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Polymorphisms in the prostaglandin E_2 receptor subtype 2 gene confer susceptibility to aspirin-intolerant asthma: a candidate gene approach

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Aspirin-intolerant asthma (AIA) is a subtype of bronchial asthma characterized by development of bronchoconstriction evoked by non-steroidal anti-inflammatory drugs (NSAIDs). NSAIDs inhibit the cyclooxygenase pathway, leading to enhancement of the lipoxygenase pathway. We evaluated allelic association of 370 single nucleotide polymorphisms (SNPs) of 63 candidate genes, mostly from the arachidonic acid metabolic cascade, with AIA. After two rounds of screening with 198 AIA patients, multiple SNPs in the prostaglandin E_2 receptor subtype 2 (*EP2*) gene were associated with AIA ($P < 0.05$). Among the 77 SNPs identified in the *EP2* gene, we selected 17 SNPs on the basis of linkage disequilibrium and allelic frequencies (minor allele frequency >0.1) for further association study. SNPs in the promoter region of the *EP2* gene, uS5, uS5b, and uS7, were significantly associated with AIA (permutation $P = 0.039–0.001$). Analysis of haplotypes constructed according to the LD pattern showed a significant association with AIA (permutation $P = 0.001$). The most significantly associated SNP, uS5, located in the regulatory region of the *EP2* gene, was in a STATs-binding consensus sequence [AIA 31.1% versus control 22.1% (permutation $P = 0.0016$) or versus aspirin-tolerant asthma 22.2% (permutation $P = 0.0017$)]. Although STAT1 binding was not observed in gel mobility shift assay with HeLa nuclear extract, an unidentified protein was specifically bound to the allelic sequence. In *in vitro* reporter assay in HCT116 cells, the site containing the uS5 allele showed reduced transcription activity. Taken together, these results suggest that uS5 allele serves as a target of a transcription repressor protein. A functional SNP of the *EP2* gene associated with risk of AIA should decrease the transcription level, resulting in reduction of the PGE_2 braking mechanism of inflammation and involvement in the molecular mechanism underlying AIA.

INTRODUCTION

In a subset of asthmatic patients, aspirin and several other non-steroidal anti-inflammatory drugs (NSAIDs) that inhibit cyclooxygenase enzymes (COXs) induce severe asthmatic attack, generally termed aspirin-intolerant asthma (AIA) (1). AIA constitutes 5–15% of asthmatic patients, and is more prevalent in women. AIA is usually more severe, has a later onset than allergic asthma and frequently is associated with nasal polyp or sinusitis. Despite the well-defined pharmacological trigger, the molecular pathogenesis underlying AIA is still obscure. The cyclooxygenase theory is the widely

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accepted pathogenesis of AIA: a pharmacological action of NSAIDs, inhibition of COXs in the respiratory tract, alters arachidonic acid metabolism in AIA patients (2–4). Thus, aspirin and most other NSAIDs lead to a decrease in the level of PGE_2, an anti-inflammatory PG generated as one of the various oxygenated metabolites in the COX pathway, which increases the number of cysteinyl leukotrienes (cys-LTs) that can mediate bronchoconstriction, mucus secretion, vascular permeability, cellular infiltration and eosinophil survival (5–7). An imbalance toward the lipoxygenase (LO) pathway is thought to play a role in accelerating the inflammation reaction in the airway tract. Although AIA is precipitated by inhibition of the COX pathway, it remains unclear why a similar adverse reaction to NSAIDs is not seen in patients with aspirin tolerant asthma (ATA) or in healthy individuals.

There is moderate genetic background in AIA; the European Network on Aspirin-Induced Asthma found that 5.1% of 365 AIA patients had family history of aspirin sensitivity (8). A polymorphism in the promoter of LTC_4 synthase, A-444C single nucleotide polymorphism (SNP), has been reported to be associated with AIA in Polish patients (9,10). However, conflicting results were reported in US and Japanese populations (11,12). A recent report showed that a haplotype of the 5-LO gene was weakly associated with AIA in Korean population (13). In the present study, an extensive candidate gene analysis was applied to identify susceptibilities to AIA. On the basis of the well-defined pharmacological actions of NSAIDs, 63 candidate genes for AIA were catalogued and screened for allelic association study in a total of 198 AIA patients. The 833 SNPs of 63 candidate genes were initially identified; 370 SNPs were selected on the basis of linkage disequilibrium and allelic frequency, and evaluated for allelic association with AIA. SNPs in the prostaglandin E_2 receptor subtype 2 (*EP2*) gene were found to be significantly associated with AIA, and the functional impact of a promoter variant was further investigated.

RESULTS

Screening of candidate genes for AIA

On the basis of the pharmacological actions of NSAIDs and the bronchial hyper-responsiveness of AIA, 63 candidate genes were selected for evaluation of association with AIA as follows: 43 genes from the arachidonic acid metabolic cascade, such as LOs, COXs, various leukotriene (LT) and PG synthases and receptors acting on the LO- and COX-pathways, and 20 genes from the immune system and other factors likely to be involved in asthma such as lymphokines, transcription factors of immune cells, matrix metalloproteinases and the platelet activating factor pathway (summarized in Table 1). The 833 SNPs of 63 genes, identified from the public database or by direct sequencing, were genotyped in 96 control subjects; among these, 370 SNPs with minor allele frequency >0.1 or location in the coding region were genotyped for the first screening (Table 1). Distributions of allele frequency of the SNPs were compared in 87 patients with AIA, 192 with ATA [96 atopic asthmatic (AT) and 96 non-atopic asthmatic (NAT) patients] and 96 with non-asthmatic controls (CTR) by a simple chi-square test. Forty-nine SNPs in 15 genes were associated with AIA in comparison with CTR in the first screening ($P < 0.05$). Fifteen SNPs in five genes showed significant differences in allele frequencies in comparisons of both AIA with ATA and AIA with CTR (data not shown).

All the subjects including the individuals in the first screening, 198 AIA, 282 ATA and 274 CTR, were then subjected to genotyping of the 49 SNPs. After increasing the sample size, the association results of most of the SNPs tested by permutation analysis with 10 000 iterations were weakened or disappeared (data not shown), except for the SNPs of *EP2* gene. SNPs uS7, S3, S4 and S5 of *EP2* gene demonstrated significant associations with AIA in comparison with ATA or with CTR (Table 2). In the second screening, uS7 showed the most significant association in both comparisons of AIA with ATA (permutation $P = 0.0025$) and AIA with CTR (permutation $P = 0.039$).

Linkage disequilibrium mapping of *EP2* gene

As multiple SNPs of the *EP2* gene were associated with AIA, extensive screening of *EP2* was undertaken. We identified 77 SNPs covering the entire gene and the regions spanning 35 kb upstream of exon 1 and 43 kb downstream of exon 2 (Fig. 1). The 77 SNPs were categorized into 24 subgroups on the basis of genotype identity after genotyping 12 individuals; SNPs expected to be in tight LD (assuming r^2-LD) were grouped together (Fig. 1). Twenty-four representative SNPs of subgroups uS1–uS10, S1–S10 and dS1–dS4 were genotyped in all the patients and controls, and pair-wise linkage disequilibrium was estimated. A highly structured LD pattern, a major LD block structure ($|D'| > 0.7$) covered by uS5–S10 and two minor LD blocks covered by uS1–uS4 and dS1–dS4, were observed (Fig. 2A). SNPs uS5–uS10, S1–S10, and dS1, associated with AIA in the second screening, were located in the major LD block. The 17 SNPs representing the major LD block then were evaluated for association with AIA by genotyping the AIA patients. SNPs in the major LD block were evaluated by r^2-statistic, and weak LD was observed (Fig. 2A). In addition to SNPs uS7, S3, S4 and S5, significant associations also were observed for uS5 and uS10 using simple chi-square test and permutation test (Table 2). The minor allele frequency of uS5 was 31.1% for AIA, 22.1% for CTR and 22.2% for ATA, showing significant association (AIA versus CTR: $\chi^2 = 9.67$, $P = 0.0019$, permutation $P = 0.0016$; AIA versus ATA: $\chi^2 = 9.61$, $P = 0.0019$, permutation $P = 0.0017$) (Table 2).

UPGMA-based 'LD tree' and haplotype analysis

A UPGMA (unweighted pair-group method using arithmetic averages)-based 'LD tree' was developed as a tool for visual inspection of subgroupings of SNPs on the basis of LD structure as described in Materials and Methods (Fig. 2B), and subsequently applied to haplotype analysis. UPGMA is a method for designing a diagram to compare the sequence

Table 1. SNPs applied to the first screening

No.	Gene	SNP	Sequence position	Variation	Localization	rs ID	Minor allele frequncies (96 CTR)
1	*ADAM33*	ADAM33S1	11 513	C/A	Intron19	rs44707	0.467
2		ADAM33S2	12 505	T/C	Exon20-non-synonymous (M764T)	rs2280091	0.098
3		ADAM33S3	12 534	T/C	Exon20-non-synonymous (M774S)	rs2280090	0.102
4		ADAM33S4	12 612	T/C	Intron20	rs2280089	0.092
5		ADAM33S5	13 018	G/A	Intron21	rs628977	0.362
6		ADAM33S6	13 026	T/C	Intron21	rs628965	0.362
7		ADAM33S7	13 060	A/C	Intron21	rs543749	0.181
8	*ALOX12B*	ALOX12BS1	1963	G/A	Intron2	rs3027303	0.189
9		ALOX12BS4	3276	G/T	Intron2	rs3027294	0.391
10		ALOX12BS5	7053	G/C	Intron4	rs2304908	0.392
11		ALOX12BS6	7223	C/T	Intron4	rs2304907	0.396
12		ALOX12BS7	7341	C/T	Intron4	rs2304906	0.130
13	*ALOX5*	ALOX5S1	9223	A/G	Intron2	rs4769060	0.151
14		ALOX5S2	26 904	G/A	Intron3	New	0.401
15		ALOX5S3	50 760	G/A	Intron4	New	0.198
16		ALOX5S4	59 443	A/G	Intron6	New	0.193
17		ALOX5S5	69 751	G/C	Intron7	New	0.354
18	*CLP*	CLPS1	1152	G/A	Intron2	rs2966305	0.401
19		CLPS2	3607	C/A	Intron2	New	0.032
20		CLPS3	3688	A/G	Intron2	rs2967871	0.315
21		CLPS4	9097	C/T	Intron2	rs2925050	0.495
22		CLPS5	9197	T/G	Intron2	New	0.457
23		CLPS6	11 744	T/C	Intron2	rs1835156	0.333
24		CLPS7	17 612	T/C	Intron2	New	0.229
25		CLPS8	17 865	G/C	Intron2	New	0.356
26		CLPS9	17 923	G/C	Intron2	rs2967876	0.284
27		CLPS10	20 308	C/T	Intron2	rs2925064	0.263
28		CLPS11	20 380	T/C	Intron2	rs934166	0.468
29		CLPS12	20 412	G/A	Intron2	rs201854	0.447
30		CLPS13	23 054	T/C	Intron2	New	0.104
31		CLPS17	27 698	A/G	Intron2	rs2288584	0.250
32		CLPS18	31 174	T/C	Intron3	rs2967855	0.283
33		CLPS19	31 262	C/G	Intron3	New	0.214
34		CLPS20	31 615	C/T	Intron3	New	0.208
35		CLPS21	43 186	A/T	Intron3	rs2914823	0.185
36		CLPS22	43 360	T/G	Intron3	New	0.404
37		CLPS23	43 394	A/G	Intron3	New	0.250
38		CLPS24	47 961	G/A	Intron3	New	0.116
39		CLPS25	48 577	C/T	Intron3	New	0.347
40		CLPS26	51794	C/T	Exon4-UTR	rs247862	0.297
41	*CNOT3*	CNOT3S1	328	C/T	Intron1	rs42318	0.115
42	*CTSG*	CTSGS1	308	G/A	Intron1	rs2236742	0.212
43		CTSGS2	527	A/G	Intron1	New	0.034
44		CTSGS3	726	A/G	Intron1	rs1957523	0.477
45		CTSGS4	1226	C/T	Intron2	New	0.034
46		CTSGS5	1349	G/A	Intron2	rs2070697	0.314
47		CTSGS6	1767	A/G	Exon4-synonymous	New	0.182
48	*CYP4F2*	CYP4F2S1	4937	A/T	Intron1	New	0.349
49		CYP4F2S4	18 733	T/C	Intron1	rs2072269	0.335
50		CYP4F2S5	18 810	G/C	Intron1	rs1064796	0.495
51		CYP4F2S6	30 715	G/A	Intron1	rs2018460	0.170
52	*CYP4F3*	CYP4F3S0	3187	G/A	Intron2	rs2203998	0.328
53		CYP4F3S0.5	6221	C/T	Intron4	rs1290626	0.141
54		CYP4F3S1	8507	C/T	Intron6	rs2283612	0.189
55		CYP4F3S2	11 871	A/C	Intron8	rs2733750	0.183
56		CYP4F3S3	12 015	A/G	Exon9-synonymous	New	0.363
57		CYP4F3S4	12 808	G/A	Intron9	rs2733752	0.136
58		CYP4F3S5	12 877	T/A	Intron9	New	0.140
59		CYP4F3S6	17 772	T/C	Intron11	New	0.453
60	*CYP4F8*	CYP4F8S1	3610	C/T	Intron2	rs2072599	0.168
61		CYP4F8S2	6771	G/T	Intron5	rs2072601	0.276
62		CYP4F8S3	13 568	C/T	Intron11	rs2239366	0.276

Continued

Table 1. *Continued*

No.	Gene	SNP	Sequence position	Variation	Localization	rs ID	Minor allele frequncies (96 CTR)
63	*CYSLT1R*	CYSLT1RS1	927	C/T	Exon1-synonymous	rs320995	0.474
64	*CYSLT2R*	CYSLT2RS1	2797	A/G	Exon1-UTR	rs912277	0.417
65		CYSLT2RS2	3078	A/C	Exon1-UTR	rs1323552	0.489
66		CYSLT2RS3	3105	A/G	Exon1-UTR	New	0.116
67	*FLAP*	FLAPS1	162	C/A	Intron1	rs4769055	0.522
68		FLAPS2	838	T/G	Intron1	rs9579645	0.096
69		FLAPS3	7272	A/G	Intron1	rs9551960	0.339
70		FLAPS4	8640	A/C	Intron2	rs3803277	0.406
71		FLAPS5	8733	T/C	Intron2	rs3803278	0.370
72		FLAPS6	13 674	G/A	Intron2	rs4075692	0.286
73		FLAPS7	20 616	G/C	Intron3	New	0.286
74		FLAPS8	20 648	C/T	Intron3	rs4468448	0.240
75		FLAPS9	23 849	T/A	Intron3	rs9551964	0.277
76		FLAPS9.5	24 348	G/A	Intron3	New	0.006
77		FLAPS10	28 209	A/G	Intron3	rs4769060	0.401
78	*HPGD*	HPGDS1	464	G/A	Exon2-synonymous	rs1050145	0.220
79		HPGDS2	798	A/G	Intron2	rs1365613	0.447
80		HPGDS3	13 332	A/C	Intron3	rs2555629	0.405
81		HPGDS4	13 430	A/T	Intron3	New	0.253
82		HPGDS5	20 442	A/G	Intron4	New	0.446
83		HPGDS6	20 516	G/A	Intron4	New	0.110
84	*IGF1*	IGF1S1	3083	A/G	Intron2	rs2162679	0.368
85		IGF1S2	9917	G/T	Intron3	rs1019731	0.021
86		IGF1S3	17 640	G/C	Intron3	rs2195239	0.426
87		IGF1S4	17 695	T/C	Intron3	rs2195240	0.443
88		IGF1S5	49 421	G/A	Intron3	rs972936	0.484
89		IGF1S6	60 710	G/A	Intron3	rs2072592	0.245
90		IGF1S7	72 103	G/A	Intron5	rs978458	0.135
91		IGF1S8	84 150	G/A	Exon6-UTR	rs6219	0.271
92	*IL13*	IL13S1	−978	C/T	Promoter	rs11575055	0.179
93		IL13S2	571	C/A	Intron1	rs2066960	0.281
94		IL13S3	598	G/C	Intron1	rs1295987	0.120
95		IL13S4	805	C/T	Intron1	rs2069744	0.120
96		IL13S5	2100	G/A	Exon4-non-synonymous (R144Q)	rs20541	0.333
97	*IL4*	IL4S1	−219	T/C	Promoter	rs2243250	0.328
98		IL4S2	3353	A/C	Intron2	rs2227284	0.255
99		IL4S3	3927	C/G	Intron2	rs2243263	0.073
100	*LTA4H*	LTA4HS1	9153	A/G	Intron3	rs763842	0.226
101		LTA4HS4	20 165	T/C	Intron11	rs1978331	0.335
102	*LTB4DH*	LTB4DHS1	4435	A/T	Intron2	New	0.506
103		LTB4DHS2	8896	A/G	Intron4	New	0.283
104		LTB4DHS2.5	9182	A/G	Intron4	New	0.092
105		LTB4DHS5	9502	C/T	Intron4	rs1053968	0.005
106		LTB4DHS6	13 234	C/G	Intron4	New	0.319
107		LTB4DHS6.5	13 249	A/G	Intron4	New	0.231
108		LTB4DHS7	20 718	G/A	Intron6	New	0.253
109		LTB4DHS8	25 971	A/G	Intron8	rs1322258	0.347
110		LTB4DHS9	26 221	A/G	Intron8	New	0.406
111		LTB4DHS10	35 589	A/C	Intron9	rs2146078	0.229
112		LTB4DHS11	35 819	G/A	Intron9	New	0.354
113	*LTB4R*	LTB4RS1	1165	G/C	Exon1-UTR	New	0.140
114	*LTC4S*	LTC4SS1.5	−348	A/C	Promoter	New	0.208
115		LTC4SS2	289	G/C	Intron1	New	0.026
116		LTC4SS3	1296	C/T	Intron1	New	0.208
117		LTC4SS4	2659	A/G	Intron5	New	0.201
118	*MGST2*	MGST2S1	815	A/G	Intron1	rs1000222	0.292
119		MGST2S2	12 971	C/T	Intron2	rs795589	0.198
120		MGST2S3	12 977	A/C	Intron2	rs795588	0.077
121	*MMP1*	MMP1S1	193	G/A	Intron1	rs470358	0.292
122		MMP1S2	2579	G/A	Exon5-synonymous	rs470558	0.130
123		MMP1S3	7300	T/C	Intron8	rs470747	0.137
124		MMP1S4	7815	C/T	Exon10-UTR	rs2239008	0.319
125		MMP1S5	7936	T/C	Exon10-UTR	rs2071230	0.298

Continued

Table 1. *Continued*

No.	Gene	SNP	Sequence position	Variation	Localization	rs ID	Minor allele frequncies (96 CTR)
126	*MMP2*	MMP2S1	3606	A/T	Intron1	rs857403	0.214
127		MMP2S2	3665	G/A	Intron1	rs1030868	0.229
128		MMP2S3	6505	C/T	Exon5-synonymous	rs1053605	0.208
129		MMP2S4	6730	T/G	Intron5	New	0.064
130		MMP2S5	6958	A/G	Intron5	rs866770	0.151
131		MMP2S6	10 603	C/T	Exon7-synonymous	rs243849	0.151
132		MMP2S7	10 896	T/C	Intron7	rs243847	0.443
133		MMP2S8	14 011	G/A	Exon9-synonymous	rs2287074	0.326
134		MMP2S9	14 196	G/A	Intron9	rs243843	0.306
135		MMP2S10	14 281	G/A	Intron9	New	0.065
136		MMP2S11	14 320	T/C	Intron9	rs243842	0.364
137		MMP2S12	17 660	G/T	Intron9	rs171498	0.363
138		MMP2S13	17 670	G/T	Intron9	rs243838	0.153
139		MMP2S14	17 928	C/T	Intron10	New	0.057
140		MMP2S15	20 976	G/A	Intron11	New	0.115
141		MMP2S16	21 134	G/A	Intron11	rs243836	0.386
142		MMP2S17	26 345	C/T	Exon13-UTR	New	0.021
143		MMP2S18	26 512	A/C	Exon13-UTR	rs7201	0.234
144	*MMP8*	MMP8S1	524	G/A	Intron1	rs1939012	0.359
145		MMP8S2	5458	A/G	Intron4	rs1940051	0.266
146		MMP8S3	11 357	T/A	Intron9	New	0.287
147		MMP8S4	11 473	T/C	Exon10-synonymous	New	0.016
148	*MMP9*	MMP9S1	2679	G/A	Exon6-non-synonymous (R279Q)	rs2664538	0.382
149		MMP9S2	3029	A/C	Intron6	rs2236416	0.179
150		MMP9S3	5565	G/A	Exon12-non-synonymous (R668Q)	rs2274756	0.214
151		MMP9S4	7419	G/A	Exon13-synonymous	rs13925	0.292
152	*P2Y10*	P2Y10S1	1192	A/G	Intron1	New	0.016
153		P2Y10S2	5030	C/T	Intron1	rs2858570	0.110
154		P2Y10S3	5318	A/T	Intron1	rs2251477	0.184
155		P2Y10S4	9417	T/C	Intron1	rs2742205	0.115
156	*PAFAH1B1*	PAFAH1B1S2	12 145	G/T	Intron1	New	0.099
157		PAFAH1B1S3	21 146	C/G	Intron1	New	0.080
158		PAFAH1B1S4	37 780	A/G	Intron1	rs1266474	0.084
159		PAFAH1B1S5	38 033	C/T	Intron1	New	0.047
160		PAFAH1B1S6	40 522	G/A	Intron1	New	0.130
161		PAFAH1B1S7	60 034	C/T	Intron2	New	0.266
162		PAFAH1B1S8	68 545	G/C	Intron2	New	0.078
163		PAFAH1B1S9	76 403	T/G	Intron5	New	0.032
164	*PAFAH1B2*	PAFAH1B2S1	3971	T/C	Intron1	rs2008908	0.446
165	*PAFAH1B3*	PAFAH1B3S1	2538	C/T	Exon4-synonymous	New	0.016
166	*PAFAH2*	PAFAH2S1	1279	G/T	Intron1	rs3008423	0.172
167		PAFAH2S2	4171	C/T	Intron1	New	0.036
168		PAFAH2S3	15456	G/T	Intron5	rs1469512	0.214
169	*PAI1m2*	PAI1m2S1	4484	G/A	Intron1	rs840088	0.401
170		PAI1m2S2	5094	A/G	Intron1	New	0.089
171		PAI1m2S3	19 022	C/A	Intron1	rs2099601	0.201
172		PAI1m2S4	19 047	A/C	Intron1	New	0.201
173		PAI1m2S5	19 120	T/C	Intron1	rs2083120	0.201
174		PAI1m2S6	19 348	A/C	Intron1	New	0.393
175		PAI1m2S7	62 178	A/G	Intron8	New	0.271
176	*PDGFB*	PDGFBS1	581	G/A	Intron1	rs758588	0.184
177		PDGFBS3	20 237	C/T	Intron3	rs740750	0.310
178		PDGFBS4	26 041	C/T	Intron10	rs1864972	0.198
179		PDGFBS5	35 170	G/A	Intron18	rs1432878	0.108
180	*PDGFRL*	PDGFRLS1	978	G/C	Intron1	rs2720576	0.365
181		PDGFRLS2	1187	C/T	Intron1	rs2588164	0.245
182		PDGFRLS3	1416	T/C	Intron1	rs2588163	0.391
183		PDGFRLS4	2963	G/C	Intron1	rs2517267	0.458
184		PDGFRLS5	3794	C/G	Intron1	rs2517268	0.394
185		PDGFRLS6	3882	G/A	Intron1	New	0.216
186		PDGFRLS7	3999	A/G	Intron1	rs2517269	0.220
187		PDGFRLS8	8893	G/A	Intron1	New	0.226

Continued

Table 1. *Continued*

No.	Gene	SNP	Sequence position	Variation	Localization	rs ID	Minor allele frequncies (96 CTR)
188		PDGFRLS9	9922	G/A	Intron1	New	0.232
189		PDGFRLS10	14 053	G/C	Intron2	rs2720583	0.167
190		PDGFRLS11	14 194	A/C	Intron2	New	0.396
191		PDGFRLS12	18 220	T/C	Intron2	rs2427709	0.140
192		PDGFRLS13	18 473	G/A	Intron2	rs2588144	0.068
193		PDGFRLS15	19 592	T/G	Intron2	rs2246488	0.339
194		PDGFRLS16	28 052	G/A	Intron2	rs2517187	0.104
195		PDGFRLS17	28 449	C/T	Intron2	rs2237823	0.104
196		PDGFRLS18	31 111	T/C	Intron2	rs2517198	0.094
197		PDGFRLS19	31 230	A/T	Intron2	New	0.260
198		PDGFRLS20	31 258	A/G	Intron2	New	0.031
199		PDGFRLS21	31 499	T/C	Intron2	rs2427715	0.094
200		PDGFRLS22	31 600	T/C	Intron2	New	0.167
201		PDGFRLS23	33 975	G/C	Intron2	rs2517208	0.037
202		PDGFRLS24	41 991	A/G	Intron2	rs2237831	0.189
203		PDGFRLS25	42 131	G/A	Intron2	rs2237831	0.205
204		PDGFRLS26	43 810	G/T	Intron2	New	0.073
205		PDGFRLS27	46 759	T/C	Intron3	New	0.120
206		PDGFRLS28	46 888	G/A	Intron3	rs2237835	0.226
207		PDGFRLS29	46 936	C/G	Intron3	New	0.074
208		PDGFRLS30	47 097	C/G	Intron3	New	0.197
209		PDGFRLS31	49 411	A/C	Intron3	rs2237836	0.548
210		PDGFRLS32	49 937	G/T	Intron3	rs2237837	0.103
211		PDGFRLS33	53 887	T/C	Intron4	rs2237842	0.188
212		PDGFRLS34	56 021	C/T	Intron4	rs2427719	0.063
213		PDGFRLS35	56 410	T/C	Intron4	rs2237845	0.240
214		PDGFRLS36	65 408	C/T	Intron5	New	0.083
215		PDGFRLS37	65 512	T/C	Exon6-synonymous	rs4705	0.521
216	*PGDS*	PGDSS1	5446	T/C	Intron1	rs2129595	0.191
217	*PGIS*	PGISS1	4650	T/C	Intron1	rs477627	0.063
218		PGISS2	6734	A/C	Intron1	rs927068	0.226
219		PGISS3	6840	T/C	Intron1	New	0.226
220		PGISS4	6870	G/C	Intron1	rs498646	0.276
221		PGISS4.5	6924	T/C	Intron1	rs476496	0.280
222		PGISS6	28 941	A/G	Intron5	rs501908	0.033
223		PGISS7	41 990	A/G	Intron5	New	0.093
224		PGISS8	44 026	G/A	Exon6-synonymous	rs5628	0.036
225		PGISS9	50 926	C/T	Intron6	New	0.104
226		PGISS10	55 002	C/A	Intron8	rs5629	0.281
227		PGISS11	57 532	A/G	Intron9	rs729824	0.226
228		PGISS12	62 730	T/C	Exon10-UTR	rs5602	0.438
229	*PLA2G7*	PLA2G7S1	2007	G/T	Intron1	New	0.214
230		PLA2G7S2	2338	G/A	Intron1	rs1421369	0.468
231		PLA2G7S3	6203	G/A	Intron1	New	0.141
232		PLA2G7S4	20 965	G/T	Intron5	rs1362931	0.042
233		PLA2G7S5	23 741	T/C	Exon7-non-synonymous (I198T)	rs1805018	0.281
234		PLA2G7S6	27 025	C/G	Intron9	rs2216465	0.542
235		PLA2G7S7	30 101	C/T	Exon11-non-synonymous (A379V)	rs1051931	0.042
236	*PTAFR*	PTAFRS3	36 031	C/A	Exon2-non-synonymous (A224D)	rs5938	0.068
237	*PTGDR*	PTGDRS1	408	G/A	Intron1	New	0.021
238		PTGDRS2	3290	A/G	Intron1	rs1254609	0.194
239		PTGDRS3	5754	T/C	Intron1	New	0.075
240		PTGDRS4	5793	A/G	Intron1	rs708486	0.081
241	*PTGDS*	PTGDSS1	4186	C/A	Exon7-UTR	rs6926	0.198
242	*EP1*	PTGER1S1	−267	G/C	Promoter	New	0.109
243	*EP2*	PTGER2uS1	−26 643	T/C	5′-Upstream	rs988209	0.232
244		PTGER2uS2	−18 461	T/G	5′-Upstream	rs1390375	0.077
245		PTGER2uS3	−18 360	G/A	5′-Upstream	rs1390374	0.022
246		PTGER2uS4	−15 332	A/T	5′-Upstream	rs708490	0.444
247		PTGER2uS5	−12 813	G/A	5′-Upstream	New	0.223
248		PTGER2uS6	−10 918	A/G	5′-Upstream	New	0.449

Continued

Table 1. *Continued*

No.	Gene	SNP	Sequence position	Variation	Localization	rs ID	Minor allele frequncies (96 CTR)
249		PTGER2uS7	−10 814	T/A	5′-upstream	New	0.427
250		PTGER2uS8	−10 250	A/G	5′-upstream	rs714366	0.433
251		PTGER2uS9	−7075	A/G	5′-upstream	New	0.452
252		PTGER2uS10	−6179	A/G	5′-upstream	New	0.479
253		PTGER2S1	−609	G/A	Promoter	rs1254601	0.288
254		PTGER2S2	300	G/A	Exon1-UTR	rs1254600	0.462
255		PTGER2S3	498	C/G	Exon1-UTR	rs2075797	0.441
256		PTGER2S4	948	A/G	Exon1-UTR	rs1353411	0.417
257		PTGER2S5	1042	G/A	Exon1-UTR	rs1254598	0.350
258		PTGER2S6	2803	G/A	Intron1	New	0.164
259		PTGER2S7	2988	C/T	Intron1	New	0.339
260		PTGER2S8	6063	C/T	Intron1	New	0.116
261		PTGER2S9	10 927	T/G	Intron1	rs1254585	0.446
262		PTGER2S10	14 081	C/T	Exon2-UTR	rs708502	0.398
263		PTGER2dS1	29 784	C/T	3′-Downstream	rs708511	0.219
264		PTGER2dS2	47 461	A/G	3′-Downstream	New	0.226
265		PTGER2dS3	57 931	C/T	3′-Downstream	rs708531	0.750
266		PTGER2dS4	58 051	C/T	3′-Downstream	rs708532	0.329
267	*EP3*	PTGER3S1	27 837	G/A	Intron1	rs1008484	0.058
268		PTGER3S2	28 078	G/A	Intron1	rs1569593	0.258
269		PTGER3S5	36 177	A/T	Intron2	rs5680	0.313
270		PTGER3S7	44 999	T/G	Intron2	rs1983588	0.268
271		PTGER3S8	45 040	A/C	Intron2	rs1983587	0.059
272		PTGER3S9	54 444	G/C	Intron2	rs1883461	0.054
273		PTGER3S10	54 634	T/A	Intron2	rs1883460	0.094
274		PTGER3S11	58 525	C/T	Intron2	New	0.271
275		PTGER3S12	65 699	G/C	Intron2	rs647921	0.048
276		PTGER3S13	65 973	A/T	Intron2	rs646621	0.443
277		PTGER3S14	70 342	G/A	Intron2	rs909842	0.442
278		PTGER3S15	70 357	A/C	Intron2	New	0.300
279		PTGER3S17	70 409	G/A	Intron2	New	0.442
280		PTGER3S20	70 755	A/T	Intron2	rs484675	0.441
281		PTGER3S24	86 923	A/G	Intron2	rs573688	0.082
282		PTGER3S25	95 146	A/C	Intron3	New	0.037
283		PTGER3S27	1 00 052	T/C	Intron3	rs1409984	0.255
284		PTGER3S29	1 14 078	G/A	Intron3	rs625617	0.371
285		PTGER3S30	1 21 798	C/A	Intron3	New	0.328
286		PTGER3S31	1 21 846	G/A	Intron3	rs602383	0.297
287		PTGER3S32	1 49 402	C/T	Intron3	rs1409165	0.370
288		PTGER3S33	1 49 626	G/A	Intron3	rs1409166	0.398
289		PTGER3S35	1 60 372	A/G	Intron3	New	0.214
290		PTGER3S36	1 60 403	A/G	Intron3	rs1409978	0.319
291		PTGER3S37	1 60 432	C/G	Intron3	New	0.214
292	*EP4*	PTGER4S1	5748	C/T	Intron2	New	0.389
293		PTGER4S2	7984	A/G	Intron2	New	0.198
294		PTGER4S3	8012	G/A	Intron2	New	0.385
295	*PTGES*	PTGESS1	214	A/G	Intron1	rs2241271	0.135
296		PTGESS2	406	G/A	Intron1	rs2241270	0.120
297		PTGESS3	9636	T/C	Intron2	New	0.326
298		PTGESS4	9669	G/A	Intron2	New	0.152
299	*PTGFR*	PTGFRS1	714	A/C	Intron1	rs3766355	0.425
300		PTGFRS2	823	G/A	Intron1	rs3766354	0.204
301		PTGFRS3	1132	G/T	Intron1	rs3766353	0.242
302		PTGFRS4	3617	G/A	Intron2	rs1830763	0.489
303		PTGFRS5	6578	A/C	Intron2	rs1322935	0.031
304		PTGFRS6	8734	A/G	Intron2	rs2057423	0.452
305		PTGFRS10	24 552	G/A	Intron2	rs3766346	0.036
306		PTGFRS12	33 123	A/C	Intron2	rs520171	0.276
307		PTGFRS13	33 548	G/C	Intron2	rs3766345	0.516
308		PTGFRS14	38 195	T/C	Intron2	rs3766338	0.266
309		PTGFRS15	38 202	C/T	Intron2	rs590309	0.511
310		PTGFRS16	40 734	G/C	Intron2	rs622346	0.202
311		PTGFRS19	46 572	A/G	Exon3-UTR	rs3766331	0.115
312		PTGFRS20	46 628	A/G	Exon3-UTR	rs899	0.005

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Table 1. *Continued*

No.	Gene	SNP	Sequence position	Variation	Localization	rs ID	Minor allele frequncies (96 CTR)
313	*PTGS1*	PTGS1S1	2850	C/T	Intron2	rs1213264	0.063
314		PTGS1S2	3219	T/C	Intron2	rs1213265	0.067
315		PTGS1S2.5	7468	C/G	Intron3	rs2282169	0.079
316		PTGS1S4	23 970	C/A	Exon11-UTR	rs10306194	0.028
317	*SCYA5*	SCYA5S1	328	T/C	Intron1	rs2280789	0.349
318	*SCYB14*	SCYB14S1	313	T/C	Intron1	rs2072347	0.311
319		SCYB14S2	2099	G/C	Intron2	rs2237062	0.307
320		SCYB14S3	6261	C/T	Intron3	rs1016666	0.370
321	*SLC21A9*	SLC21A9S1	196	T/C	Exon1-UTR	New	0.158
322		SLC21A9S2	231	G/A	Exon1-UTR	rs1944612	0.055
323		SLC21A9S3	4589	C/T	Intron1	New	0.443
324		SLC21A9S5	10 724	A/G	Intron1	New	0.374
325		SLC21A9S6	13 387	G/C	Intron3	New	0.453
326		SLC21A9S7	15 413	G/A	Intron4	rs949069	0.391
327		SLC21A9S8	15 916	A/G	Intron4	New	0.161
328		SLC21A9S9	17 493	G/A	Intron4	rs1676878	0.136
329		SLC21A9S10	24 698	A/G	Intron7	rs1676881	0.226
330		SLC21A9S10.5	24 880	C/T	Intron7	rs1612859	0.458
331		SLC21A9S11	25 005	T/A	Intron7	rs1789693	0.401
332		SLC21A9S13	26 261	T/G	Intron7	New	0.376
333		SLC21A9S14	39 441	G/A	Intron8	rs1789692	0.479
334		SLC21A9S15	43 575	C/T	Intron9	New	0.430
335		SLC21A9S16	45 422	C/T	Exon10	New	0.432
336	*STAT2*	STAT2S1	10 525	A/G	Intron14	rs2020854	0.021
337	*STAT4*	STAT4S1	5564	C/A	Intron3	rs1031509	0.396
338		STAT4S2	19 235	G/A	Intron3	rs1551443	0.214
339		STAT4S3	91 147	A/T	Intron10	New	0.516
340		STAT4S4	100 038	G/C	Intron14	rs1400655	0.095
341		STAT4S5	118 213	A/G	Intron21	rs925847	0.553
342		STAT4S6	119 708	A/C	Intron22	rs1517351	0.495
343		STAT4S7	119 711	G/A	Intron22	New	0.165
344	*TBX21*	TBX21S1	8941	T/C	Intron1	rs2158079	0.201
345	*TBXA2R*	TBXA2RS1	10937	T/C	Exon3-synonymous	rs4523	0.184
346	*TXAs*	TXAsS2	17778	A/C	Intron1	rs41708	0.302
347		TXAsS3	24573	T/C	Intron1	rs41706	0.355
348		TXAsS4	29218	A/T	Intron1	rs194150	0.443
349		TXAsS5	57546	T/G	Intron3	rs1015571	0.447
350		TXAsS7	63115	C/A	Intron3	rs2013219	0.426
351		TXAsS9	69322	G/A	Intron3	rs757762	0.447
352		TXAsS11	80249	C/T	Intron3	rs1978180	0.028
353		TXAsS13	1 06 385	T/C	Intron4	rs41733	0.117
354		TXAsS14	1 06 401	G/A	Intron4	rs41732	0.117
355		TXAsS15	1 12 521	T/C	Intron5	New	0.247
356		TXAsS16	1 12 694	C/T	Intron5	rs42335	0.134
357		TXAsS17	1 15 453	G/A	Intron5	New	0.011
358		TXAsS21	1 28 260	G/T	Intron7	New	0.165
359		TXAsS22	1 39 370	T/C	Intron9	rs41718	0.146
360		TXAsS23	1 46 442	T/C	Intron9	rs740150	0.389
361		TXAsS24	1 52 303	A/G	Intron9	rs193949	0.177
362		TXAsS25	1 52 455	C/T	Intron9	New	0.308
363		TXAsS29	1 78 072	G/A	Intron10	rs740204	0.234
364		TXAsS30	1 86 274	G/A	Intron10	New	0.479
365	*UPAR*	UPARS1	11 475	A/C	Intron3	New	0.019
366		UPARS2	11 639	G/A	Intron3	rs2283628	0.335
367		UPARS3	11 667	A/C	Intron3	rs2239373	0.385
368		UPARS4	11 746	C/T	Intron3	rs2239372	0.375
369		UPARS5	18 100	C/T	Intron5	rs2302525	0.074
370		UPARS6	18 228	A/G	Exon6-non-synonymous (K220R)	rs2302524	0.114

The SNPs applied to the first screening are listed. The sequence position indicates the location of the SNP relative to the transcription initiation site of exon1of each gene. The variation of allele and the localization in gene structure are shown. Each given SNP is referenced an accession number in dbSNP created by NCBI, rs ID (http://www.ncbi.nlm.nih.gov/SNP/). The New findings are mentioned as 'new'. The minor allele frequency of each SNP typed in the control sample of 96 individuals is shown.

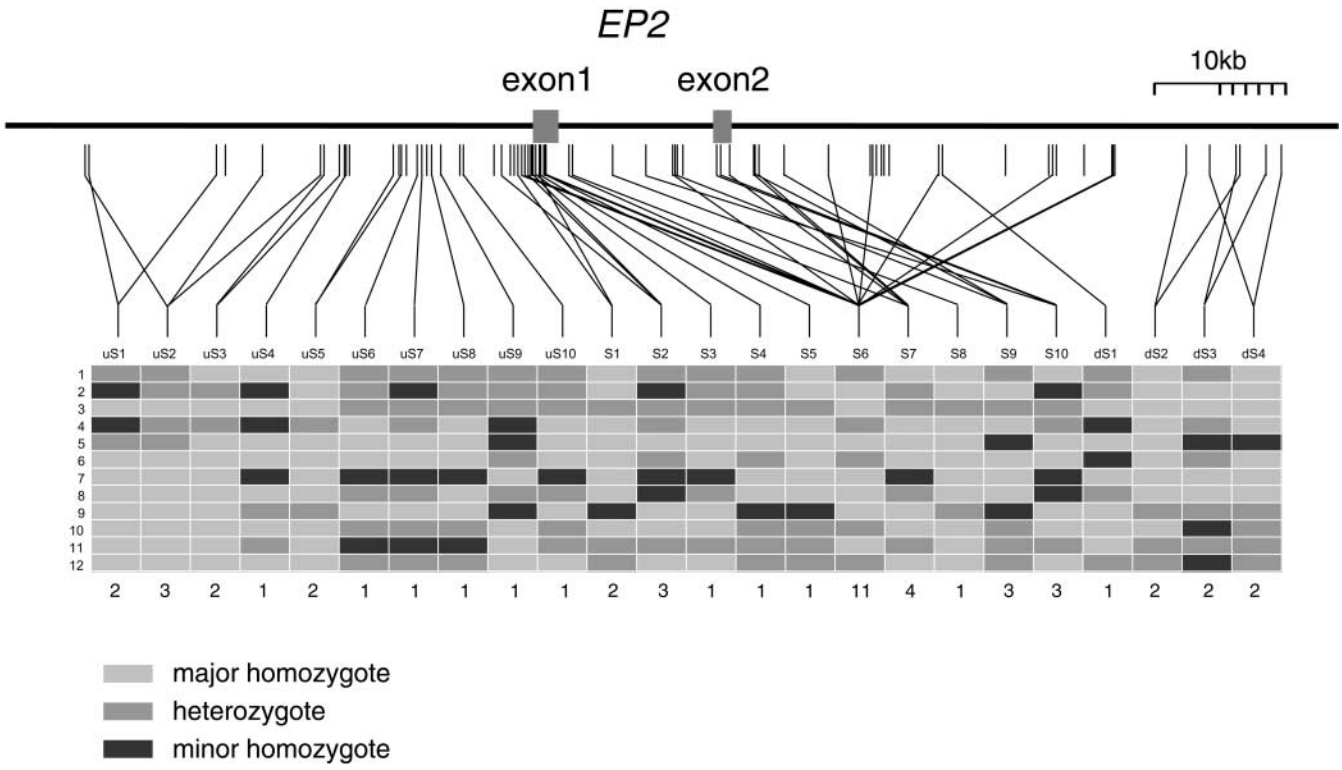

Figure 1. SNP location in *EP2* gene and subgrouping for screening. Locations of 77 SNPs in the genomic region from 35 kb upstream of exon 1 to 43 kb downstream of exon 2 are depicted. The 77 SNPs were categorized into 12 groups on the basis of the rule of perfect matching of genotypes with 12 individuals (SNP-group is connected by line). SNPs <5% of minor allele frequency were not subgrouped. In the block table, light gray box shows homozygote for major allele, mid-gray box heterozygote and dark gray box homozygote for minor allele. The numbers of SNPs belonging to each group are shown under the block tables.

Table 2. Allele frequencies of SNPs of *EP2* and comparisons between AIA and ATA and AIA and CTR

	Position	Variation	Localization	Allele count			Minor allele frequency			Permutation *P* value	
				AIA	CTR	ATA	AIA	CTR	ATA	AIA versus CTR	AIA versus ATA
uS5	−12 813	G/A	5′-Upstream	396	548	564	0.311	0.221	0.222	0.0016**	0.0017**
uS6	−10 918	A/G	5′-Upstream	392	528	526	0.395	0.449	0.451	0.0844	0.0912
uS7	−10 814	T/A	5′-Upstream	396	540	562	0.492	0.428	0.397	0.0393*	0.0025**
uS8	−10 250	A/G	5′-Upstream	384	522	516	0.393	0.433	0.438	0.1767	0.1716
uS9	−7075	A/G	5′-Upstream	384	504	522	0.432	0.452	0.469	0.5329	0.2594
uS10	−6179	A/G	5′-Upstream	374	526	564	0.374	0.401	0.452	0.4213	0.0199*
S1	−609	G/A	Promoter	396	538	564	0.348	0.288	0.303	0.0554	0.1175
S2	300	G/A	Exon1(UTR)	384	530	558	0.445	0.462	0.504	0.6037	0.0757
S3	498	C/G	Exon1(UTR)	392	534	558	0.401	0.427	0.468	0.4121	0.0393*
S4	948	A/G	Exon1(UTR)	396	532	564	0.477	0.417	0.415	0.0665	0.0480*
S5	1042	G/A	Exon1(UTR)	396	514	564	0.422	0.350	0.367	0.0276*	0.0844
S6	2803	G/A	Intron1	390	538	554	0.141	0.164	0.132	0.3396	0.6785
S7	2988	C/T	Intron1	388	528	554	0.327	0.339	0.377	0.7125	0.1169
S8	6063	C/T	Intron1	396	536	562	0.109	0.119	0.117	0.5876	0.6338
S9	10 927	T/G	Intron1	390	534	548	0.474	0.446	0.451	0.3808	0.4641
S10	14 081	C/T	Exon2(UTR)	378	516	554	0.373	0.399	0.419	0.4484	0.1632
dS1	29 784	C/T	3′-Downstream	372	522	542	0.194	0.218	0.173	0.3565	0.4316

*Significant difference at the 5% level, **At the 1% level.

similarities of a gene between species that is used to evaluate evolutionary processes (14). In the LD tree, $(1 - r^2)$ value was used to calculate a distance matrix for construction of the tree structure. Seventeen SNPs categorized into six subgroups ($r^2 - a$ to $-f$) were in LD estimated by r^2-statistic ($r^2 > 0.5$), as shown in Figure 2B. SNPs uS5, uS7, S1, S3, S6 and S8 represented six subgroups, and were combined to construct haplotypes; the haplotype-based associations were tested with 10 000 iterated permutations. Six major haplotypes (each frequency >5%) were observed in 274 CTR (Table 3).

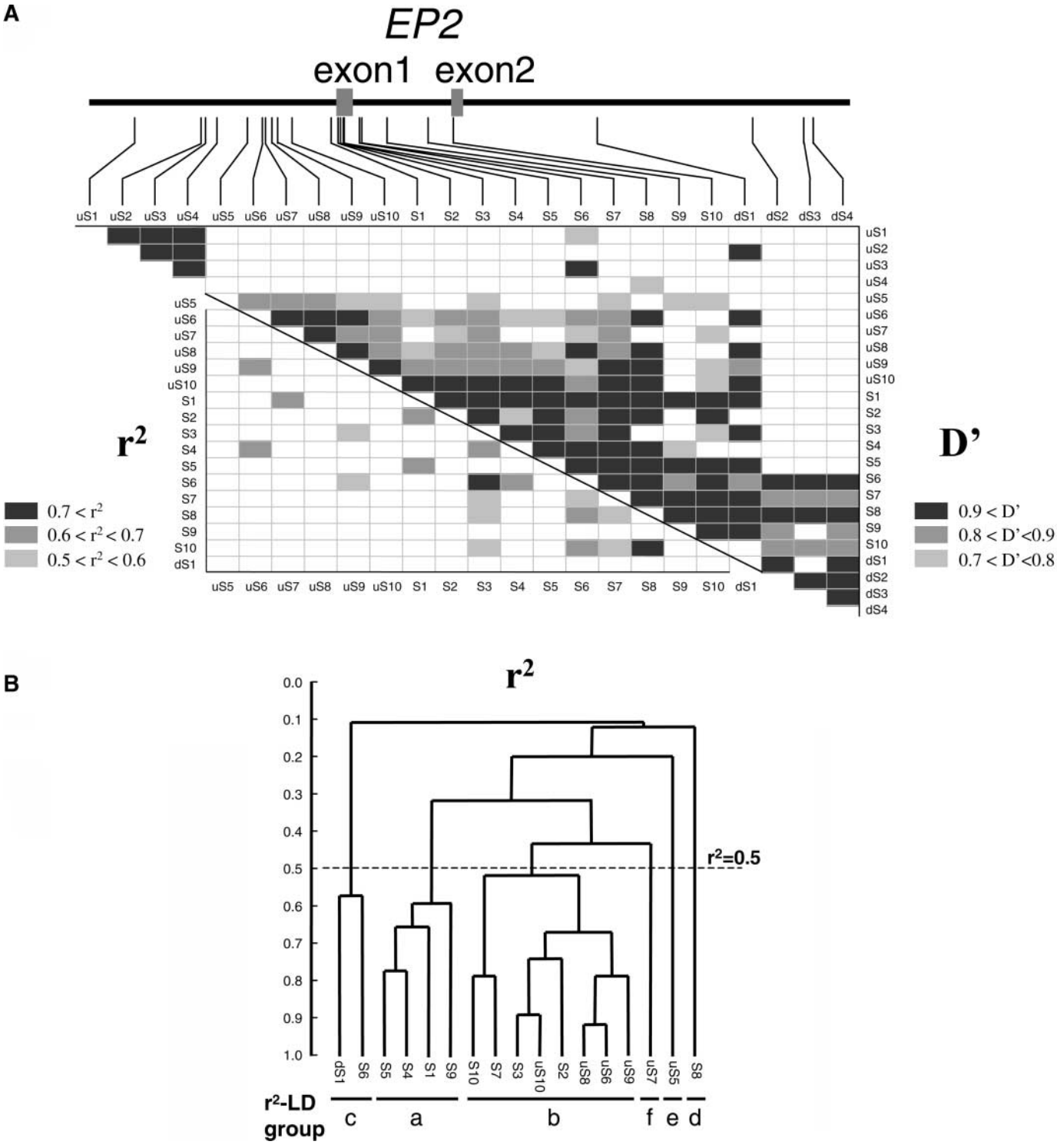

Figure 2. LD pattern of *EP2* gene. Pair-wise LD coefficients, D' and r^2, were determined and expressed as block structure (**A**) and UPGMA-based tree structure (**B**). (A) In the schematic block, shaded boxes of dark gray show pair-wise LD of $D' > 0.9$, medium gray $0.8 < D' < 0.9$ and light gray $0.7 < D' < 0.8$. Blank boxes represent $D' < 0.7$. For all the pairs of SNPs in the major D′-LD block (uS5–dS1), pair-wise LD coefficients r^2 are also presented. (B) The major LD block (uS5–dS1) was further analyzed with LD tree constructed according to UPGMA method as described in Materials and Methods. For subgrouping of SNPs based on LD structure, $r^2 = 0.5$ was utilized for cut-off.

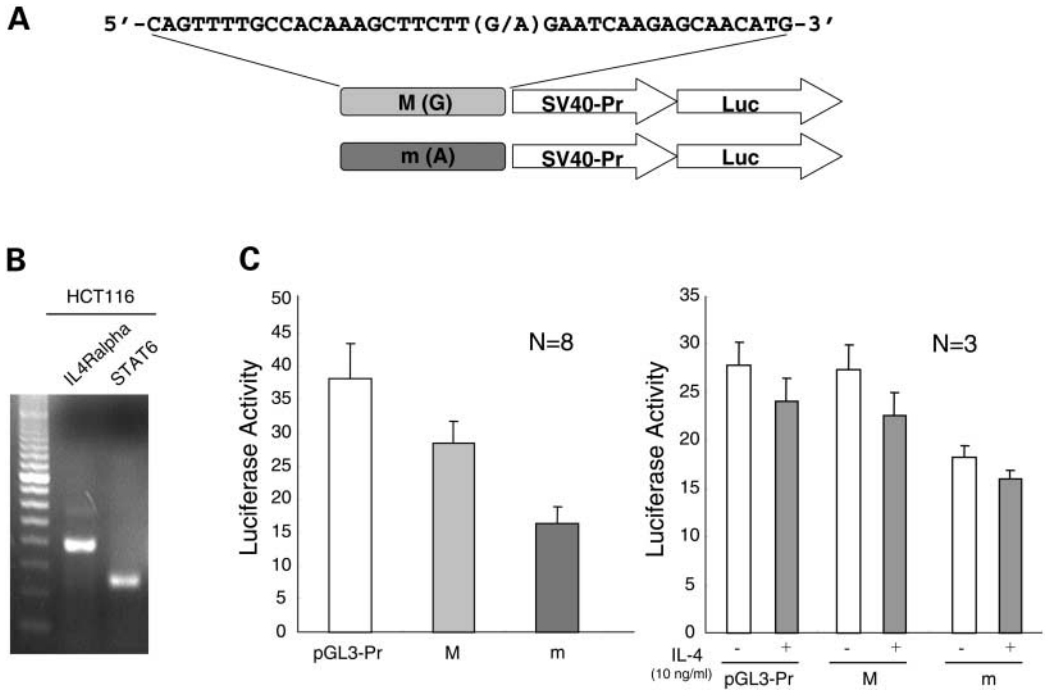

Figure 3. uS5 Allele-dependent transcription activity in HCT116 cells. (**A**) The 41 bp sequences centering on uS5 SNP were subcloned into the reporter vector as described in Materials and Methods. (**B**) HCT116 cells that endogenously expressed *IL4Rα* and *STAT6* genes were used for the experiments. (**C**) Each reporter vector was transfected into HCT116 cells, and the firefly luciferase activity was normalized with the *Renilla* luciferase activity of co-transfected pRL-TK (left). IL-4 stimulation (10 ng/ml) on the effect of transcription was monitored (right).

Table 3. Haplotype-based association study with AIA

Haplotype: uS5/uS7/S1/S3/S6/S8	Haplotype frequency				Permutation *P* value	
	Total	AIA	CTR	ATA	AIA versus CTR	AIA versus ATA
M/M/M/m/M/M	0.381	0.341	0.375	0.415	0.3074	0.0295*
m/m/m/M/M/M	0.160	0.212	0.130	0.155	0.0012**	0.0259*
M/m/M/M/m/M	0.104	0.101	0.120	0.092	0.3515	0.6218
M/m/M/M/M/M	0.064	0.062	0.072	0.057	0.5298	0.7388
M/M/M/M/M/M	0.059	0.056	0.060	0.058	0.8965	0.9032
M/M/m/M/M/m	0.055	0.034	0.061	0.066	0.0636	0.0488*

M and m denotes major and minor alleles, respectively.
*Significant difference at the 5% level, **At the 1% level.

One haplotype, m/m/m/M/M/M at uS5/uS7/S1/S3/S6/S8, showed highly significant difference between AIA and CTR ($\chi^2 = 11.03$, df = 1, $P = 0.0009$, permutation $P = 0.0012$) (Table 3). The most common haplotype, M/M/M/m/M/M, and the m/m/m/M/M/M haplotype showed significant differences in frequency between AIA and ATA, further supporting the involvement of *EP2* in susceptibility to AIA. The m/m/m/M/M/M haplotype was an at-risk haplotype for AIA, and uS5 a tag-SNP for the haplotype. Similar association evidence with uS5 was observed, indicating that uS5 is likely a causal SNP for AIA.

Transcriptional regulatory motif on uS5 site

The 5′-upstream region of the *EP2* gene was surveyed with a TFSEARCH program (15). The sequence surrounding uS5 was predicted to a STATs-binding motif with a possibility to bind subtypes 1, 2, 3, 4 and 6 of the human STAT proteins that play key roles in cytokine signaling (16). Variation of uS5 could affect STAT protein binding affinity, resulting in altered *EP2* transcription activity.

To examine the impact on transcriptional regulation due to the allelic difference of uS5, we cloned the two types of allelic sequence surrounding the uS5 site (41 bp) into upstream of an SV40 promoter-luciferase gene transcriptional unit (Fig. 3A). The effects on transcriptional activity of the 41 bp sequences centering either G allele or A allele of uS5 were investigated in HCT116 cells in which endogenous *IL4R alpha* and *STAT6* gene expressions were confirmed with RT–PCR method (Fig. 3B). Both inserted sequences showed suppressive effects on reporter gene transcription: G allele had 74.6% and A allele had 42.7%

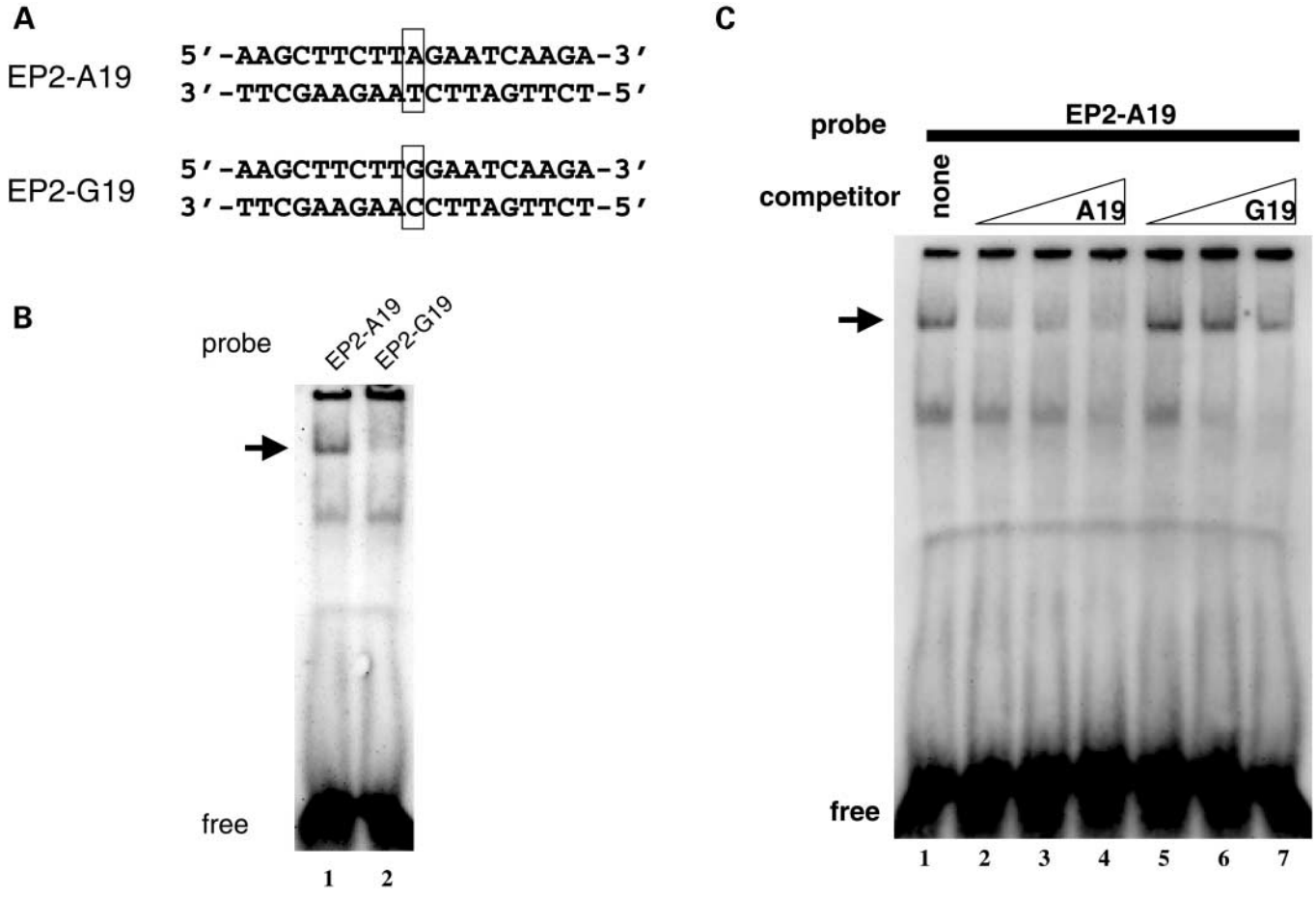

Figure 4. EMSA with ologonucleotide containing A allele or G allele of uS5. (**A**) Double-stranded oligonucleotide probes (*EP2*-A19 and *EP2*-G19) labeled with digoxigenin for EMSA are shown. (**B**) Nuclear extract from HeLa cells was incubated with *EP2*-A19 or *EP2*-G19 probes. Arrowhead points to specific binding. (**C**) Specific interaction with *EP2*-A19 competed with various amounts of non-labeled *EP2*-A19 or *EP2*-G19 competitor (6.25-, 12.5- and 25-fold from left to right).

activity of control (Fig. 3C, left). If STAT binding was responsible for repressive transcriptional activity, it should be altered by addition of IL-4. However, the reduced luciferase activities due to the inserted sequences were not affected by the addition of IL-4 (Fig. 3C, right). A allele, the susceptibility allele, showed 2.26-fold greater suppressive effect than G allele, suggesting that the uS5 variant affects the rate of transcription due to differences in nuclear factor interaction.

Accordingly, two digoxigenin-labeled double-stranded oligonucleotides (Fig. 4A) containing A allele (*EP2*-A19) and G allele (*EP2*-G19) were designed for electrophoretic mobility shift assay (EMSA) using HeLa nuclear extract. DNA–protein binding was observed with higher intensity in *EP2*-A19 than in *EP2*-G19 after incubation with the HeLa nuclear extracts (Fig. 4B), and the DNA–protein interaction was more efficiently competed by unlabeled *EP2*-A19 than by unlabeled *EP2*-G19 (Fig. 4C). The binding was not observed with purified STAT1 protein (data not shown), and *EP2*-A19 had a mismatch for STAT consensus motif, suggesting that a nuclear factor other than STAT proteins is involved in the interaction.

DISCUSSION

SNPs are being identified and assembled in large SNP databases at a rapid pace that should facilitate clarification of the genetic basis of complex diseases and drug responses. AIA is a distinct entity of asthma triggered by aspirin or NSAIDs, but little is known of its genetic basis. Identification of genetic susceptibilities to AIA might both clarify its molecular mechanism and reveal promising clinical and therapeutic targets.

Overproduction of cys-LTs in bronchial epithelium and inhibition of prostaglandin synthesis by COX inhibition apparently underlie the pathogenesis AIA (17–19). Cowburn *et al.* demonstrated that bronchial biopsies from AIA patients exhibited 4-fold increase in eosinophils compared with specimens from ATA patients, and baseline concentrations of cys-LTs measured in BALF of AIA patients correlate well with counts of inflammatory cells having positive immunoreactivity with LTC_4 synthase in the bronchial mucosa (6). A similar observation in nasal polyp was reported demonstrating that the number of cells expressing cys-LT_1 receptor and the level of cys-LTs in nasal polyp from AIA patients were

significantly higher than those from ATA patients, indicating that overexpression of cys-LT_1 receptor is involved in the pathogenesis of aspirin sensitivity (20,21). Considering these results together, we speculate that genetic predisposition directed to the regulation of LTC_4 synthase expression may play a key role in the pathogenesis of AIA. Sanak *et al.* reported that a SNP of LTC_4 synthase was associated with AIA, and that it functioned to reduce expression of the gene (9,10). The allelic association was not confirmed in Japanese AIA (12), and could not be replicated in the present study with much larger samples. In the current study, we applied an extensive candidate gene approach to identify susceptibility SNPs to AIA. Sixty-three candidate genes were selected on the basis of knowledge of the pharmacological action of aspirin and the plausible factors involving asthma (Table 1). SNPs in the *EP2* gene were screened through an extensive two rounds of association studies of the 370 selected SNPs. *EP2* is a receptor for PGE_2, the well-known inhibitory mediator of inflammation released from mast cells, eosinophils and macrophages that acts as a 'brake' on the inflammatory process and thus has a broncho-protective role in the airways, in part by inhibiting the release of chemoattaractants such as LTB_4 from alveolar macrophages and airway epithelium and the production of LTC_4 in eosinophils (22). PGE_2 likely modulates airway tone by inhibiting acetylcholine release of cholinergic nerve endings and mast cell histamine release. More importantly, pre-inhalation of exogenous PGE_2 has shown that PGE_2 directly suppresses aspirin-induced LT synthesis, most likely from eosinophils and mast cells infiltrating the bronchial mucosa (23). The ability of endogenous and exogenous PGE_2 to suppress LTC_4 synthesis also has been found *in vitro* in human eosinophils and other cells (24). Thus, PGE_2 may well act to reduce LT synthesis via *EP2* receptors on airway leukocytes. At least four subtypes of PGE_2 receptor (EPs 1-4), which differ in tissue distribution, ligand-binding affinity and coupling to intracellular signaling pathways, have been cloned to date (25). Although a specific role in blood pressure control was demonstrated in mice lacking *EP2* (26,27), involvement in the asthmatic phenotype of *EP2* was not known.

The allele frequency of uS5 of the *EP2* gene in AIA patients was quite different from the frequency both in controls and ATA patients [31.3% (AIA) versus 22.3% (CTR) and versus 21.7% (ATA)] (Table 2). The haplotype m/m/m/M/M/M, containing uS5, was most significantly associated with AIA (permutation $P = 0.0012$) (Table 3). uS5 locates in the 5′-upstream of the gene on the STAT-binding motif, the allele likely having impact on transcriptional activity. The 41 bp sequence centered by either G or A allele of uS5 was subcloned into upstream of the SV40 promoter reporter vector. *In vitro* reporter assay demonstrated stronger transcription repression with A allele of uS5, which is associated with AIA. Despite the site being consensus for STAT binding (G allele of uS5), STAT1 protein did not bind to the sequence; instead, an unknown nuclear factor, presumably a negative regulator, bound particularly to the A allele sequence. Considered together, these data suggest that the low level of *EP2* gene expression caused by the uS5 allele could lead to a low response to PGE_2, skewed LT activation and bronchoconstriction in response to numerous stimuli, thereby influencing individual susceptibility to AIA. Consistent with the finding that uS5 allele leads to quantitative differences, the homozygous carriers for A allele of uS5 showed the highest odds ratio (OR = 3.21, 95% C.I. = 1.53–6.75). A modest but persistent failure in the PGE_2 braking mechanism together with increased sensitivity to NSAIDs has been postulated to explain why AIA patients overproduce cyc-LTs even without ingestion of NSAIDs or after low doses of NSAIDs. Our hypothesis is consistent with the cyclooxygenase theory in suggesting that a persistent failure of the suppressive activity of PGE_2 in AIA patients allows over-activity of the LT and other pathways, both after NSAID exposure and chronically. Thus, the impaired suppressive activity of PGE_2 in at least some AIA patients may be related to reduced expression of *EP2* receptors due to the polymorphic allele uS5, the transcription of which is reduced by an unidentified repressor protein.

In conclusion, genetic screening of a candidate gene for AIA suggests that variants in the promoter of the *EP2* gene are significantly associated with AIA, and are functional by reducing transcriptional activity of the *EP2* gene. The functional analysis constitutes only *in vitro* evidence, so further investigation is required. In addition, this study is preliminary in that it included only Japanese individuals.

MATERIALS AND METHODS

Subjects

Diagnosis of AIA was made on the basis of self-reported history of more than one episode of moderate to severe asthmatic reaction after aspirin or other NSAID ingestion that had been identified by a physician. The oral provocation test was not performed in most patients because of the theoretical risk and the very low likelihood of significant occult disease. (28). ATA was defined as adult asthma diagnosed by expert physicians according to the American Thoracic Society criteria (29) and no history of aspirin or NSAID-induced asthmatic attack. The controls were outpatients with diseases other than asthma and who self-reported no history of aspirin sensitivity. The 198 unrelated individuals with AIA (age: 54.7 $\pm$ 13.2 years; 63 males/135 females),282 with ATA (age: 56.0 $\pm$ 16.1 years; 132 males/150 females) and 274 non-asthmatic controls (CTR) (age: 50.3 $\pm$ 24.6 years; 111 males/163 females) were recruited at Niigata University Hospital, University of Tokyo Hospital, Nagoya University Hospital, Doai Memorial Hospital and Kyushu University Hospital. ATA included 154 AT (age: 48.0 $\pm$ 15.6 years; 80 male/74 female) subjects and 128 NAT (age: 65.9 $\pm$ 10.0 years; 52 male/76 female) subjects who were genotyped. The patients and controls were all of Japanese ethnicity. Although the Japanese population is considered genetically homogenous, similar numbers of patients and controls from the various locations were used to avoid possible geographical differences in allelic frequencies. For the first screening, 87 randomly selected patients with AIA, 192 patients with ATA and 96 controls were genotyped. All the subjects gave written, informed consent and the study was performed with the approval of the Ethical Committee of Tokyo University. Blood samples of each subject were collected for isolation of genomic DNA.

Selection of SNPs for association study

SNPs were obtained from the two public databases; NCBI dbSNP (http://www.ncbi.nlm.nih.gov/SNP/) and IMS-JST JSNP DATABASE (http://snp.ims.u-tokyo.ac.jp/). Additional gene-based SNPs were identified by direct sequencing to cover each gene within a 3 kb SNP-interval. Ninety-six control subjects were genotyped for each SNP and SNPs with minor allele frequencies greater than 0.1 were subjected to further analysis. SNPs in the coding region that might affect gene function were given priority regardless of the allele frequencies. The 833 SNPs of 63 genes were validated and the 370 SNPs then were used for the subsequent study, according to the criteria. Direct sequencing was first performed on the *EP2* gene, and 77 SNPs identified were validated; 24 SNPs then were selected and used in the association study.

SNP genotyping

SNPs were genotyped using either the pyrosequencing method on a PSQ96 Instrument (Pyrosequencing AB, Uppsala, Sweden) or direct sequencing using BigDye Terminator cycle sequencing on an ABI PRISM 3700 DNA analyzer (Applied Biosystems, Tokyo, Japan). PCR was performed with a standard protocol except a biotin-labeled primer was used when the pyrosequencing method was applied.

Statistical analysis

Differences in allelic frequencies were evaluated by case–control design with chi-square test. Haplotype frequencies for multiple loci were estimated using the expectation-maximization method with SNPAlyze v3.0 software (DYNACOM, Mobara, Japan). In addition, the permutation test was performed to test deviation of allelic frequencies of SNPs and haplotypes of the *EP2* gene (30). Distribution of a test statistic was estimated by evaluating the statistics for a random sampling of 10 000 iterated permutations at fixing the total numbers of both the cases and controls, which is incorporated in SNPAlyze v3.0 software. *P*-value is estimated by the proportion of permutations for which the permutated data test statistic (P_{permuted}) is greater than the initially observed test statistic (P_{observed}), so permutation $P = P$ ($P_{\text{observed}} > P_{\text{permuted}}$).

Pair-wise LD was estimated as $D = x_{11} - p_1q_1$, where x_{11} is the frequency of haplotype A_1B_1, and p_1 and q_1 are the frequencies of alleles A_1 and B_1 at loci A and B, respectively. A standardized LD coefficient, r, is given by $D/(p_1p_2q_1q_2)^{1/2}$, where p_2 and q_2 are the frequencies of the other alleles at loci A and B, respectively (31). Lewontin's coefficient, D', is given by $D' = D/D_{\max}$, where $D_{\max} = \min(p_1q_2,\ p_2q_1)$ when $D < 0$ or $D_{\max} = \min(p_1q_1,\ p_2q_2)$ when $D > 0$ (32).

UPGMA-based LD tree

LD according to r^2-statistics was visualized by an 'LD tree' constructed on the basis of the UPGMA method of Neighbor program from the PHYLIP package v3.57c, available at web site (http://evolution.gs.washington.edu/phylip.html). $(1 - r^2)$ was calculated and converted to a distance matrix. Calculated coefficients were within 0–1, and the smaller values represent high LD against uncalculated coefficients.

Transcription regulatory motif

The computer program TFSEARCH based on TRANSFAC databases, available at web site (http://www.cbrc.jp/research/db/TFSEARCH.html), was used to predict potential binding sites of transcription factors in the regulatory region.

Transfection and reporter assays

The complementary oligonucleotide spanning the uS5 promoter allelic sequence, 5′-CCAGTTTTGCCACAAAGCTTCT(G/A)GAATCAAGAGCAACATGC-3′ or 5′-TCGAGCATGTTGCTCTTGATTC(C/T)AAGAAGCTTTGTGGCAAAACTGGGTAC-3′, was annealed and ligated into the *Kpn*I/*Xho*I-digested pGL3-promoter vector (Promega, Tokyo, Japan), and sequenced. HCT116 cells were cultured in McCoy's 5A medium supplemented with antibiotics and 10% fetal bovine serum. HCT116 cells were transfected with FuGENE6 (Roche Diagnostics, Tokyo, Japan) according to the manufacturer's instructions. Briefly, 200 ng of firefly luciferase reporter plasmid, and 0.6 ng of *Renilla* luciferase reporter plasmid (pRL-TK, Promega) per 24-well dish were used for each transfection. The cells were harvested 48 h after the transfection, and luciferase assay using the Dual-Luciferase Reporter Assay System was performed in accordance with the manufacturer's protocol (Promega). Experiments were performed at least twice in triplicate, and the relative activities of luciferase were expressed as mean ± S.E, after normalizing with the *Renilla* luciferase activities.

The total RNA was extracted using Trizol reagent (Invitrogen, Tokyo, Japan) and RT–PCR was performed with the SuperScript One-Step RT–PCR system (Invitrogen) based on the manufacturer's protocol.

Electrophoretic mobility shift assay

EMSA was performed with a DIG Gel Shift Kit (Roche Diagnostics) using digoxigenin (DIG)-labeled double-stranded 19 mer oligonucleotides specific to A allele (*EP2*-A19) and G allele (*EP2*-G19) of uS5. DIG-labeled probe was incubated with HeLa nuclear extracts (Promega) for 30 min at 4°C and separated by electrophoresis on a 5% non-denaturing polyacrylamide gel with 0.5× TBE running buffer. DNA–protein complexes were electroblotted onto nylon membrane and the band shift was visualized according to the user's manual for DIG Gel Shift Kit. For the competition assay, we incubated HeLa nuclear extracts with non-labeled competitors for 15 min at 4°C before incubation with labeled *EP2*-A19.

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REFERENCES

1. Szczeklik, A. and Stevenson, D.D. (1999) Aspirin-induced asthma: advances in pathogenesis, diagnosis and management. *J. Allergy Clin. Immunol.*, **104**, 5–13.
2. Picado, C. (2002) Aspirin-intolerant asthma: role of cyclo-oxygenase enzymes. *Allergy*, **S72**, 58–60.
3. Sampson, A., Holgate, S., Austen, K.F. and Szczeklik, A. (1998) Cyclo-oxygenase. *Thorax*, **53**, 719–720.
4. Szczeklik, A., Sanak, M., Nizankowska-Mogilnicka, E. and Kielbasa, B. (2004) Aspirin intolerance and the cyclooxygenase-leukotriene pathways. *Curr. Opin. Pulm. Med.*, **10**, 51–56.
5. Pierzchalska, M., Szabo, Z., Sanak, M., Soja, J. and Szczeklik, A. (2003) Deficient prostaglandin E2 production by bronchial fibroblasts of asthmatic patients, with special reference to aspirin-induced asthma. *J. Allergy Clin. Immunol.*, **111**, 1041–1048.
6. Cowburn, A.S., Sladek, K., Soja, J., Adamek, L., Nizankowska, E., Szczeklik, A., Lam, B.K., Penrose, J.F., Austen, F.K., Holgate, S.T. *et al.* (1998) Overexpression of leukotriene C_4 synthase in bronchial biopsies from patients with aspirin-intolerant asthma. *J. Clin. Invest.*, **101**, 834–846.
7. Lam, B.K. and Frank Austen, K. (2000) Leukotriene C_4 synthase. A pivotal enzyme in the biosynthesis of the cysteinyl leukotrienes. *Am. J. Respir. Crit. Care Med.*, **161**, S16–S19.
8. Szczeklik, A., Nizankowska, E. and Duplaga, M. (2000) Natural history of aspirin-induced asthma. AIANE Investigators. European Network on Aspirin-Induced Asthma. *Eur. Respir. J.*, **16**, 432–436.
9. Sanak, M., Simon, H.U. and Szczeklik, A. (1997) Leukotriene C_4 synthase promoter polymorphism and risk of aspirin-induced asthma. *Lancet*, **350**, 1599–1600.
10. Sanak, M., Pierzchalska, M., Bazan-Socha, S. and Szczeklik, A. (2000) Enhanced expression of the leukotriene C_4 synthase due to overactive transcription of an allelic variant associated with aspirin-intolerant asthma. *Am. J. Respir. Cell Mol. Biol.*, **23**, 290–296.
11. Van Sambeek, R., Stevenson, D.D., Baldasaro, M., Lam, B.K., Zhao, J., Yoshida, S., Yandora, C., Drazen, J.M. and Penrose, J.F. (2000) 5′ Flanking region polymorphism of the gene encoding leukotriene C_4 synthase does not correlate with the aspirin-intolerant asthma phenotype in the United States. *J. Allergy Clin. Immunol.*, **106**, 72–76.
12. Kawagishi, Y., Mita, H., Taniguchi, M., Maruyama, M., Oosaki, R., Higashi, N., Kashii, T., Kobayashi, M. and Akiyama, K. (2002) Leukotriene C_4 synthase promoter polymorphism in Japanese patients with aspirin-induced asthma. *J. Allergy Clin. Immunol.*, **109**, 936–942.
13. Choi, J.H., Park, H.S., Oh, H.B., Lee, J.H., Suh, Y.J., Park, C.S. and Shin, H.D. (2004) Leukotriene-related gene polymorphisms in ASA-intolerant asthma: an association with a haplotype of 5-lipoxygenase. *Hum. Genet.*, **114**, 337–344.
14. Nei, M. and Kumar, S. (2000) *Molecular Evolution and Phylogenetics.* Oxford University Press, New York.
15. Heinemeyer, T., Wingender, E., Reuter, I., Hermjakob, H., Kel, A.E., Kel, O.V., Ignatieva, E.V., Ananko, E.A., Podkolodnaya, O.A., Kolpakov, F.A. *et al.* (1998) Databases on transcriptional regulation: TRANSFAC, TRRD and COMPEL. *Nucl. Acids Res.*, **26**, 362–367.
16. Kotanides, H. and Reich, N.C. (1996) Interleukin-4-induced STAT6 recognizes and activates a target site in the promoter of the interleukin-4 receptor gene. *J. Biol. Chem.*, **271**, 25555–25561.
17. Sampson, A.P., Cowburn, A.S., Sladek, K., Adamek, L., Nizankowska, E., Szczeklik, A., Lam, B.K., Penrose, J.F., Austen, K.F. and Holgate, S.T. (1997) Profound overexpression of leukotriene C4 synthase in bronchial biopsies from aspirin-intolerant asthmatic patients. *Int. Arch. Allergy Immunol.*, **113**, 355–357.
18. Pierzchalska, M., Mastalerz, L., Sanak, M., Zazula, M. and Szczeklik, A. (2000) A moderate and unspecific release of cysteinyl leukotrienes by aspirin from peripheral blood leucocytes precludes its value for aspirin sensitivity testing in asthma. *Clin. Exp. Allergy*, **30**, 1785–1791.
19. Antczak, A., Montuschi, P., Kharitonov, S., Gorski, P. and Barnes, P.J. (2002) Increased exhaled cysteinyl-leukotrienes and 8-isoprostane in aspirin-induced asthma. *Am. J. Respir. Crit. Care Med.*, **166**, 301–306.
20. Sousa, A.R., Parikh, A., Scadding, G., Corrigan, C.J. and Lee, T.H. (2002) Leukotriene-receptor expression on nasal mucosal inflammatory cells in aspirin-sensitive rhinosinusitis. *N. Engl. J. Med.*, **347**, 1493–1499.
21. Steinke, J.W., Bradley, D., Arango, P., Crouse, C.D., Frierson, H., Kountakis, S.E., Kraft, M. and Borish, L. (2003) Cysteinyl leukotriene expression in chronic hyperplastic sinusitis-nasal polyposis: importance to eosinophilia and asthma. *J. Allergy Clin. Immunol.*, **111**, 342–349.
22. Celik, G., Bavbek, S., Misirligil, Z. and Melli, M. (2001) Release of cysteinyl leukotrienes with aspirin stimulation and the effect of prostaglandin E_2 on this release from peripheral blood leucocytes in aspirin-induced asthmatic patients. *Clin. Exp. Allergy*, **31**, 1615–1622.
23. Sestini, P., Armetti, L., Gambaro, G., Pieroni, M.G., Refini, R.M., Sala, A., Vaghi, A., Folco, G.C., Bianco, S. and Robuschi, M. (1996) Inhaled PGE2 prevents aspirin-induced bronchoconstriction and urinary LTE4 excretion in aspirin-sensitive asthma. *Am. J. Respir. Crit. Care Med.*, **153**, 572–575.
24. Tenor, H., Hatzelmann, A., Church, M.K., Schudt, C. and Shute, J.K. (1996) Effects of theophylline and rolipram on leukotriene C4 (LTC4) synthesis and chemotaxis of human eosinophils from normal and atopic subjects. *Br. J. Pharmacol.*, **118**, 1727–1735.
25. Coleman, R.A., Smith, W.L. and Narumiya, S. (1994) International Union of Pharmacology classification of prostanoid receptors: properties, distribution and structure of the receptors and their subtypes. *Pharmacol. Rev.*, **46**, 205–229.
26. Kennedy, C.R., Zhang, Y., Brandon, S., Guan, Y., Coffee, K., Funk, C.D., Magnuson, M.A., Oates, J.A., Breyer, M.D. and Breyer, R.M. (1999) Salt-sensitive hypertension and reduced fertility in mice lacking the prostaglandin *EP2* receptor. *Nat. Med.*, **5**, 217–220.
27. Tilley, S.L., Audoly, L.P., Hicks, E.H., Kim, H.S., Flannery, P.J., Coffman, T.M. and Koller, B.H. (1999) Reproductive failure and reduced blood pressure in mice lacking the *EP2* prostaglandin E2 receptor. *J. Clin. Invest.*, **103**, 1539–1545.
28. Jenkins, C., Costello, J. and Hodge, L. (2004) Systematic review of prevalence of aspirin induced asthma and its implications for clinical practice. *BMJ*, **328**, 434.
29. National Heart, Lung and Blood Institute (1991) Guidelines for the diagnosis and management of asthma. National Heart, Lung, Blood Institute. National Asthma Education Program. Expert panel report. *J. Allergy Clin. Immunol.*, **88**, 425–534.
30. Zhao, J.H., Curtis, D. and Sham, P.C. (2000) Model-free analysis and permutation tests for allelic associations. *Hum. Hered.*, **50**, 133–139.
31. Hill, W.G. and Robertson, A. (1968) Linkage disequilibrium in finite populations. *Theor. Appl. Genet.*, **38**, 226–231.
32. Lewontin, R.C. (1964) The interaction of selection and linkage. I. General considerations; Heterotic models. *Genetics*, **49**, 49–67.