]

There are several epidemiological evidences suggesting the relation between allergic airway inflammation and increased FeNO. Saito *et al.* examined FeNO levels, pulmonary function, and serum total and antigen specific IgE levels in 278 normal school children aged 10 to 12.[59] There are statistically significant positive correlations between FeNO and total IgE or mite specific IgE, and significant negative correlations between FeNO and FEV1/FVC. In addition, FeNO was determined by means of multiple logistic regression analysis to be the best predictor for recurrent wheeze, suggesting the relationship between allergic airway inflammation and FeNO levels. They also obtained the same results when they examined 280 normal adults aged 18 to 82 who received annual health check.[60] Moody *et al.* revealed that the increase in FeNO is associated with house dust mite sensitivity in asymptomatic subjects.[61] Additionally, some atopic subjects without symptoms and airway hyperresponsiveness have airway eosinophilc inflammation.[62] These findings support the tight relationship between increase of FeNO levels and allergic airway inflammation.

NO AS A DIAGNOSTIC TOOL OF ASTHMA

Traditionally, asthma has been characterized by respiratory symptoms such as cough, wheeze, and dyspnea, reversible airflow limitation, and non-specific airway hyperresponsiveness.[63,64] These are evaluated by pulmonary function tests before and after inhalation of bronchodilators such as β-adrenergic receptor agonists, and bronchial challenge test with bronchoconstrictors such as histamine and acetylcholine.[64] For the airway inflammation, trans-bronchial biopsy (TBB) and brochoalveolar lavage (BAL) under the fi-

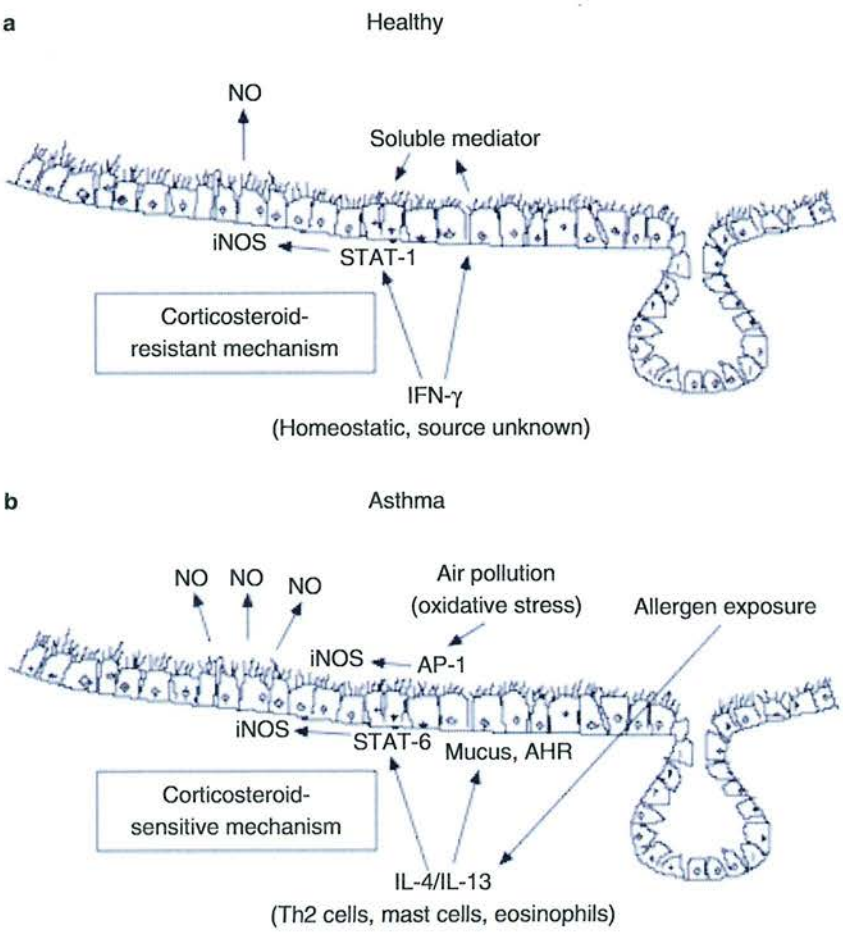

Fig. 2 A possible model of iNOS regulation of human airway. **a)** normal healthy airway, **b)** asthmatic airway (Adapted from reference 54).

beroptic bronchoscopic examination are applied. Recently, cell sorting of sputum induced by the inhalation of hypertonic saline has also been utilized. However, these methods are relatively invasive and sometimes induce asthma attacks. It is therefore difficult to apply widely in general clinical practice.

Because of the tight relation between FeNO and allergic airway inflammation and its non-invasiveness, attempts to use FeNO as a non-invasive tool for asthma diagnosis have been carried out in various clinical settings. Sato *et al.* examined 71 consecutive patients who visited out-patient clinics by complaining chronic cough continuing more than 3 weeks.[65] They examined FeNO, pulmonary function, serum IgE, methacholine airway responsiveness and induced sputum. FeNO is significantly higher in patients with asthma and cough variant asthma compared to other diseases including COPD and eosinophilic pneumonia without asthma, suggesting the usefulness of FeNO measurement in the diagnosis of asthma in patients with chronic cough. Cut-off value for FeNO for the diagnosis of asthma was 38.8 ppb with sensitivity of 79.2% and specificity of 91.3%. Similar results were also reported in patients with chronic cough by Chatkin *et al.*, Fujimura *et al.*, and Kowal *et al.*.[66-68]

For the patients with non-specific respiratory symptoms and suspected to having asthma, Smith *et al.* examined FeNO and sputum eosinophils in addition to conventional peak expiratory flow and spirometric parameters before and after bronchodilator treatment.[69] They observed the overall superiority of FeNO measurements and induced sputum analysis in the diagnosis of asthma compared with conventional tests. Dupont *et al.* also reported the usefulness of FeNO in 160 asthmatic patients diagnosed by the presence of reversible airflow obstruction (delta FEV1 > 12%) and histamine airway hyperresponsiveness (PC20 < 8.0 mg/ml).[70] Their cut-off level of FeNO at expiratory flow rate of 200 ml/s (FeNO200) was 16.0 ppb with the sensitivity of 69.4% and specificity of 90.0%. Fortuna *et al.* also reported that the diagnostic accuracy of FeNO measurement was superior to that of the standard diagnostic spirometry in patients with symptoms suggestive of asthma.[71] Fukuhara *et al.* recently reported the results of their prospective validation

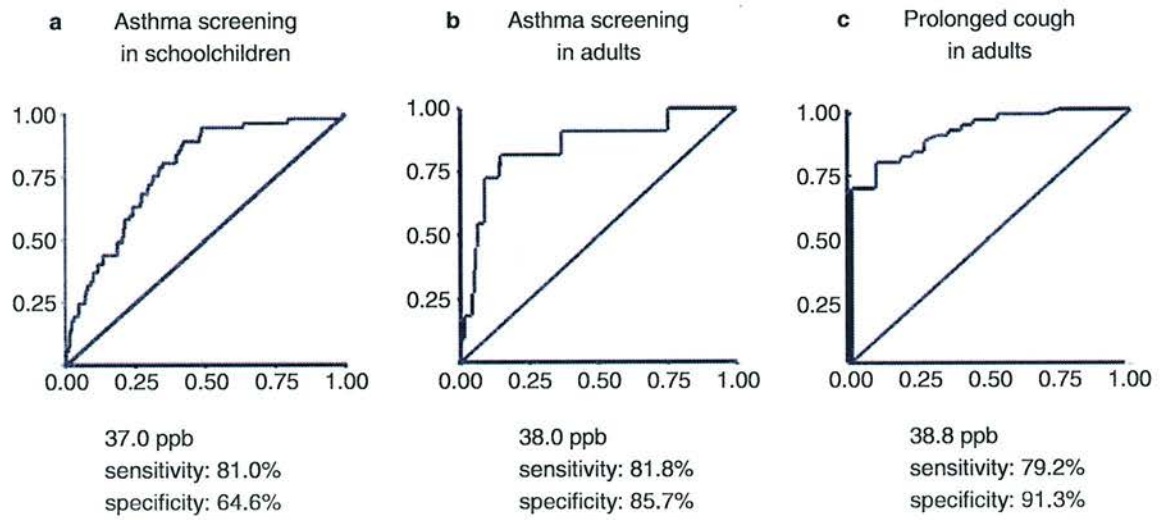

Fig. 3 The cut-off levels of fractional exhaled nitric oxide (FeNO) for diagnosing asthma obtained from 3 independent studies. **a)** for 277 school children, **b)** for 280 adult volunteers, **c)** for 71 patients with chronic cough (Adapted from reference 42).

study of asthma screening criteria based on subjective symptoms and FeNO at expiratory flow rate of 50 ml/sec ($FeNO_{50}$); i) recurrent cough, wheezeing, or dyspnea; ii) $FeNO_{50} > 40$ ppb, iii) exclusion of other lung diseases.[42] A cut-off value of 40 ppb was determined by the results of their 3 previous independent studies on school children, normal adults, and patients with prolonged cough (Fig. 3).[59,60,65] When compared to conventional asthma diagnostic criteria based on GINA and JGL guidelines,[63,64] FeNO based criteria showed good sensitivity, specificity, and a concordance rate (k) (78.6%, 89.5%, and 0.62, respectively). However, 9 of 42 patients were misdiagnosed as not having asthma by FeNO based criteria, and 7 of these 9 patients were non-atopic according to their IgE levels. From these results, they suggested that FeNO could be used as a tool for the non-invasive accurate diagnosis of asthma, particularly in atopic patients in daily clinical practice.

NO AS A CONTROL TOOL OF ASTHMA

Understanding that the most basic event in asthma is airway inflammation and the tight correlation between FeNO and airway inflammation has motivated the application of FeNO as a monitoring tool for asthma control. In 2005, Smith *et al.* did a single-blind, placebo-controlled trial of adult asthmatics to examine the usefulness of FeNO measurements for the adjustment of ICS doses. With the FeNO based strategy, the maintenance doses of ICS were significantly reduced without compromising asthma control compared to those with an algorithm based on conventional guidelines.[72] In the same year, Pijnenburg *et al.* did a randomized controlled trial to examine the usefulness of FeNO for the titration of ICS in atopic children with asthma. They also found that FeNO improved airway hyperresponsiveness and inflammation without elevating the ICS doses.[73] Following these studies, several consecutive studies were conducted and controversial results were reported. For the adult asthmatics, Shaw *et al.* reported that a treatment strategy based on FeNO measurement did not result in a large reduction in asthma exacerbation or in the total amount of ICS therapy used over 12 months, compared with the current asthma guideline.[74] But when the results were pored over precisely, in the initial several months, the required dose of ICS was higher in the FeNO based group compared to the control group, the dose gradually declined and the final daily dose of ICS was significantly lower in the FeNO based group compared to the control group (average; 557 ug/day and 895 ug/day, respectively, $p < 0.028$). More recently, Powel *et al.* carried out a double-blinded, randomized controlled trial to examined the usefulness of asthma management in pregnancy guided by FeNO. They revealed that asthma exacerbations during pregnancy can be significantly reduce with a validated FeNO-based treatment algorithm.[75] For adolescents and young adults, Szefler *et al.* did the largest randomized controlled trial to date with 780 patients with asthma to examine the usefulness of FeNO-based asthma management in addition to guideline-based treatment. They concluded that the addition of FeNO as an indicator of asthma control resulted in higher doses of ICS, without clinically important improvements in symptomatic asthma control.[76] But the subgroup analyses of the patients with a higher number of positive skin tests or those with serum nonspecific IgE higher than 460 kU/L revealed that the FeNO monitoring group had significantly fewer maximum days with symptoms in 2 weeks than that of the control group (0.84 and 0.51, p

< 0.024 and $p < 0.007$, respectively). As shown in the previous section, FeNO is suggested to be a very useful tool to monitor airway inflammation in atopic subjects. From these facts, it is suggested that application of FeNO as a tool for asthma control might be limited to the patients with atopic asthma.

Another point that should be borne in mind is the fact that the period of previously introduced studies were only up to 12 months. Sont *et al.* compared the difference in histopathologic outcome of asthma when using airway hyperesponsiveness as an additional guide to long-term treatment. They demonstrated that the examined strategy group showed a greater reduction in thickness of the subepithelial reticular layer compared to the reference strategy group, suggesting a role for the monitoring of airway hyperresponsiveness or other surrogate makers of inflammation in preventing airway remodeling.[77] Long-term usefulness of FeNO as a monitoring tool for asthma control, whether it could be helpful in prevention of airway remodeling or in decreasing annual decline in FEV1, needs to be examined.

FUTURE DIRECTIONS

As noted above, FeNO is a very useful diagnostic tool and control monitoring maker of asthma. Usefulness of FeNO in asthma management is probably better than spirogram, induced sputum, and AHR test, because of its non-invasiveness, effort independency, measurement simplicity, and reproducibility. Although the FeNO analyzer has not been approved as a medical device, it will be widely used as a convenient clinical tool for asthma management in the near future in Japan.

The FeNO analyzer has been used as a clinical research tools and wide application of FeNO in the clinical setting revealed the issues that should be solved before its clinical application. Now, many researchers recognize that there is a minor population of subjects with very high levels of FeNO without respiratory symptoms, and that there are asthmatics with all asthmatic symptoms but with normal FeNO levels. The mechanisms behind these phenomena are unclear, meaning that the production mechanisms of NO in the airway and lung parenchyma have not been thoroughly clarified. Additional point is that, although the ATS/ERS guideline was established to standardize the FeNO measurements,[43] there are variations in FeNO values measured by different analyzers.[41,42] It also affects the determination FeNO cut-off levels for the diagnosis and control of asthma. In addition, to be used widely in the clinical practice, the development of handier, more accurate, less expensive measurement systems is required. Furthermore, not only more practical studies but also more basic studies will be warranted.

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Functional assessment of the mutational effects of human *IRAK4* and *MyD88* genes

Takahiro Yamamoto[a,1], Naotaka Tsutsumi[b,1], Hidehito Tochio[b,**], Hidenori Ohnishi[a,*], Kazuo Kubota[a], Zenichiro Kato[a], Masahiro Shirakawa[b,c], Naomi Kondo[a,d]

[a] Department of Pediatrics, Graduate School of Medicine, Gifu University, 1-1 Yanagido, Gifu 501-1194, Japan
[b] Department of Molecular Engineering, Graduate School of Engineering, Kyoto University, Katsura, Nishikyo-ku, Kyoto 615-8510, Japan
[c] Core Research for Evolutional Science and Technology, Japan Science and Technology Corporation, 4-1-8 Hon-cho, Kawaguchi, Saitama 332-0012, Japan
[d] Heisei College of Health Sciences, 180 Kurono, Gifu 501-1131, Japan

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ABSTRACT

Human interleukin-1 receptor-associated kinase 4 (IRAK4) deficiency and myeloid differentiating factor 88 (MyD88) deficiency syndromes are two primary immune-deficiency disorders with innate immune defects. Although new genetic variations of *IRAK4* and *MyD88* have recently been deposited in the single nucleotide polymorphism (SNP) database, the clinical significance of these variants has not yet been established. Therefore, it is important to establish methods for assessing the association of each gene variation with human diseases. Because cell-based assays, western blotting and an NF-κB reporter gene assay, showed no difference in protein expression and NF-κB activity between R12C and wild-type IRAK4, we examined protein–protein interactions of purified recombinant IRAK4 and MyD88 proteins by analytical gel filtration and NMR titration. We found that the variant of IRAK4, R12C, as well as R20W, located in the death domain of IRAK4 and regarded as a SNP, caused a loss of interaction with MyD88. Our studies suggest that not only the loss of protein expression but also the defect of Myddosome formation could cause IRAK4 and MyD88 deficiency syndromes. Moreover a combination of *in vitro* functional assays is effective for confirming the pathogenicity of mutants found in IRAK4 and MyD88-deficiency patients.

1. Introduction

Interleukin-1 receptor-associated kinase (IRAK) 4 is the one of the essential molecules of the Toll/interleukin-1 receptor signaling pathway (Suzuki et al., 2002). In this pathway, ligand-induced hetero- or homodimerization of receptors recruits the Toll/interleukin-1 receptor homology domain (TIR domain) containing adaptor oligomers. One of these adaptors, MyD88, then binds IRAK4 (Burns et al., 2003). Recently, defects in the innate immune system have been shown to cause newly categorized human primary immune-deficiency syndromes (Al-Herz et al., 2011) such as human IRAK4 deficiency (Picard et al., 2003).

In affected IRAK4 deficient patients, invasive infections such as bacterial meningitis, sepsis, arthritis, or osteomyelitis are caused by *Streptococcus pneumonia*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* (Picard et al., 2010). Human MyD88 deficiency (von Bernuth et al., 2008) has remarkably similar clinical features to human IRAK4 deficiency. Interestingly, the life-threatening infections in IRAK4 or MyD88 deficient patients first occur during early infancy, but their frequency and severity reduce after the teenage years (Picard et al., 2011). Therefore, it is necessary for them to be diagnosed quickly.

IRAK4 and MyD88 proteins both consist of two major functional domains. In IRAK4, the death domain (DD) interacts with MyD88, while the kinase domain phosphorylates downstream signaling factors such as IRAK1, IRAK2, and subsequently causes activation of TNF Receptor Associated Factor 6 (TRAF6). In MyD88, both the DD and TIR domains interact in homotypic binding to similar domain structures. The domain–domain interactions are critical for these signaling pathways. IRAK4 and MyD88 form a hetero-oligomeric signaling complex via a shared DD, so-called Myddosome (Motshwene et al., 2009). Appropriate Myddosome

Abbreviations: DD, death domain; HEK, human embryonic kidney; ID, internal domain; IRAK, Interleukin-1 receptor-associated kinase; MyD88, myeloid differentiating factor 88; Mal, MyD88 adaptor-like; NMR, nuclear magnetic resonance; ELISA, enzyme-linked immunosorbent assay; TIR domain, Toll/interleukin-1 receptor homology domain; WT, wild type; SNP, single nucleotide polymorphism; IRAK4-DD, death domain of IRAK4; IRAK4-DD + ID, death domain and internal domain of IRAK4; MyD88-DD, death domain of MyD88; MyD88-DD + ID, death domain and internal domain of MyD88; MyD88-TIR, TIR domain of MyD88; Mal-TIR, TIR domain of Mal; TRAF, TNF receptor associated factor.

* Corresponding author. Tel.: +81 58 230 6386; fax: +81 58 230 6387.
** Corresponding author. Tel.: +81 75 383 2536; fax: +81 75 383 2541.
E-mail addresses: tochio@moleng.kyoto-u.ac.jp (H. Tochio), ohnishih@gifu-u.ac.jp (H. Ohnishi).
[1] Contributed equally as first authors.

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formation can induce activation of the downstream signaling pathway, which eventually leads to the activation of NF-κB and activator protein 1 (AP-1).

Most previously identified causative mutations of human IRAK4 deficiency are nonsense or frame shift mutations that create early stop codons (Cardenes et al., 2006; Davidson et al., 2006; Enders et al., 2004; Krause et al., 2009; Ku et al., 2007; Medvedev et al., 2003; Picard et al., 2010; Takada et al., 2006; Yoshikawa et al., 2010), however, three missense mutations (M1V, R12C, and G298D) have been reported (Bouma et al., 2009; de Beaucoudrey et al., 2008; Hoarau et al., 2007). In human MyD88 deficiency, one nonsense mutation (E53X) and three missense mutations (E52del, L93P, and R196C) were reported as causative mutations (Conway et al., 2010; von Bernuth et al., 2008). Recently, new gene variations of *IRAK4* and *MyD88* have been deposited in the single nucleotide polymorphism (SNP) database following next-generation DNA sequencing, but the significance of these variants has not been evaluated. It is therefore important to establish methods to determine the association of gene variations with human diseases. For example, about MyD88, previous attempts have used western blotting, reporter gene assays, immunoprecipitation, and size exclusion chromatography of recombinant proteins to show that the SNPs MyD88 S34Y and R98C were loss-of-function variants (George et al., 2011), while another study used immunofluorescence to determine that S34Y fails to interact with IRAK4 (Nagpal et al., 2011).

Methods to detect the impaired responses to the Toll/interleukin-1 receptor agonists, such as enzyme-linked immunosorbent assay (ELISA) and flow-cytometry, are useful for rapid screening of innate immune deficiency syndromes (Davidson et al., 2006; Ohnishi et al., 2012a; Takada et al., 2006; von Bernuth et al., 2006). However, no *in vitro* method to assess the pathogenicity of novel variants of human *IRAK4*, *MyD88* and the other possible signaling components has been established. Therefore, when novel gene variants are found in that possible cases of IRAK4 or MyD88 deficiency syndromes, it is difficult to analyze the pathogenetic significance of these variants. In this study, we used a cell-based assay as well as *in vitro* protein-interaction analyses to show that IRAK4 R12C and R20W caused a loss of interaction with MyD88. This suggested that not only the loss of full-length IRAK4 and MyD88 protein expression but also the loss of Myddosome formation could cause IRAK4 and MyD88 deficiency syndromes.

2. Materials and methods

2.1. Cell culture

Human embryonic kidney (HEK) 293T cells were cultured in high glucose-containing DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated FBS (Sigma–Aldrich, St. Louis, MO), penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2.

2.2. Vector preparations

cDNA encoding full-length IRAK4 (amino acid residues 1–460) or the DD and the internal domain (ID) of IRAK4 (IRAK4-DD + ID, amino acid residues 1–150) were tagged at the N terminus with a FLAG-epitope and cloned into the plasmid vector pcDNA3.1+ (Invitrogen). M1V was tagged at the C terminus because of a substitution of the start codon, and wild type (WT) tagged at the C terminus was prepared as a reference. Similarly, cDNA encoding full-length MyD88 (amino acid residues 1–296) or the TIR domain of MyD88 (MyD88-TIR, amino acid residues 148–296) tagged at the N terminus with a myc-epitope were cloned into the plasmid vector pcDNA3.1+ (Nada et al., 2012; Ohnishi et al., 2009). IRAK4 mutants and SNPs taken from dbSNP135 of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/snp) were generated using the GeneEditor *in vitro* Site-Directed Mutagenesis System (Promega, Fitchburg, WI). The pUNO hIL1R1(mb) vector (InvivoGen, San Diego, CA) was purchased and cDNA encoding IL-1RAcP and IL-18RAcPL were cloned into the plasmid vector pcDNA3.1+. The pGL4.32[luc2P/NF-κB-RE/Hygro] vector, used as an NF-κB luciferase reporter vector, and the pGL4.70[hRluc] vector, used as an internal control Renilla luciferase reporter vector, were purchased from Promega.

2.3. Western blot analysis

To detect protein expression, HEK293T cells were seeded on six-well plates at a density of 2×10^5/ml and transfected with 1 μg of expression plasmids of FLAG-tagged IRAK4 full length, FLAG-tagged IRAK4-DD, and myc-tagged MyD88 full length using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 48 h incubation, cells were harvested, washed with PBS, and lysed using CytoBuster Protein Extraction Reagent (Novagen, Darmstadt, Germany) containing a protease inhibitor mix (Roche Applied Science, Indianapolis, IN). All extracts were adjusted to contain equal amounts of total cellular proteins, as determined using the Bradford method. Supernatants and whole cell lysates were separated by electrophoresis on SDS polyacrylamide gels and transferred to nitrocellulose membranes using an iBlot Gel Transfer Device (Invitrogen). Membranes were blocked for 1 h in 5% BSA in TBST (pH 8.0, 10 mM Tris buffer containing 0.15 M NaCl and 0.1% Tween 20), then incubated at room temperature for 2 h with an anti-FLAG M2 monoclonal antibody (Sigma–Aldrich), anti-myc antibody (Invitrogen), or anti-β-actin antibody (Sigma–Aldrich) followed by incubation with anti-mouse IgG HRP conjugate (Promega) at room temperature for 30 min. Detection was performed using the ECL Chemiluminescent Substrate Reagent Kit (Invitrogen) and LightCapture system AE6970CP (ATTO, Tokyo, Japan).

2.4. NF-κB reporter gene activity

For the functional assessment of IRAK4, HEK293T cells, HEK293-hTLR1/2 cells (InvivoGen), HEK293-hTLR4-MD2-CD14 cells (InvivoGen) and HEK293-hTLR5 cells (InvivoGen) were transfected with NF-κB luciferase reporter vector, Renilla luciferase reporter vector, pcDNA3.1+ empty vector or pcDNA3.1+ FLAG-IRAK4 WT using Lipofectamine 2000. After transfection, cells were incubated for 24 h then stimulated with recombinant IL-1β (10 ng/ml) prepared as previously described (Wang et al., 2010), Pam3CSK4 (10 ng/ml, InvivoGen), LPS (10 ng/ml, Sigma–Aldrich), and recFLA-ST (10 ng/ml, InvivoGen) for 6 h. In a similar way, HEK293T cells were transfected as described above and with IL-18RAcPL, and stimulated with recombinant IL-18 (50 ng/ml) prepared as previously described (Kato et al., 2003; Li et al., 2003) for 6 h. Luciferase reporter gene activities were analyzed using the Dual-Luciferase Reporter Assay System (Promega). Similarly, HEK293T cells were transfected with pUNO-hIL1R1 vector, pcDNA3.1+ IL-1RAcP vector, NF-κB luciferase reporter vector, Renilla luciferase reporter vector, pcDNA3.1+ empty vector or pcDNA3.1+ FLAG-IRAK4 WT or variants, as described above. After transfection, cells were incubated for 24 h and luciferase reporter gene activities were analyzed.

For the functional assessment of MyD88, HEK293T cells were transfected with NF-κB luciferase reporter vector, Renilla luciferase reporter vector, and different amounts of pcDNA3.1+ myc-MyD88

WT or variants (5, 15 or 50 ng). The amounts of transfected plasmid were adjusted to an equal amount with pcDNA3.1+ empty vector. After transfection, cells were incubated for 24 h. To compare the dominant negative effect of MyD88, pcDNA3.1+ myc-MyD88-TIR WT or variants (5, 15 or 50 ng), pcDNA3.1+ IL-18RAcPL, NF-κB luciferase reporter vector, and Renilla luciferase reporter vector were co-transfected, and then, cells were incubated for 24 h and stimulated with/without IL-18 (10 ng/ml) for 6 h. Luciferase reporter gene activities were analyzed as described above.

The NF-κB activation of each condition was assessed in at least three independent experiments. The statistical significance of the differences in luciferase activities was determined using one-way ANOVA with Bonferroni's post-hoc test. The statistical significance was defined as $P < 0.05$.

2.5. Protein preparation

The portion of the human *IRAK4* gene encoding the DD + ID (amino acid residues 1–150) and the human *MyD88* gene encoding the DD and the ID (MyD88-DD + ID, amino acid residues 1–152) were cloned into vector pGEX-6P-1 (GE Healthcare, Little Chalfont, UK). These vectors were transformed into *Escherichia coli* BL-21 (DE3) (Novagen). IRAK4-DD + ID variants and MyD88-DD + ID, which were expressed as GST fusion proteins, were first purified by glutathione Sepharose 4B FF (GE Healthcare) affinity chromatography, and the GST-tag was removed by digestion with PreScission protease (GE Healthcare). Subsequently, the DD + IDs were purified by anion exchange chromatography (Q-Sepharose column; GE Healthcare) and gel filtration (Superdex 75 HR 26/60 column; GE Healthcare). Using a similar purification protocol, ^{1}H–^{15}N-labeled MyD88-DD + ID was prepared. All nuclear magnetic resonance (NMR) samples were uniformly ^{15}N-labeled and prepared in 210 μl solutions of H_2O/D_2O (95%/5%) containing 20 mM potassium phosphate buffer at pH 6.0 with 10 mM DTT. The portions of the human TIR domains of MyD88 WT and its mutants (M178I, R196C) and MyD88 adaptor-like (Mal) (Mal-TIR, amino acid residues 75–235) were cloned into pGEX-5X-1 and pGEX5X-3 vectors (GE Healthcare), respectively. The proteins were purified as previously described (Ohnishi et al., 2009).

2.6. Analytical gel filtration

Molecular masses of the purified recombinant proteins IRAK4-DD + ID and MyD88-DD + ID were evaluated by size exclusion chromatography. Gel filtration analysis was performed using a Superdex-200 10/300 GL column (GE Healthcare) attached to an AKTA purifier (GE Healthcare) at 10 °C. The column was equilibrated with 20 mM HEPES buffer (pH 7.0), 100 mM KCl, 10 mM DTT and 1 mM EDTA. The column was calibrated using a gel filtration standard kit (Bio-Rad, Hercules, CA). A total of 100 μl of 100 μM IRAK4-DD + ID and 100 μM MyD88-DD + ID proteins was applied to the gel filtration column. Protein elution was monitored by UV absorption at 280 nm. The molecular masses of these proteins were estimated using a calibration curve.

2.7. GST pull-down assays

GST-fusion proteins of MyD88-TIR and purified proteins of the TIR domain of Mal were incubated with Glutathione Sepharose 4B (GE Healthcare) in binding buffers (20 mM potassium phosphate buffer (pH 6.0), 0.1 mM EDTA, 10 mM DTT, and 0.2% Triton X-100) for 16 h. After four wash steps using 20 mM potassium phosphate buffer (pH 6.0), 100 mM KCl, 0.1 mM EDTA, 10 mM DTT, and 0.2% Triton X-100, the resin was analyzed by SDS-PAGE and Coomassie Brilliant Blue staining.

2.8. NMR titration

An aliquot of 0.25 equivalent amounts of non-labeled IRAK4 WT or its variants was added to 210 μl of 50 μM ^{15}N-labeled MyD88-DD + ID, with the exception for R20W, up to its 2.0 equivalent amounts. For the titration with R20W IRAK4-DD + ID, 25 μM MyD88-DD + ID was used. The samples were in 20 mM potassium phosphate (pH 6.0) and 10 mM DTT in H2O/D2O (95%/5%). At each titration point, 2D ^{1}H–^{15}N SOFAST-HMQC spectra were recorded at 298 K on Bruker Avance II 700 MHz spectrometer equipped with cryogenic probes. The 2D spectra were processed using NMRPipe (Delaglio et al., 1995) and analyzed using the Sparky (Goddard and Kneller, 1999) analysis software, whereas 1D projections were generated using Bruker TopSpin 3.1. A well-resolved NMR signal derived from a Trp sidechain aromatic ^{1}H–^{15}N pair in the projection was selected (supplementary Fig. S1B) at each titration point, and then intensities were normalized with the intensity of the corresponding NMR signal of ^{15}N MyD88-DD + ID recorded in the absence of IRAK4-DD + ID (Ohnishi et al., 2009). The normalized intensities were plotted as a function of the equivalent molar amounts of the titrant.

2.9. Protein stability assay

HEK293T cells were seeded on six-well plates at a density of 2×10^5/ml and transfected with 1 μg of expression plasmids Flag-tagged IRAK4-DD using Lipofectamine 2000. After 48 h, cells were treated with 25 μM cycloheximide for 0, 24, 48, and 72 h (Fukao et al., 1999). Cellular extracts were prepared in CytoBuster Protein Extraction Reagent containing complete protease inhibitor mix. All extracts were adjusted to contain equal amounts of total cellular proteins, as determined using the Bradford method. Western blot analysis with anti-FLAG antibody was carried out using standard protocols as described above.

3. Results

3.1. Cell-based assays of IRAK4 variants

3.1.1. Protein expression of IRAK4

To functionally characterize the genetic variants of *IRAK4*, FLAG-tagged full-length expression constructs corresponding to the loss-of-function mutations that were previously reported as pathogenic mutations ('*IRAK4* mutants') and nonsynonymous SNPs ('*IRAK4* SNPs') were generated. In this study, we selected the six IRAK4 mutants (M1V, a missense mutation of start codon reported in Slovenia (de Beaucoudrey et al., 2008); R12C, a missense mutation reported in France and located in the DD (Hoarau et al., 2007); c.118insA and R183X, mutations reported in Japan that include a frame shift mutation (Picard et al., 2010; Takada et al., 2006); Q293X, the most common mutation in Europe (Picard et al., 2010); G298D, a missense mutation reported in UK and located in the kinase domain (Bouma et al., 2009)) and five SNPs in the DD (Fig. 1A). Our five SNPs were all within the DD as we focused on the interaction between IRAK4 and MyD88.

No protein expression could be detected of the three *IRAK4* mutations M1V, Q293X, and c.118insA. R183X expressed a smaller protein than WT, while G298D protein expression levels were decreased (Fig. 1B). The expression of R12C was comparable with WT as were expression levels of all SNPs (Fig. 1C).

3.1.2. Inhibition of NF-κB activation of IRAK4

Next, *IRAK4* variants were tested for NF-κB reporter gene activity using a dual luciferase assay system. As Medvedev et al.

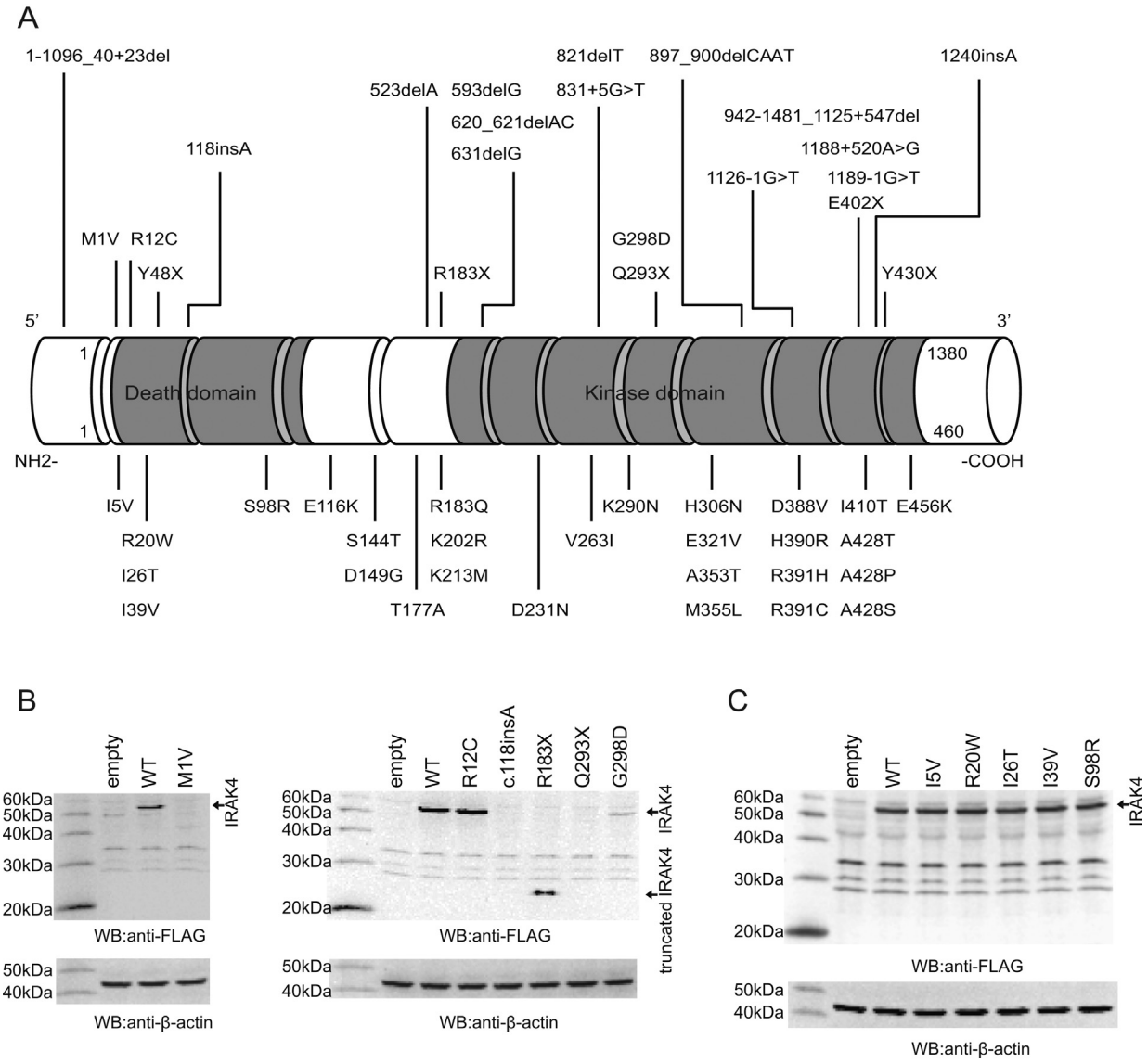

Fig. 1. Protein expression of *IRAK4* variants. (A) Schematic representation of *IRAK4* showing all identified mutations and nonsynonymous SNPs. *IRAK4* consists of 12 exons and the protein is composed of an N-terminal death domain and C-terminal kinase domain. Mutations are annotated at the upper side of this schema, and SNPs at the lower. (B) Expression levels of *IRAK4* mutants in HEK293T cells. Protein expression of M1V, Q293X, and c.118insA could not be detected. R183X expressed a truncated protein and the expression level of G298D was low. (C) Expression levels of *IRAK4* SNPs in HEK293T cells. All SNPs examined in this study expressed protein at the same level as WT.

(2003) previously reported that IL-1β-induced NF-κB activation was inhibited by overexpressed IRAK4 in HEK293T cells, we repeated their method with five different HEK293 cell lines and their appropriate ligands to compare the inhibition of NF-κB activation (Fig. 2). IL-1β, IL-18 and the ligands of TLR1/2, TLR4, and TLR5-induced NF-κB activations were not significantly inhibited by overexpressed IRAK4 (Fig. 2A–E), but the NF-κB activity enhanced by both transiently transfected IL-1R1 and IL-1RAcP could be significantly inhibited by overexpressed IRAK4 (Fig. 2F).

This system was used to compare *IRAK4* variants. Fig. 2G shows that NF-κB activation of the mutants c.118insA, R183X, Q293X, and G298D was less inhibited than WT. However, R12C showed a similar activity level to WT, although this mutation was previously reported to be a loss-of-function mutant in a human IRAK4 deficiency patient (Hoarau et al., 2007). Fig. 2H shows that all *IRAK4* SNPs significantly inhibited NF-κB activity to the same extent as WT. Only R20W showed a stronger inhibitory effect.

3.2. Cell-based assays of MyD88 variants

3.2.1. Protein expression of MyD88

We analyzed four previously reported loss-of-function mutations of MyD88: E52del, E53X, and L93P located in the DD, and R196C located in the TIR domain, as well as three SNPs: S34Y and R98C (loss-of-function variants (George et al., 2011; Nagpal et al., 2011) located in the DD, and M178I in the TIR domain (Fig. 3A). To functionally characterize the *MyD88* genetic variants, myc-tagged full-length expression constructs corresponding to the loss-of-function mutations (‘*MyD88* mutants’) and nonsynonymous SNPs (‘*MyD88* SNPs’) were generated. No protein expression was detected of S34Y and E53X, while expression of E52del and L93P was very low. On the other hand, expression levels of R98C, M178I, and R196C were similar to WT (Fig. 3B).

3.2.2. NF-κB activity of MyD88

Next, we assessed the abilities of *MyD88* variants to activate the NF-κB signaling pathway using a dual luciferase assay system in

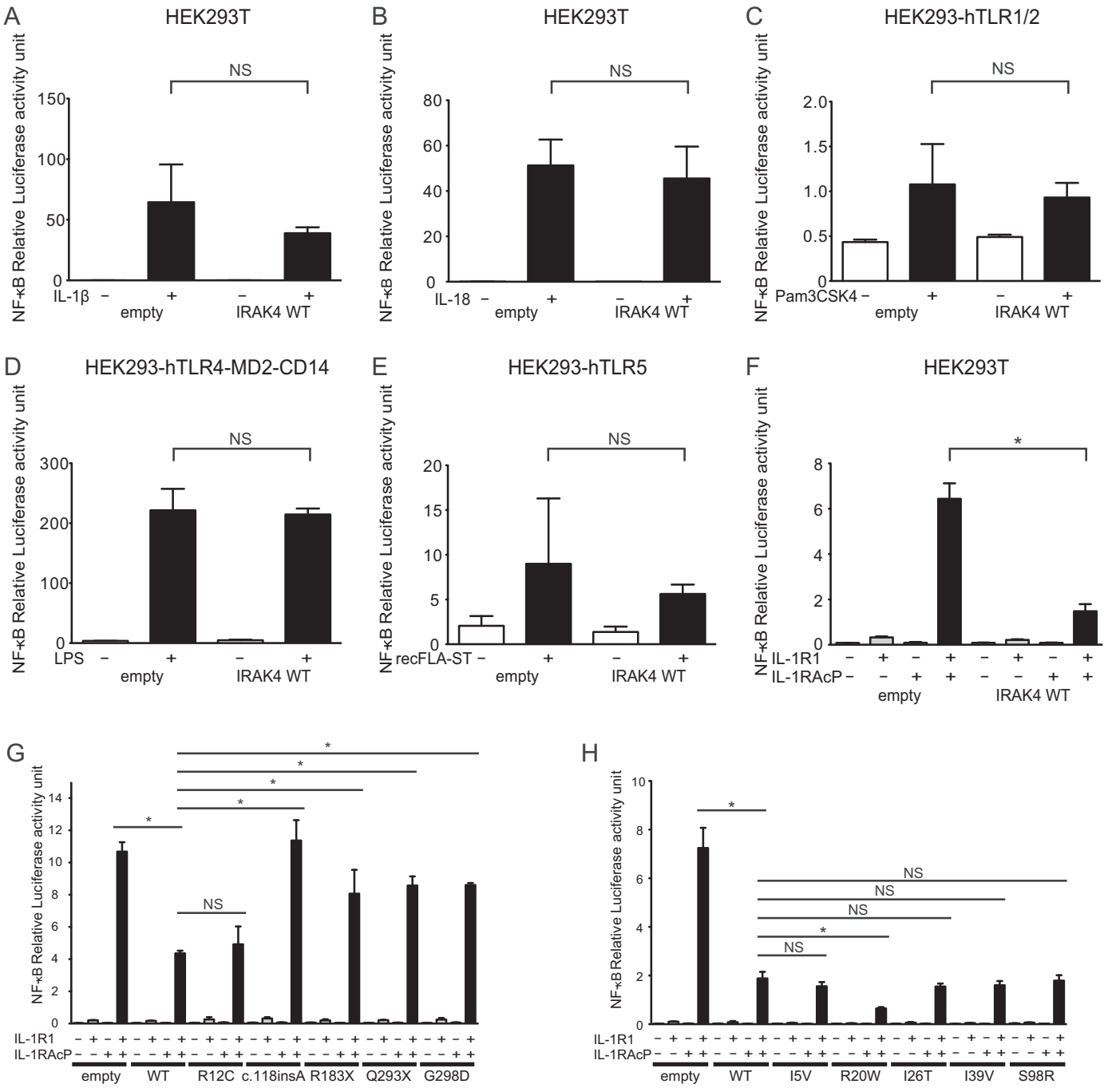

Fig. 2. Cell-based NF-κB activity assays of *IRAK4* variants. (A-F) The inhibition of NF-κB activation in five different cell lines by IRAK4. IL-1β- (10 ng/ml), IL-18- (50 ng/ml), Pam3CSK4- (10 ng/ml), LPS- (10 ng/ml) and recFLA-ST- (10 ng/ml) induced NF-κB activations was not significantly inhibited by overexpressed IRAK4 WT in HEK293T cells (A, B, F), HEK293-hTLR1/2 cells (C), HEK293-hTLR4-MD2-CD14 cells (D), and HEK293-hTLR5 cells (E). Transient transfection of both IL-1R1 and IL-1RAcP significantly enhanced NF-κB activity in HEK293T cells, which could be significantly inhibited by overexpressed IRAK4. (G and H) The inhibition of NF-κB activation by *IRAK4* variants in HEK293T cells. The inhibition by mutants c.118insA, R183X, Q293X, and G298D of NF-κB activation induced by co-transfection both IL-1R1 and IL-1RAcP was significantly less than in WT. R12C showed a similar inhibition level to WT. All *IRAK4* SNPs significantly inhibited NF-κB activity as well as WT. Only R20W showed a stronger inhibition of NF-κB activity than WT. Data represent the mean ± SD of a representative experiment ($n = 3$). Asterisk indicates a statistically significant difference between WT and the others. NS means "not-significant".

HEK293T cells. Fig. 3C shows that overexpression of S34Y, E52del, E53X, L93P, R98C, and R196C resulted in lower NF-κB activation than that of WT. We previously reported that the truncated MyD88 lacking a DD (MyD88-TIR) inhibited IL-18-stimulated NF-κB activation by means of a dominant negative effect (Ohnishi et al., 2012b). Therefore, in the present study, we examined NF-κB activation inhibition from a dominant-negative effect in HEK293T cells transiently co-transfected with IL-18RAcP and MyD88-TIR WT, or M178I and R196C (Fig. 3D). MyD88-TIR M178I inhibited NF-κB activation to a similar level as WT, but MyD88-TIR R196C was compromised in its effect to inhibit NF-κB activation.

3.3. GST pull-down assay of MyD88-TIR to Mal-TIR

MyD88 interacts with Mal via a shared TIR domain and activates a downstream signaling pathway. To analyze the mutations

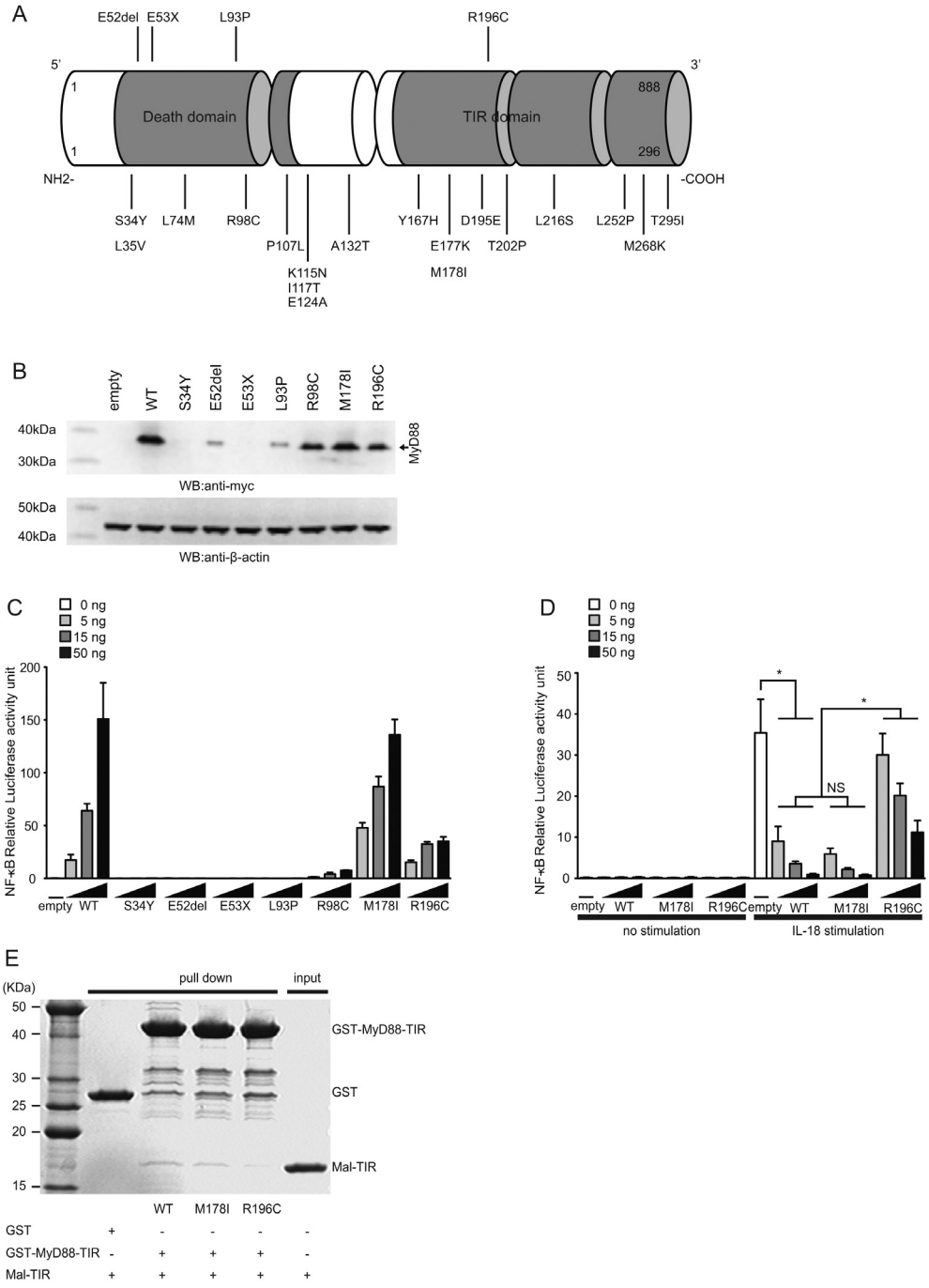

Fig. 3. Cell-based assays of *MyD88* variants. (A) Schematic representation of *MyD88* with all identified mutations and nonsynonymous SNPs. *MyD88* consists of five exons and the protein is composed of an N terminal death domain and C-terminal TIR domain. Mutations are annotated at the upper side of this schema, and SNPs at the lower. (B) Expression levels of *MyD88* variants in HEK293T cells. The protein expression of S34Y and E53X could not be detected, and that of E52del and L93P was very low. The expression levels of R98C, M178I, and R196C were similar to that of WT. (C) NF-κB reporter gene activities of *MyD88* variants in HEK293T cells. S34Y, E52del, E53X, L93P, R98C, and R196C were compromised in the ability to enhance NF-κB activation, with the exception of M178I. (D) Dominant negative inhibitory effects of MyD88-TIR variants in HEK293T cells. MyD88-TIR R196C failed to inhibit NF-κB activation. Data represent the mean ± SD of a representative experiment ($n = 3$). All data were compared at each transfection dose. Asterisk indicates statistically significant difference between WT and the others. NS means "not-significant". (E) Binding assays for WT or mutant MyD88-TIRs with Mal-TIR using GST pull-down assays. M178I interacted with Mal-TIR as well as WT. R196C showed a reduced interaction with Mal-TIR.

located in the TIR domain of MyD88, we carried out a GST pull-down assay of MyD88-TIR and Mal-TIR (Fig. 3E). As we reported previously (Nada et al., 2012; Ohnishi et al., 2009), MyD88-TIR R196C showed a significant decrease in its ability to directly bind to Mal-TIR, while MyD88-TIR M178I interacted with Mal-TIR as well as WT.

3.4. Analytical gel filtration of IRAK4 and MyD88

To compare the interaction between MyD88 and IRAK4, analytical gel filtration was carried out. We purified MyD88-DD + IDs and IRAK4-DD + IDs recombinant proteins, and analyzed the elution profiles of mixtures of MyD88 WT or mutants in the presence

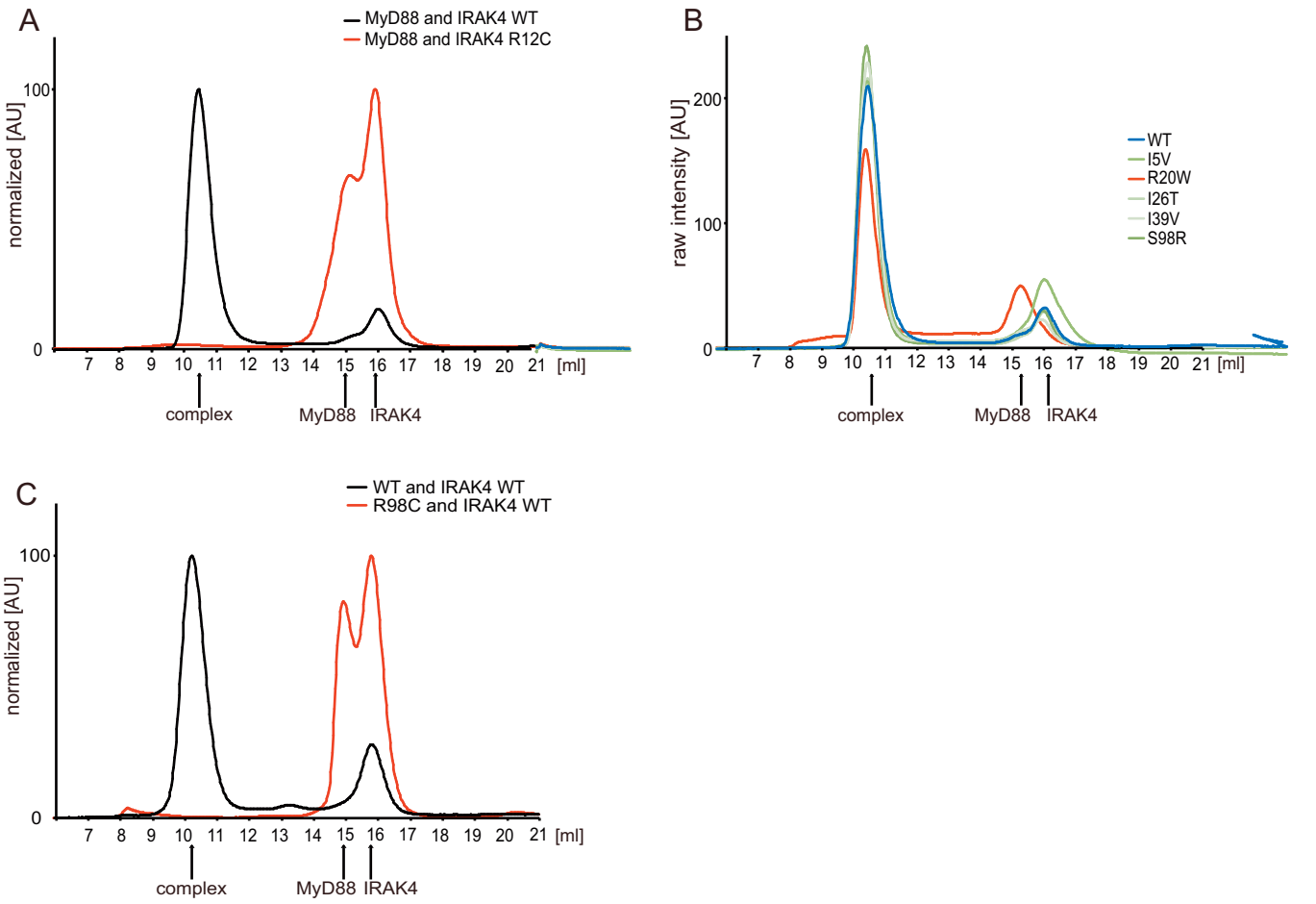

Fig. 4. Analytical gel filtration of IRAK4 and MyD88. (A) IRAK4-R12C failed to interact with MyD88. R12C was unable to assemble into a Myddosome as shown by size exclusion chromatography of mixtures of bacterially purified MyD88-DD + IDs and IRAK4-DD + IDs (added in excess). MyD88-DD + ID WT + IRAK4-DD + ID WT mixture eluted in a discrete complex peak that was absent from R12C mixtures. (B) IRAK4 SNPs interacted with MyD88 WT. Only IRAK4 R20W showed a decreased peak intensity of complete complex and residual peak of MyD88-DD + ID despite mixing an excess of IRAK4. (C) MyD88-R98C failed to interact with IRAK4. Individual peak fractions from gel filtration, purified MyD88-DD + ID or IRAK4-DD + ID alone (for size comparison) were analyzed by reducing SDS-PAGE (data not shown).

of a molar excess of IRAK4-DD + ID WT or mutants. Individual fractions or purified reference proteins were analyzed by SDS-PAGE (data not shown). The estimated molecular weights were calculated using a calibration curve, and were 30.6 kDa, 48.3 kDa, and 423 kDa for IRAK4-DD + ID, MyD88-DD + ID, and their complex, respectively. Although IRAK4-DD was eluted as a 1mer (George et al., 2011; Lin et al., 2010; Motshwene et al., 2009), IRAK4-DD + ID was mainly eluted as a 2mer. While MyD88 was mainly eluted as a 3mer. IRAK4 WT formed a characteristic oligomer by mixing with MyD88 WT, but not R98C, as previously reported (Fig. 4C) (Nagpal et al., 2011). Moreover, IRAK4 R12C also failed to form a complex (Fig. 4A). By contrast, *IRAK4* SNPs interacted with MyD88 WT (Fig. 4B), but only IRAK4 R20W showed a decreased intensity of the complex and residual peak of MyD88-DD + ID, despite an excess amount of IRAK4.

3.5. NMR titration

The IRAK4–MyD88 interaction was also examined using NMR spectroscopy for which 2D ^{1}H–^{15}N correlation NMR spectra of ^{15}N-labeled MyD88-DD + ID were recorded in the presence or absence of various concentrations of IRAK4-DD + ID or its derivatives. Changes of NMR signal intensities of a Trp residue in ^{15}N-labeled MyD88-DD + ID following titration were used to monitor the interaction (Fig. 5). The normalized NMR signal intensity steeply decreased upon IRAK4 titration. Attenuation of the NMR signal could be

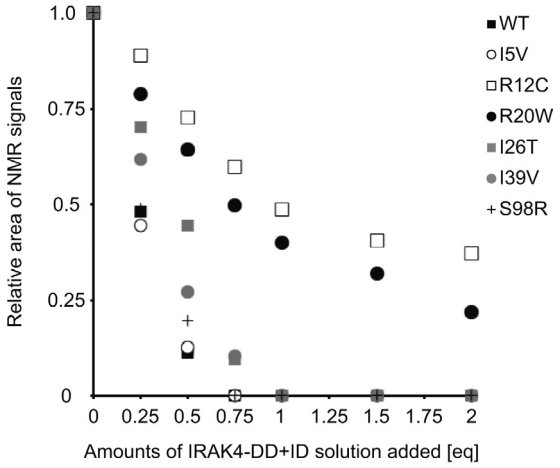

Fig. 5. NMR titration study of ^{1}H–^{15}N-MyD88-DD + ID and IRAK4-DD + ID. Normalized intensities of NMR signals, obtained from the NMR titration experiment (Supplemental Fig. S1), were plotted in a function of equivalent moles (eq) of IRAK4-DD + ID added to ^{15}N-MyD88-DD + ID. The attenuation of signal intensities was presumably caused by formation of complexes between MyD88-DD + ID and IRAK4-DD + ID. NMR signal attenuation of R12C and R20W was significantly suppressed relative to WT, indicating a weak affinity for MyD88-DD + ID. Black box, white circle, white box, black circle, gray box, gray circle, and black cross indicate the normalized intensities of WT, I5V, R12C, R20W, I26T, I39V, and S98R, respectively.

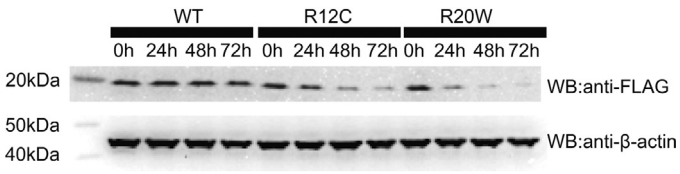

Fig. 6. Stability of IRAK4-DD variants in HEK293T cells. Cells were incubated in cycloheximide (25 μM) for the indicated times before preparation of cell extracts for SDS-PAGE separation and immunoblotting with an anti-FLAG antibody. IRAK4-DD WT maintained a steady state of protein structure, but R20W began to collapse after 24 h.

interpreted as a result of the formation of large protein complexes involving ^{15}N-labeled MyD88-DD + ID, indicating an interaction of the titrated IRAK4-DD + ID with ^{15}N-MyD88-DD + ID. Four IRAK4 derivatives, I5V, I26T, I39V, and S98R, showed a similar attenuation pattern to that of WT. By contrast, attenuation of NMR signal intensities was significantly suppressed when one of two derivatives, R12C or R20W, was used as a titrant. These observations suggest that the affinity of R12C and R20W towards MyD88-DD + ID was weaker than that of WT.

3.6. Instability of IRAK4 R12C and R20W

In the analytical gel filtration and NMR titration assays, IRAK4 R12C failed to interact with MyD88. On the other hand, IRAK4 R20W could interact with MyD88, but the amount of complete complex was lower than WT and other SNPs. Additionally, R12C and R20W were predicted to be "probably damaging" with scores of 1.000 and 0.998, respectively, by the PolyPhen-2 algorithm (http://genetics.bwh.harvard.edu/pph2/) (Adzhubei et al., 2010). Therefore, we next evaluated the protein stability of IRAK4-DD + ID R12C and R20W compared with WT following treatment with cycloheximide (Fig. 6). IRAK4-DD + ID WT protein levels did not change during 72 h of cycloheximide treatment, but R12C protein levels slightly decreased after 48 h of treatment, and R20W protein levels decreased after 24 h of treatment.

4. Discussion

4.1. In vitro assays for assessments of the mutational effects of human IRAK4 gene

Several cell-based functional assays of *IRAK4* mutants have previously been described, but the mutational effects of novel mutations have not been confirmed. For example, Lye et al. examined the NF-κB activation of *IRAK4* mutants using IRAK4-knocked out murine fibroblasts (Lye et al., 2004), while Qin et al. (2004) used human fibroblasts derived from an IRAK4 deficiency patient. Medvedev et al. (2005) examined the IL-1 signaling complex formation of an *IRAK4* mutant using immunoprecipitation. In the present study, we examined selected *IRAK4* mutations, including three previously reported missense mutations (Fig. 1A). Protein expression levels of WT and R12C were similar (Fig. 1B), so we then assessed the signaling function using HEK293T cells (Medvedev et al., 2003) as it is difficult to obtain an IRAK4-deficient human cell line. IRAK4 Q293X protein expression was undetectable and it did not appear to inhibit NF-κB activity (Figs. 1B and 2G), which agrees with the results of Medvedev et al. (2003).

The other *IRAK4* mutants, with the exception of R12C which expressed undetectable or low level IRAK4 protein, also did not inhibit NF-κB activity. On the other hand, R12C inhibited NF-κB activity to almost the same extent as WT. This suggests that overexpressed IRAK4 protein only inhibits NF-κB activity when full-length IRAK4 is expressed at levels above a certain threshold. All SNPs analyzed expressed similar protein levels and inhibited NF-κB activity to the same level as WT (Fig. 1C and E). Interestingly, R20W showed a significantly stronger inhibition of NF-κB activity than WT and other SNPs, while the PolyPhen-2 algorithm "probably damaging" prediction for R12C and R20W meant that the behavior of both was uncertain.

To clarify this, we focused on these two variants. From information about the Myddosome protein structure (PDB code: 3MOP) (Lin et al., 2010), residues R12 and R20 appeared to be located on the surface of IRAK4-DD, in the interface between IRAK4 and MyD88, and to directly interact with MyD88-DD E102 and D46, respectively (Fig. 7B). A protein–protein interaction study was used to assess the mutational effect of these residues. MyD88-DD R98 was located in the interface to IRAK4-DD (George et al., 2011), while MyD88-TIR R196 was located in the interface to Mal-TIR (Ohnishi et al., 2009). These arginine to cysteine substitutions caused a change in protein–protein interaction abilities. The recombinant proteins of IRAK4-DD + ID WT and MyD88-DD + ID WT formed a higher order oligomeric complex, but IRAK4 R12C failed to interact with MyD88.

While we were preparing this manuscript, T77del, a novel mutation of human IRAK4 deficiency, was reported as a loss-of-expression variant following a western blot of a patient's fibroblasts (Andres et al., 2013). Lin et al. (2010) used immunoprecipitation to show that both T76 and N78 are critical residues for the interaction with MyD88. Therefore T77 might be critical not only for protein expression but also the interaction with MyD88. It should be noted that although IRAK4 S98 is located on the surface of IRAK4-DD (Fig. 7A), it is distant from the interface with MyD88-DD. From the complex structural information, S98 might be located in the interface between IRAK4 and IRAK2. Future work should carry out a protein interaction study between IRAK4 S98R and IRAK2 or IRAK1 to evaluate the pathogenicity of IRAK4 S98R.

All *IRAK4* SNPs examined in the present study formed a complex in analytical gel filtration. Interestingly, R20W also formed a complex, albeit less than WT and the other SNPs. In addition, an incomplete complex of IRAK4 R20W and MyD88 was observed between the peak of unbound proteins and complete complex (Fig. 4B). Moreover, the NMR signal attenuation titrated with IRAK4 R12C and R20W was reduced compared with WT (Fig. 5), suggesting that the interaction was weakened by amino acid substitutions. From these results, we speculated that not only the mild loss of protein–protein interaction, but also the loss of IRAK4-DD R20W protein stability inhibits the formation of a complete complex of IRAK4 and MyD88. Therefore, we examined protein stability using cycloheximide. The stability of IRAK4-DD R12C was slightly lower, but that of R20W was much lower than WT and R12C (Fig. 6). Thus it is conceivable that the hydrophilic Arg20 substitution to hydrophobic tryptophan reduces the stability of the protein structure. Finally, we propose the possibility that not only IRAK4 R12C but also R20W has an impact on human IRAK4 deficiency (Table 1).

4.2. In vitro assays for assessments of the mutational effects of human MyD88 gene

Recently, the loss-of-function variants S34Y and R98C were found in naturally occurring *MyD88* SNPs (George et al., 2011; Nagpal et al., 2011). The functions of these variants were also shown by a luciferase reporter gene assay in HEK293 I3A cells, and interaction analysis with recombinant proteins. On the other hand, Napgal et al. carried out an initial functional assay of *MyD88* variants using a luciferase reporter gene assay in HEK293T cells, which are not MyD88-deficient. In addition, Loiarro et al. (2009) used immunoprecipitation in HEK293T cells to indicate that E52 and Y58 were key residues that interact with both IRAK1 and IRAK4, and that K95 was an important residue to interact with IRAK4. In this

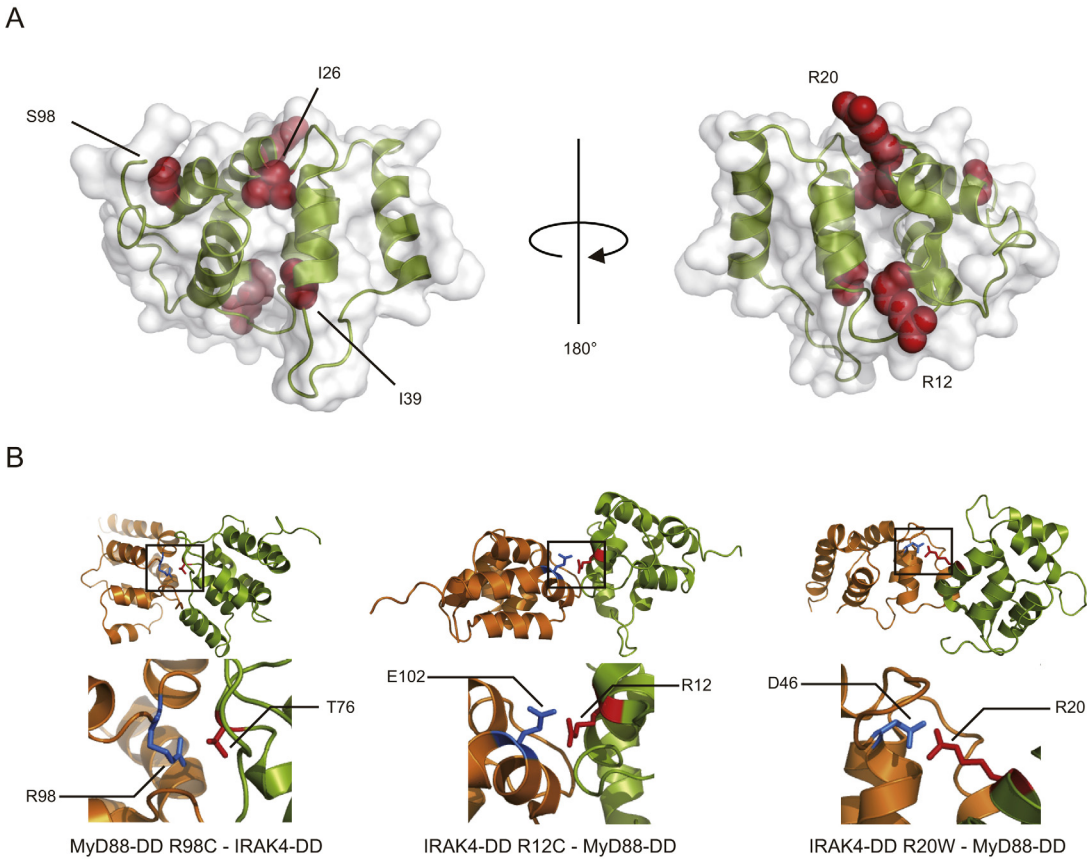

Fig. 7. Protein structure of IRAK4-DD. (A) Schematic representation of IRAK4-DD (Protein Data Bank accession code 2A9I) generated with PyMOL (DeLano Scientific, www.pymol.org). Mutants and variants are shown as red full-surface amino acid residues. R12, R20, and S98 are located on the surface of IRAK4-DD. (B) 3D interaction models of IRAK4-DD (green) with MyD88-DD (orange). MyD88-DD R98 (side chain shown as a blue stick) interacts with IRAK4-DD T76. IRAK4-DD R12 and R20 (side chains shown as red sticks) interact with MyD88-DD E102 and D46, respectively.

study, we revealed decreased protein expression levels of *MyD88* variants (S34Y, E52del, and L93P) with HEK293T cells, indicating that they have unstable protein structures (Fig. 3B). Moreover, R98C as well as the loss-of-expression variants had lower NF-κB activity than MyD88 WT (Fig. 3C), while MyD88-DD R98C had an impaired direct interaction with IRAK4-DD WT (Fig. 4C). Consequently, MyD88 R98C can be a risk-allele for MyD88 deficiency, because it showed similar to IRAK4 R12C about the results of interaction assay between MyD88-DD and IRAK4-DD. Thus, the results of MyD88-DD gene variation from a cell-based assay using a MyD88-non-deficient cell line are consistent with the protein interaction study, unlike the *IRAK4* variants.

MyD88 interacts with Mal via a shared TIR domain. We previously found that MyD88-TIR R196C had stable protein folding but a significant decrease in its ability to directly bind Mal-TIR (Nada et al., 2012; Ohnishi et al., 2009). TIR domains have an important functional region, the BB loop, which interacts with other TIR domain-containing proteins. As the R196C mutation and M178I SNP are located in or near the BB loop of the MyD88 TIR domain, we tested MyD88 full-length-induced NF-κB activation and the inhibition of ligand-induced NF-κB activity caused by a dominant-negative effect to assess the functional effect of M178I. MyD88 full-length M178I significantly enhanced NF-κB activity, as seen in WT (Fig. 3C). MyD88-TIR M178I inhibited NF-κB activation to the same extent as WT, but R196C did not (Fig. 3D). Furthermore, a GST pull-down assay using recombinant purified proteins found that MyD88-TIR M178I interacted with Mal-TIR as well as WT (Fig. 3E), which was consistent with the cell-based assays. Therefore we determined M178I to be a variant, although the mutation of a neighboring residue, I179N also called the Poc

Table 1
Summary of the expression and functional analysis of *IRAK4* variants.

Gene	Variant	Protein expression	NF-κB activity	Interaction	Protein stability	Pathogenicity
IRAK4	M1V	Absent	–	–	–	Reported
	I5V	Normal	Inhibited	Normal	–	Polymorphism
	R12C	Normal	Inhibited	Reduced	Mild reduced	Reported
	R20W	Normal	Inhibited	Mild reduced	Reduced	Probable
	I26T	Normal	Inhibited	Normal	–	Polymorphism
	I39V	Normal	Inhibited	Normal	–	Polymorphism
	c.118insA	Severely reduced	Not inhibited	–	–	Reported
	S98R	Normal	Inhibited	Normal	–	Polymorphism
	R183X	Truncated	Not inhibited	–	–	Reported
	Q293X	Severely reduced	Not inhibited	–	–	Reported
	G298D	Reduced	Not inhibited	–	–	Reported

Table 2
Summary of the expression and functional analysis of *MyD88* variants.

Gene	Variant	Protein expression	NF-κB activity	Interaction	Pathogenicity
MyD88	S34Y	Severely reduced	Not enhanced	–	Probable
	E52del	Reduced	Not enhanced	–	Reported
	E53X	Severely reduced	Not enhanced	–	Reported
	L93P	Reduced	Not enhanced	–	Reported
	R98C	Normal	Not enhanced	Reduced	Probable
	M178I	Normal	Enhanced	Normal	Polymorphism
	R196C	Normal	Not enhanced	Reduced	Reported

mutation, is associated with a loss-of-function of MyD88 (Jiang et al., 2006).

In Table 2, we summarize the functional phenotypes of *MyD88* mutations and SNPs described in this study. Our results suggest that the mutational effects of *MyD88* variants, at least those located in DD and TIR domains, can only be assessed by cell-based reporter gene assays using widely available cell lines such as HEK293 cells, unlike *IRAK4* variants.

4.3. The additional discussion about another components of Myddosome, Mal

Recently, several functional assays of variants of Mal, located in the TIR domain have been reported. Nagpal et al. (2009) found that Mal D96N was unable to interact with MyD88 using a reporter gene assay and immunoprecipitation, while George et al. (2010) confirmed this by immunofluorescence. We used a reporter gene assay to show that E132K, R143Q, and E190D are loss of functional variants (An et al., 2011). E132K is of particular importance as it is located in the BB loop of the Mal TIR domain, so we speculate that it might be a pathogenic mutation of Mal deficiency. More recently, Weller et al. (2012) reported that Mal R121W, which is also located in the Mal BB loop at a similar site to R196C of *MyD88*, causes human Mal deficiency. Therefore, future work should examine the functions of the gene variants of not only IRAK4 and MyD88 but also Mal deposited in the SNP database.

5. Conclusion

Not only previously reported loss-of-function mutations but also several SNPs are considered likely to be pathogenic for human diseases, because of their loss of functions proved by *in vitro* methods. Loss of protein stability and defect of interaction between the components of Myddosome may cause IRAK4 and MyD88 deficiencies as a result of a failure to form a precise Myddosome structure. Our findings indicate that the analysis of Myddosome formation with recombinant proteins is useful to distinguish whether missense mutations, especially those located in the DD of IRAK4, are causative. Thus, the combination of *in vitro* functional assays is effective to confirm pathogenicity of mutants found in IRAK4 and MyD88 deficiency patients.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molimm.2013.11.008.

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Epidemiology of virus-induced wheezing/asthma in children

Yuzaburo Inoue* and Naoki Shimojo

Department of Pediatrics, Graduate School of Medicine, Chiba University, Chiba, Japan

Edited by:
Hirokazu Kimura, National Institute of Infectious Diseases, Japan

Reviewed by:
Linfa Wang, Commonwealth Scientific and Industrial Research Organisation Livestock Industries, Australia
Takeshi Saraya, Kyorin University School of Medicine, Japan

****Correspondence:***
Yuzaburo Inoue, Department of Pediatrics, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuou-ku, Chiba, Chiba 260-8670, Japan
e-mail: yuzaburo@chiba-u.jp

Wheezing is a lower respiratory tract symptom induced by various viral respiratory infections. Epidemiological studies have revealed the phenotypes of wheezing in early childhood which have different risk factors for the development of asthma among school age children. The major viral species causing wheezing in children include respiratory syncytial virus, rhinovirus, human metapneumovirus and influenza viruses. It has been shown that the impact on the development of asthma is different between those virus species. Moreover, recent studies have also focused on the interaction between virus infection and other risk factors in the development of asthma, such as genetic factors or allergic sensitization. In this review, we summarize the previous findings and discuss how clinicians can effectively intervene in these viral infections to prevent the development of asthma.

Keywords: wheezing, viral respiratory infection, cohort study, interferon, respiratory syncytial virus, rhinovirus

INTRODUCTION

Wheezing is a lower respiratory tract symptom induced by various viral respiratory infections. It is in common in children, with approximately one-third of the children having at least one wheezing episode by age nine. However, about 1 to 2% of affected infants need to be hospitalized due to respiratory distress. Moreover, although the condition is transient in the majority of cases, some children develop recurrent wheezing and are diagnosed to have asthma when they reach school age. In those children, the virus-induced wheezing in early childhood may be associated with the subsequent development of recurrent wheezing and/or asthma in connection to the pathology of asthma, including chronic airway inflammation, thus leading to airway hyperresponsiveness and airway remodeling.

Epidemiological studies are therefore considered to be important for clarifying which populations are at risk for developing virus-induced wheezing accompanied with other severe symptoms, recurrent wheezing and especially asthma. Moreover, recent studies have also focused on the interaction between virus infection and other risk factors for the development of asthma, such as genetic factors or allergic sensitization.

In this review, we summarize the previous findings and discuss how clinicians can effectively intervene in these viral infections to prevent the development of asthma.

PHENOTYPES OF VIRUS-INDUCED WHEEZING

Birth cohort studies have been conducted to clarify the natural history of wheezing in early childhood and to assess the risk factors for the development of wheezing and subsequent asthma. The first large prospective study focusing on the wheezing history was performed by the Tucson Children's Respiratory Study group (Halonen et al., 1999; Sherrill et al., 1999; Stein et al., 1999; Taussig et al., 2003; Morgan et al., 2005). They followed 828 infants until the age of 6 years and identified four different patterns of wheezing in early childhood (never wheeze, transient early wheeze, late-onset wheeze, and persistent wheeze) on the basis of clinical observations. The "never wheeze" phenotype (51.5% of the cohort) was defined as children with no episodes of wheezing during the first 6 years of the life. The "transient early wheeze" phenotype (19.9% of the cohort) was defined as children having at least one lower respiratory tract illness with wheezing during the first 3 years of the life, but no wheezing at 6 years of age. The children with this phenotype had a diminished airway function both before the age of 1 year and at the age of 6 years, were more likely than the other children to have mothers who smoked but not mothers with asthma, and did not have elevated serum IgE levels or skin-test reactivity. The "late-onset wheeze" phenotype (15.0% of the cohort) was defined as children having no wheezing before the age of the 3 years, but having wheezing at 6 years of age. The "persistent wheeze" phenotype (13.7% of the cohort) was defined as children having wheezing both before 3 years of age and at 6 years of age. The children of this phenotype were more likely than the "never wheeze" children to have mothers with a history of asthma, to have elevated serum IgE levels and normal lung function in the first year of life, and to have elevated serum IgE levels and diminished airway function at 6 years of age. Interestingly, these phenotypes have been shown to be associated with different risk factors for the number of encountered viral infections in early childhood (Kusel et al., 2007) and the development of asthma (Taussig et al., 2003; Stein and Martinez, 2004).

Birth cohort studies from Europe using latent class analysis identified more complicated wheezing phenotypes, including an intermediate-onset wheezing phenotype. A population-based birth cohort study of 6265 children in the United Kingdom (the ALSPAC study) identified six wheezing phenotypes in childhood, from birth to age 7 years, and demonstrated that these phenotypes differed in the atopy prevalence and lung function levels at 7–8 years of age (Henderson et al., 2008). Another multicenter birth cohort study of 2810 children in the Netherlands (the PIAMA

study) also identified five wheezing phenotypes in childhood from birth to age 8 years. Interestingly, the wheezing phenotypes identified by the two birth cohort studies were comparable (Savenije et al., 2011).

VIRAL SPECIES CAUSING WHEEZING

The major viral species causing wheezing in children include respiratory syncytial virus (RSV), rhinovirus (HRV), human metapneumovirus (hMPV) and influenza viruses.

RSV

Respiratory syncytial virus is a medium-sized negative-stranded RNA virus of the family *Paramyxoviridae*, which causes respiratory infections mainly in children. Interestingly, the clinical symptoms of RSV infection in infancy and early childhood are extremely variable. Most infants experience an RSV infection before 3 years of age (Ruuskanen and Ogra, 1993), normally escaping with only upper respiratory diseases, whereas approximately 1–2% of them require hospitalization because of severe RSV bronchiolitis (Green et al., 1989; Stretton et al., 1992). This is particularly common in those who are premature or who have chronic lung disease or congenital heart disease. Recently, a humanized monoclonal antibody designed to provide passive immunity against an epitope in the A antigenic site of the F protein of RSV has been widely used for the prophylaxis of severe RSV lower respiratory infection in those children.

HRV

Rhinovirus is a small-sized positive-strand RNA virus of the family *Picornaviridae*, which is well known as the predominant cause of the common cold. Because of the development of PCR techniques, it has been recognized that HRVs cause not only upper respiratory infections, but also lower respiratory infections or asthma exacerbation. HRVs consist of over 100 types classified into one of three species (A, B, and C) according to the phylogenetic sequence criteria. HRV C (HRV-C) is a recently classified group and has been shown to be associated with severe asthma attacks more frequently than other groups of HRV. The prevalence of HRV-associated wheezing increases by age, and it is significantly more common in children with recurrent wheezing episodes than in first-time wheezers in age categories of <24 and <36 months (Jartti et al., 2009).

HMPV

The hMPV is a medium-sized negative-stranded RNA virus of the family *Paramyxoviridae*, which was recently discovered (van den Hoogen et al., 2001), the clinical course of which resembles RSV infection. Similar to RSV, it has been reported that hMPV infection was associated with wheezing among children younger than 3 years, especially during the winter, while hMPV was not significantly associated with wheezing requiring hospitalization among children 3 years of age and older (Williams et al., 2005).

INFLUENZA VIRUSES

Influenza viruses are a medium-sized negative-stranded RNA virus of the family of *Orthomyxoviridae*. Influenza viruses cause severe lower respiratory tract complications, such as bronchitis or pneumonia. In addition, influenza is significantly associated with wheezing during the winter among children younger than 3 years of age although the detection percentage of the influenza virus is lower than that of RSV (Heymann et al., 2004).

RISK FACTORS FOR THE DEVELOPMENT OF VIRUS-INDUCED WHEEZING

BEHAVIORAL OR ENVIRONMENTAL FACTORS

In the Tucson study, it was reported that breast-feeding at early infancy for at least 1 month was associated with lower rates of virus-induced wheezing during the first 4 months of the life (Wright et al., 1989). However, the results of the subsequent studies have been conflicting. A meta-analysis study finally showed that there was no association between any or exclusive breast feeding and wheezing illness (Brew et al., 2011).

Infants exposed to more children at home or day care experienced more frequent wheezing when they were 2 years old, but less frequent wheezing from years eight through year thirteen. Therefore, although exposure to children at home or in day care during infancy increased wheezing in early life, it appears to be protective against the development of frequent wheezing in school age children (Ball et al., 2000).

HOST IMMUNOLOGICAL FEATURES

Interferon (IFN) secretion is important in the clearance of viral pathogens. Therefore, IFN deficiency has been supposed to lead to lower respiratory viral infections. There are three types of interferons: Type I (IFN-α/β), Type II (IFN-γ) and Type III (IFN-λ). It was shown that low IFN-γ production in cord bloods (Copenhaver et al., 2004) or PBMCs in the first year of life (Stern et al., 2007) was a risk factor for wheezing during childhood, in addition to a risk factor for the development of asthma and allergies (Tang et al., 1994). It has recently been clarified that the deficiency of IFN production is related to atopy. It was reported that allergic asthmatic children had an impaired HRV-induced IFN-α and IFN-λ1 production that correlated with an increased FcεRI expression on plasmacytoid dendritic cells in PBMCs, which were reduced by FcεRI cross-linking (Durrani et al., 2012). In addition, it was reported that bronchial epithelial cells from asthmatic individuals produced less IFN-β in response to HRV, leading to impaired apoptosis and increased HRV replication (Wark et al., 2005). Interestingly, it was revealed that allergic sensitization precedes HRV-induced wheezing, but the converse is not true (Jackson et al., 2012). The results of that study suggested that allergic sensitization can lead to more severe HRV-induced lower respiratory illnesses, which is considered to be a risk factor for the subsequent development of asthma.

GENETIC FACTORS

There have been many genetic risk factors reported to be associated with the development of RSV bronchiolitis. Two large scale genetic association studies were performed using a candidate gene approach (Janssen et al., 2007; Siezen et al., 2009). They analyzed 384 single-nucleotide polymorphisms (SNPs) in 220 candidate genes involved in the airway mucosal responses, innate immunity, chemotaxis, adaptive immunity, and allergic asthma. They found that SNPs in genes of the innate immune responses (the transcriptional regulator Jun, alpha interferon, IFN-α, nitric oxide synthase

and the vitamin D receptor) are important for determining the susceptibility to RSV bronchiolitis in term children. As RSV is recognized by Toll-like receptor (TLR) 4, SNPs in the genes of molecules related to TLR4 signaling have also been studied (Tal et al., 2004; Inoue et al., 2007).

In contrast, the genetic factors related to the development of HRV-induced wheezing are less well known (Helminen et al., 2008; Caliskan et al., 2013). However, the 17q21 variants, which were found to be related to childhood-onset asthma in a genomewide association study (Moffatt et al., 2007), were associated with HRV wheezing illnesses in early life, but not with RSV wheezing illnesses (Caliskan et al., 2013).

ASSOCIATION BETWEEN VIRUS-INDUCED WHEEZING AND THE DEVELOPMENT OF ASTHMA, AND EFFECTIVE TYPES OF INTERVENTION TO PREVENT THE SUBSEQUENT DEVELOPMENT OF ASTHMA

It is still unclear whether lower respiratory viral infections are causal factors, or instead serve as indicators, of a predisposition to asthma. Moreover, recent studies have indicated that the impact on the development of subsequent recurrent wheezing or asthma is different between virus species.

It was reported that infant birth approximately 4 months before the winter virus peak, which is the peak of bronchiolitis hospitalizations for that winter season, carried the highest risk for the development of asthma, thus suggesting that a lower respiratory infection with winter viruses, including RSV, in early childhood may be an important factor in the development of asthma (Wu et al., 2008). In a birth cohort study, Sigurs et al. (2010) followed 47 children aged <1 year hospitalized with RSV lower respiratory infection (RSV group) and 93 age- and gender-matched controls (Control group) for 18 years. They found that the RSV group had an increased prevalence of asthma/recurrent wheezing, clinical allergy and sensitization to perennial allergens, compared to the Control group (Sigurs et al., 2010). Meanwhile, it was shown that RSV prophylaxis using Palivizumab, a humanized monoclonal antibody against the RSV fusion protein that prevents severe RSV lower respiratory infection, in non-atopic children decreased the relative risk of recurrent wheezing by 80%, but did not have any effect in infants with an atopic family history (Simoes et al., 2010). These results suggest that RSV predisposes to recurrent wheezing via an atopy-independent mechanism.

Rhinovirus has been implicated as an important pathogen in asthma pathogenesis due to the improvement of PCR for HRV detection. In the Childhood Origins of ASThma (COAST) cohort, HRV in nasal lavage samples were evaluated by PCR. They found that, by age 3 years, wheezing in those with HRV-positive samples (OR, 25.6) was more strongly associated with asthma at age 6 years than aeroallergen sensitization (OR, 3.4; Jackson et al., 2008). As IFN deficiency is related to both atopy and the susceptibility to HRV infection, the inhalation of IFN by HRV-infected children with risk factors for asthma might thus help to prevent the development of asthma.

Recently, pandemic H1N1 influenza virus has been reported to increase the risk of lower respiratory tract complications including asthma attack, pneumonia, and atelectasis even in atopic children without any history of either an asthma attack or asthma treatment, compared to the seasonal influenza virus (Hasegawa et al., 2011). This observation suggests that the pandemic H1N1 influenza virus may be a strong risk factor contributing to the development or exacerbation of asthma.

LIMITATIONS OF EPIDEMIOLOGY STUDIES

The correct diagnosis of individual viral infections is necessary for assessing which virus infection is important for the development of wheezing or the subsequent development of asthma. The principal diagnostic methods for respiratory viruses are virus culture, serology, immunofluorescence/antigen detection, and nucleic acid/PCR-based tests (Tregoning and Schwarze, 2010).

Although virus culture proves that the virus detected in clinically obtained samples is able to infect human cells, viral culture is time-consuming and is not appropriate for analyzing many samples in epidemiological studies. Viral serologic testing is also time-consuming, and generally requires at least two rounds of blood sampling, because viral serological testing can diagnose infections by an increase of a virus-specific antibody in the blood, which usually takes 2 weeks to develop. Most previous epidemiological studies thus evaluated viral infections by immunofluorescence/antigen detection or nucleic acid/PCR-based tests. Antigen detection is based on the use of virus-specific monoclonal antibodies. There are a variety of diagnostic test kits that use nasopharyngeal aspirate, nasopharyngeal wash or nasal swab specimens as the test material, and detect viral antigen by using either a conjugated enzyme or fluorescence. Immunofluorescence/antigen detection is appropriate for epidemiological studies because it is convenient, cheap and possible to use when handling for many samples. However, there is a limitation to the species of target viruses. Nucleic acid tests are significantly more sensitive than the other methods, and are now being multiplexed, allowing for the rapid detection of many viruses concurrently. The PCR method has greatly increased the recovery rates of viruses (Johnston et al., 1995; Rakes et al., 1999). However, the PCR-based diagnosis of viruses, especially HRV, may not necessarily indicate that the virus is causing the observed disease, because virus RNAs can be detected by PCR for several weeks after the onset of clinical symptoms (van Benten et al., 2003; Jartti et al., 2004; Wright et al., 2007).

Another limitation in epidemiological studies assessing wheezing/wheezes in early childhood is the difficulty of diagnosing these conditions in young children based on the clinical assessment of symptoms by both guardians and clinicians. It was reported that the parents of children aged 4 months to 15 years and clinicians agreed on only 45% of occasions that the patient was wheezy or had asthma (Cane et al., 2000), thus suggesting that epidemiological studies using symptom records kept by guardians may sometimes lead to a wrong conclusion. Moreover, it has been shown that even specialists might not always correctly recognize wheezing (Bisgaard and Bonnelykke, 2010).

FUTURE PROSPECTIVE

To our knowledge, previous epidemiological studies regarding the association between viral infections and wheezing/asthma did not evaluate all other risk factors for the development

of wheezing/asthma in childhood, including behavioral factors, environmental factors, host immunological features and genetic factors. Large birth cohort studies evaluating viral infections and these factors in childhood could thus better elucidate the impact of viral infections on the development of wheezing/asthma. However, epidemiological studies only reveal an association, but not a causal relationship between some viral infections and the development of wheezing or asthma. Therefore, in the future, intervention trials with preventative intervention or therapies on a specific virus would be needed to clearly identify when and how clinicians should intervene in such viral infections to thereby prevent the development of wheezing or asthma.

CONCLUSION

Virus-induced wheezing is not only a burden in early childhood, but also may be one of causes or signs of childhood asthma. Therefore, clarifying the risk factors for virus-induced wheezing in epidemiological studies can and have provided clues about the pathogenesis of asthma. Further studies are needed to clarify which virus(es) in which population should be the major target of early intervention for preventing the subsequent development of asthma.

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