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ORIGINAL ARTICLE

A whole-genome association study of major determinants for allopurinol-related Stevens–Johnson syndrome and toxic epidermal necrolysis in Japanese patients

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Stevens–Johnson syndrome and toxic epidermal necrolysis (SJS/TEN) are severe, cutaneous adverse drug reactions that are rare but life threatening. Genetic biomarkers for allopurinol-related SJS/TEN in Japanese were examined in a genome-wide association study in which Japanese patients ($n=14$) were compared with ethnically matched healthy controls ($n=991$). Associations between 890 321 single nucleotide polymorphisms and allopurinol-related SJS/TEN were analyzed by the Fisher's exact test (dominant genotype mode). A total of 21 polymorphisms on chromosome 6 were significantly associated with allopurinol-related SJS/TEN. The strongest association was found at rs2734583 in *BAT1*, rs3094011 in *HCP5* and GA005234 in *MICC* ($P=2.44\times10^{-8}$; odds ratio = 66.8; 95% confidence interval, 19.8–225.0). rs9263726 in *PSORS1C1*, also significantly associated with allopurinol-related SJS/TEN, is in absolute linkage disequilibrium with *human leukocyte antigen-B*5801*, which is in strong association with allopurinol-induced SJS/TEN. The ease of typing rs9263726 makes it a useful biomarker for allopurinol-related SJS/TEN in Japanese.
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Keywords: allopurinol; Stevens–Johnson syndrome; toxic epidermal necrolysis; human lymphocyte antigen; single nucleotide polymorphism; genome-wide association study

Introduction

Allopurinol is a xanthine oxidase inhibitor that prevents the production of uric acid to reduce plasma uric acid levels to a normal range. It is the most frequently used anti-hyperuricemic agent in the world due to its long-term pharmacological effect.[1] However, allopurinol is also one of the most frequent causes of a variety of delayed severe cutaneous adverse drug reactions (SCARs).[2] According to spontaneous reports of severe adverse drug reactions to the Ministry of Health, Labor, and Welfare of Japan, allopurinol-related SCARs accounted for about 11% of all reported SCAR cases in Japan in 2008.[3] Allopurinol-related SCARs include the drug-induced hypersensitivity syndrome, Stevens–Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN).[4] SJS/TEN are characterized by high fever, malaise and rapid development of blistering exanthema, with macules and target-like lesions, accompanied by mucosal involvement.[5] Even though the incidence of SJS/TEN is extremely low, the mortality rate of TEN can be as high as 26%.[5] Therefore, SJS/TEN is a serious problem in allopurinol therapy, in spite of the ideal anti-hyperuricemic effect of allopurinol.

Although previous works have suggested that the development of SJS/TEN depends on an immune mechanism involving a drug-dependent cytotoxic cell response against epidermal cells,[5,6] the pathophysiology of SJS/TEN remains largely unknown. Susceptibility to such idiosyncratic reactions is thought to be genetically determined, and familial predisposition to allopurinol-induced SJS/TEN has been reported.[6] Therefore, the exploratory studies for genetic risk factors related to SJS/TEN are needed. A strong association has been observed between allopurinol-induced SCAR and the human lymphocyte antigen (*HLA*) allele B variant (*HLA-B*5801*) in the Han Chinese in Taiwan[7] and in the Thai population.[8] These studies showed that the *HLA-B*5801* allele is present in all patients with allopurinol-induced SCAR (51/51 of Han Chinese and 27/27 of Thai patients) and in only 12–15% of tolerant patients (20/135 and 7/54, respectively). The odds ratio (OR) was 580 (95% confidence interval , 34–9781; $P = 4.7 \times 10^{24}$) for the Han-Chinese data[7] and 348.3 (95% confidence interval, 19.2–6336.9; $P = 1.61 \times 10^{13}$) for the Thai study.[8] Although the association was confirmed in both Caucasian and Japanese subjects,[9,10] the OR in the Han-Chinese and Thai populations were much higher than those in the Caucasian (OR = 80) and Japanese (OR = 40) groups. These reports indicated that *HLA-B*5801* is the valid genetic biomarker for allopurinol-induced SJS/TEN in various ethnic groups, but the mechanisms by which *HLA-B*5801* is specifically involved in allopurinol-induced SJS/TEN progression and the strength of the association showed ethnic differences are unknown.

Currently, genotyping by high-density array scanning of the whole genome allows discovery of previously unsuspected genetic risk factors that influence the pathogenesis of serious adverse drug reactions.[11–13] Genome-wide association studies (GWASs) provide opportunities to uncover polymorphisms that influence susceptibility to allopurinol-induced SJS/TEN free of mechanistic hypotheses. Therefore, in addition to *HLA-B* typing as shown in our previous study,[10] we further conducted a retrospective pharmacogenetic case–control study using whole-genome single nucleotide polymorphism (SNP) data from high-density DNA microarrays in order to identify new and effective genetic biomarkers for allopurinol-related SJS/TEN in Japanese patients.

Materials and methods

Recruitment of study subjects

A total of 141 Japanese SJS/TEN patients from unrelated families were recruited from July 2006 to April 2010 from participating institutes of the Japan Severe Adverse Reactions (JSAR) research group and through a nationwide blood-sampling network system in Japan for SJS/TEN onset patients, operated by the National Institute of Health Sciences.[10] In all, 121 of these patients were diagnosed as defined SJS or TEN by JSAR research group's dermatological experts based on diagnostic criteria[4] that are currently used in Japan. Information was collected using a standardized case report form that includes medical records, co-administrated drug records, disease progress and involvement of systemic complications, as well as SJS/TEN treatment. Among the 141 SJS/TEN patients, 20 were diagnosed as probable SJS due to atypical or mild symptoms. TEN and SJS were defined as mucocutaneous disorders characterized by extensive erythema, blisters, epidermal detachment, erosions, enanthema and high fever. SJS was defined as skin detachment of 10% or less of the body surface area, and TEN as skin detachment of more than 10%, excluding staphylococcal scaled skin syndrome.[5] In all enrolled cases defined as SJS or TEN, allopurinol was regarded as the drug responsible for SJS or TEN if the onset of SJS/TEN symptoms occurred within the first 2 months of allopurinol exposure. For the retrospective pharmacogenetic case–control study, 991 healthy, ethnically matched subjects in the Tokyo metropolitan area were used as the control group. Healthy subjects were used as the control group instead of allopurinol-tolerant patients because the incidence of SJS/TEN is extremely low (0.4–6 per million per year).[3]

The ethics committees of the National Institute of Health Sciences, each participating institute of the JSAR research group and the Japan Pharmacogenomics Data Science Consortium (JPDSC) approved this study. Written informed consent was obtained from all cases and ethnically matched controls.

Whole-genome genotyping of SNPs

Genome-wide genotyping of the 14 allopurinol-related SJS/TEN patients and 991 ethnically matched controls was conducted using the Illumina Human 1M-Duo BeadChip (Illumina, San Diego, CA, USA), which contained 1 163 218 SNPs. SNPs were discarded from case–control association analysis if they exhibited a minor allele frequency <0.001 in the control group (237 890 SNPs), a call rate <0.95 for each SNP (32 640 SNPs) or a P-value <0.001 in the test of Hardy–Weinberg equilibrium among controls (2 368 SNPs). These quality control steps removed a total of 272 897 SNPs. All samples had a call rate for each microarray above 0.99. Sample duplicates and hidden relatedness were investigated on the basis of pairwise identity-by-state analysis via PLINK;[14] however, there was no duplicate or hidden relatedness in the samples. This quality-control procedure ensured reliable genotyping data.

HLA genotyping and TaqMan genotyping of SNPs on chromosome 6

HLA A, *B* and *Cw* types were determined using sequencing-based methods, as described previously.[10] Representative SNPs of 6p21 (rs2734583, rs3099844, rs9263726 and rs3131643) were re-genotyped using TaqMan SNP Genotyping Assays (Life Technologies, Carlsbad, CA, USA) (ID; C__27465749_10, C__27455402_10, C__30352071_10, C__26778946_20) according to the manufacturer's instruction using 5 ng of genomic DNA. We did not genotype rs9267445 and rs1634776 because TaqMan SNP genotyping assays for these SNPs were not available. Measurement of the linkage disequilibrium (LD) coefficient was performed using

the *HLA* types and 6p21 SNPs of the 141 Japanese SJS/TEN cases and an additional 65 Japanese individuals (non-SJS/TEN patients). The LD coefficient was calculated as previously described.[15,16]

Association analysis

Genome-wide SNPs data from allopurinol-related SJS/TEN cases and ethnically matched controls were used for association analysis using the Fisher's exact test based on the dominant genotype mode and minor allele frequencies of each SNP. Because there are no homozygotes of minor alleles of SNPs, which have significantly related to allopurinol-related SJS/TEN except rs3099844 and rs3131643 in 'Case group', other association analysis models such as trend test (Cochran–Armitage analysis) or recessive model analysis were not applied in this study. All association analyses were carried out with PLINK.[14] *P*-values were corrected for multiple testing according to the Bonferroni's correction. *P*-values $<5.62\times10^{-8}$ were regarded as statistically significant.

Results

Characteristics of study subjects

A total of 14 allopurinol-treated Japanese patients, who were diagnosed with definite SJS/TEN were recruited for the whole-genome association study (IDs 1–14 in Table 1). Patients, IDs 1, 2, 3, 9, 10, 13 and 14 were reported in our previous paper.[10] After the GWAS, an additional four allopurinol-treated Japanese SJS/TEN patients were recruited for *HLA* typing (IDs 15–18). Therefore, a total of 18 allopurinol-treated Japanese SJS/TEN patients participated in the study (Table 1). In all, 12 of 18 patients were male and 6 were female, and the average age was 72.3 ± 10.0 (mean $\pm$ s.d.) years. In all, 12 of 18 cases showed systemic complications of liver and/or renal dysfunction, and most patients had high fever. The average period of SJS/TEN onset after allopurinol treatment was 21.7 ± 11.9 days. Drug-induced lymphocyte stimulation tests were examined in 13 of 18 patients to determine the causative agent; however, in these tests, only two cases (IDs 1 and 5) were positive for allopurinol and only one (ID 16) was positive for oxipurinol, a metabolite of allopurinol. The patient (ID 1) who was positive for the drug-induced lymphocyte stimulation test for allopurinol was also positive for other co-administrated drugs (Table 1). On the other hand, patients who received a patch test showed positive reactions for allopurinol although only two patients were examined (ID 4, 10). The patient who was patch test positive for allopurinol (ID 4) was also patch test positive for other co-administrated drugs (Table 1). Four patients (ID 1, 2, 4 and 14) were co-administrated non-steroidal anti-inflammatory drugs, four (ID 7, 8, 11 and 15) were co-administrated angiotensin II receptor antagonists and three (ID 4, 7 and 17) were co-administrated statin anti-hyperlipemic agents.

Whole-genome association study of major determinants for allopurinol-related SJS/TEN

A total of 14 allopurinol-related SJS/TEN patients (IDs 1–14), who were diagnosed with definite SJS/TEN, and 991 ethnically matched controls, were genotyped with the use of the Illumina Human 1M-Duo BeadChip containing 1 163 218 SNPs. A series of quality-control steps resulted in the elimination of 272 897 polymorphisms. For each SNP, Fisher's exact tests were performed to compare the dominant genotype distributions and minor allelic frequencies in the allopurinol-related SJS/TEN patients (the case group) versus those in the ethnically matched healthy control group. The resulting *P*-values were adjusted with the Bonferroni's correction ($P<5.62\times10^{-8}$). The distribution of *P*-values from the Fisher's exact tests (dominant genotype mode) along each chromosome indicated that 21 SNPs were significantly associated with the cases, all of which were located on the chromosome 6: 6p21.3, 6p22.1 and 6p21.1 (Figures 1a and b). The quantile–quantile (Q–Q) plot for the distribution of *P*-values showed that observed *P*-values matched the expected *P*-values over the range of $0<-\log_{10}(p)<4.0$ (Figure 2). A departure was observed at the extreme tail ($-\log_{10}(p)>4.0$) of the distribution of test statistics for the allopurinol-related Japanese SJS/TEN, suggesting that the identified associations are likely due to true variants rather than potential biases such as genotyping error. These SNPs, with their associated genes, are described in Table 2. As is observed in all SNPs in Table 2, minor allele frequencies in the controls were quite small, ranging around 0.5–0.6%. The genotypic distributions of the case and control groups are identical among groups with the same *P*-value, suggesting that these SNPs might be linked. These SNPs also have ORs that are much higher than the ORs of SNPs commonly observed in sporadic cancer and other complex diseases, suggesting they are of higher penetrance. For example, the most significant SNPs (rs2734583, rs3094011 and GA005234) had an OR of 66.8 (95% confidence interval, 19.8–225.0), and the twentieth most significant SNPs (rs9263827 and rs1634776) had an OR of 60.9 (95% confidence interval, 18.3–202.5). Most SNPs in Table 2 are associated with known or predicted genes; of these, 13 are in known genes. Three SNPs (rs17190526, rs9263726 and rs2233945) were found in *PSORS1C1* (psoriasis susceptibility 1 candidate 1), which is considered as one of the potential psoriasis genes.[17–19] The *CCHCR1* (coiled coil α helical rod protein 1), which is a regulator of keratinocyte proliferation or differentiation and is over-expressed in keratinocytes in psoriatic lesions,[20–23] contained four SNPs (rs9263745, rs130077, rs9263781 and rs9263785). *HCP5* (HLA complex P5), which is involved in hypersensitivity to abacavir,[24–26] had three SNPs (rs3094011, rs3099844 and rs31431643). *TCF19* (transcription factor 19), which is a potential trans-activating factor that might play an important role in the transcription of genes required for the later stages of cell cycle progression,[27] contained two SNPs (rs9263794 and rs10448701). Two SNPs (rs9263796 and rs9263800) were also found in *POU5F1* (POU class 5 homeobox; alternative names for Oct4). *BAT1* (HLA-B

Table 1 Summary of clinical characteristics of Japanese patients with allopurinol-related Stevens–Johnson syndrome or toxic epidermal necrolysis

								Co-administered drugs	
Patient ID[a]	*ADR type*	*Sex/age (years)*	*Highest BT (°C)*	*Total area of blistering skin (%)*	*Systemic complications*	*DLST to allopurinol (PT)*	*Period of onset (days) by allopurinol*	*Drug name*	*DLST result/period of onset*
1	SJS	F/53	38.1	0.5	liver dysfunction renal dysfunction	+	26	loxoprofen clarithromycin	+/9 days +/26 days
2	TEN	M/58	37.1	15	neutropenia liver dysfunction	–	ca 10 days	loxoprofen levofloxacin	–/1 day –/1 day
3	SJS	M/77	unknown	unknown	none	not tested	16	none	
4	TEN	F/72	>37	20	none	–(PT+)	16	pitavastatin lansoprazole salicylamide, acetaminophen, caffeine, promethazine, methylenedisalicylate serrapeptase loxoprofen acetaminophen	–/16 days –/179 days –(PT+)/8 days –/1 day –/8 days (PT+)/8 days
5	TEN	M/82	39	35	none	+	52	none	
6	SJS	M/67	1	1	liver dysfunction	not tested	14	none	
7	SJS	M/76	38.8	unknown	GI tract disturbance liver dysfunction renal dysfunction	not tested	<26 days	losartan furosemide carbon atorvastatin	not tested/8 days not tested/3 days not tested/7 days not tested/8 days
8	SJS	M/83	>38	10	renal dysfunction	–	20	amlodipine olmesartan medoxomil	not tested/very long not tested/very long
9	TEN	M/75	>38	20	neutropenia liver dysfunction renal dysfunction	–	6	none	
10	SJS	M/75	38.4	6	neutropenia liver dysfunction renal dysfunction	–(PT+)	14	none	
11	SJS	M/74	37.8	8	neutropenia liver dysfunction renal dysfunction	–	38	cefazolin Furosemide Sodium polystyrene sulfonate olmesartan medoxomil	not tested/1 day not tested/53 day not tested/51 day not tested/59 day
12	SJS	M/67	38.9	2	liver dysfunction	not tested	17	none	
13	SJS	F/81	39.2	0.5	renal dysfunction	–	28	spironolactone	–/24 days
14	SJS	M/83	39	0	respiratory involvement	–	29	diclofenac	–/1 day
15	TEN	F/73	38	10	liver dysfunction renal dysfunction	–	27	valsartan epoetin β	–/18 days –/2 days
16	SJS	M/53	40	5	liver dysfunction	–(oxipurinol +)	19	none	
17	SJS	F/86	38	0	liver dysfunction renal dysfunction	–	30	rosuvastatin	–/43 days
18	TEN	F/66	37.8	15	none	not tested	2	none	

Abbreviations: ADR, adverse drug reaction; BT, body temperature; DLST; drug-induced lymphocyte stimulation test; F, female; M, male; PT, patch test; SJS, Stevens–Johnson syndrome; TEN, toxic epidermal necrolysis.

[a]Patients ID 1–14 were applied for whole genome analysis. ID 1–18 were for the *HLA* typing and the analysis of linkage disequilibrium.

Patients IDs 1, 2, 3, 9, 10, 13, and 14 were reported in our previous paper.[10]

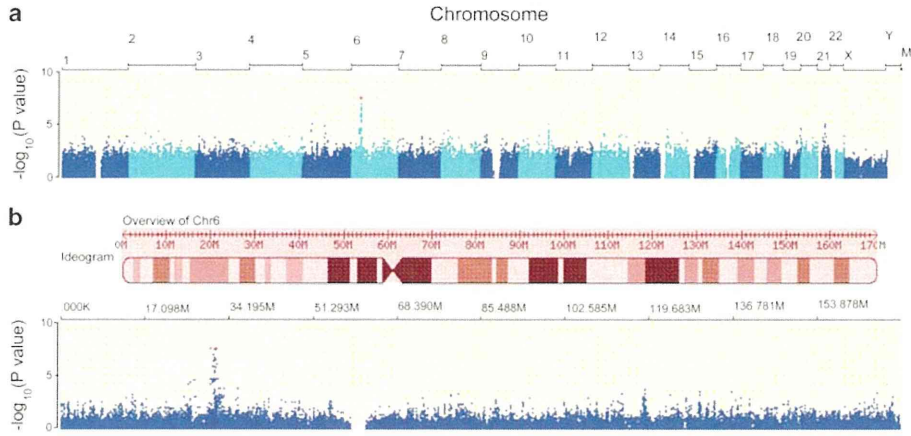

Figure 1 Genome-wide association study of allopurinol-related Stevens–Johnson syndrome or toxic epidermal necrolysis. Each dot represents a single nucleotide polymorphism (SNP). The *x* axis: the position of the SNP on chromosomes. The *y* axis: the $-\log_{10}$ of Fisher's exact test *P*-values (dominant genotype mode) of the SNP in the case–control association study. SNPs with *P*-values $< 5.62 \times 10^{-8}$ are highlighted in red. (**a**) Whole genome. (**b**) Chromosome 6.

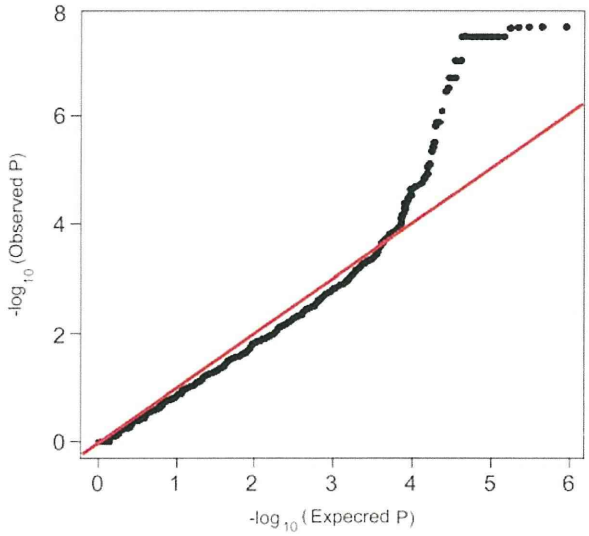

Figure 2 Quantile–quantile plot of Fisher's exact test statistics obtained from the genome-wide association study for allopurinol-related Stevens–Johnson syndrome or toxic epidermal necrolysis under dominant genotype mode. The solid red line represents the null model where observed Fisher's exact test values match the expected values. The dots represent observed versus the expected values from the case–control study.

associated transcript 1) and *PSORS1C3* each carried one SNP (rs2734583 and rs9263827). The SNPs, rs1634776 and rs4084090, were located in more than 10 kb away from the *HLA-B* and *HLA-C* genes, respectively. Two pseudo genes, *MICC* (major histocompatibility complex class I polypeptide-related sequence) and *PPIAP9* (peptidylprolyl isomerase A (cyclophilin A) pseudogene 9), had one SNP each (GA005234 and rs9267445). Previous report using Han-Chinese patients with allopurinol-induced SCAR indicated rs3117583 of *BAT3*, rs1150793 of *MSH5* and rs2855804 of *MICB*, which are located in *HLA* region, showed significant *P*-values ($P < 1 \times 10^{-7}$).[7] In this study using Japanese patients, both rs3117583 and rs1150793 showed $P = 6.34 \times 10^{-3}$ (allele frequency mode) and $P = 6.14 \times 10^{-3}$ (dominant genotype mode). There was no data of rs2855804 in the Illumina Human 1M-Duo BeadChip.

HLA types of allopurinol-related SJS/TEN patients

Classical class I *HLA* types (*A*, *B* and *Cw*) of allopurinol-related SJS/TEN patients were determined because the *HLA-B*5801* type is associated with allopurinol-related SCARs in Han Chinese,[7] Caucasians[9] and Japanese[10] (Table 3). In this analysis, four patients with allopurinol-related SJS/TEN (IDs 15–18), who were recruited after BeadChip analysis, joined the case group (total of 18 allopurinol-related SJS/TEN patients). Eight cases of *HLA-A*3303* (allele frequency = 22.2%), 10 cases of *HLA-B*5801* (allele frequency = 27.8%) and 10 cases of *HLA-Cw*0302* (allele frequency = 27.8%) were found in 18 allopurinol-related SJS/TEN patients (Table 3). By comparison, the allelic frequencies of *HLA-A*3303*, *HLA-B*5801* and *HLA-Cw*0302* were 7.9%, 0.6% and 0%, respectively in Japanese general population (Tables 4a–c). The OR of *HLA-A*3303* was calculated as 3.32 (Table 4a). The OR of *HLA-B*5801* was calculated as 62.8 (Table 4b), which was a little larger than the previously reported OR in Japanese patients.[10] *HLA-Cw*0302* also showed significant association with allopurinol-related SJS/TEN (Table 4c). *HLA-A*3303* and *HLA-Cw*0302* are in LD with *HLA-B*5801* in the Japanese although the general frequency of *HLA-A*3303* is higher than other two types. Other *HLA-A*, *B* and *Cw* types, which were not listed in Tables 4a–c, showed very low frequencies in the general Japanese population, or were not found in 18 allopurinol-related SJS/TEN patients.

Table 2 The association of single nucleotide polymorphism with allopurinol-related Japanese patients with Stevens–Johnson syndrome or toxic epidermal necrolysis

							Dominant genotype mode		Allelic frequency mode	
Order	*SNP*	*Chromosome*	*Closest gene*	*Distance to gene (bp)*	*Case*[a]	*Control*[a]	P	*Odds ratio (95% CI)*	P	*MAF (%)*
1	rs2734583	6p21.3	*BAT1*	0	0/6/8	0/11/980	2.44×10^{-8}	66.8 (19.8–225.0)	4.62×10^{-8}	0.55
1	rs3094011	6p21.3	*HCP5*	6553	0/6/8	0/11/980	2.44×10^{-8}	66.8 (19.8–225.0)	4.62×10^{-8}	0.55
1	GA005234	6p22.1	*MICC*	0	0/6/8	0/11/980	2.44×10^{-8}	66.8 (19.8–225.0)	4.62×10^{-8}	0.55
4	rs3099844	6p21.3	*HCP5*	3693	1/5/8	0/11/978	2.47×10^{-8}	66.7 (19.8–224.5)	1.33×10^{-9}	0.56
5	rs9267445	6p21.1	*PPIAP9*	3776	0/6/8	0/11/971	2.58×10^{-8}	66.2 (19.7–222.9)	4.87×10^{-8}	0.56
6	rs17190526	6p21.3	*PSORS1C1*	−446	0/6/8	0/12/979	3.64×10^{-8}	61.2 (18.4–203.5)	6.87×10^{-8}	0.61
6	rs9263726	6p21.3	*PSORS1C1*	0	0/6/8	0/12/979	3.64×10^{-8}	61.2 (18.4–203.5)	6.87×10^{-8}	0.61
6	rs2233945	6p21.3	*PSORS1C1*	0	0/6/8	0/12/979	3.64×10^{-8}	61.2 (18.4–203.5)	6.87×10^{-8}	0.61
6	rs9263733	6p21.3	*POLR2LP*	139	0/6/8	0/12/979	3.64×10^{-8}	61.2 (18.4–203.5)	6.87×10^{-8}	0.61
6	rs9263745	6p21.3	*CCHCR1*	0	0/6/8	0/12/979	3.64×10^{-8}	61.2 (18.4–203.5)	6.87×10^{-8}	0.61
6	rs130077	6p21.3	*CCHCR1*	0	0/6/8	0/12/979	2.44×10^{-8}	61.2 (18.4–203.5)	6.87×10^{-8}	0.61
6	rs9263781	6p21.3	*CCHCR1*	0	0/6/8	0/12/979	2.44×10^{-8}	61.2 (18.4–203.5)	6.87×10^{-8}	0.61
6	rs9263785	6p21.3	*CCHCR1*	0	0/6/8	0/12/979	2.44×10^{-8}	61.2 (18.4–203.5)	6.87×10^{-8}	0.61
6	rs9263794	6p21.3	*TCF19*	0	0/6/8	0/12/979	2.47×10^{-8}	61.2 (18.4–203.5)	6.87×10^{-8}	0.61
6	rs1044870	6p21.3	*TCF19*	0	0/6/8	0/12/979	2.58×10^{-8}	61.2 (18.4–203.5)	6.87×10^{-8}	0.61
6	rs9263796	6p21.3	*POU5F1*	0	0/6/8	0/12/979	3.64×10^{-8}	61.2 (18.4–203.5)	6.87×10^{-8}	0.61
6	rs9263800	6p21.3	*POU5F1*	0	0/6/8	0/12/979	3.64×10^{-8}	61.2 (18.4–203.5)	6.87×10^{-8}	0.61
6	rs4084090	6p21.3	*HLA-C*	17691	0/6/8	0/12/979	3.64×10^{-8}	61.2 (18.4–203.5)	6.87×10^{-8}	0.61
19	rs3131643	6p21.3	*HCP5*	0	1/5/8	0/12/977	3.68×10^{-8}	61.1 (18.4–203.1)	2.08×10^{-9}	0.61
20	rs9263827	6p21.3	*PSORS1C3*	−3369	0/6/8	0/12/974	3.75×10^{-8}	60.9 (18.3–202.5)	7.07×10^{-8}	0.61
20	rs1634776	6p21.3	*HLA-B*	12661	0/6/8	0/12/974	3.75×10^{-8}	60.9 (18.3–202.5)	7.07×10^{-8}	0.61

Abbreviations: CI, confidence interval; MAF; minor allelic frequency; SNP, ingle nucleotide polymorphism.
[a]Number of subjects in minor homo/hetero/major homo.

Table 3 *HLA* types and representative genotypes in 6p21 of allopurinol-related Japanese patients with Stevens–Johnson syndrome or toxic epidermal necrolysis

ID	HLA-A		HLA-B		HLA-Cw		*rs2734583*	*rs3099844*	*rs9267445*	*rs9263726*	*rs3131643*	*rs1634776*
1	2402	3303	4002	**5801**	0302	0304	T/**C**	C/A	G/**C**	G/A	C/**T**	G/A
2	2402	3101	1501	5601	0303	0401	T/T	C/C	G/G	G/G	C/C	G/G
3	2402	3101	5201	**5801**	0302	1202	T/**C**	C/A	G/**C**	G/A	C/**T**	G/A
4	1101	1101	4801	**5801**	0302	0803	T/**C**	A/A	G/**C**	G/A	T/**T**	G/A
5	2402	2602	4006	5101	0801	1402	T/T	C/C	G/G	G/G	C/C	G/G
6	0201	1101	1518	3501	0401	0801	T/T	C/C	G/G	G/G	C/C	G/G
7	2402	3303	5201	**5801**	0302	1202	T/**C**	C/A	G/**C**	G/A	C/**T**	G/A
8	0201	2402	1527	4003	0304	0401	T/T	C/C	G/G	G/G	C/C	G/G
9	2402	2402	3501	5201	0303	1202	T/T	C/C	G/G	G/G	C/C	G/G
10	0210	1101	4002	4006	0401	0801	T/T	C/C	G/G	G/G	C/C	G/G
11	0207	2402	4601	5101	0102	1402	T/T	C/C	G/G	G/G	C/C	G/G
12	2402	3101	3901	4001	0304	0702	T/T	C/C	G/G	G/G	C/C	G/G
13	0207	3303	4601	**5801**	0102	0302	T/**C**	C/A	G/**C**	G/A	C/**T**	G/A
14	3101	3303	3901	**5801**	0302	0702	T/**C**	C/A	G/**C**	G/A	C/**T**	G/A
15	2402	3303	5101	**5801**	0302	1402	T/**C**	C/A	NA	G/A	T/**T**	NA
16	0201	3303	3802	**5801**	0302	0702	T/**C**	C/A	NA	G/A	T/**T**	NA
17	2402	3303	0702	**5801**	0302	0702	T/**C**	C/A	NA	G/A	C/**T**	NA
18	2402	3303	5101	**5801**	0302	0304	T/**C**	C/A	NA	G/A	T/**T**	NA

Abbreviations: HLA, human leukocyte antigen; NA, not available.
Single nucleotide polymorphisms data of rs2734583, rs3099844, rs9263726 and rs3131643 are from BeadChip analysis and TaqMan genotyping analysis. Single nucleotide polymorphisms data of rs9267445 and rs1634776 are from BeadChip analysis.
Underlines of HLA types mean that these types are in linkage disequilibrium. HLA-B*5801s are expressed by bold types.
Bold types of the nucleotide mean the variant allele.

Table 4a Association between *HLA-A* alleles and allopurinol-induced Stevens–Johnson syndrome or toxic epidermal necrolysis

HLA-A allele	*Number of alleles detected (allele frequency)*		P	*Odds ratio (95% CI)*
	Case, n = 36 (%)	*General population control (n = 986)[a] (%)*		
0201	3 (8.3)	10.9	0.7895	
0206	0 (0)	10.4	0.0426	
0207	2 (5.6)	3.4	0.3650	
0210	1 (2.8)	0.1	0.0692	
1101	4 (11.1)	8.1	0.5299	
2402	13 (36.1)	35.6	1.000	1.02 (0.51–2.04)
2601	0 (0)	9.8	0.0417	
2602	1 (2.8)	2.2	0.5657	
3101	4 (11.1)	7.7	0.5195	
3303	8 (22.2)	7.9	0.0077	3.32 (1.46–7.54)

Abbreviations: CI, confidence interval; HLA, human leukocyte antigen.
We listed the *HLA-A* types of which the allele frequencies in the Japanese population are more than 9% or which were detected in this study.
[a]General population control data are cited from Tanaka *et al.*[40]

Table 4b Association between *HLA-B* alleles and allopurinol-induced Stevens–Johnson syndrome or toxic epidermal necrolysis

HLA-B allele	*Number of alleles detected (allele frequency)*		P	*Odds ratio (95% CI)*
	Case, n = 36 (%)	*General population control (n = 986)[a] (%)*		
0702	1 (2.8)	5.2	1.000	
1501	1 (2.8)	7.2	0.5076	
1518	1 (2.8)	0.9	0.3025	
1527	1 (2.8)	0	0.0352	
3501	2 (5.6)	8.6	0.7621	
3802	1 (2.8)	0.3	0.1338	
3901	2 (5.6)	4.0	0.6520	
4001	1 (2.8)	5.1	1.0000	
4002	2 (5.6)	8.2	0.7620	
4003	1 (2.8)	1.1	0.3512	
4006	2 (5.6)	5.3	0.7150	
4403	0 (0)	6.9	0.1648	
4601	2 (5.6)	3.8	0.6441	
4801	1 (2.8)	2.7	1.0000	
5101	4 (11.1)	7.9	0.5244	
5201	3 (8.3)	13.7	0.4624	
5401	0 (0)	6.5	0.1620	
5601	1 (2.8)	1.0	0.3273	
5801	10 (27.8)	0.6	5.388×10^{-12}	62.8 (21.2–185.8)

Abbreviations: CI, confidence interval; HLA, human leukocyte antigen.
We listed the *HLA-B* types of which the allele frequencies in the Japanese population are more than 6.5% or which were detected in this study.
[a]General population control data are cited from Tanaka *et al.*[40]

*LD of HLA-B*5801 with SNPs on chromosome 6*

We compared the genotypic distributions of six SNPs, which were significantly associated with SJS/TEN (Table 2), with *HLA* types because these SNPs are located near the *HLA-B* gene. These 6 SNPs listed in Table 3 represent 21 SNPs in Table 2 because the other 15 SNPs are in absolute LD with 1 of the 6 SNPs. Representative six variants of the significant SNPs on chromosome 6 were found in all of the SJS/TEN patients who carried the *HLA-B*5801* (10 patients) (Table 3). Therefore, in order to evaluate LD in the Japanese

Table 4c Association between *HLA-Cw* alleles and allopurinol-induced Stevens–Johnson syndrome or toxic epidermal necrolysis

HLA-Cw allele	*Number of alleles detected (allele frequency)*		P	*Odds ratio (95% CI)*
	Case, n = 36 (%)	*General population control (n = 234)[a] (%)*		
0102	2 (5.6)	17.0	0.0859	
0302	10 (27.8)	0	5.303×10^{-10}	
0303	2 (5.6)	7.8	1.000	
0304	4 (11.1)	11.3	1.000	
0401	4 (11.1)	6.5	0.2961	
0702	4 (11.1)	11.3	1.000	
0801	3 (8.3)	10.9	0.7777	
0803	1 (2.8)	2.6	1.000	
1202	3 (8.3)	10.4	1.000	
1402	3 (8.3)	5.7	0.4559	
1403	0 (0)	12.2	0.0192	

Abbreviations: CI, confidence interval; HLA, human leukocyte antigen.
We listed the *HLA-Cw* types of which the allele frequencies in the Japanese population are more than 10% or which were detected in this study.
[a]General population control data are cited from Tokunaga *et al.*[41]

Table 5 The linkage disequilibrium between *HLA* types and representative single nucleotide polymorphisms on 6p21 of 206 Japanese individuals

HLA	*rs3099844*	*rs3131643*	*rs2734583*	*rs9267445*	*rs9263726*	*rs1634776*
A	0.821	0.621	0.835	0.798	0.847	0.803
B	0.973	0.873	1.000	1.000	1.000	0.996
Cw	0.984	0.773	1.000	1.000	1.000	0.909

Abbreviation: HLA, human leukocyte antigen.
Data are expressed in D'.

Table 6 The linkage disequilibrium between representative single nucleotide polymorphisms on 6p21 and *HLA-B*5801* of 206 Japanese individuals

SNP	D'	r^2
rs3099844	0.930	0.866
rs3131643	0.929	0.674
rs2734583	1.000	0.931
rs9267445	1.000	0.896
rs9263726	1.000	1.000
rs1634776	1.000	0.905

Abbreviation: SNP, single nucleotide polymorphism.

population, LD coefficients (D') were calculated between classical class 1 *HLA* types and six representative SNPs at 6p21, using the *HLA*-type and SNPs genotype data of 206 Japanese individuals, including 141 SJS/TEN cases and an additional 65 non-SJS/TEN Japanese subjects. As shown in Tables 5 and 6 representative SNPs on chromosome 6 showed LD for the *HLAs*. In particular, three SNPs (rs2734583, rs9267445 and rs9263726) showed a strong linkage with *HLA-B* and *Cw* alleles (Table 5). LD between six representative SNPs in 6p21 and *HLA-B*5801* are shown in Table 6. A novel observation was the absolute LD ($D' = 1$, $r^2 = 1$) between rs9263726 in *PSORS1C1* and the *HLA-B*5801* allele.

Discussion

In order to explore new genetic biomarkers associated with the occurrence of allopurinol-related SJS/TEN Japanese patients, we conducted a GWAS using 890321 SNPs from patients with allopurinol-related SJS/TEN and an ethnically matched control group. The GWAS data indicated that most SNPs significantly associated with allopurinol-related SJS/TEN are located on or close to genes that overlap the 6p21 region, especially the genes neighboring *HLA-B*. There was no significantly associated SNP in any other region of the genome (Figures 1 and 2 and Table 2), indicating that the 6p21 region has the most important role in the progress of allopurinol-related SJS/TEN. We expected to find SJS/TEN-associated SNPs, which are unrelated to *HLA-B*5801* from this GWAS study because the association of *HLA-B*5801* with SJS/TEN is incomplete (10/18) in Japanese patients in contrast to Han Chinese[7] and Thai patients.[8] However, most

of significant SNPs were closely linked with *HLA-B*5801* (Table 6). Previous studies have indicated that a SNP (rs2395029) in the *HCP5*, which is on 6p21.3, is strongly associated with human immunodeficiency virus-1 set points,[28–30] abacavir-induced hypersensitivity[24–26] and flucloxacillin-induced liver injury.[31] This SNP is in strong LD with *HLA-B*5701* in Caucasians.[25] Another SNP in 6p21 in *PSORS1C1*, a psoriasis-susceptibility candidate gene, was related with psoriasis in Swedish and Canadian populations[17,18] and exhibits LD with *HLA-Cw*0602* in Canadian populations.[18] These reports suggest that SNPs located in 6p21 link with a specific type of classical class I *HLA* that could be an alternative biomarker for the physiological phenomenon. Therefore, we examined the LD between these SNPs, shown in Table 2, and *HLA-B*5801*, which has been regarded as a genetic biomarker of SJS/TEN not only in Han Chinese,[7] but also in Caucasians[9] and Japanese.[10] We found that all of the Japanese patients with the allopurinol-related SJS/TEN who had the *HLA-B*5801* (10 patients) also had variant SNPs of genes that are located in 6p21, including *BAT1*, *HCP5*, *PPIAP9*, *PSORS1C1* and *HLA-B* (Table 3). The analysis of the LD coefficients between SNPs located in 6p21 and *HLA* types in the Japanese population indicated that these SNPs are in strong LD with *HLA* types (Table 5), and an absolute LD between rs9263726 in *PSORS1C1* and *HLA-B*5801* was observed in the Japanese population (Table 6). These results mean that all subjects (14 individuals including 10 with allopurinol-related SJS/TEN) who carry *HLA-B*5801* are in complete accord with all subjects with minor A allele of rs9263726 in the Japanese population. Therefore, rs9263726 in *PSORS1C1* is an alternative biomarker for *HLA-B*5801* in the Japanese population. Conventional genotyping of rs9263726 based on allelic discrimination offers several advantages over *HLA-B* typing, which is determined by genotyping of several SNPs forming the *HLA-B*5801* haplotype. Various broadly used technologies (for example, TaqMan genotyping) allow the standardized identification of two distinct alleles in one reaction tube, limiting the risk of contamination and allowing high-throughput genotyping with high sensitivity and specificity. In addition, the test is largely independent of both the performance of and interpretation by laboratory personnel. SNP genotyping is also less time consuming and cheaper than sequence-based *HLA* typing, and it does not require specialized laboratories. Therefore, the easy detection of these SNPs has a practical and economical advantage in clinical application for predicting the onset of allopurinol-related SJS/TEN. Although the previous report revealed that three SNPs in *HLA* region strongly associated with allopurinol-related SCAR in Han Chinese,[7] the two SNPs analyzed by the Illumina Human 1M-DUO BeadChip showed only weak association in the Japanese. This ethnic difference might be due to the difference of LD.

The functional analysis of genes that carry these SNPs—including *HCP5*, *BAT1*, *PSORS1C1*, *CCHCR1*, *TCF19* and *POU5F1*—in the pathogenesis of allopurinol-related SJS/TEN might be useful for determining their relevance. *CCHCR1* is a regulator of keratinocyte proliferation or differentiation and is overexpressed in keratinocytes in psoriatic lesions.[20–23] *TCF19* is a potential trans-activating factor that could play an important role in the transcription of genes required for the later stages of cell cycle progression.[27] Possible psoriasis candidate genes near *HLA-B* include *PSORS1C1*,[17–19] *CCHCR1*,[22,23] and *POU5F1*.[32,33] Mutations in *BAT1* may be associated with rheumatoid arthritis.[34–36] *HCP5* encodes an endogenous retroviral element mainly that is expressed in immune cells and there is evidence that the SNP in this gene is protective against human immunodeficiency virus-1 infection.[37–39] The functions and relevance of these genes suggest that the pathogenesis of allopurinol-related SJS/TEN might involve not only an immune system disorder, but also processes of cell proliferation and differentiation.

In conclusion, the results of this GWAS of allopurinol-related SJS/TEN in Japanese patients show that SNPs in genes located in 6p21, which are in LD with *HLA-B*5801*, are strongly associated with the cutaneous adverse reaction. Therefore, these SNPs, especially rs9263726, prove to be predictors for allopurinol-related SJS/TEN in Japanese, and their genes might be involved in the pathogenesis of allopurinol-related SJS/TEN. The OR of rs9263726 is extremely high from this case–control study and the typing cost of SNP is much cheaper than that of *HLA* typing. Moreover, the SJS/TEN has a very severe adverse reaction of allopurinol, which is high mortality. Therefore, we believe that the screening of rs9263726 genotype before allopurinol administration is necessary to prevent SJS/TEN in allopurinol-treated Japanese patients, although its allele frequency is very low in the Japanese. Association analyses of other ethnic populations are needed for confirming and comparing the results obtained in this study. *In vitro* functional studies of these genes are also necessary for identification of the physiological and molecular pathways leading to allopurinol-related SJS/TEN.

Conflict of interest

The authors declare no conflict of interest except one member of JPDSC, Mitsubishi Tanabe Pharma, which is a distributor of allopurinol in Japan.

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Appendix

Japan Pharmacogenomics Data Science Consortium (JPDSC)

The Japan Pharmacogenomics Data Science Consortium is composed of Astellas Pharma, Otsuka Pharmaceutical, Daiichi Sankyo, Taisho Pharmaceutical, Takeda Pharmaceutical and Mitsubishi Tanabe Pharma, and is chaired by Ichiro Nakaoka (Takeda Pharmaceutical).

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薬物動態研究におけるヒト多能性幹細胞の活用

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1. はじめに

幹細胞（stem cells）とは，近年よく聞く言葉であるが，自分と同じ細胞を作る能力（自己複製能）と，組織や臓器を構成する多種類の細胞に分化する能力（多分化能）を併せ持った細胞のことである．幹細胞には階層性があり，上位の未分化な幹細胞は自己複製能が高く様々な細胞系列に分化できるが，下位になるに従い自己複製能は失われていき，特定の細胞系列にしか分化できなくなる．胚性幹細胞（embryonic stem cells, ES 細胞）や人工多能性幹細胞（induced pluripotent stem cells, iPS 細胞）は最上位に位置し，生体を構成する全ての細胞に分化する能力を持っていることから多能性幹細胞あるいは万能細胞とも呼ばれている（図 1）．下位には造血幹細胞，間葉系幹細胞，小腸上皮幹細胞，肝幹細胞などの組織幹細胞（体性幹細胞，成体幹細胞とも呼ばれる）がある．組織幹細胞は分化の方向性が決まっているため分化が容易ではあるが，新鮮な組織をヒトから採取し，しかも極わずかしか含まれていない幹細胞を単離しなければならない．一方，多能性幹細胞は，遺伝子操作が可能であり，安定供給にも優れていることから，分化誘導法が確立さえできれば，実験材料としての利用価値は組織幹細胞よりも遥かに大きいことが容易に想像できる．

医薬品の効果や毒性は体内動態に大きく左右されることから，創薬研究において薬物動態試験の重要性はますます高まってきている．従来，薬物動態試験にはラット等の実験動物が多用されてきたが，種差の問題があり，ヒトへの外挿は容易ではない．そこで，より効率よく評価するために，医薬品開発の早い段階からヒトの細胞やオルガネラが薬物動態試験に使用されるようになった．しかし，肝細胞，小腸上皮細胞，腎尿細管上皮細胞等のヒトの試料は，薬物動態試験や毒性試験において有用な実験材料であるが，新鮮な組織や細胞は入手が困難であり，入手できたとしてもロット間差が大きく，数量も限られている．一方，iPS 細胞は目的とする細胞への分

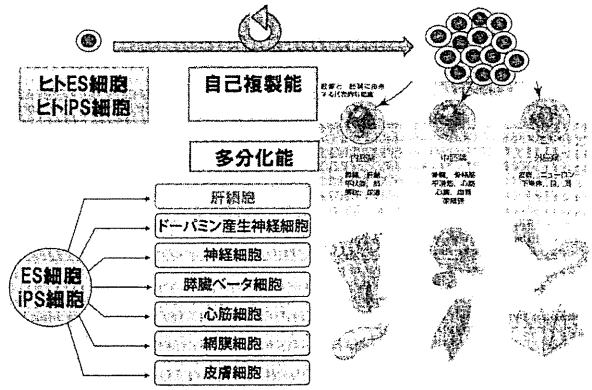

図 1 多能性幹細胞（ES 細胞及び iPS 細胞）の特性

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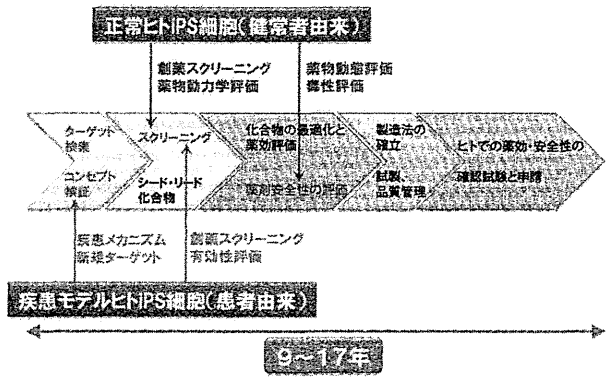

図2　創薬プロセスにおけるヒトiPS細胞の利用

化が確立され，評価系としてのバリデーションが確認できれば，創薬研究の薬物動態評価のみならず新しい実験材料として極めて有用である（図2）．

iPS細胞については，その性質や再生医療等への利用について知る機会が多い半面，規制については誤解されている点も多い．そこで，本稿では，iPS細胞に関する規制と薬物動態研究への応用に向けた取り組みを紹介する．

2. iPS細胞に関する規制

iPS細胞がES細胞と非常に類似した性質を持つことはよく知られている．そのため，法的あるいは倫理的な規制も同じと勘違いされている方が意外と多い．しかし，ES細胞は樹立の際に生命の萌芽である受精卵を壊す（滅失する）のに対し，iPS細胞は線維芽細胞等の体細胞を材料として樹立される点が，法的あるいは倫理的な規制を考える上で根本的に異なる．

ヒトiPS細胞を用いた研究に関わる規制について，①iPS細胞樹立まで，②iPS細胞を用いた研究（非臨床），③iPS細胞を用いた研究（臨床）に分けられるが，本稿では①と②について概略を説明する．

2.1 iPS細胞樹立までに関わる規制

ドナー（患者/健常者）から採取した組織・細胞を用いてiPS細胞を樹立する場合は，被験者の自己決定権，プライバシー及び個人情報の秘密保持，試料の収集・分析・保存/再利用に対する同意，インフォームドコンセント，倫理委員会による審議など「ヘルシンキ宣言」（世界医師会）の人間を対象とする医学研究の倫理的原則に従って行われなければならない．また，ドナー由来のiPS細胞について遺伝子解析を行う場合には，「ヒトゲノム・遺伝子解析研究に関する倫理指針」及び「個人情報の保護に関する法律」を遵守し，解析に当たっては匿名化が原則となる．その際には，連結可能匿名化（結果と提供者を結び付けることが可能）と連結不可能匿名化（結果と提供者を結び付けることが不可能）があり，どちらも倫理委員会の承認が必要である．

一方，ヒト皮膚線維芽細胞等iPS細胞の樹立に用いる細胞を表1の様な公的機関や会社等から入手することも可能である．ただし，このような細胞を使用する際には，契約書を交わす場合があり，また細胞によっては寄託者の承認や使用目的に制限がある場合もある．

近年は，iPS細胞樹立にウイルスを用いない方法も開発されているが，最も一般的なレトロウイルスを用いた場合を例に挙げると，以下の規制が関わってくる．すなわち，大腸菌によるプラスミドの増幅（ウイルス/非ウイルス）とウイルスベクター感染によるiPS細胞誘導がある．そのために，「遺伝子組換え生物等の使用等の規制による生物多様性の確保に関する法律」（カルタヘナ法）（研究での使用は「二種使用」）と「研究開発等に係る遺伝子組換え生物等の第二種使用等に当たって執るべき拡散防止措置等を定める省令」及び「研究開発段階における遺伝子組換え生物等の第二種使用等の手引」が関与することになる．なお，レトロウイルスベクター（増殖力等欠損株）感染によるiPS細胞誘導時には，P2レベルの核酸防止措置が必須となるが，樹立後は通常P2レベルで行う必要はない．

2.2 樹立したヒトiPS細胞を用いた非臨床研究に関わる規制

樹立されたiPS細胞を非臨床研究に用いる場合，生殖細胞への分化誘導と特定胚（クローン胚を含む）

表1　実験用細胞の入手先

Cell applications Inc.	(http://www.cellapplications.com/)
Lonza	(http://www.lonza.com/group/en.html)
American Type Culture Collection	(ATCC：http://www.atcc.org/)
理研バイオリソースセンター	(http://www.brc.riken.jp/)
医薬基盤研究所	(JCRB：http://www.nibio.go.jp/index.shtml)
ヒューマンサイエンス研究資源バンク	(HSRRB：http://www.jhsf.or.jp/bank/cell.html)

の作成に関する研究を除き，その扱いはHepG2細胞やHEK293細胞等，通常研究で用いられている培養細胞と同じである．この点は，「ヒトES細胞の使用に関する指針」に規定されている様に，例え使用研究であっても機関内又は他の使用機関の倫理審査委員会の審査を受けた後に使用計画の届け出を文部科学大臣に出さなければならないES細胞研究とは大きく異なる．

生殖細胞への分化誘導には，「ヒトiPS細胞又はヒト組織幹細胞からの生殖細胞の作成を行う研究に関する指針」，「ヒトiPS細胞又はヒト組織幹細胞からの生殖細胞作成における研究計画の実施の手引き」及び「ヒトiPS細胞又はヒト組織幹細胞からの生殖細胞の作成を行う研究に関する指針」があり，作成した生殖細胞でヒト胚を作成しないことや大臣への届け出等が義務付けられている．また，特定胚の作成に関する規制については，「ヒトに関するクローン技術等の規制に関する法律」(クローン規制法)，「ヒトに関するクローン技術等の規制に関する法律施行規則」及び「特定胚の取扱いに関する指針」がある．

特定胚のうち，現時点で作成が可能なのは，人クローン胚と動物性集合胚であるが，どちらも特定胚を作成することについての提供者による同意が必要である．また，これら研究の胚の取扱期間は，原始線条が現れるまで，または14日以内のどちらか短い方となっている．したがって，ヒトの特定胚を使った薬物動態試験などは余程発生初期段階での解析でなければ現段階では不可能である．

3.　薬物動態試験への応用と期待

ヒトiPS細胞の薬物動態試験への応用という観点から，薬物動態において重要な細胞である肝細胞様細胞および小腸上皮細胞様細胞への分化誘導に関して，最近の報告に著者らの結果を踏まえて紹介したい．

3.1　ヒトiPS細胞の肝細胞様細胞への分化誘導法

ヒトiPS細胞の肝細胞様細胞への分化誘導研究は，先行していたES細胞の分化誘導法を参考として，胚体内胚葉への分化，肝芽細胞様細胞への分化，肝細胞様細胞への分化・成熟の3つの段階に大きく分けて，複数の因子を段階的に添加していくことで行われている．ヒトES細胞から肝細胞様細胞への分化誘導は，2003年にRambhatlaらによって初めて報告された[1]．その後，より効率的に分化させるために改良された方法が現在までに多数報告されている[2,3]．ヒトiPS細胞の肝細胞様細胞への分化については，2009年のSongら[4]の報告が最初であり，それに引き続いていくつか報告されている[5]．

動物の体を構成する臓器が発生過程でどのようなメカニズムで統一された形づくりをするのかをツメガエルやイモリ卵を使って*in vitro*で研究されてきた．胞胚期のアニマルキャップ（未分化細胞塊）にtransforming growth factor-β（TGF-β）superfamilyのひとつであるactivin Aを処理すると濃度依存的に様々な器官や組織を分化誘導する．低濃度では血球や体腔内上皮，中濃度は筋肉，高濃度では脊索を分化誘導する．さらに高濃度では心臓や小腸，肝臓といった内胚葉性器官も分化誘導する．また，ヒトES細胞の場合でも同様に高濃度のactivin A(100 ng/mL）を処理することで効率的に胚体内胚葉へ誘導される．このような知見から，ヒトES細胞やiPS細胞の胚体内胚葉への分化のほとんどにactivin Aが用いられる．このとき，Wnt familyのひとつであるWnt3aは内胚葉や中胚葉の分化に重要であり，効率よくかつ速やかに肝細胞様細胞への分化が進むとして，activin Aと併用される場合もある．しかし，著者らはWnt3aが特に分化を促進するとの結果を得ることは出来なかったことから，通常activin Aのみを使用している．

肝臓の初期発生において，腹側前腸内胚葉が近接する心臓中胚葉からのfibroblast growth factor(FGF)

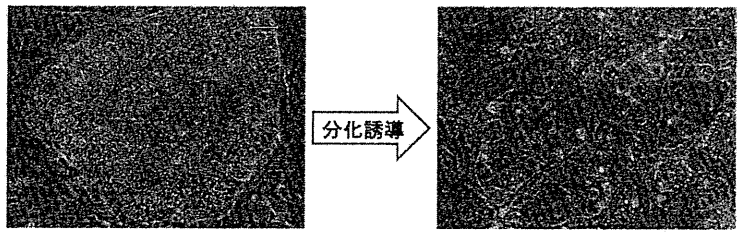

図3　ヒトiPS細胞とiPS細胞由来肝細胞様細胞の顕微鏡写真

シグナルや横中隔からのbone morphogenetic protein（BMP）シグナルによって肝芽細胞への分化が誘導される．そのため，胚体内胚葉から肝芽細胞様細胞への分化の過程にFGFやBMPがよく用いられる．一方，興味深いことに溶媒として多用されるdimethyl sulfoxide（DMSO）も肝芽細胞様細胞や肝細胞様細胞へ分化させることが知られている[2]．

この段階は研究者により様々であるが，多くは上記因子の複数あるfamilyを色々と組み合わせて行われている．著者らもこれら液性因子を組み合わせて分化を試みたが，分化効率にあまり差がなく，DMSOのみを使用した場合とも大きな差は認められなかった．また，DMSOは液性因子よりはるかに安価で取扱いも容易なことから，今では専らDMSOを使用している．この方法で分化した場合でも，ヒトES細胞での結果であるが成人様の薬物応答性を示す肝細胞様細胞を得ることができた[6]．

Hepatocyte growth factor（HGF）は，肝再生において成熟肝細胞の増殖因子として発見されたものであるが，肝臓形成中間期（mid-stage hepatogenesis）において胎児肝細胞の増殖・維持に関係している．また，マウス胎仔初代肝細胞をinterleukin-6（IL-6）familyのサイトカインであるoncostatin M（OSM）とdexamethasone（DEX）を含む培地で培養すると未熟な肝細胞を成熟させることが示された．このようなことから，最終の成熟段階において多くの研究者はHGF, OSM, DEXを添加している．しかし，成熟と言ってもこの組み合わせで胎児様の肝細胞には誘導されるが，それ以降の成熟は難しい．近年，著者らは様々な分化誘導法の工夫を行い，肝細胞マーカーのalbumin（ALB）のmRNA発現レベルは，成人肝培養細胞と同程度の発現が認められるまでになっている．しかし，胎児肝に特異的に発現するα-fetoprotein（AFP）やCYP3A7も高発現しているため，現段階では胎児肝レベルであり十分に成熟しているとは言えない．これは著者らの研究に限らず肝細胞への分化に関する報告はどれも成熟化が問題となっており，今後薬物動態試験に使用するために克服しなければならない必須の課題である．

液性因子だけでは限界があるとして近年新たな試みがなされている．それは肝発生において重要な転写因子のひとつであるhematopoietically expressed homeobox（HEX）[7]や肝の機能獲得に重要なhepatocyte nuclear factor 4α（HNF-4α）をヒトiPS細胞の分化のある時期に各々一過性に過剰発現する方法であり，従来の方法に比べ，短期間かつ高効率に肝細胞様細胞を作成している．この場合も，胎児様の性質を残していることが課題として挙げられているが，肝発生において重要な役割を果たしている遺伝子の導入は，効率的な肝細胞様細胞の新たな分化誘導手段として今後利用されることが予想される．

3.2　ヒトiPS細胞由来肝細胞様細胞の特徴

形態学的な特徴として，未分化なiPS細胞は細胞質がほとんどないため核の占める割合が大きく，核小体が明瞭である．また，ヒトiPS細胞は単層から成るコロニーを形成し，そのコロニーの境界線は滑らかで明確である．ヒトiPS細胞にactivin Aを処理すると非常に多くの細胞が死ぬが，残った細胞は大きくなり，縁が尖った形態を示す．分化に伴い更に大きくなり，最終段階では肝細胞に特徴的な多核の細胞が出現する（図3）．

遺伝子の発現は，activin Aの処理により未分化マーカーの発現が低下し，内胚葉のマーカーであるforkhead box A2（FOXA2），sex determining region Y box 17（SOX17）の発現が誘導される．さらに分化が進むとAFP, ALB, tyrosine aminotransferase（TAT）などの肝細胞マーカーに加えて，薬物代謝の主要酵素であるCYP3A4の発現量が増加する．ま

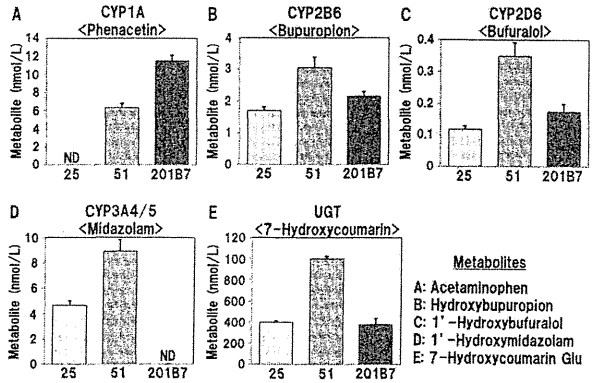

図4　ヒトiPS細胞から分化させた肝細胞様細胞の薬物代謝活性

Mean+S.D.($n=3$)，25：ヒトiPS細胞#25株，51：ヒトiPS細胞#51株，201B7：ヒトiPS細胞201B7株

た，肝細胞に特異的な機能であるALBの分泌やインドシアニングリーンの取り込み等も認められる．

3.3　ヒトiPS細胞由来肝細胞様細胞の薬物代謝活性

ヒトiPS細胞から誘導した肝細胞様細胞におけるCYPの発現やその代謝活性は成人肝培養細胞と比較して顕著に低いがmethylcholanthrene（CYP1Aの誘導剤），phenobarbital（CYP2Bの誘導剤），rifampicin（CYP3A4の誘導剤）により誘導され，各々代謝活性の上昇がみられる[1,4,7]．その程度はCYP分子種によっても異なるが，2〜5倍程度である．Bufuralolを基質としてヒトiPS細胞から分化誘導した肝細胞様細胞の代謝特性について検討を行った報告では，初代培養肝細胞と同様の代謝物が生成され，その代謝物はCYPによるものだけでなく，UDP-glucuronosyltransferase（UGT）やglutathione *S*-transferase（GST）などphase IIの代謝酵素によって生成されるグルクロン酸抱合体やグルタチオン抱合体なども検出されている[3]．また，pregnane X receptor（PXR），constitutive androstane receptor（CAR），aryl hydrocarbon receptor（AhR），liver X receptor α（LXRα）などの核内受容体に加え，multidrug resistance protein 1（MDR1），organic anion transporting polypeptide 2（OATP2）等の薬物トランスポーターの発現もみられていることから，薬物動態関連の機能をある程度獲得していると考えられる．

著者らはヒトiPS細胞の肝細胞様細胞への分化誘導を第一段階としてactivin Aで内胚葉，肝芽細胞への分化にDMSOを用い，成熟段階をHGF, OSM, DEX，インスリンで行っている．図4はヒトiPS細胞3株を同じ条件で同時に分化させた細胞における薬物代謝活性を比較したものである[8]．株によって差はあるものの，CYP1A，CYP2B6，CYP2D6，CYP3A4およびUGTの活性が検出された．さらに，結果は示していないがsulfotransferase（SLT）活性も検出されており，薬物動態試験への利用の可能性が示唆された．

3.4　ヒトiPS細胞から小腸上皮細胞様細胞への分化

現在使用されている医薬品の大部分は経口薬であり，経口投与された医薬品が全身循環に移行するためには小腸粘膜を通過する必要がある．小腸上皮には薬物トランスポーターや薬物代謝酵素が存在し，これらが医薬品のバイオアベイラビリティーに影響を及ぼすことから，小腸は肝臓と同様に薬物動態に影響する主要な臓器となっている．したがって，小腸における医薬品の吸収・排泄や代謝を解析・評価することは非常に重要である．現在，肝臓については，ヒト凍結肝細胞や初代肝細胞など組織由来の細胞が利用可能であるが，医薬品候補化合物の吸収・排泄特性を調べるためにイヌ腎由来MDCK(Madin-Darby canine kidney）細胞株やヒト結腸ガン由来Caco-2細胞単層膜による薬物透過試験が行われている．また，腸管は医薬品の吸収部位でもあるが，CYP3A4やUGTなど薬物代謝酵素等も多く発現しており，グレープフルーツジュース等による阻害やリファンピシン等による誘導等の食品や医薬品との相互作用が問題となる点でも重要である．しかし，MDCK細胞やCaco-2細胞は小腸上皮細胞とは本来異なる性質を持つ細胞であるために，適正な評価と予測が困難である．

肝細胞と同様にヒトiPS細胞から小腸上皮細胞へ分化誘導し，薬物動態の評価系としての利用が可能となれば有用なツールとなるが，腸管への分化に関する報告は肝細胞への分化の報告に比べて極めて少ない．2009年にマウスES細胞での方法を応用し，初めてマウスiPS細胞から三胚葉よりなる胚様体を形成して腸管組織への分化誘導に成功している．また，ヒトiPS細胞を用いた分化誘導に関しても最近報告されている[9]．しかし，これらは形態学的に腸管組織としての特徴を有するが，薬物動態学的な解

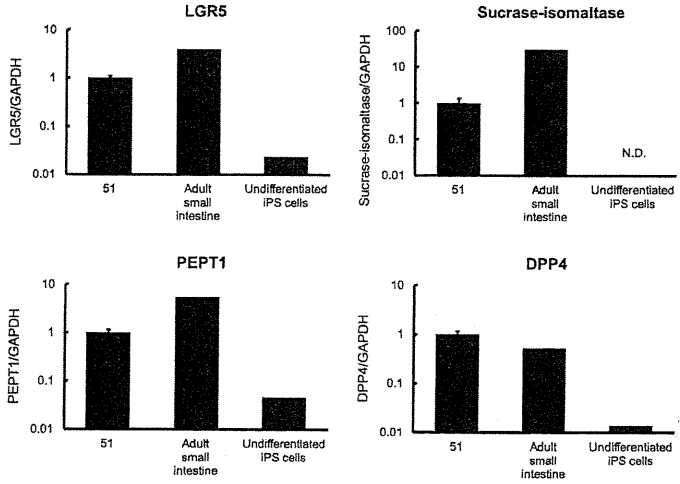

図5　ヒトiPS細胞から分化させた腸管上皮細胞様細胞のmRNA発現量
Mean＋S.D.($n=3$), 51：ヒトiPS細胞#51株由来腸管上皮細胞様細胞, N.D.: not detected. LGR5: leucine-rich repeat containing G protein-coupled receptor 5, PEPT1: oligopeptide transporter 1, DPP4: dipeptidyl-peptidase 4.

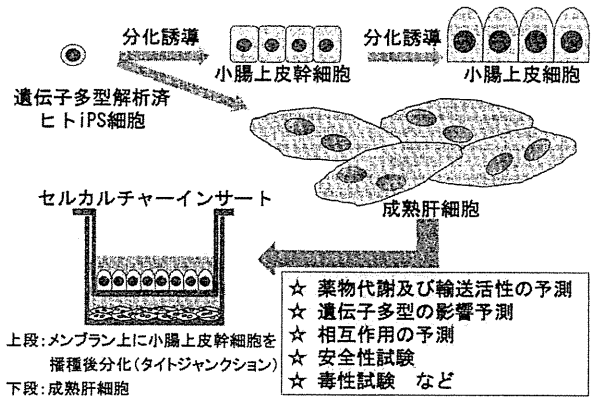

図6　ヒトiPS細胞から小腸上皮細胞及び肝細胞の分化誘導と薬物動態試験

析に応用できるまでの機能評価はされておらず，ほとんど研究が進んでいないのが現状である．

著者らもヒトiPS細胞から内胚葉を経由した腸管上皮細胞様細胞への分化について検討を行っている．その結果，腸管幹細胞に局在するとされるleucine-rich repeat containing G protein-coupled receptor 5（LGR5），腸管上皮細胞に特異的に存在するsucrase-isomaltaseやdipeptidyl-peptidase 4（DPP4）の発現に加え，ペプチドトランスポーターであるoligopeptide transporter 1（PEPT1）の発現も確認されたことから，腸管組織細胞への分化が確認された（図5）[8]．今後は，より効率的な分化法の検討に加え，薬物代謝能や輸送能に関する機能解析を行っていく予定である．

4. さいごに

多くの場合医薬品は経口投与されることから，薬物の吸収及び代謝に関与する主要な臓器である小腸や肝臓での薬物動態をより正確かつ簡便に評価することは，薬物の有効性や安全性の予測に極めて重要である．したがって，小腸から肝臓までの薬物吸収・代謝の評価を一体化して評価できる実験モデル系の構築が望まれる．しかし，小腸上皮細胞と肝細胞を用いて，小腸と肝臓を結ぶモデル系を構築することは，これまでヒト小腸上皮細胞が容易に入手できないために困難であった．しかし，肝細胞同様ヒトiPS細胞から小腸上皮細胞を分化誘導にて作成することが出来ればセルカルチャーインサートを用いた一体型のモデル系も可能と考える（図6）．さらに，将来的にはチップ上にiPS細胞由来の小腸上皮細胞と肝細胞をつなぐ微小な流路や反応室，混合室を設け，細胞間相互作用を解析する細胞機能解析チップが出来れば，生化学分析デバイスとして，基礎研究や創薬分野において，有用なツールとなると期待される．小腸上皮細胞への分化は世界的にも始まったばかりではあるが，これが実現できればより優れた動態特性を有する安全で有用な医薬品の開発にも貢献できるものと考えられる．また，薬物代謝酵素や薬物トランスポーターの遺伝子多型が薬物動態に影響し，

薬効や副作用発現の個人差の原因の1つとなっていることが明らかになっている．これまでの評価系では遺伝子多型の評価は困難であったが，遺伝子多型が明らかな体細胞からiPS細胞を樹立すれば，遺伝子多型の薬物動態に及ぼす影響の予測も容易になるものと考えられる．著者らはこのようなことを夢見て，肝細胞と小腸上皮細胞への分化について研究を行っている．

著者らが使用したヒトiPS細胞は，国立成育医療研究センターおよび京都大学より供与していただきました．また，研究を遂行するにあたりご協力いただいた国立医薬品食品衛生研究所　医薬安全科学部　黒瀬光一室長，田辺三菱製薬株式会社　研究本部　薬物動態研究所　丹羽卓朗氏に感謝致します．

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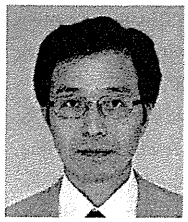
アドメノート

薬物動態研究における実験材料及び評価系開発の最近の動向

名古屋市立大学大学院薬学研究科
松永民秀



肝細胞，腎尿細管上皮細胞，小腸上皮細胞，脳血管内皮細胞，心筋細胞，神経細胞などのヒトの試料は，創薬研究の薬物動態試験や毒性試験において極めて有用な実験材料であるが，入手が困難な場合が多く，たとえ入手できたとしてもロット間差が大きいうえに，量も限られている．近年，胚性幹細胞(ES細胞)や人工多能性幹細胞(iPS細胞)などの多能性幹細胞がヒトより樹立され，生体を構成する全ての細胞に分化する能力を秘めていることから，創薬研究における新しい実験材料として注目されている．特に，肝細胞への分化については数多くの研究が行われており，薬物動態試験や毒性試験への利用が大いに期待されている[1,2]．一方，生体の様々な組織にある組織幹細胞(体性幹細胞，成体幹細胞)は，分化の方向性がある程度決まっているため多能性幹細胞と比較して分化能は限られているが，高い増殖性を維持しており，目的とする細胞への分化が比較的容易であるという利点がある．その中で，間葉系幹細胞(mesenchymal stem cell)は，本来間葉系に属する細胞(骨細胞，心筋細胞，軟骨細胞，腱細胞，脂肪細胞など)への分化能を持っているが，最近グリア細胞(外胚葉由来)や肝細胞(内胚葉由来)など，胚葉の差をこえて中胚葉性でない組織にまで分化できる可塑性を持っていることが示された[3]．間葉系幹細胞から分化誘導された肝細胞様細胞は，薬物代謝活性や薬物代謝酵素の誘導能を有しており，薬物動態研究の有望な材料の1つとして興味が高まっている[4,5]．

肝臓は再生能力に優れた代表的な臓器である．しかし，成人の肝細胞を体外で培養した場合，増殖能力が殆ど無いばかりか，肝細胞特異的な機能が急激に低下し，それを維持することさえ困難である[6]．種差があることから，臨床でのヒトでの薬物の挙動を予測するためにヒト細胞を用いた試験が必要不可欠とされている．そのためヒト凍結肝細胞を使用するケースが年々増加している．しかしながら，通常の培養方法あるいは装置でヒト肝細胞を培養すると，プレートに接着しにくく，生体に近い機能を維持することができないことから長期の薬物代謝や毒性試験には不向きである．これまで，三次元細胞培養装置，浮遊(スフェロイド)培養法，サンドイッチ培養法など生体環境に少しでも近づけるための培養技術の開発が進んできた[7-9]．このような技術は，貴重で高価なヒト試料の有効利用に繋がるだけでなく，幹細胞の肝細胞への分化誘導において，肝特異的機能を獲得・維持することにも利用できる大変貴重な技術である．

一方，細胞培養技術を用いた *in vitro* 研究ではなく，ヒト化動物を作成することによる *in vivo* での薬物動態試験を行う系も確立されている．その中で最も代表的なものがヒト肝細胞を持つキメラマウスであろう．このマウスは，肝臓に障害を持つ albumin enhancer/promoter urokinase plasminogen activator トランスジェニックマウス(uPAマウス)と免疫不全の SCID マウスを掛け合わせ，どちらの形質もホモ接合体である uPA/SCID マウスであり，ヒトの肝細胞を移植することで80％がヒト肝細胞に置換した肝臓を持つ[10]．このヒト肝細胞を持つキメラマウスは，既に薬物動態研究において高い評価を得ている[11-13]．一方，ヒト人工染色体(HAC)技術を基にさまざまな遺伝子の機能を解析するツールとして HAC ベクターが開発された．この HAC ベクターシステム技術を利用し，ヒトの医薬品代謝において最も重要な酵素である CYP3A 遺伝子クラスターを導入した CYP3A ヒト型マウスが作製された．本マウスは，肝臓と小腸にヒト CYP3A を発現したモデル動物としてヒトにおける CYP3A を介した薬物相互作用や薬物代謝が血中動態に及ぼす影響を *in vivo* で予測する新規モデルとして注目されている．

薬物の多くは複数の代謝酵素により代謝を受けると共に，薬物や代謝物の細胞内への取り込みや排泄にはトランスポーターが関与している．また，発現には多くの転写因子が複雑に関与しており，受容体には種差があることも知られている．ヒト肝細胞を持つキメラマウスはヒト肝細胞そのものであることから，薬物動態試験や毒性試験において薬物代謝酵素や薬物トランスポーター等の関与や相互作用を総合的に評価できる実験材料として優れている．しかし，少量残っているマウスの肝細胞の影響を無視できない場合もあることが知られているし，*in vivo* と言っても経口投与で問題となる小腸での評価は出来ない．一方，CYP3A－HAC マウスの場合では薬物代謝で重要な CYP3A が小腸と肝臓に発現していることから，小腸での代謝も解析できる特徴がある．しかし，HAC マウスでは人工染色体を導入して発現した酵素により解析することになり，その他の代謝酵素との関わりや誘導評価における転写因子あるいは受容体については今後の課題かと思われる．

各種オミックス技術は，創薬研究において重要な地位を占めてきた．その中でメタボロミクスは，大規模な発現プロファイリングやプロテオーム研究を論理的に補完するものとして急速に浸透している手法である．この技術は，あ

る遺伝子，または生理的・病理的環境が異なった複数の系において多数の構成分子を網羅的・包括的に分析し，そのプロファイルを比較することにより，細胞のある瞬間の生理的・病理的現象に最も関与する可能性の高い因子を明らかにすることができる[14,15]．しかし，代謝物は化学的にきわめて多様であることから，メタボロミクスには分析上の大きな困難が伴う．その困難を克服する技術として，高感度と高分離能を兼ね備えたLC-MS/MSなど質量分析装置があげられる．

新しい実験材料や評価系の開発は，これまで困難だった薬物動態研究を容易にすることで，その予測精度を増すことになり，延いては安全で有効な医薬品の開発に資することになる．そこで，本シリーズは各分野の専門の先生により，第1回「多能性幹細胞と組織幹細胞」，第2回「3次元培養・ミクロ組織形成技術」，第3回「ヒト化モデル動物」，第4回「質量分析装置」で紹介して頂く予定である．

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