Development of a rapid and inexpensive assay for detecting a surrogate genetic polymorphism of *HLA-B*58:01*: a partially predictive but useful biomarker for allopurinol-related Stevens-Johnson syndrome/toxic epidermal necrolysis in Japanese.

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Running title: PCR-RFLP assay for detecting a surrogate SNP of *HLA-B*58:01*

Footnotes

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Supplementary Figure: 2 pages

Abstract

Allopurinol-induced Stevens-Johnson syndrome (SJS)/toxic epidermal necrolysis (TEN) are strongly associated with *HLA-B*58:01* in various populations including Japanese. We demonstrated that several single nucleotide polymorphisms (SNPs) around the *HLA* region on chromosome 6 were strongly linked with *HLA-B*58:01* in a previous study using Japanese allopurinol-related SJS/TEN patients. Their very strong linkage suggests that these SNPs could be used as surrogate biomarkers to find carriers of *HLA-B*58:01* to avoid these serious adverse effects. In the present study, to expedite the application of this pharmacogenomic information to the proper usage of allopurinol in the clinical situation, we developed a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay for the genotyping of rs9263726 in the *psoriasis susceptibility 1 candidate 1* (*PSORS1C1*) gene, which is in absolute linkage disequilibrium (r^2=1, D'=1) with *HLA-B*58:01*. Developed PCR-RFLP assay using FokI restriction enzyme was able to detect three different genotypes, GG, GA, and AA of rs9263726 robustly, and thus enabled to find *HLA-B*58:01* carriers. This robust and inexpensive assay would be useful for pre-screening the subjects with *HLA-B*58:01*, a genetically high risk factor for allopurinol-induced SJS/TEN.

Keywords: allopurinol, PCR-RFLP, screening test, Stevens-Johnson syndrome, toxic epidermal necrolysis.

Introduction

Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) are severe cutaneous adverse reactions (SCARs).[1)] SJS and TEN, considered variants of the same skin disorder, are characterized by the development of limited (in SJS) or widespread (in TEN) detachment and blistering of the skin epidermis and mucous epithelium, often with organ involvement.[1,2)] The incidence of SJS/TEN is very rare, estimated to occur at about 2 patients per million individuals per year in Caucasians[3)], but these SCARs require intensive care due to the high mortality rates (1-5% for SJS and 20-30% for TEN) and long-term treatments for subsequent complications, especially ocular pathologies.[1)] SJS/TEN are idiosyncratic SCARs that have considered, for a long time, to be difficult to predict, but human lymphocyte antigen (HLA) types have recently been reported to be associated with the onset of SJS/TEN in a drug-specific manner.[1,4)]

Allopurinol is a widely-prescribed urate-lowering drug and has known to be the most common causative drug for SJS/TEN in Japan.[4,5)] In 2005, Hung *et al.* reported that an *HLA* allele *B* variant, *HLA-B*58:01*, is strongly associated with allopurinol-induced SCARs consisting of SJS, TEN and hypersensitivity syndrome in Han Chinese population.[6)] They found that 100% (51/51 patients) of the case patients had this *HLA* type, while only 15% (20/135 patients) of the tolerant control had, giving an odds ratio (OR) of 580.3 (sensitivity = 100%, specificity = 85%). This association was later confirmed in Thai (SJS/TEN patients, OR = 348.3, sensitivity = 100%, specificity = 87%)[7)], Korean (SJS/TEN/drug-induced hypersensitivity syndrome patients, OR = 97.8, sensitivity = 92%, specificity = 89%)[8)], European (SJS/TEN patients, OR = 80, sensitivity = 56%)[9)] and Japanese (SJS/TEN patients, OR = 62.8, sensitivity = 56%)[10, 11)] populations. Although the associations have been partial, especially in Europeans

and Japanese, *HLA-B*58:01* is thought to be a useful biomarker for allopurinol-induced SJS/TEN.

Recent report showed that based on the very strong association of *HLA-B*15:02* allele with SJS/TEN in Han Chinese population (sensitivity = 98%, specificity = 96%),[12] prospective testing for *HLA-B*15:02* and subsequent avoidance of carbamazepine therapy result in zero occurrence of SJS/TEN in Taiwan.[13] Based on this result and severity of these adverse reactions, pre-screening test is now mandatory and covered by the National Health Insurance in Taiwan, although its positive predictive value could be estimated at around 3% using the values of their study. Thus, examining *HLA-B*58:01* prior to allopurinol administration may be also valuable to avoid allopurinol-induced SJS/TEN. However, testing *HLA* types is relatively laborious, time-consuming and expensive. Very recently, we found that several single nucleotide polymorphisms (SNPs) around the *HLA* region on chromosome 6 were strongly linked with *HLA-B*58:01* in a group of SJS/TEN patients.[11] In general, single SNP can be easily genotyped and inexpensively compared to *HLA* type. Thus, the linked SNPs could be used as alternatives to testing for *HLA-B*58:01* when deciding on the application of drug therapies involving allopurinol. To expedite the application of this pharmacogenomic information for the proper usage of allopurinol in clinical settings, we developed a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method that can genotype SNPs easily without high skills and inexpensively.

Materials and Methods

Patients

Japanese SJS/TEN patients from unrelated families were recruited from July 2006 through April 2010 at participating institutes of the Japan Severe Adverse Reactions (JSAR) research group.[11] In addition, SJS/TEN patients were recruited through a nationwide blood-sampling network system in Japan for severe drug adverse reactions operated by the National Institute of Health Sciences under the auspices of the Ministry of Wealth, Labour and Welfare and the Federation of Pharmaceutical Manufacturers' Associations of Japan. Genomic DNA was extracted from blood leukocytes as described previously.[10] DNA samples extracted from the cord blood of healthy Chinese-Americans were purchased from AllCells (Emeryville, CA, USA). The ethics committees of the National Institute of Health Sciences and each participating institute of the JSAR research group approved this study. Written informed consent was obtained from all cases and healthy Chinese-American subjects.

Genotyping of Single Nucleotide Polymorphism by TaqMan assay and *HLA* types

HLA-B types were determined by the sequencing-based method as reported in a previous paper.[11] Of the several SNPs linked with *HLA-B*58:01*, we selected rs9263726 (110G>A, Arg37His) in *psoriasis susceptibility 1 candidate 1* (*PSORS1C1*) as a surrogate marker for *HLA-B*58:01*, because this SNP was in absolute linkage disequilibrium ($r^2 = 1$, D' = 1) with *HLA-B*58:01* and associated with SJS/TEN with an odds ratio of 61.2 ($p = 3.64 \times 10^{-8}$) in the dominant genotype mode.[11] This variation was located *ca.*, 215 kb away from the *HLA-B* gene, detected at minor allele frequency of 0.006 (12/1982 alleles), which was the same as that of the reported Japanese frequency of *HLA-B*58:01* (0.006),[14] and in Hardy-Weinberg equilibrium (p=0.847).[11]

In allopurinol-related SJS/TEN patients, the minor allele frequency of *HLA-B*58:01* and rs9263726 was 0.278[11]. Rs9263726 was genotyped using TaqMan SNP Genotyping Assays (C_30352071_10, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instruction using 5 ng of genomic DNA from Japanese SJS/TEN patients or healthy Chinese-Americans. Hardy-Weinberg equilibrium was analyzed by Fisher's exact test using SNPAlyze ver. 3.1 software (Dynacom, Chiba, Japan).

Genotyping of rs9263726 by PCR-RFLP

PCR primers (forward: 5'-AAGCTCCATCCACCCCTGGT-3' and reverse: 5'-ACACATTGGGTGGGGGACAT-3') were designed to amplify a *PSORS1C1* genomic fragment containing the rs9263726 SNP locus. PCR was performed using *Ex*-Taq (0.625 units) (Takara Bio Inc., Shiga, Japan) with a pair of primers (0.2 μM) and genomic DNA (50 ng). The PCR conditions were 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 60°C for 1 min, 72°C for 1.5 min, and a final extension at 72°C for 7 min. Aliquots of PCR products (5 μl) were then digested by the addition of 0.4 units of FokI restriction endonuclease (New England Biolabs, Beverly, MA, U.S.A.) in the presence of 1 x Buffer 4 (New England Biolabs) at 37°C for 2 h. Restriction mixtures were incubated at 65°C for 20 min to inactivate FokI, and then electrophoresed through a 15-25% gradient acrylamide gel (MULTIGEL II Mini, Cosmo Bio Co., Ltd, Tokyo, Japan). Following electrophoresis, the gels were stained with ethidium bromide, and DNA was visualized by placing the gel on an UV transilluminator.

Results and Discussion

First, we compared the results of genotyping rs9263726 in *PSORS1C1* with the PCR-RFLP and TaqMan SNP Genotyping assays (C_30352071_10). DNAs from Chinese-Americans were used since the frequency of *HLA-B*58:01* in this population is reportedly higher than in the Japanese population.[6,10] Preliminary, experiments using the TaqMan assay showed that the 200 DNA samples from Chinese-Americans contained 161 homozygotes of the major allele (GG), 36 heterozygotes (GA), and 3 homozygotes of the minor allele (AA) of rs9263726 (data not shown), which distribution was in Hardy-Weinberg equilibrium (p=0.550). In addition, we confirmed that the 3 subjects with homozygous AA surely had homozygous *HLA-B*58:01* (data not shown). From the DNAs from Chinese-Americans genotyped by TaqMan assay, 5 samples with GG, 4 with GA, and 2 with AA of rs9263726 were selected to establish the PCR-RFLP method. In the developed assay, the 260 bp PCR products derived from the A allele of rs9263726 were digested with Fok I produced two bands (141 bp and 119 bp), while those derived from the G allele remained as the parent single band (260 bp) (supplementary Fig. 1A). Genotypes of these samples by PCR-RFLP assay were 100% in concordance with those from the TaqMan SNP assay, indicating that this is a robust method of genotyping rs9263726.

Next, in order to validate this PCR-RFLP assay, the rs9263726 locus was genotyped for the DNA samples with or without *HLA-B*58:01* of 27 Japanese SJS/TEN patients for whom *HLA-B* types had been previously determined.[10,11] The following SJS/TEN samples were selected: 5 *HLA-B*58:01* heterozygous carriers and 22 other *HLA-B* allele carriers. The other *HLA* types were selected based on an allele frequency $\geq$ 0.01 in Japanese control populations,[14,15] although a *HLA-B*44:02* sample (allele

frequency = 0.01) was not available. As shown in Table 1 and supplementary Fig. 1B, the 5 patients with heterozygous *HLA-B*58:01* were also heterozygotes for rs9263726 (GA), and the remaining 22 patients with the other *HLA-B* types were major homozygotes for this SNP (GG). Thus, our developed PCR-RFLP assay can robustly predict the *HLA-B*58:01* status of SJS/TEN patients.

Very recently, Kostenko *et al.* generated a monoclonal antibody to recombinant HLA-B*57:01 protein and developed an flow cytometric assay for the detection of HLA-B57-positive peripheral blood mononuclear cells.[16] This antibody can cross-react with HLA-B58 proteins and thus could be used to pre-screen for *HLA-B*58:01* carriers. However, this assay method cannot discriminate *HLA-B*57:01* from *B*58:01* and uses blood cells, making it laborious and expensive (i.e., a flow cytometer is necessary). In contrast, our PCR-RFLP method does not require a high skill set, and at a low cost without use of specific machines, although a DNA extraction step is necessary.

Although the testing of rs9263726 or *HLA-B*58:01* cannot perfectly predict allopurinol-induced SJS/TEN, the *HLA-B*58:01*-positive patients may be better to avoid the administration of allopurinol, as the *HLA-B*15:02*-positive patients for carbamazepine in Taiwan. Because allopuirol is a xantine oxidase inhibitor, febuxostat, having same pharmacological effect by different structure, might be an alternative drug for the *HLA-B*58:01*-positive patients, although further studies are clearly necessary to prove that SJS/TEN induced by febuxostat is surely not to be associated with *HLA-B*58:01*.

In conclusion, we have developed a robust PCR-RFLP genotyping assay for rs9263726 in *PSORS1C1*, which is in absolute linkage disequilibrium with

*HLA-B*58:01*, a partially predictive but useful biomarker for allopurinol-related SJS/TEN in Japanese. The genotyping of rs9263726 by this easy and inexpensive method makes it useful for the prospective screening of patients with *HLA-B*58:01* in the future.

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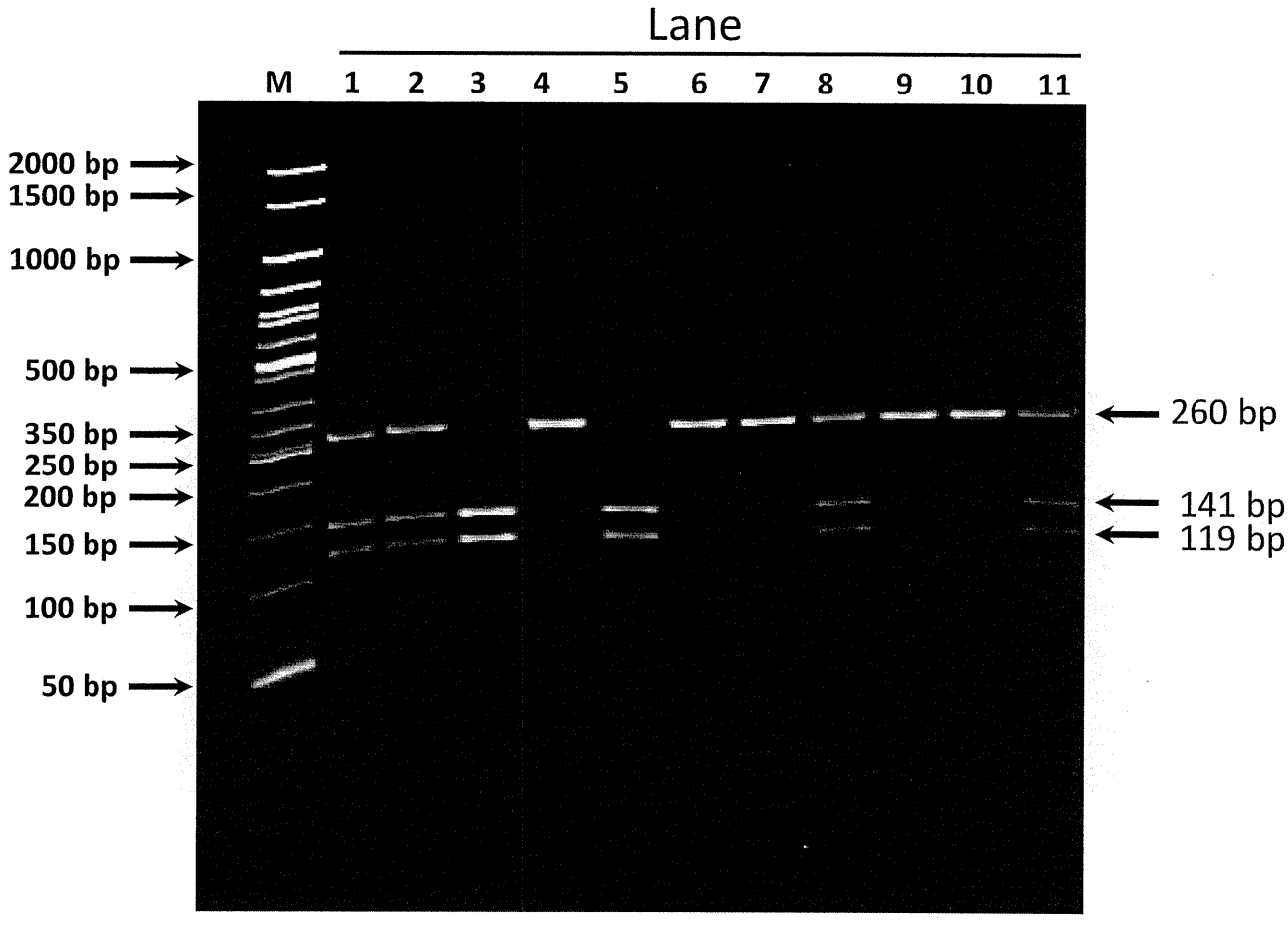
Figure Legends

Supplementary Figure 1. Establishment of PCR-RFLP assay for rs9263726 and its validation by samples with or without *HLA-B*58:01*. A. Establishment of PCR-RFLP assay for rs9263726 in *PSORS1C1* using FokI restriction digestion on DNA samples from Chinese-Americans. Genotypes of DNA samples were pre-determined by TaqMan SNP genotyping assay method. The genotypes were GG (260 bp) in lanes 4, 6, 7, 9 and 10, GA (260 bp, 141 bp and 119 bp) in lanes 1, 2, 8, and 11, and AA (141 bp and 119 bp) in lanes 3 and 5. M, DNA ladder marker (OneSTEP Ladder 50, NIPPON GENE CO., LTD, Tokyo, Japan). B. Genotyping of rs9263726 in 27 Japanese SJS/TEN patients with or without *HLA-B*58:01*. Patient ID corresponds to those in Table 1. M, DNA ladder marker (All Purpose HI-LO DNA marker, BIONEXUS Inc., Oakland, CA, USA).

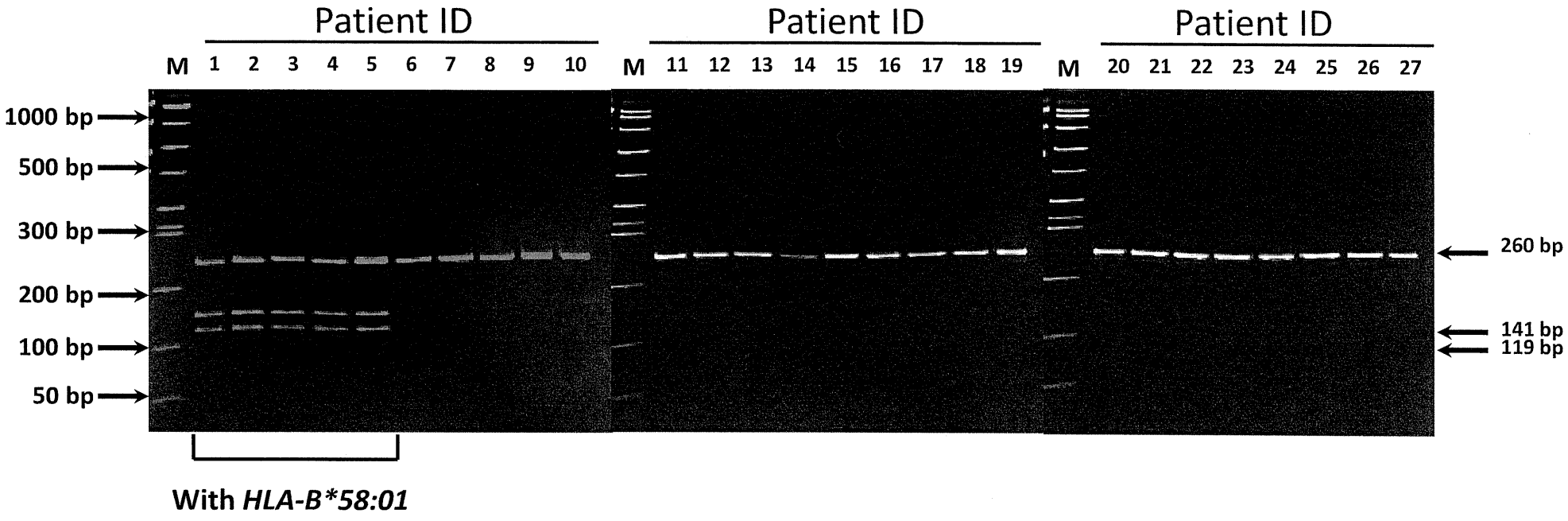
Table 1 *HLA* and rs9263726 genotypes in Japanese SJS/TEN patients.

ID	*HLA-B**		rs9263726
1	***58:01***	*46:01*	**G/A**
2	***58:01***	*51:01*	**G/A**
3	***58:01***	*51:01*	**G/A**
4	***58:01***	*38:02*	**G/A**
5	***58:01***	*07:02*	**G/A**
6	*07:02*	*51:01*	G/G
7	*13:01*	*35:01*	G/G
8	*15:01*	*40:02*	G/G
9	*15:11*	*40:02*	G/G
10	*15:18*	*38:02*	G/G
11	*35:01*	*40:02*	G/G
12	*37:01*	*40:06*	G/G
13	*39:01*	*51:01*	G/G
14	*40:01*	*40:01*	G/G
15	*48:01*	*51:02*	G/G
16	*40:02*	*40:06*	G/G
17	*40:06*	*52:01*	G/G
18	*44:03*	*44:03*	G/G
19	*46:01*	*35:01*	G/G
20	*51:01*	*35:01*	G/G
21	*52:01*	*52:01*	G/G
22	*54:01*	*54:01*	G/G
23	*55:02*	*51:01*	G/G
24	*56:01*	*46:01*	G/G
25	*59:01*	*35:01*	G/G
26	*67:01*	*39:01*	G/G
27	*40:03*	*54:01*	G/G

Suppl. Fig. 1A


Lane
M
1
2
3
4
5
6
7
8
9
10
11
2000 bp
1500 bp
1000 bp
500 bp
350 bp
250 bp
200 bp
150 bp
100 bp
50 bp
260 bp
141 bp
119 bp

Supple. Fig. 1B



メタボローム新技術が切り拓くこれからの脂質バイオロジー研究

有田 誠，斎藤嘉朗，田口 良，西島正弘

さまざまなバイオロジーや病態の背後に潜む分子メカニズムを「脂肪酸の質の違い」という観点から明らかにするためには，解析対象となる組織における脂肪酸の質の違いを定性・定量的に分析し，可視化することが必要である．そのようなことを可能にする技術として，近年急速に発展しているメタボローム解析技術について紹介する．さらにゲノム，プロテオーム解析を加えた多層的オミックスへの展開など，これからの脂質バイオロジー研究の方向性・可能性について議論したい．

キーワード● 脂質メタボロミクス，多層的オミックス，脂肪酸の質

はじめに

本特集では，「脂肪酸の質」に視点を置いて，それらのバイオロジーや病態との関連を示唆する研究について紹介した．明らかに脂肪酸の量だけでなく質が重要であることが強く示唆されるものの，それを分子レベルで検証していくうえで，実際に組織や細胞における脂肪酸の質の違いを定性・定量的に分析し，可視化することが必要である．すなわち，生体内にはさまざまな炭素数や不飽和度の異なる脂肪酸が存在しており，それらの分布や動態を正確かつ網羅的に解析するための手法が求められる．近年急速に発展している質量分析機器を用いたメタボロミクスがそれに当たる．本稿では，精度の高いメタボローム解析技術として，とくに脂肪酸の質の違いを明らかにするための具体的な分析システムについて紹介する．各項目では，LC-MSを用いた多彩な脂質の特徴に合わせた解析法（1 3 4 5），質量顕微鏡を用いた局在解析（2），さらに複数の方法を組合わせた最新の解析ストラテジーを紹介した．さらに項目6では，多層的オミックス解析を展開する脂質研究の新潮流を，進行中の大型研究プロジェクトを含めてご覧いただく．

1 リン脂質の多様性とメタボロミクス

❶ リン脂質，脂肪酸の基本構造

生体膜の主要構成成分であるリン脂質は，グリセロリン脂質とスフィンゴリン脂質に分類される（図1）．グリセロリン脂質の構造は，グリセロール-3-リン酸にコリン，エタノールアミン，セリン，イノシトールなどの水酸基を有する極性基とのリン酸ジエステルに2分子の脂肪酸がエステル結合したものであり，それぞれホスファチジルコリン，ホスファチジルエタノールアミン，ホスファチジルセリン，ホスファチジルイ

Application of lipidomics to the lipid biology research

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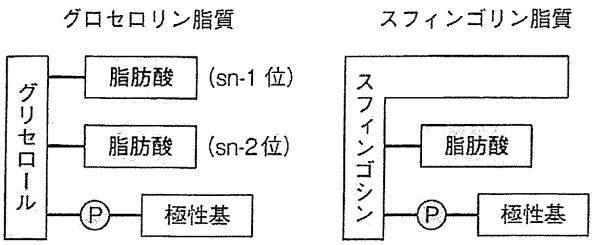

図1　グリセロリン脂質とスフィンゴリン脂質の模式図

ノシトール，ホスファチジン酸などとよばれる．また，脂肪酸の代わりに長鎖アルキル，アルケニルを有するものもある．またスフィンゴリン脂質もグリセロリン脂質と同様に主要な膜脂質である．セラミドの水酸基にホスホリルコリン基がエステル結合したものがスフィンゴミエリンであり，単糖またはオリゴ糖がグルコシド結合したものがスフィンゴ糖脂質である．

グリセロリン脂質にエステル結合する脂肪酸は，sn-1位が飽和脂肪酸あるいはオレイン酸（C18:1），sn-2位が多価不飽和脂肪酸であることが多い．一方で，網膜の膜リン脂質にはsn-1，sn-2両方に多価不飽和脂肪酸であるドコサヘキサエン酸（DHA，C22:6）が結合した分子種が多く含まれ，網膜ロドプシンの機能において重要であると考えられている．また，肺胞Ⅱ型上皮細胞が分泌する肺サーファクタントには，sn-1，sn-2両方が飽和脂肪酸のリン脂質が多く含まれている．このように，生体内には異なる脂肪酸により構成される多様なリン脂質分子種が，それぞれの部位に特徴的な分布を示している．また，炎症のメディエーターとして知られるエイコサノイド（プロスタグランジン，ロイコトリエンなど）は脂肪酸代謝物であり，リン脂質のsn-2位からホスホリパーゼA_2により切り出されたアラキドン酸（C20:4）から，シクロオキシゲナーゼ，リポキシゲナーゼなどの酵素反応により合成される．また，スフィンゴシン1リン酸やリゾホスファチジン酸などのリゾリン脂質も，免疫機能の調節や血管形成などの多彩な生理機能にかかわるメディエーターとして機能する．すなわち，リン脂質における脂肪酸分子種の多様性は，そこから生成する脂質メディエーターの種類を規定するうえでも重要な要素である．

グリセロリン脂質の生合成は，Kennedy経路とよばれるde novo合成系と，2位の脂肪酸を切り離して新たな脂肪酸を導入するリモデリング経路からなる．特にリモデリング経路がリン脂質の脂肪酸分子種を規定する重要な過程であると考えられており，脂肪酸の切り出しにかかわるホスホリパーゼA_2，そして脂肪酸の導入にかかわるアシル基転移酵素により制御されている．近年これらグリセロリン脂質の脂肪酸リモデリングにかかわる酵素の分子実体が次々と明らかになり，生化学的および遺伝学的な解析がなされている[1)~3)]．これらの知見に加え，多様なリン脂質分子種およびその代謝物が，いつ，どこに，どれだけあるのかを高感度かつ包括的に測定することが，リン脂質分子種の多様性の生物学的意義について理解を進めるうえで重要である．

❷ リン脂質の解析技術

リン脂質分子種のメタボローム解析には，主にLC-MSが用いられる[4)]．MSにおけるイオン化法のなかで，エレクトロスプレーイオン化（ESI）はソフトなイオン化法であり，LCによる分離と連動させて分子イオンを同定するLC-MSにおいて最適な方法である．リン脂質の脂肪酸鎖については，逆相系のLCとオンラインのESI，MS/MSによる分子種特異的なプロダクトイオンの検出で解析可能である．リン脂質由来のプロダクトイオンとしては，主に極性基由来のものと脂肪酸鎖に由来するものが生じ，これらを用いて個別のリン脂質分子の構造情報が得られる．さらに，特定のプロダクトイオンを生じるすべての前駆体を検出するプレカーサーイオンスキャン，また特定の部分構造や官能基由来の共通の中性フラグメントが脱離した場合，そのプレカーサーイオンをすべて検出するニュートラルロススキャンなどの手法により，極性基のクラスやある特定の脂肪酸をもつリン脂質を選択的に検出することができる．例えば，アラキドン酸のMS値のプレカーサーイオンスキャンを行うと，アラキドン酸を有するリン脂質のみを特異的に検出することができる．また，ホスファチジルセリンからは極性基であるセリンが脱離したプロダクトイオンが生成するため，セリンの質量数のニュートラルロススキャンを行うことで，ホスファチジルセリンが選択的に検出される（図2）．この手法はLCによる分離を行わずとも用いることが