眼合併症を伴う日本人Stevens-Johnson症候群のHLA class Ⅰ解析

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要 約

目 的：以前，我々は，眼合併症を伴う日本人Stevens-Johnson症候群(Stevens-Johnson syndrome：SJS)・中毒性表皮壊死融解症(toxic epidermal necrolysis：TEN)患者71人と非発症対照113人を対象に，HLA class Ⅰ-A，B，Cの解析を行い，SJS/TEN発症とHLA class Ⅰ，特にA*0206との間に強い相関があることを報告した．今回は，日本人SJS/TEN患者118人と前回と異なる日本人対照220人を対象に，HLA class Ⅰ-A，B，Cについての再検討を行ったので報告する．

対象と方法：京都府立医科大学附属病院眼科に通院するSJS/TEN患者118例を対象とし，新たに京都府立医科大学で血液を採取した日本人非発症者220人を対照として用いた．末梢血からDNAを採取し，polymerase chain reaction-sequence specific oligonucleotide probe(PCR-SSO)法を用いてHLA class Ⅰ-A，B，Cについて塩基配列レベルの解析を行い，遺伝子頻度(gene frequency：GF)と保持者頻度(carrier frequency：CF)を検討した．

結 果：A*0206が有意に増加していたが，p値は以前の報告よりさらに上昇した(p値はGF：p=0.000000007，CF：p=0.0000000002，オッズ比はGF：4.2，CF：5.2)．

結 論：眼合併症を伴うSJS/TENの発症には，前回の報告よりさらにHLA class Ⅰ A*0206が強く相関することが確認された．(日眼会誌 116：581-587，2012)

キーワード：HLA-A*0206，Stevens-Johnson症候群(SJS)，中毒性表皮壊死融解症(TEN)，眼合併症，HLA

HLA-class Ⅰ Gene Polymorphisms in Japanese Stevens-Johnson Syndrome Patients with Ocular Surface Complications

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Abstract

Purpose：Our previous study of polymorphisms in the HLA-class Ⅰ genes of 71 Japanese SJS/TEN patients with ocular surface complications and 113 Japanese healthy controls showed that in the Japanese, HLA-A*0206 was strongly associated with SJS/TEN. In this study, we examined 118 Japanese SJS/TEN patients with ocular surface complications and a new control group consisting of 220 healthy Japanese volunteers, and investigated the association between HLA class Ⅰ antigens, HLA-A, B, C, and the SJS/TEN.

Methods：For HLA genotyping we enrolled 118 Japanese patients with SJS/TEN in the chronic or sub-acute phase at Kyoto Prefectural University of Medicine；all presented with ocular surface complications. We also enrolled 220 healthy Japanese volunteers. We performed polymerase chain reaction amplification followed by hybridization with sequence-specific oligonucleotide probes(PCR-SSO).

Results：HLA-A*0206 was most strongly associated with Japanese SJS/TEN patients with ocular surface complications (carrier frequency：p=0.0000000002, OR=5.2；gene frequency：p=0.000000007, OR=4.2).

Conclusion：HLA-A*0206 is strongly associated with Japanese SJS/TEN patients with ocular surface complications.

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Key words：HLA-A*0206, Stevens-Johnson syndrome (SJS), Toxic epidermal necrolysis (TEN), Ocular surface complications, HLA

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I 緒 言

Stevens-Johnson 症候群(Stevens-Johnson syndrome：SJS)，ならびにその重症型とされる中毒性表皮壊死融解症(toxic epidermal necrolysis：TEN)は，ともに突然の高熱と皮膚および粘膜の発疹，びらんで発症し，急速に全身に拡大する疾患である[1)2)]．日本では，皮疹の範囲が10% 以下を SJS，それ以上の広範囲に及んだ場合は TEN と診断される[3)4)]．両疾患は致死率が高く，医療体制の充実した本邦にあっても SJS で数 %，TNE で約 30% が敗血症，呼吸不全，多臓器障害などで亡くなっている．SJS/TEN における眼障害合併率は約 60% とされ[5)]，高熱や発疹の出現と同時，あるいはその数日前より両眼性に結膜充血を生じ，偽膜や角膜びらん，結膜びらんを伴う[6)7)](図 a)．広範囲に眼表面のびらんを生じて角膜上皮幹細胞を喪失すると，角膜は周囲から伸展する結膜で被覆されて高度の角膜混濁，血管侵入を伴うようになる(図 b)．救命できた場合の最大の問題は眼後遺症であり，高度のドライアイ，角膜混濁による視力障害が生涯に及ぶ．

SJS/TEN のほとんどは発症前に何らかの薬剤を投与されており，両疾患は重症薬疹として分類される．発症機序は不明であるが，患者側の素因が関与すると考えられ，これまでに，SJS/TEN の発症には HLA 型が関与するということが報告されている[8)9)]．HLA 型は民族による違いが大きく，我々は日本人の眼合併症を伴う SJS/TEN 71 例，非発症対照 113 例を比較し，HLA-A*0206 が有意に相関していることを以前に報告した[10)11)]．発症に強く関与する HLA 型が明らかになれば，発症予防や発症後の迅速な診断が可能となり，予後改善に寄与することができる．

そこで今回，眼合併症を伴う日本人 SJS/TEN 症例数を 100 例以上に増やし，かつ前回とは異なった日本人対照サンプルを用いて HLA class Ⅰ-A，B，C の解析を行い興味ある結果を得たので報告する．

II 対象と方法

京都府立医科大学附属病院眼科に通院する SJS/TEN 患者 118 例を対象とし，新たに京都府立医科大学で日本人非発症者 220 人より同意を得て採取した血液を対照として用いた．対照については，前回の解析に用いた東京都赤十字血液センターで採取したものとはまったく別グループのものである．末梢血から DNA を採取し，polymerase chain reaction-sequence specific oligonucleotide probe(PCR-SSO)法に基づき HLA タイピング試薬(湧永製薬)を用いて，WAKFlow system にて HLA 遺伝子のタイピングを行った[10)11)]．HLA class Ⅰ-A，B，C について塩基配列レベルの解析を行い，遺伝子頻度(gene frequency)と保持者頻度(carrier frequency)を検討した．

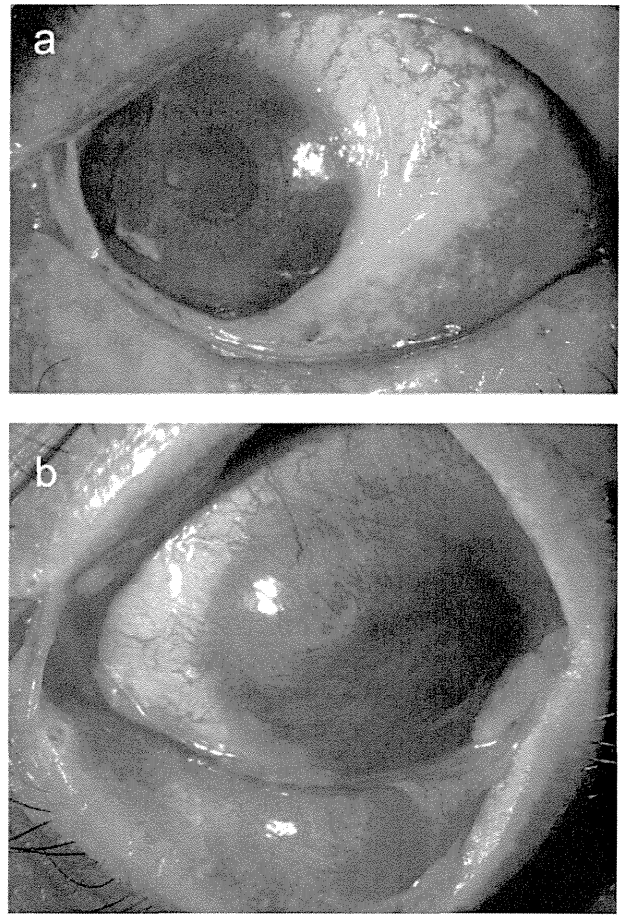

図 Stevens-Johnson 症候群(SJS)/中毒性表皮壊死融解症(TEN)の眼所見．
a：急性期 SJS/TEN の眼所見．結膜充血，偽膜，角膜びらん，結膜びらんを認める．
b：慢性期 SJS/TEN の眼所見．角膜への結膜侵入，瞼球癒着を認める．この症例ではドライアイに対して，涙点プラグを挿入している．眼脂を認めることも多い．

III 結 果

表 1 に HLA class Ⅰ-A 型の結果を示す．118 人の患者を対象とした今回の解析において，前回の 71 人の患者を対象にした解析(保持者頻度：$p=0.00004$，オッズ比 4.1．遺伝子頻度：$p=0.0001$，オッズ比 3.2)以上に[10)11)]，HLA-A*0206 と強い有意な相関が確認された(保持者頻度：$p=0.0000000002$，オッズ比 5.16．遺伝子頻度：$p=0.000000007$，オッズ比 4.15)．

また，HLA-A*1101(保持者頻度：$p=0.02$，オッズ比 0.43．遺伝子頻度：$p=0.03$，オッズ比 0.46)と HLA-A*2402(保持者頻度：$p=0.008$，オッズ比 0.54，遺伝子頻度：$p=0.001$，オッズ比 0.57)に相関を認めたが，HLA class Ⅰ-A 型の数 18 で補正すると有意差はなくなり，弱い相関を示唆するのみとなった．HLA-A*1101 については，前回の解析では，発症しにくさと相関することが推定されたが[10)]，症例数を 71 人から 118 人に増

表1　HLA class Ⅰ-Aの遺伝子頻度と保持者頻度

HLA-A alleles	保持者頻度				遺伝子頻度			
	SJS/TEN (n=118)	正常対照 (n=220)	p値 (χ^2)	オッズ比	SJS/TEN (n=236)	正常対照 (n=440)	p値 (χ^2)	オッズ比
*0101	0.0% (0/118)	1.4% (3/220)	0.20	—	0.0% (0/236)	0.7% (3/440)	0.20	—
*0201	26.3% (31/118)	20.5% (45/220)	0.22	—	14.4% (34/236)	11.1% (49/440)	0.22	—
*0206	44.9% (53/118)	13.6% (30/220)	1.91×10^{-10}	5.16	23.3% (55/236)	6.8% (30/440)	7.14×10^{-9}	4.15
*0207	8.5% (10/118)	7.7% (17/220)	0.99	—	4.2% (10/236)	3.9% (17/440)	0.81	—
*0210	0.0% (0/118)	0.9% (2/220)	0.30	—	0.0% (0/236)	0.5% (2/440)	0.30	—
*0301	2.5% (3/118)	0.9% (2/220)	0.24	—	1.3% (3/236)	0.5% (2/440)	0.24	—
*0302	0.0% (0/118)	0.5% (1/220)	0.46	—	0.0% (0/236)	0.2% (1/440)	0.46	—
*1101	8.5% (10/118)	17.7% (39/220)	0.02	0.43	4.2% (10/236)	8.9% (39/440)	0.03	0.46
*2402	45.8% (54/118)	60.9% (134/220)	0.008	0.54	25.0% (59/236)	37.0% (163/440)	0.001	0.57
*2601	11.0% (13/118)	12.3% (27/220)	0.73	—	5.5% (13/236)	6.4% (28/440)	0.66	—
*2602	5.1% (6/118)	2.7% (6/220)	0.26	—	2.5% (6/236)	1.6% (7/440)	0.39	—
*2603	1.7% (2/118)	7.3% (16/220)	0.03	0.22	0.8% (2/236)	3.6% (16/440)	0.03	0.23
*2605	0.0% (0/118)	0.5% (1/220)	0.46	—	0.0% (0/236)	0.2% (1/440)	0.46	—
*2901	0.0% (0/118)	1.8% (4/220)	0.14	—	0.0% (0/236)	0.9% (4/440)	0.14	—
*3001	0.8% (1/118)	0.5% (1/220)	0.65	—	0.4% (1/236)	0.2% (1/440)	0.65	—
*3101	14.4% (17/118)	18.1% (40/220)	0.38	—	7.2% (17/236)	9.5% (42/226)	0.30	—
*3201	0.0% (0/118)	0.5% (1/220)	0.46	—	0.0% (0/236)	0.2% (1/440)	0.46	—
*3303	22.0% (26/118)	14.1% (31/220)	0.06	—	11.0% (26/236)	7.0% (31/440)	0.06	—

やすことにより，その相関は消失した．

表2にHLA class Ⅰ-B型の結果を示す．HLA-B*1501（保持者頻度：p=0.02，オッズ比0.43．遺伝子頻度：p=0.05，オッズ比0.50），HLA-B*4403（保持者頻度：p=0.004，オッズ比2.26．遺伝子頻度：p=0.004，オッズ比2.14），HLA-B*5201（保持者頻度：p=0.01，オッズ比0.44．遺伝子頻度：p=0.03，オッズ比0.51）とHLA-B*5401（保持者頻度：p=0.01，オッズ比0.36．遺伝子頻度：p=0.01，オッズ比0.37）に弱い相関を認めたが，HLA class Ⅰ-B型の数37で補正すると有意差は消失した．結果として，HLA class Ⅰ-B型には，重篤な眼合併症を伴うSJS/TENと有意な相関を認めるものはなかった．

表3にHLA class Ⅰ-C遺伝子の結果を示す．HLA-C*0304（保持者頻度：p=0.01，オッズ比1.96．遺伝子頻度：p=0.01，オッズ比1.81），HLA-C*1403（保持者頻度：p=0.005，オッズ比2.25．遺伝子頻度：p=0.007，オッズ比2.06）に弱い相関を認めたが，HLA class Ⅰ-C型の数18で補正すると有意差は消失した．結果として，HLA class Ⅰ-C型には，重篤な眼合併症を伴うSJS/TENと有意な相関を認めるものはなかった．

Ⅳ　考　　按

日本人の眼合併症を伴うSJS/TEN患者118人ならびに対照220人の解析により，眼合併症を伴うSJS/TENの発症には，HLA class Ⅰ A*0206が強く相関することが確認された．前回の患者71人，対照113人の解析（保持者頻度：p=0.00004，オッズ比4.1．遺伝子頻度：p

表 2 HLA class I-B の遺伝子頻度と保持者頻度

HLA-B alleles	保持者頻度				遺伝子頻度			
	SJS/TEN (n=118)	正常対照(n=220)	p 値(χ^2)	オッズ比	SJS/TEN (n=118)	正常対照(n=220)	p 値(χ^2)	オッズ比
*0702	8.5% (10/118)	11.0% (24/220)	0.48	—	4.2% (10/236)	6.6% (29/440)	0.21	—
*0705	0.0% (0/118)	1.8% (4/220)	0.14	—	0.0% (0/236)	0.9% (4/440)	0.14	—
*1301	6.8% (8/118)	2.7% (6/220)	0.07	—	3.4% (8/236)	1.4% (6/440)	0.08	—
*1302	0.0% (0/118)	1.4% (3/220)	0.20	—	0.0% (0/236)	0.7% (3/440)	0.20	—
*1501	8.5% (10/118)	17.7% (39/220)	0.02	0.43	4.7% (11/236)	8.9% (39/440)	0.05	0.50
*1502	0.0% (0/118)	0.5% (1/220)	0.46	—	0.0% (0/236)	0.2% (1/440)	0.46	—
*1507	0.8% (1/118)	1.4% (3/220)	0.68	—	0.4% (1/236)	0.7% (3/440)	0.68	—
*1511	0.0% (0/118)	0.5% (1/220)	0.46	—	0.0% (0/236)	0.2% (1/440)	0.46	—
*1518	3.4% (4/118)	1.0% (2/220)	0.10	—	1.7% (4/236)	0.5% (2/440)	0.10	—
*1527	0.8% (1/118)	0.0% (0/220)	0.17	—	0.4% (1/236)	0.0% (0/440)	0.17	—
*2704	0.8% (1/118)	0.5% (1/220)	0.65	—	0.4% (1/236)	0.2% (1/440)	0.65	—
*2705	0.0% (0/118)	0.5% (1/220)	0.46	—	0.0% (0/236)	0.2% (1/440)	0.46	—
*3501	18.6% (22/118)	14.5% (32/220)	0.33	—	9.3% (22/236)	7.3% (32/440)	0.35	—
*3701	0.8% (1/118)	1.8% (4/220)	0.48	—	0.4% (1/236)	0.9% (4/440)	0.48	—
*3802	0.0% (0/118)	0.5% (1/220)	0.46	—	0.0% (0/236)	0.2% (1/440)	0.46	—
*3901	7.6% (9/118)	5.9% (13/220)	0.54	—	3.8% (9/236)	3.0% (13/440)	0.55	—
*3902	0.8% (1/118)	1.0% (2/220)	0.95	—	0.4% (1/236)	0.5% (2/440)	0.95	—
*3904	0.0% (0/118)	1.0% (1/220)	0.46	—	0.0% (0/236)	0.2% (1/440)	0.46	—
*4001	14.4% (17/118)	10.9% (24/220)	0.35	—	7.6% (18/236)	5.5% (24/440)	0.26	—
*4002	12.7% (15/118)	9.5% (21/220)	0.37	—	6.8% (16/236)	5.2% (23/440)	0.41	—
*4003	1.7% (2/118)	1.0% (2/220)	0.52	—	0.8% (2/236)	0.5% (2/440)	0.53	—
*4006	7.6% (9/118)	5.9% (13/220)	0.54	—	3.8% (9/236)	3.0% (13/440)	0.55	—
*4402	0.8% (1/118)	1.8% (4/220)	0.48	—	0.4% (1/236)	0.9% (4/440)	0.48	—
*4403	26.3% (31/118)	13.6% (30/220)	0.004	2.26	13.6% (32/236)	6.8% (30/440)	0.004	2.14
*4601	12.7% (15/118)	8.2% (18/220)	0.18	—	6.4% (15/236)	4.3% (19/440)	0.25	—
*4801	4.2% (5/118)	8.2% (18/220)	0.17	—	2.1% (5/236)	4.1% (18/440)	0.18	—
*5101	19.5% (23/118)	19.5% (43/220)	0.99	—	10.6% (25/236)	10.2% (45/440)	0.88	—
*5102	1.7% (2/118)	0.0% (0/220)	0.05	—	0.8% (2/236)	0.0% (0/440)	0.053	—
*5201	11.0% (13/118)	21.8% (48/220)	0.01	0.44	5.9% (14/236)	10.9% (48/440)	0.03	0.51
*5401	5.9% (7/118)	15.0% (33/220)	0.01	0.36	3.0% (7/236)	7.7% (34/440)	0.01	0.37
*5502	2.5% (3/118)	4.5% (10/220)	0.36	—	1.3% (3/236)	2.3% (10/440)	0.37	—
*5601	2.5% (3/118)	1.8% (4/220)	0.65	—	1.3% (3/236)	0.9% (4/440)	0.66	—
*5603	0.0% (0/118)	0.5% (1/220)	0.46	—	0.0% (0/236)	0.2% (1/440)	0.46	—
*5801	2.5% (3/118)	1.8% (4/220)	0.66	—	1.3% (3/236)	0.9% (4/440)	0.66	—
*5901	7.6% (9/118)	4.5% (10/220)	0.24	—	3.8% (9/236)	2.3% (10/440)	0.25	—
*5904	0.8% (1/118)	0.0% (0/220)	0.17	—	0.4% (1/236)	0.0% (0/440)	0.17	—
*6701	1.7% (2/118)	3.6% (8/220)	0.31	—	0.8% (2/236)	1.8% (8/440)	0.32	—

=0.0001，オッズ比 3.2)からサンプルを増やすことにより，保持者頻度での p 値が 0.0000000002，オッズ比 5.16，遺伝子頻度での p 値が 0.000000007，オッズ比 4.15 と，さらに強い相関を確認できたことは大変に意義深い．

SJS/TEN の HLA 解析について以前の報告を振り返ってみると，1982 年にアメリカ人の眼科医 Mondino らが[8]，また，1986 年にフランス人の皮膚科医 Roujeau らが[9]，白人の SJS 患者では HLA-B12 血清型を有意に多く保有すると報告した．HLA-B12 血清型は，現在の遺伝子型

表 3　HLA class Ⅰ-Cの遺伝子頻度と保持者頻度

HLA-C alleles	保持者頻度				遺伝子頻度			
	SJS/TEN (n=118)	正常対照 (n=220)	p値 (χ^2)	オッズ比	SJS/TEN (n=236)	正常対照 (n=440)	p値 (χ^2)	オッズ比
*0102	30.5% (36/118)	34.5% (76/220)	0.45	—	15.7% (37/236)	18.6% (82/440)	0.34	—
*0103	0.0% (0/118)	0.5% (1/220)	0.46	—	0.0% (0/236)	0.2% (1/440)	0.46	—
*0302	2.5% (3/118)	1.8% (4/220)	0.66	—	1.3% (3/236)	0.9% (4/440)	0.66	—
*0303	2.3% (27/118)	21.3% (47/220)	0.75	—	11.4% (27/236)	10.9% (48/440)	0.83	—
*0304	32.3% (38/118)	19.5% (43/220)	0.01	1.96	17.8% (42/236)	10.7% (47/440)	0.01	1.81
*0401	8.5% (10/118)	9.5% (21/220)	0.75	—	4.2% (10/236)	4.8% (21/440)	0.75	—
*0501	2.5% (3/118)	1.8% (4/220)	0.66	—	1.3% (3/236)	0.9% (4/440)	0.66	—
*0602	0.8% (1/118)	3.2% (7/220)	0.18	—	0.4% (1/236)	1.6% (7/440)	0.18	—
*0701	0.8% (1/118)	0.0% (0/220)	0.17	—	0.4% (1/236)	0.0% (0/440)	0.17	—
*0702	18.6% (22/118)	25.0% (55/220)	0.18	—	10.6% (25/236)	14.1% (62/440)	0.20	—
*0704	2.5% (3/118)	0.0% (0/220)	0.02	0	1.3% (3/236)	0.0% (0/440)	0.02	0
*0801	8.5% (10/118)	13.6% (30/220)	0.16	—	4.2% (10/236)	6.8% (30/440)	0.18	—
*0803	2.5% (3/118)	3.6% (8/220)	0.59	—	1.3% (3/236)	1.8% (8/440)	0.59	—
*1202	11.9% (14/118)	21.4% (47/220)	0.03	0.50	6.4% (15/236)	10.7% (47/440)	0.06	—
*1402	12.7% (15/118)	14.1% (31/220)	0.72	—	6.8% (16/236)	7.5% (33/440)	0.73	—
*1403	25.4% (30/118)	13.2% (29/220)	0.005	2.25	12.7% (30/236)	6.6% (29/440)	0.007	2.06
*1502	8.5% (10/118)	5.9% (13/220)	0.37	—	4.2% (10/236)	3.0% (13/440)	0.38	—
*1505	0.0% (0/118)	1.8% (4/220)	0.14	—	0.0% (0/236)	0.9% (4/440)	0.14	—

ではHLA-B*4402とHLA-B*4403に当てはまる．しかしながら我々の解析ではHLA-B*4402は対照で1.8%，患者で0.8%と保持者頻度が少なく，また相関も認めなかった．HLA-B*4403については，対照で13.6%，患者で26.3%の頻度で認めたが，SJS/TEN発症との相関は確認されなかった．また，2004年にChungらが，抗てんかん薬カルバマゼピンにより発症した台湾のSJS/TEN患者では，100%がHLA-B*1502を保有していることを報告した[12]．しかし，Lonjouらは，白人ではHLA-B*1502保有率が低く，カルバマゼピン発症SJS/TEN患者とHLA-B*1502には相関を認めなかったと報告している[13]．日本人でもHLA-B*1502を保有していることはごくまれであり，SJS/TENの発症と相関を示さないことが報告されている[14]．我々の解析でも，HLA-B*1502は，対照でわずか1例認めたのみであった．また，重篤な眼合併症を伴うSJS/TENにおいては，抗てんかん薬による発症は大変少ない[15]．2005年にHungらは，高尿酸血症薬であるアロプリノールにより発症したSJS/TEN患者とHLA-B*5801との間に有意な相関があると報告した[16]．このアロプリノールとHLA-B*5801との相関は，白人でも[17]，日本人でも[18]報告されている．しかし，今回解析しているSJS/TEN患者118例のなかでアロプリノールによる発症はわずか3例であり，どの症例も眼後遺症は軽度であった．これらのことから，我々は，アロプリノールによる発症では，重篤な眼合併症は生じにくい可能性を考えている．

皮膚科医は，SJS/TEN症例の多くは，抗てんかん薬またはアロプリノールによる発症であると報告してい

る[19][20]．しかし上述したように，眼障害を合併し後遺症を生じた患者では抗てんかん薬，アロプリノールによる発症は少なく，多くが総合感冒薬あるいは非ステロイド性抗炎症薬(non-steroidal anti-inflammatory drugs：NSAIDs)を契機に発症している[21]．重篤なTENで眼障害のない症例もあれば，全身的に軽症で眼障害の高度なSJS症例もある．眼合併症を伴うSJS/TENは，SJS/TEN全体からみた単なる一部の症例群ではなく，独立した疾患カテゴリーになるのではないかと我々は考えている．

我々の調査では，重篤な眼合併症を伴うSJS/TEN患者では，急性結膜炎が皮疹に先行することが多く，口唇・口腔内の出血性びらん，爪囲炎が必発である[6][7]．これらの患者の約8割は感冒様症状を最初に自覚し，その後に薬剤投与がなされて高熱，発疹を生じている[6][7][21]．SJS/TENの発症機序は不明であるが，このような共通する病歴と初期症状から，最初に何らかのウイルス感染を生じ，その後に薬剤が契機となって眼表面，口唇・口腔内，爪囲を主座とする病変を生じていると考えられる．

HLAクラスⅡは，抗原提示細胞に発現しており，T細胞を介した免疫応答に大きく関与している．一方，HLAクラスⅠは，上皮細胞を含むほぼすべての細胞に発現しており，ウイルス抗原を提示することにより，ウイルス感染に対する生体反応に大きく関与する．SJS/TENの発症素因に，HLAクラスⅡではなく，HLAクラスⅠであるHLA-A*0206が大きく関与していることは，その発症にウイルス感染が何らかの形でかかわっている可能性があるという我々の考え[22]~[24]を支持する結果であると考えられる．

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利益相反：利益相反公表基準に該当なし

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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 11 63 218 SNPs. SNPs were discarded from case–control association analysis if they exhibited a minor allele frequency <0.001 in the control group (2 37 890 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 2 72 897 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 11 63 218 SNPs. A series of quality-control steps resulted in the elimination of 2 72 897 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]

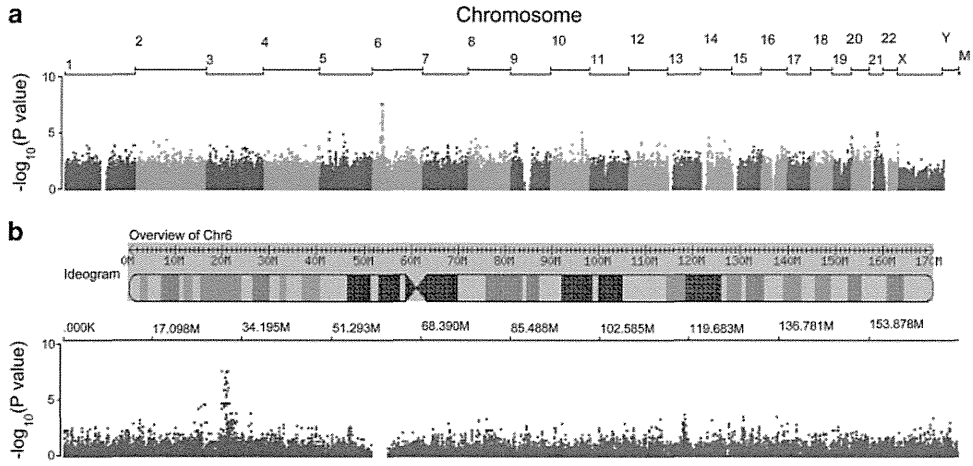

Figure 1 Genome-wide association study of allopurinol-related Stevens–Johnson syndrome or toxic epidermal necrolysis. Each dot represents a single nucleotide polymorphism (SNP). The *x* axis: the position of the SNP on chromosomes. The *y* axis: the $-\log_{10}$ of Fisher's exact test *P*-values (dominant genotype mode) of the SNP in the case–control association study. SNPs with *P*-values $<5.62\times10^{-8}$ are highlighted in red. (**a**) Whole genome. (**b**) Chromosome 6.

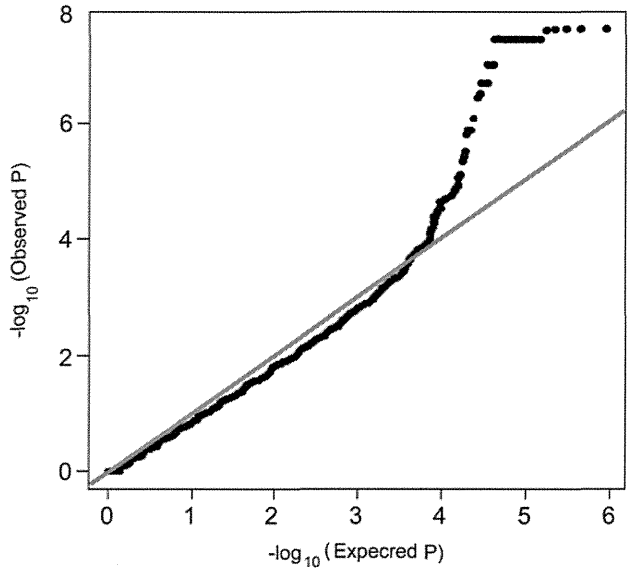

Figure 2 Quantile–quantile plot of Fisher's exact test statistics obtained from the genome-wide association study for allopurinol-related Stevens–Johnson syndrome or toxic epidermal necrolysis under dominant genotype mode. The solid red line represents the null model where observed Fisher's exact test 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 ($P<1\times10^{-7}$).[7] In this study using Japanese patients, both rs3117583 and rs1150793 showed $P=6.34\times10^{-3}$ (allele frequency mode) and $P=6.14\times10^{-3}$ (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.

Acknowledgments

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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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Identification of a novel gene by whole human genome tiling array

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ABSTRACT

When the whole human genome sequence was determined by the Human Genome Project, the number of identified genes was fewer than expected. However, recent studies suggest that undiscovered transcripts still exist in the human genome. Furthermore, a new technology, the DNA microarray, which can simultaneously characterize huge amounts of genome sequence data, has become a useful tool for analyzing genetic changes in various diseases. A version of this tool, the tiling DNA microarray, was designed to search all the transcripts of the entire human genome, and provides huge amounts of data, including both exon and intron sequences, by a simple process. Although some previous studies using tiling DNA microarray analysis have indicated that numerous novel transcripts can be found in the human genome, none of them has reported any novel full-length human genes. Here, to find novel genes, we analyzed all the transcripts expressed in normal human prostate cells using this microarray. Because the optimal analytical parameters for using tiling DNA microarray data for this purpose had not been established, we established parameters for extracting the most likely regions for novel transcripts. The three parameters we optimized were the threshold for positive signal intensity, the Max gap, and the Min run, which we set to detect all transcriptional regions that were above the average length of known exons and had a signal intensity in the top 5%. We succeeded in obtaining the full-length sequence of one novel gene, located on chromosome 12q24.13. We named the novel gene "POTAGE". Its 5841-bp mRNA consists of 26 exons. We detected part of exon 2 in the tiling data analysis. The full-length sequence was then obtained by RT-PCR and RACE. Although the function of POTAGE is unclear, its sequence showed high homology with genes in other species, suggesting it might have an important or essential function. This study demonstrates that the tiling DNA microarray can be useful for identifying novel human genes.

1. Introduction

DNA microarray analysis has been established as one of the most useful technologies for investigating the underlying pathogenesis of various diseases (Castellano et al., 2009; Heintzman et al., 2009; Hussain et al., 2009; Takata et al., 2010; Xu et al., 2005; Yeager et al., 2007; Yeager et al., 2009). Annotated gene expression levels and single nucleotide polymorphisms can be conveniently evaluated using this technique. The tiling DNA microarray is a variation that was developed for investigating all the transcripts of the whole genome, including those of undiscovered genes (Bertone et al., 2004; Johnson et al., 2005; Kapranov et al., 2002; Mockler et al., 2005; Royce et al., 2005; Schadt et al., 2004; Shoemaker et al., 2001). This innovation also allows us to investigate the pathogenesis of various diseases.

The Human Genome Project reported the first complete sequence of the human genome in 2003. This project found 30,000 fewer expressed genes than had been expected (International Human Genome Sequencing Consortium, 2004; Lander et al., 2001; Venter et al., 2001). However, even though the findings also suggested that more than 98% of all genomic sequences are not transcribed (Cheng et al., 2005), recent studies on these "non-coding" DNA regions have revealed that they have many functions. In addition, these regions contain computationally predicted genes that may encode functional DNA and/or proteins. These observations suggest that novel genes that are transcribed into RNA may be found in these regions.

Because the original DNA microarray technology, used for evaluating annotated gene expression levels, was designed with relatively few probes, usually covering only the 5′-ends of annotated genes, it is not very useful for finding undiscovered transcripts in unexplored genomic regions. Specifically, the number and location of the probes meant that transcribed regions that lay between the probes could

Abbreviations: mRNA, messenger RNA; RT-PCR, reverse transcription-polymerase chain reaction; RACE, rapid amplification of cDNA ends; kb, kilobases; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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not be detected. In contrast, tiling DNA microarrays are useful for mapping novel transcripts, because the "tiling" feature consists of 25-mer oligonucleotide probes that are tiled at approximately 35-bp intervals, as measured from the central position of the adjacent probe. Therefore, a gap of only approximately 10 bp lies between probes, which cover the entire genome except the telomeres and centromeres (Sasaki et al., 2007). Tiling DNA microarray data have improved gene annotations and revealed the extensive transcriptions of non-coding RNAs. The closely spaced probes allow for the accurate measurement of small transcriptional features, such as single exons or small introns. This technology is now allowing us to investigate undiscovered transcripts as well as the expression of annotated genes. In this regard, the tiling DNA microarray is one of the most powerful and fruitful tools for evaluating both annotated genes and novel transcripts that have unclear functions. Previous reports using tiling DNA microarray have demonstrated novel transcripts in the human genome. However, full-length novel genes have not been reported (Kampa et al., 2004; Kapranov et al., 2005; Nelson et al., 2008; Weile et al., 2007).

In this study, we used tiling DNA microarray to seek undiscovered transcripts, and we demonstrated its usefulness for identifying a novel coding gene.

2. Materials and methods

2.1. Cell culture

Primary normal prostate epithelial cells (PrECs) were purchased from Lonza (Walkersville, MD) and maintained in prostate epithelial cell media (PrEGM Bullet Kit-Lonza) supplemented with a mixture of various growth factors (Single Quots-Lonza). Cells were seeded at recommended densities and cultured at 37 °C at 5% CO_2. Media were changed every 48 h.

2.2. RNA and DNA preparation

Total RNA was extracted from PrECs with the RNeasy Plus Mini Kit (Qiagen, Valencia, CA). RNA quality was evaluated by spectrophotometry with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and by gel electrophoresis.

Genomic DNA was extracted from cancerous and normal frozen prostate tissue. The samples were minced and mixed well in lysis buffer with proteinase-K (to 0.2 mg/mL) and SDS (to 0.1%) at 55 °C. DNA was separated from the proteinaceous component by two extractions with an equal volume of phenol/chloroform isoamyl alcohol. The aqueous phase was mixed with 2.5 volumes of 100% ethanol and 0.1 volumes of 3 M sodium acetate and centrifuged at 12,000 ×*g* for 20 min at 4 °C. The DNA pellet was washed with cold 70% ethanol and allowed to air dry before resuspension in TE (10 mM Tris–HCl pH 7.5, 1 mM EDTA).

2.3. Affymetrix GeneChip® hybridization

Affymetrix Human Tiling 1.0R Array GeneChip® (Tiling array; Affymetrix, Santa Clara, CA) arrays were used for triplicate hybridizations. For the microarray hybridization, we followed the protocol described in the Affymetrix GeneChip® Whole Transcript Double-Stranded Target Assay Manual (Affymetrix, Santa Clara, CA). In brief, 6 μg of total RNA was purified by ribosomal RNA reduction using the RiboMinus Human/Mouse Transcriptome Isolation Kit (Invitrogen Co., Carlsbad, CA) and cleaned up. A single-stranded cDNA was synthesized using a T7-$(N)_6$ primer, and the cDNA was made double-stranded. The ds cDNA was amplified by in vitro transcription into complementary RNA (cRNA) and cleaned up. The second cycle ds cDNA was synthesized using the amplified cRNA as a template. The ds DNA was cleaned up, fragmented, and labeled with biotin. The fragmented ds DNA was used for hybridization to the microarrays at 45 °C for 16 h with a rotation rate of 60 rpm using a GeneChip® Hybridization Oven (Affymetrix, Santa Clara, CA). The microarrays were washed and stained using an Affymetrix GeneChip® Fluidics Station 450 and scanned by an Affymetrix GeneChip® Scanner 3000.

2.4. Tiling array data analysis

To handle the data generated by using probes that hybridize throughout the whole genome, we extracted the positive data as follows (Supplemental Fig. 1). As the distance used to locally group positional data for statistical analysis, we set the bandwidth at the maximum recommended level (73 bp). This setting increases the reliability of the signal intensity derived from a perfectly matched probe vs. a mismatched probe. After removing data that showed no signal intensity (43.9% of all probes in 14 arrays), the threshold for each array was set to filter out all but the top 5% of probe intensities. A positive probe was defined as one having a signal intensity greater than threshold (Supplemental Table 1, Supplemental Fig. 2) (Eisenberg and Levanon, 2003). To evaluate the DNA regions hybridizing with positive probes, we used a Max gap parameter (the maximum tolerated gap between positive positions in the derivation of detected regions) of 70 bp, to permit the hybridization of a negative probe between two positive probes. The Min run parameter (the minimum size of a detected region) was set at 140 bp, which is the approximate average length of all exons identified among the annotated genes of NCBI (http://www.ncbi.nlm.nih.gov/) Build 36.2 (Supplemental Table 1, Supplemental Fig. 3). The regions whose signals passed our three parameter settings were compared to those of annotated genes by the probe position, according to the information provided by NCBI Build 36 in the Affymetrix Integrated Genome Browser (IGB) (Nicol et al., 2009), to remove regions that overlapped with annotated genes. Next, the data were carefully divided into known or unknown transcripts by checking each sequence against the latest annotations. New transcripts that appeared within an annotated gene, even if not in the exonic sequences, were also considered to be gene-related transcripts, and we excluded them from further analysis. Finally, cases of two or more novel regions lying within 5-kbp on the genome were defined as "zones," and were investigated further.

2.5. RT-PCR and rapid amplification of cDNA ends (RACE)

Total RNA was extracted from the specimens using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA). First-strand cDNA synthesis was performed using the SuperScript III First-Strand Synthesis System with an oligo $(dT)_{20}$ primer for RT-PCR (Invitrogen Co., Carlsbad CA), according to standard procedures.

Rapid amplification of cDNA ends (RACE) was performed with a GeneRacer kit (Invitrogen Co., Carlsbad, CA) and SMART RACE cDNA Amplification Kit (Clontech Laboratories, CA, USA), according to the manufacturer's instructions.

2.6. Sequencing analysis

Amplified RT-PCR and RACE products of target regions were sequenced with a BigDye terminator v1.1 or v3.1 Cycle Sequencing kit (Applied Biosystems, CA, USA) using Applied Biosystems 3130 Genetic Analyzers. The primers for the sequencing analysis were designed according to the results of each RACE analysis. The primer sequences are described in the supplementary information.

2.7. Quantitative RT-PCR assay in multiple human tissues

To evaluate the levels of POTAGE expression in human tissues, quantitative PCR (QPCR) was performed using the Stratagene Mx3005P

real-time QPCR system with the Brilliant II Fast SYBR Green QPCR Master Mix (Agilent Technologies, CA, USA) and Human MTC Panels I and II, which include heart, brain, placenta, lung, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon, and peripheral leukocytes (Clontech Laboratories, CA, USA). These assays were performed at least four times in duplicate. To normalize the values in the quantitative assays, the level of *beta-Actin* was assessed as a control. The primer sequences for *beta-actin* were (forward) 5′-ATTGCCGACAGGATGCAGAA-3′ and (reverse) 5′-ACATC TGCTGGAAGGTGGACAG-3′. After the QRT-PCR assay, each sample was examined by agarose gel electrophoresis to evaluate the amplification of a single RT-PCR product.

2.8. Methylation assay with normal and cancerous human prostate tissue

DNA methylation is an important epigenetic mechanism of gene regulation. To investigate the methylation status of POTAGE in the human prostate, we performed methylation PCR using human prostate tissues. Normal and cancerous prostate samples were obtained from six patients, with their informed consent, during radical retropubic prostatectomy for clinically localized prostate adenocarcinoma, performed at the Kyoto Prefectural University of Medicine. All procedures were conducted in accordance with the Helsinki declaration. This study was approved by the Institutional Review Board of Kyoto Prefectural University of Medicine.

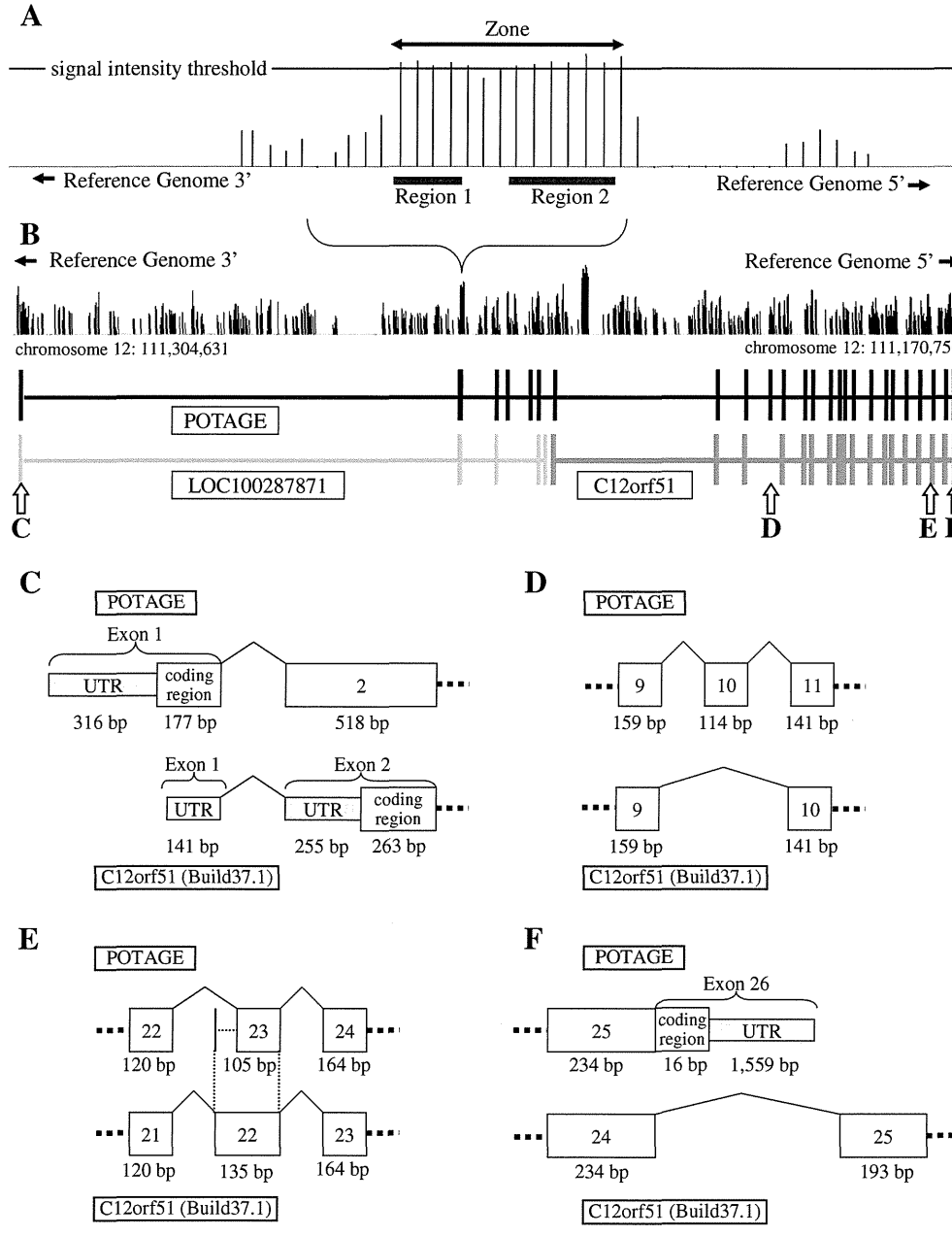

Fig. 1. (A) For this study, we defined two concepts: the region and the zone. A region was the genomic area that passed our settings for all three parameters and did not overlap with annotated genes. Zones were two or more regions that lay within 5-kbp of one another, on the chromosome. Zones were assumed to encode at least some portion of a novel gene. (B) Each novel zone was investigated in detail by RT-PCR and RACE. Shown is the region containing the novel protein, which overlapped with exon 2 of LOC100287871, a predicted gene in NCBI Build 36.3. We obtained the full-length mRNA sequence encoded by this region. (C, D, F) Although 22 of the 26 exons of the novel gene also served as exons of C12orf51, exons 1, 10, and 26 were new. (E) Exon 23 of the novel gene had a 30-bp deletion.