表５　男女比

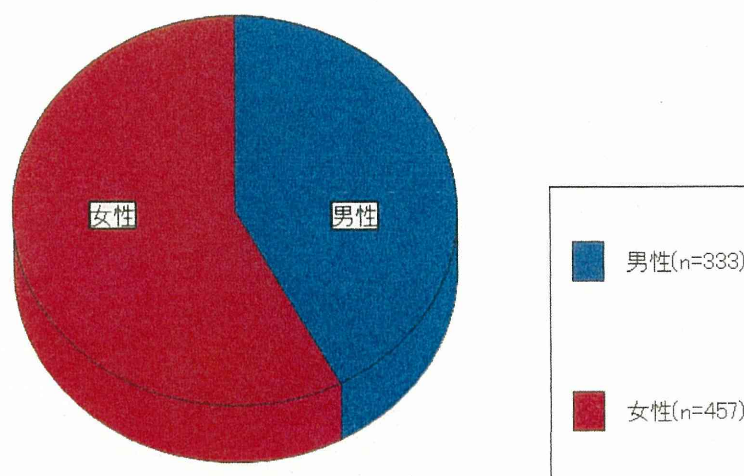

表６　健康食品の摂取に対する性別の影響

全体(n=790)	女性（n=457）	男性(n=333)
グルコサミン[15.8]	グルコサミン[15.5]	グルコサミン[16.2]
ブルーベリー類[13.5]	コラーゲン[13.8]	ブルーベリー類[16.0]
コラーゲン[12.0]	黒酢（香醋）[12.3]	DHA・EPA[13.0]
黒酢（香醋）[11.6]	ブルーベリー類[10.9]	にんにく[11.4]
DHA・EPA[10.1]	ヒアルロン酸[10.5]	黒酢（香醋）[10.8]
にんにく[9.9]	コンドロイチン[9.0]	コラーゲン[9.6]
ヒアルロン酸[9.2]	にんにく[8.8]	コンドロイチン[8.1]
コンドロイチン[8.6]	葉酸[8.8]	ヒアルロン酸[7.2]
デキストリン[7.8]	DHA・EPA[7.9]	葉酸[6.0]
葉酸[7.6]	胚芽[5.3]	大麦若葉[5.4]

[%]

表７　健康食品の摂取に対する年齢の影響

全体(n=790)	15 歳以上 65 未満(339)	65 歳以上 75 歳未満(255)	75 歳以上 (188)
グルコサミン（125）	コラーゲン(42)	グルコサミン(49)	グルコサミン(51)
ブルーベリー類(107)	ブルーベリー類(42)	ブルーベリー類(38)	コンドロイチン(28)
コラーゲン(95)	葉酸(42)	黒酢（香醋）(36)	ヒアルロン酸(28)
黒酢（香醋）(92)	黒酢（香醋）(30)	DHA・EPA(33)	にんにく(27)
DHA・EPA(80)	グルコサミン(25)	にんにく(32)	黒酢（香醋）(26)

表８　健康食品の摂取に対する疾病による影響

悪性腫瘍(219)	眼瞼下垂症（27）	狭心症（18）
黒酢（香醋）[12.3]	DHA・EPA（7）	グルコサミン（3）
コラーゲン[12.3]	ベリー系（4）	コラーゲン（3）
グルコサミン[12.3]	コラーゲン（4）	DHA・EPA（3）
ブルーベリー類[11.9]	イチョウ葉エキス（4）	コンドロイチン（3）
DHA・EPA[10.5]	グルコサミン（3）	ヒアルロン酸（2）

表９　悪性腫瘍患者で特に使用率が高い健康食品

フコイダン	(9/9)
霊芝	(7/10)
マイタケ	(5/7)
雲南紅豆杉	(5/6)
しいたけ	(4/6)
オルニチン	(4/4)

厚生労働科学研究費補助金（食品の安全確保推進研究事業）
研究課題：いわゆる「健康食品」と医薬品との併用に関わる 安全性評価に関する研究

研究分担報告書

ヒト iPS 細胞から肝・腸管上皮細胞への分化および誘導評価

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研究協力者　近藤　祐樹　名古屋市立大学大学院薬学研究科・博士後期課程 2 年

研究要旨：　本研究では、薬物代謝および毒性試験への利用を目指し、ヒト人工多能性幹細胞（iPS 細胞）から、肝細胞および腸管細胞への分化誘導を行った。その結果、ヒト iPS 細胞から肝細胞様細胞の分化誘導に成功した。さらに、mRNA 発現解析および機能解析の結果から、今回用いた低分子化合物 1 はヒト iPS 細胞から肝細胞への分化促進剤として有用であることが明らかとなった。また、腸管上皮細胞様の機能を有する細胞への分化誘導にも成功し、低分子化合物 A および B は腸管幹細胞への分化を促進することも明らかとなった。

A. 研究目的

医薬品開発の薬物動態試験において実験動物が多用されているが、種差があるため結果をヒトへ外挿することが困難である。一方、ヒト初代肝細胞や凍結肝細胞ではロット間差が大きく、良質の肝細胞を安定して入手することが難しいため、研究の障害となっている。ヒト人工多能性幹（induced pluripotent stem：iPS）細胞は、2007 年に山中らによって樹立された。このヒト iPS 細胞は、多分化能とほぼ無限の増殖能をもつ細胞であり、医薬品開発のための安定した細胞供給源として期待される。現在、iPS 細胞から肝細胞への分化誘導方法がいくつか報告されている。その主な方法として分化誘導に増殖因子等の液性因子が用いられているが、非常に高価であること等が課題となっている。そのため、安価で取り扱いの容易な低分子化合物を分化誘導因子として用いることは非常に有用であると考えられる。本研究では、低分子化合物がヒト iPS 細胞から肝細胞への分化誘導に与える影響を明らかにすることを目的とした。また、腸管も肝と同様、医薬品の薬物動態への関与が大きいことから、ヒト iPS 細胞から腸管細胞への分化も行った。

B. 研究方法

本研究では、国立成育医療研究センターにおいて樹立されたヒト iPS 細胞株（Windy）を用いた。

ヒト iPS 細胞から肝細胞への分化

ヒト iPS 細胞をアクチビン A 処理することで内胚葉に、続いてジメチルスルホキシドにて肝芽細胞に分化させ、オンコスタチン M、デキサメタゾン、肝細胞増殖因子によって成熟させる方法にて肝細胞に分化誘導した。薬物代謝酵素の誘導剤処理は 40 μM リファンピシン（RIF）を含む培地で回収前 48 時間培養することで行った。また、分化

の際に低分子化合物1もしくは2を添加し、肝細胞への分化に及ぼす影響について検討した。

分化後の細胞は real-time PCR 法により mRNA 発現解析を行った。低分子化合物1を添加した細胞においては、免疫蛍光染色にてアルブミン（ALB）陽性細胞の分布を、インドシアニングリーン（ICG）染色により ICG 取り込み及び排泄能を、過ヨウ素酸シッフ（PAS）染色によりグリコーゲン貯蔵能を評価した。

ヒト iPS 細胞から腸管細胞への分化

ヒト iPS 細胞をアクチビン A 処理することで内胚葉に、続いて線維芽細胞増殖因子 2 にて腸管系譜細胞に分化させ、上皮細胞成長因子よって腸管細胞に分化誘導した。CYP3A4 の誘導剤処理は 10 nM 1α,25-ジヒドロキシビタミンD_3（VD_3）を含む培地で回収前 48 時間培養することで行った。また、分化の際に低分子化合物 A および B を添加し、腸管幹細胞への分化に及ぼす影響について検討した。

分化後の細胞は real-time PCR 法により mRNA 発現解析を行った。また、β-Ala-Lys-AMCA（ジペプチド）の取り込みおよび sucrase- isomaltase（腸管上皮細胞の特異的マーカー）の免疫蛍光染色を行うことで機能を評価した。

C. 研究結果

ヒト iPS 細胞から肝細胞への分化

分化後の細胞において、形態学的に肝細胞に特徴的な多核の細胞が観察され、肝細胞マーカーの発現が認められた（Fig. 1）。また、ALB、プレグナン X 受容体（PXR）、CYP2C9、CYP2C19、CYP3A4 及び UGT1A1 に関して、これらの mRNA 発現は低分子化合物 1 の添加により増加した（Fig. 2）。一方、低分子化合物 2 の添加によるこれら肝細胞マーカーの mRNA 発現に顕著な差は認められなかった。CYP3A4 の誘導剤である RIF 処理を行ったところ、低分子化合物 1 を添加することで、増加した CYP3A4 がさらに 5 倍の mRNA 発現の誘導が認められた（Fig. 3）。しかし、低分子化合物 2 を添加した場合には RIF による CYP3A4 の mRNA 発現の誘導自体全く認められなくなった。

低分子化合物 1 を添加して分化させた細胞において、ALB 免疫蛍光染色を行ったところ、ほぼ全ての細胞で陽性を示した（Fig. 4）。また、この細胞は ICG 取り込み及び排泄能が認められ、PAS 染色にも陽性像を示した。

ヒト iPS 細胞から腸管細胞への分化

我々が行った検討で最も効率が良かった分化条件で分化させた細胞において、β-Ala-Lys-AMCA の取り込みがみとめられ、この細胞は sucrase- isomaltase が発現していた（Fig. 5）。また、VD_3で処理することで、7 倍程度の CYP3A4 の発現誘導が認められた（Fig. 6）。さらに、低分子化合物 A および B を分化の過程で処理することにより、腸管幹細胞マーカーの LGR5 および EphB2 の発現レベルの上昇が認められた（Fig. 7）。

D. 考察

肝細胞への分化において、我々が行った分化誘導法で分化させたヒト iPS 細胞は、敷石状で多核など形態学的な肝細胞の特徴を示し、各種肝細胞マーカー遺伝子を発現したことから、分化した細胞は肝細胞様細胞であることが示唆された。また、低分子化合物 1 の添加により、ヒト iPS 細胞由来肝細胞の肝細胞マーカーの mRNA 発現量が増加し、低分子化合物 1 はヒト iPS 細胞から肝細胞への分化誘導において、分化を促進することが示唆された。さらに、RIF の

添加によって CYP3A4 が誘導され、薬物代謝酵素の誘導剤に対する応答性が肝細胞に類似していることが明らかとなった。低分子化合物 2 を添加して分化させた細胞は、肝細胞マーカーの mRNA 発現の上昇は認められず、分化に影響を与えない、もしくはむしろ抑制することが明らかとなった。なお、RIF による CYP3A4 の mRNA 発現誘導が認められなかった可能性として、CYP3A4 の誘導に関与する核内受容体 PXR の発現が低下したためと考えられた。

低分子化合物 1 を用いて分化させたヒト iPS 細胞の ALB 免疫蛍光染色の結果より、ほぼ全ての細胞が肝細胞様細胞に分化したことが示唆された。また、ヒト肝細胞と同様に、ICG 取り込み及び排泄能並びにグリコーゲン貯蔵能も有していた。これらの結果より、低分子化合物 1 を添加し分化させた細胞が肝細胞の機能を有する肝細胞様細胞であることが示唆された。

腸管細胞への分化において、ヒト iPS 細胞から分化させた細胞は腸管上皮マーカーである sucrase- isomaltase が発現していた。また、本細胞において腸管上皮細胞に特異的に発現しているペプチドトランスポーター SLC15A1/PEPT1 の基質薬物（β-Ala-Lys-AMCA）の取り込みが認められた。さらに、VD_3によりビタミン D 受容体（VDR）を介した CYP3A4 の発現誘導がみとめられたことから、ヒト iPS 細胞より腸管上皮細胞様の機能を有する細胞が作製できたことが示唆された。また、低分子化合物 A および B の添加は、腸管幹細胞への分化を促進することも明らかとなった。

E. 結論

我々はヒト iPS 細胞から肝細胞様細胞の分化誘導に成功した。さらに、今回用いた低分子化合物 1 はヒト iPS 細胞から肝細胞への分化促進剤として有用であることが明らかとなった。また、腸管細胞への分化において、腸管上皮細胞様の機能を有する細胞への分化誘導に成功した。さらに、低分子化合物 A および B は腸管幹細胞への分化を促進することが明らかとなった。

F. 研究発表

1. 論文発表

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2. 著書

該当なし

3. 学会発表

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2. Iwao T, Nakamura K, Nagata K, Matsunaga T. Generation of human induced pluripotent stem cell derived enterocytes with peptide transport function. 26th JSSX Annual Meeting, November 2012 (Chiba).
3. Kondo Y, Iwao T, Yoshihashi S, Mimori K, Sugiyama R, Sasaki T, Nagata K, Kurose K, Niwa T, Yamaor S, Ohmori S, Nakamura K, Matsunaga T. Small molecule compounds enhance differentiation to hepatocytes from human induced pluripotent stem cells. 26th JSSX Annual Meeting, November 2012 (Chiba).

G. 知的財産権の出願・登録状況

1. 特許取得

1. 発明の名称：人工多能性幹細胞を肝細胞へ分化誘導する方法

発明者：松永民秀、岩尾岳洋、近藤祐樹、吉橋幸美

出願番号：特願 2012-247010

出願日：2012 年 11 月 19 日

特許出願人：公立大学法人名古屋市立大学

2. 発明の名称：人工多能性幹細胞を腸管上皮細胞へ分化誘導する方法

発明者：松永民秀、岩尾岳洋

出願番号：特願 2013-036434

出願日：2013 年 2 月 26 日

特許出願人：公立大学法人名古屋市立大学

2. 実用新案登録

該当なし

3. その他

該当なし

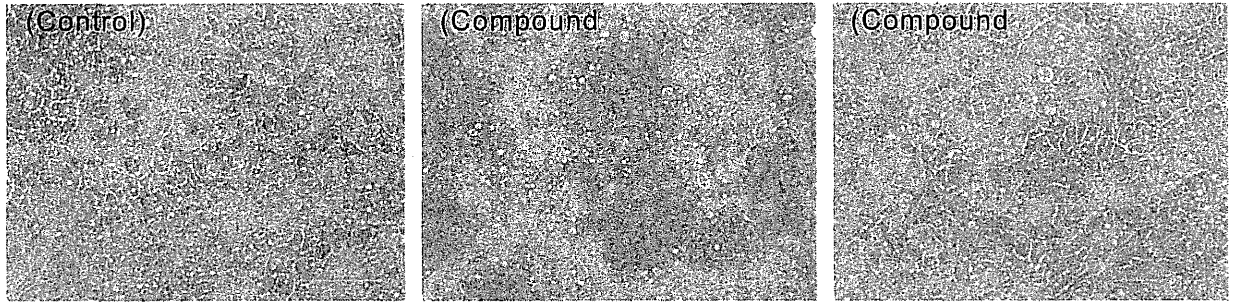

Fig. 1. 分化させたヒト iPS 細胞由来肝細胞様細胞の形態学的変化

Relative Gene Expression

ALB
1000
100
10
1
0.1
Ctrl. 1 2 Adult Fetal Adult (pool) 0h 48h iPS cells
Compound Human Liver Hepatocytes

AFP
1000000
100000
10000
1000
100
10
1
0.1
Ctrl. 1 2 Adult Fetal Adult (pool) 0h 48h iPS cells
Compound Human Liver Hepatocytes

TAT
1000
100
10
1
0.1
0.01
Ctrl. 1 2 Adult Fetal Adult (pool) 0h 48h iPS cells
Compound Human Liver Hepatocytes

PXR
100
10
1
0.1
0.01
Ctrl. 1 2 Adult Fetal Adult (pool) 0h 48h iPS cells
Compound Human Liver Hepatocytes

CYP1A2
1000
100
10
1
0.1
0.01
Ctrl. 1 2 Adult Fetal Adult (pool) 0h 48h iPS cells
Compound Human Liver Hepatocytes

CYP2C9
100
10
1
0.1
0.01
0.001
Ctrl. 1 2 Adult Fetal Adult (pool) 0h 48h iPS cells
Compound Human Liver Hepatocytes

CYP2C19
100
10
1
0.1
0.01
0.001
Ctrl. 1 2 Adult Fetal Adult (pool) 0h 48h iPS cells
Compound Human Liver Hepatocytes

CYP3A4
10000
1000
100
10
1
0.1
0.01
0.001
Ctrl. 1 2 Adult Fetal Adult (pool) 0h 48h iPS cells
Compound Human Liver Hepatocytes

UGT1A1
100
10
1
0.1
0.01
0.001
Ctrl. 1 2 Adult Fetal Adult (pool) 0h 48h iPS cells
Compound Human Liver Hepatocytes

Fig. 2. 分化させたヒト iPS 細胞由来肝細胞様細胞における肝細胞マーカーの発現

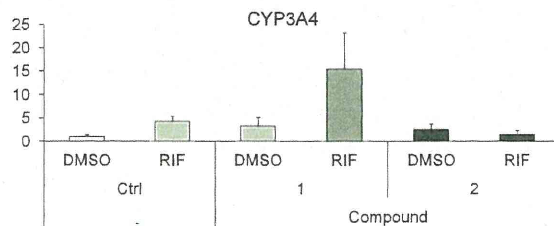

Fig. 3. 分化させたヒト iPS 細胞由来肝細胞様細胞の RIF に対する CYP3A4 の応答性

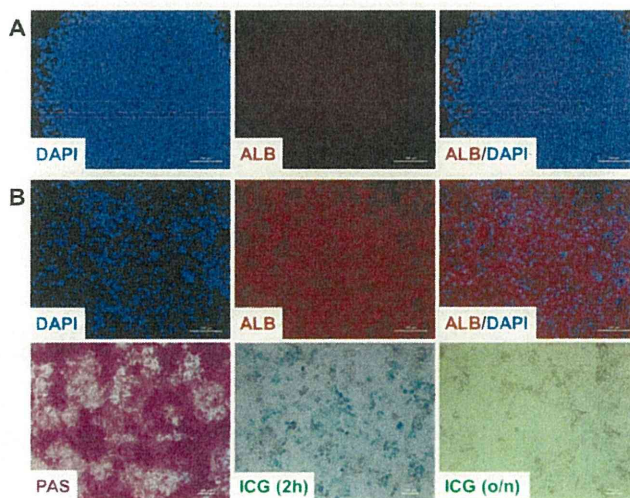

Fig. 4. 低分子化合物 1 を用いて分化させたヒト iPS 細胞由来肝細胞様細胞の ALB 免疫蛍光染色、ICG 染色及び PAS 染色
(A) 未分化のヒト iPS 細胞の ALB 免疫蛍光染色
(B)低分子化合物 1 を用いて分化させたヒト iPS 細胞由来肝細胞様細胞

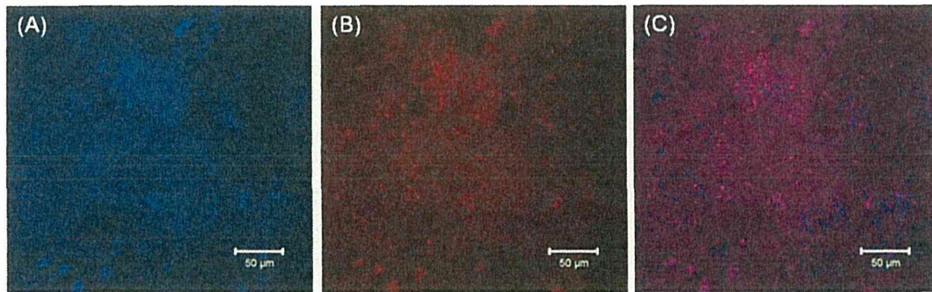

Fig. 5. ヒト iPS 細胞由来腸管細胞における β-Ala-Lys-AMCA 取り込みおよび sucrase- isomaltase 免疫蛍光染色

(A) β-Ala-Lys-AMCA の取り込み

(B) sucrase- isomaltase 免疫蛍光染色

(C) (A) と (B) をマージした画像

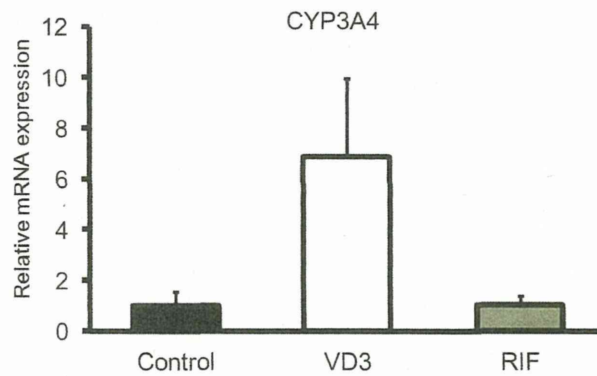

Fig. 6. ヒト iPS 細胞由来腸管細胞の VD_3 に対する CYP3A4 の応答性

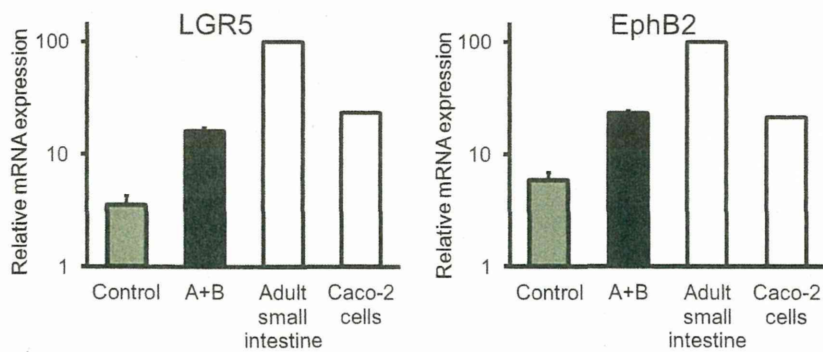

Fig. 7. ヒト iPS 細胞から腸管幹細胞への分化に対する低分子化合物の効果

Ⅲ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

書籍

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永田　清	薬物相互作用	川西正祐、小野秀樹、賀川義之	薬害・副作用学　第１版	南山堂	東京		2012
坂口修平、高橋昌吾、熊谷　健、佐々木崇光、永田清	永田　清、敗血症モデルとしての TNF-a誘導肝細胞死と酸化ストレス　－シグナル伝達機構からのアプローチ	自然免疫研究会編	エンドトキシン	医学書点	東京	53-57	2013

雑誌

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Maekawa K, Nishikawa J, Kaniwa N, Sugiyama E, Koizumi T, Kurose K, Tohkin M, Saito Y.	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	*Drug Metab Pharmacokinet*	27	447-450	2012

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Suzaki Y, Uemura N, Hosokawa M, Ohashi K.	Gly143Glu polymorphism of the human carboxylesterase1 gene in an Asian population.	*Eur J Clin Pharmacol*	79	735-736	2013
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Ⅳ．研究成果の刊行物・別刷

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Note

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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Summary: Allopurinol-induced Stevens-Johnson syndrome (SJS)/toxic epidermal necrolysis (TEN) is 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 a 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*. The developed PCR-RFLP assay using FokI restriction enzyme was able to detect three different genotypes, GG, GA, and AA of rs9263726 robustly, and thus 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 been 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

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hypersensitivity syndrome in a 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.

A recent report showed that based on the very strong association of the *HLA-B*15:02* allele with SJS/TEN in the Han Chinese population (sensitivity = 98%, specificity = 96%),[12] prospective testing for *HLA-B*15:02* and subsequent avoidance of carbamazepine therapy resulted in zero occurrence of SJS/TEN in Taiwan.[13] Based on this result and the severity of these adverse reactions, a 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, a single SNP can be easily genotyped and inexpensively compared to the *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 a 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 instructions 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 s, 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 × 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 a UV transilluminator.

Results and Discussion

First, we compared the results of genotyping rs9263726 in *PSORS1C1* with the PCR-RFLP and TaqMan SNP Geno-

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typing assays (C_30352071_10). DNA from Chinese-Americans was 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 DNA 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 ≥ 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 a 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 can be done 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, it may be better for the *HLA-B*58:01*-positive patients to avoid the administration of allopurinol, as do the *HLA-B*15:02*-positive patients for carbamazepine in Taiwan. Because allopuirol is a xantine oxidase inhibitor, febuxostat, having the same pharmacological effect through a 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.

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

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LETTER TO THE EDITORS

Gly143Glu polymorphism of the human carboxylesterase1 gene in an Asian population

Yuki Suzaki · Naoto Uemura · Masakiyo Hosokawa · Kyoichi Ohashi

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Dear Editor,
As Drs Zhu and Markowitz commented in their letter, it is widely recognized that carboxylesterase (CES) is an important enzyme that activates prodrugs in vivo. In our report, the interindividual variability observed in the metabolism of oseltamivir could not be explained on the basis of the CES*1A1* genotypes and the number of *CES1* functional genes [1]. However, the area under the curve (AUC) for oseltamivir for one subject in our study was approximately 10-fold higher than the corresponding AUCs for the rest of the subjects.

A previous report has shown that two *CES1* variants, Gly143Glu (rs71647871) and Asp260fs (rs7164782), reduce the hydrolytic activity of CES1 [2]. Moreover, the Gly143Glu variant has been shown to be associated with impaired oseltamivir metabolism [3, 4]. Although we are very interested in these polymorphisms, the Gly143Glu polymorphism appears to be extremely rare in the Asian population, as indicated by Zhu and Markowitz [2, 5], and the Asp260fs polymorphism is even rarer [2]. Gly143Glu is located in *CES1A1* rather than *CES1A2/CES1A3* [5], whereas Asp260fs is a mutation in the *CES1A2* gene [5]. The transcriptional efficiency of *CES1A1* is markedly higher than that of *CES1A2*. Therefore, the Gly143Glu mutation may be more important in CES1 metabolism.

After submitting the manuscript, we examined the frequency of the Gly143Glu and Asp260fs polymorphisms in nearly 400 Japanese subjects. As expected, no such Glu variants were found in the DNA of the subjects, including those who participated in the reported study [1]. In addition, no Asp260fs variants were observed in our samples. Therefore, another hitherto unknown mutation in the *CES1* gene may be involved in the slow oseltamivir metabolism in the poor metabolizer identified in our study. Additional studies are required to further elucidate the cause of the individual differences observed in CES1 substrate drug metabolism.

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