and ES patients were compared at the transcriptome level [19]. $P<0.05$ was considered significant in every statistical analysis.

Quantitative real-time reverse transcription polymerase chain reaction analysis

Two transcripts, *INDO* and *IL1R2*, that were differentially expressed between AIA and CS nasal polyps were subjected to real-time reverse transcription polymerase chain reaction (RT-PCR) for verification of the microarray data, using a validation set of total RNAs from AIA ($n=10$) and CS ($n=4$) nasal polyps including nine AIA and two CS samples for the present microarray experiment. Total RNA from each nasal polyp was used as a template in first-strand cDNA synthesis with the SuperScript III First-Strand Synthesis System (Invitrogen). Real-time PCR was performed using TaqMan Gene Expression Assays (Applied Biosystems, Tokyo, Japan) with TaqMan Universal PCR Master Mix (Applied Biosystems) on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) according to the manufacturer's instructions. The relative quantification method [20] was used to measure the amounts of the respective genes in nasal polyps, normalized to *GAPDH* as an endogenous control. The statistical significance in gene expression between the AIA and the CS samples was determined by the Welch t-test; $P<0.05$ was considered significant.

Single nucleotide polymorphism genotyping

For gene-based association analysis, SNPs of AIA candidate genes were obtained from the NCBI dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/) using SNPbrowser Software (Applied Biosystems), to cover the entire regions of the genes positionally and genetically. SNPs were genotyped using the TaqMan SNP Genotyping assay (Applied Biosystems) with the allelic discrimination software SDS version 2.1 (Applied Biosystems) on the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) according to the manufacturer's instructions.

Statistical analysis of association study

Differences in allelic frequencies were evaluated by a case–control design with a χ^2 test. Haplotype frequencies for multiple loci were estimated using the expectation-maximization method with SNPAlyze v6.0 software (DYNACOM, Mobara, Japan). Bonferroni's correction was adopted for each gene and haplotype for multiple test correction.

Pairwise 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 A1 and B1 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 [21]. 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 $D<0$ [22].

The power of the present association analysis was calculated using 'Genetic Power Calculator [23] (http://pngu.mgh.harvard.edu/~purcell/gpc/)'. Using our sample sizes in the AIA–CTR comparison, the study has had 80% power to detect common alleles (risk allele frequency = 0.1) with a relative risk of 1.65, and 50% power to detect the alleles with a relative risk of 1.44 at a threshold of nominal P-value = 0.05 under an additive model in the log-odds scale.

Results and discussion

Microarray analysis of nasal polyp tissues of Aspirin-Intolerant Asthma patients

Bronchial biopsy specimens from AIA patients exhibit a fourfold increase in eosinophils compared with those from ATA patients [24]. The increased influx of eosinophils into the airway mucosa of AIA patients is likely a result of an inflammatory rather than an atopic mechanism. It is noteworthy that the nasal polyps of AIA patients show very similar pathological characteristics such as infiltration of eosinophils into the bronchial mucosa [12, 13]. These observations led us to postulate a common molecular mechanism in the development of a polyp and AIA. In such a case, genes related to nasal polyp development in AIA patients might suggest both potential susceptibility genes and pathways involved in aspirin hypersensitivity and the development of AIA. Because it is not practical to apply bronchial tissues for microarray analysis, we used nasal polyp tissues from AIA patients that were under resection for therapeutic purpose and monitored global gene expressions to demonstrate AIA-specific gene expression profiles. ES is known to be a related disorder of AIA; ES is typically characterized by a nasal polyp with inflammatory cell infiltration similar to that in an AIA polyp but without aspirin sensitivity, thus being an appropriate reference for the selection of AIA-specific genes.

The global gene expression profiles of AIA nasal polyps and those of ES nasal polyps were then compared. Similar expression profiles were expected in polyps of AIA and ES patients due to the similar histological and biochemical characteristics such as extensive infiltration of eosinophils. Figure 1a shows a hierarchical clustering (HC) dendrogram for the profiles of nasal polyps from nine AIA and five ES patients. Unexpectedly, two discrete clusters appeared, representing AIA and ES nasal polyps, respectively, with the exception of one (ES#5) of the ES tissues, from a patient who was aspirin tolerant and had clinical characteristics similar to those of other ES patients

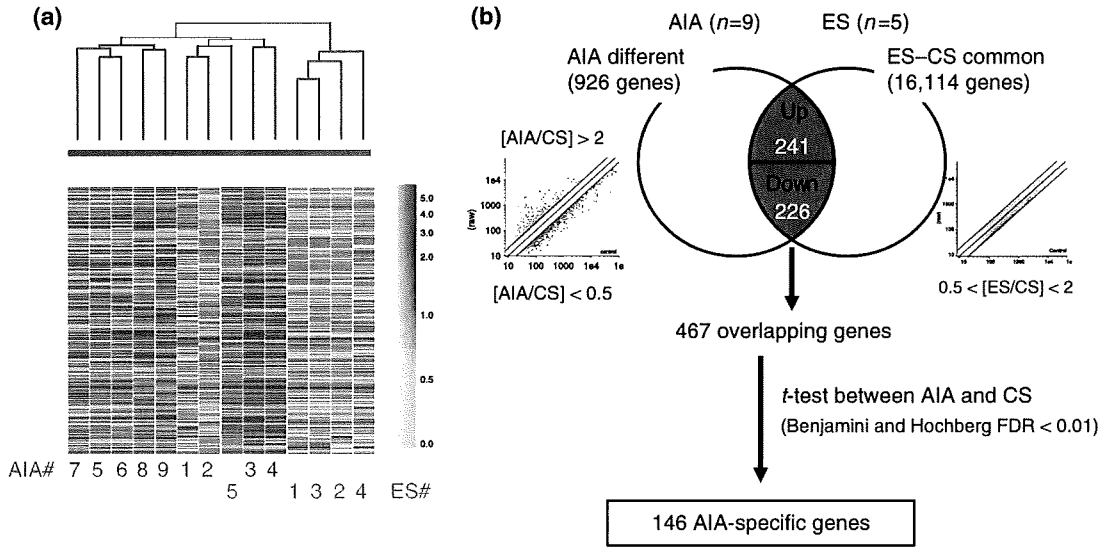

Fig. 1. Experimental design to extract aspirin-intolerant asthma (AIA)-specific genes with microarray analysis. (a) Hierarchical clustering (HC)-based classification of nasal polyps from AIA and eosinophilic sinusitis (ES) patients. Based on the gene expression in nasal polyps, HC clustering shows distinct expression profiles in AIA and ES patients. The clinical characteristics of the patients for the expression analysis are summarized in Table 1. (b) Strategy for discovering AIA-specific gene expression profiles, referred to as 'AIA-specific genes'. From 16,114 genes representing no change (less than twofold) in gene expression between ES and chronic sinusitis (CS) polyps, 146 AIA candidate genes were extracted at the threshold of twofold differences in expression with statistical significance (FDR < 0.01) between AIA and CS polyps.

(Table 1). Thus, AIA and ES nasal polyps appear to exhibit distinct expression profiles. The HC analysis was supported statistically in that 4012 of 18 716 transcripts surveyed by microarray displayed significant differences in expression between the AIA and the ES polyps using a permutation test, followed by Student's *t*-test at a significance level of 0.05. While the expression differences between the two groups could be due to an inter-group variation in cell composition within the nasal polyp tissues, they could not have been due to aspirin sensitivity, and so a two-step selection process was used to extract an AIA-specific expression profile (Fig. 1b). We first obtained genes (16 114 genes) common to polyp formation, i.e., genes showing no difference (less than twofold) between ES and chronic sinusitis (CS) polyps, in which a difference in the pathological state of the polyps such as infiltration of inflammatory cells would be minimized. We then selected 926 genes differentially expressed between AIA and CS polyps showing twofold differences, which could be related to the pathophysiology of aspirin sensitivity. From the overlapping genes (467 genes) between the two gene lists, 146 genes were statistically extracted including 143 elevated and three decreased transcripts that were defined as AIA-specific genes.

To examine the biological features of these AIA-specific genes, we assigned 146 genes to the GO classification using the web-accessible DAVID program. As shown in Table 2, nine GO terms were highly associated with the AIA-specific genes. It is notable that the genes involved in cell proliferation and immune response were enriched in the AIA candidate genes, indicating successful extraction of the genes related to nasal polyp formation, because both nasal cell growth and acute inflammation in the respiratory tract are clinical characteristics during the development of nasal polyp in AIA patients.

We then applied the *k*-means algorithm [17], an unsupervised partitional clustering approach, to organize AIA-specific genes into functionally meaningful groups. The *k*-means method has been efficient in showing a significant enrichment of genes belonging to given functional categories in the *k*-means-based clusters [25]. In this analysis, we selected an optimal number of clusters (*k*) in which the number of unclassified genes was minimized. As shown in Table 2, four distinct clusters, subsets 1, 2, 3, and 4, in gene expression were generated for the AIA-specific genes using the *k*-means method (figure not shown). Thus, four types of distinct expression patterns across samples were observed using the dataset of 146 AIA-specific genes. The three genes with decreased expression in AIA nasal polyps were categorized into subset 4, and the 143 elevated genes were classified into three subsets, 1, 2, and 3 (Table 2). Interestingly, genes involved in immune response (18/21 genes) and response to external signal (11/15 genes) were highly enriched in subset 2, while another enrichment of cell proliferation-related genes (17/24 genes) was observed in subset 1 (Table 2). These features of the gene enrichments indicate the biological significance of the present *k*-means-based clusters for AIA candidate genes. According to the cluster–GO correlation, the most notable functional patterning occurred for genes relevant to an immune response owing to the highest concentration (85.7% of the genes

Table 2. Enrichements of genes involved in GO-functional categories within *k*-means-based clusters

GO TERM (biological process; level 3)	Count	*P*-value	*k*-means clusters (no. of genes assigned) Subset 1 ($n = 57$)	Subset 2 ($n = 57$)	Subset 3 ($n = 29$)	Subset 4 ($n = 3$)
Cell proliferation	24	0.0000028	17	7		
Immune response	21	0.068		18	3	
Biopolymer metabolism	20	0.034	9	7	3	
Response to stress	17	0.00097	4	11	2	
Response to external stimulus	15	0.083		11	4	
Catabolism	14	0.017	5	5	4	
Cell organization and biogenesis	12	0.031	2	8		1
Cell motility	6	0.012	2	4		
Cellular defense response	4	0.024		3	1	

DAVID v2.1 (http://david.abcc.ncifcrf.gov/) was used to classify 146 AIA-specific genes functionally according to Gene ontology (GO) classification for biological process. Genes in the respective GO categories were mapped to four *k*-means-based clusters for gene expression.

extracted) in one subset (subset 2) of clusters, indicating that their expression might be highly coordinated in nasal polyps.

Association study with candidate genes for AIA

Based on functional clustering of the AIA-specific genes in the *k*-means clustering, immune response-related genes might serve as candidate genes for susceptibility underlying AIA because the AIA-specific changes in gene expression reflect elevated immune and inflammatory reactions in the nasal polyps of AIA patients. Table 3 shows 21 immune response-related genes in descending order based on the expression ratios in the microarray analysis. We focused on the three top-ranked genes, *INDO*, *IL1R2*, and *CLECSF6*, and screened 17 SNPs of these three genes (three SNPs for *INDO*, 11 SNPs for *IL1R2*, and three SNPs for *CLECSF6*) for an allelic association study between 219 AIA patients and 178 non-asthmatic controls (CTR) in the first screening. One SNP of *INDO* and four SNPs of *IL1R2* were significantly associated with AIA evaluated by a simple χ^2 test (data not shown) based on nominal *P*-values. Differential expressions of the two genes, *INDO* and *IL1R2*, in AIA nasal polyps were confirmed by real-time RT-PCR (Fig. 2). Because the three SNPs of *CLECSF6* examined were not associated with AIA, the gene was not pursued, and other ranked genes were also not screened further.

After increasing the sample size of CTR to 374 subjects, we further examined the allelic associations of a total of 14 SNPs (three SNPs for *INDO* and 11 SNPs for *IL1R2*) with AIA in a second screening. As shown in Tables 4 and 5, one SNP of *INDO* (INDO-SNP2: rs7820268) and one SNP of *IL1R2* (IL1R2-SNP10: rs11688145) showed significant associations with AIA after a multiple test correction using Bonferroni's correction (corrected $P = 0.011$ for the INDO-SNP2 and corrected $P = 0.026$ for the IL1R2-SNP10). None of the SNPs in the controls showed deviation from Hardy–Weinberg's equilibrium (data not shown). Both the significant SNPs observed were located in non-coding regions of the respective genes, and so the functional impacts of the SNPs were not demonstrated. In order to examine whether *INDO* and *IL1R2* were genetic

Table 3. Twenty-one genes involved in *immune response*

	Expression ratio (normalized)	Gene Symbol	Name
1	3.70	INDO	Indoleamine-pyrrole 2,3 dioxygenase
2	3.31	IL1R2	Interleukin 1 receptor, type II
3	2.75	CLECSF6	C-type lectin, superfamily member 6
4	2.68	CCL11	Chemokine (C-C motif) ligand 11
5	2.65	CD163	CD163 antigen
6	2.63	TNFSF10	Tumour necrosis factor (ligand) superfamily, member 10
7	2.53	AIF1	Allograft inflammatory factor 1
8	2.46	NCF2	Neutrophil cytosolic factor 2
9	2.32	ALOX5AP	Arachidonate 5-lipoxygenase-activating protein
10	2.31	FPR1	Formyl peptide receptor 1
11	2.21	TYROBP	TYRO protein tyrosine kinase-binding protein
12	2.21	CTSC	Cathepsin C
13	2.11	IFI30	Interferon, gamma-inducible protein 30
14	2.03	MICB	MHC class I polypeptide-related sequence B
15	1.90	LCP2	Lymphocyte cytosolic protein 2
16	1.86	NCK1	NCK adaptor protein 1
17	1.84	LST1	Leukocyte-specific transcript 1
18	1.83	TLR2	Toll-like receptor 2
19	1.76	PTAFR	Platelet-activating factor receptor
20	1.71	CKLF	Chemokine-like factor
21	1.65	EDG6	Endothelial differentiation, G-protein-coupled receptor 6

susceptibility genes underlying aspirin hypersensitivity, we further genotyped the two significant SNPs in 282 ATA patients for comparison. In the AIA–ATA association study, the INDO-SNP2 also showed a statistically significant association with AIA ($P = 0.038$) (Table 4), whereas an association of the IL1R2-SNP10 with AIA was marginal ($P = 0.073$) with the same direction of genetic effect of the associated allele on AIA susceptibility (Table 5). In contrast, no significant differences in allele frequencies at the two SNPs were observed between CTR and ATA groups (statistical data not shown). These SNP-based association results indicate that the two SNPs in *INDO* and *IL1R2* are associated with the risk of aspirin hypersensitivity rather than an asthmatic reaction in Japanese population.

Figure 3 shows D'- and r^2-based LD block structures in the genomic regions around *INDO* and *IL1R2*, respectively. We observed a strong LD ($|D'| = 0.98$) between INDO-SNP1 and -SNP2 in the *INDO* region. A highly structured LD pattern, a major LD block structure ($|D'| > 0.7$) covered by IL1R2-SNP6 to -SNP11, was observed in *IL1R2* (Fig. 3). Next, we conducted a haplotype-based association study within the respective LD blocks (Tables 6 and 7). We found that one haplotype of *INDO*, m/m (double minor haplotype) at INDO-SNP1 and -SNP2, was underrepresented in AIA with statistical significance after multiple test correction with Bonferroni's correction (Table 6; $\chi^2 = 6.74$, df = 1, corrected $P = 0.038$), indicating a protective effect of the m/m haplotype of *INDO*. One haplotype of *IL1R2*, M/M/M (triple major haplotype) at IL1R2-SNP6, -SNP10, and -SNP11, showed a highly significant difference between AIA and CTR (Table 7; $\chi^2 = 8.94$, df = 1, corrected $P = 0.011$), indicating that the M/M/M haplotype represented a risk for AIA.

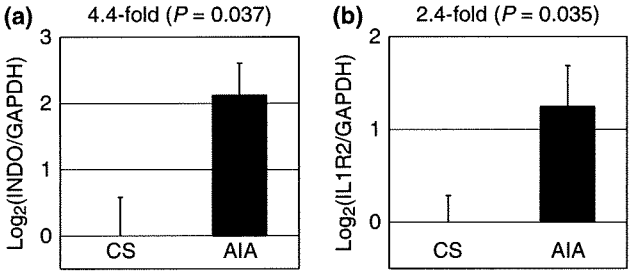

Fig. 2. Real-time reverse transcription polymerase chain reaction (RT-PCR) analysis validates expression differences for two genes, *INDO* and *IL1R2*, in nasal polyps from aspirin-intolerant asthma (AIA) and chronic sinusitis (CS) patients. Relative amounts of the respective genes in nasal polyps (AIA, $n = 10$; CS, $n = 4$) were measured by real-time RT-RCR using TaqMan Gene Expression Assays. *Y*-axes indicate the $\log_2$ relative expression levels, normalized to the amount of *GAPDH* and relative to the averaged expression levels in CS groups. The expression levels of *INDO* and *IL1R2* were significantly higher in AIA than CS nasal polyps by the Welch *t*-test.

INDO encodes indoleamine 2, 3-dioxygenase, which is a rate-limiting enzyme of tryptophan catabolism and is expressed in various cell types such as fibroblasts, macrophages, and dendritic cells [26]. INDO activity is induced by interferons (IFNs) and further enhanced by inflammatory cytokines such as IL-1 [27] but suppressed by anti-inflammatory cytokines such as TGF-β and IL-4 [28, 29]. INDO-induced tryptophan degradation in macrophages results in inhibition of T cell proliferation [30], suggesting that INDO plays an important role in the regulation of T cell-mediated immune responses. Aspirin inhibits INDO activity in stimulated peripheral blood mononuclear cells indirectly, via its inhibitory effect on the production of IFN-γ [31]. Therefore, the functional disturbance of INDO activity due to the INDO-SNP2 might play a role in the pathogenesis of aspirin sensitivity or AIA. A possibility remains that an unknown SNP in tight LD with the INDO-SNP2 or on the m/m haplotype could serve as a *bona fide* causality, which could prevent AIA induction by keeping INDO activity normal. Further studies are needed to resolve the functional significance of the INDO-SNP2 and the m/m haplotype in the genetic aetiology of AIA.

Interleukin 1 receptor type II (IL1R2) acts as a soluble decoy receptor that inhibits IL-1 signalling [32]. The inhibition of IL-1 binding to the receptor in human monocytes results in a reduction of COX-2 activity but not COX-1 activity [33]. As an imbalance in arachidonate

Table 4. Allelic association of *INDO* SNPs with AIA in Japanese population

					MAF		AIA vs. CTR				MAF	AIA vs. ATA	
SNP no.	Position*	Localization	dbSNP ID	Alleles (M/m)†	AIA $n = 219$	CTR $n = 374$	χ^2	Odds ratio (95% CI)	P	Corrected P‡	ATA $n = 282$	χ^2	P
INDO-SNP1	−1953	5′-upstream	rs3808606	T/C	0.414	0.472	3.65	0.79 (0.62–1.01)	0.056	0.17	ND		
INDO-SNP2	6202	intron4	rs7820268	C/T	0.101	0.163	8.47	0.58 (0.40–0.84)	0.0036	0.011	0.145	4.29	0.038
INDO-SNP3	13994	intron9	rs3739319	A/G	0.474	0.436	1.63	1.17 (0.92–1.49)	0.20	0.60	ND		

*Numbers indicate the nucleotide position from the first nucleotide of exon 1.

†M and m denote major and minor alleles, respectively, at each SNP site.

‡Corrected *P* values were obtained using Bonferroni's correction.

MAF, minor allele frequency; AIA, aspirin intolerant asthma; CTR, non-asthmatic control; ATA, aspirin tolerant asthma; ND, not determined; SNP, single nucleotide polymorphism.

Table 5. Allelic association of *IL1R2* SNPs with AIA in Japanese population

SNP No.	Position*	Localization	dbSNP ID	Alleles (M/m)†	MAF AIA $n=219$	MAF CTR $n=374$	AIA vs. CTR χ^2	Odds ratio	P	MAF Corrected P‡	AIA vs. ATA ATA $n=282$	χ^2	P
IL1R2-SNP1	−6913	5′-upstream	rs4851519	C/T	0.272	0.219	4.27	1.34 (1.01-1.76)	0.039	0.43	ND		
IL1R2-SNP2	−4381	5′-upstream	rs35789178	T/G	0.368	0.358	0.12	1.05 (0.81-1.34)	0.73	1	ND		
IL1R2-SNP3	−3657	5′-upstream	rs12467316	A/C	0.299	0.376	7.21	0.71 (0.55-0.91)	0.0072	0.080	ND		
IL1R2-SNP4	−3145	5′-upstream	rs12468239	C/T	0.090	0.093	0.02	0.97 (0.64-1.47)	0.89	1	ND		
IL1R2-SNP5	9147	intron1	rs11691240	C/T	0.439	0.479	1.73	0.85 (0.67-1.08)	0.19	1	ND		
IL1R2-SNP6	14513	intron1	rs3755482	A/G	0.273	0.340	5.58	0.73 (0.56-0.95)	0.018	0.20	ND		
IL1R2-SNP7	15413	intron1	rs719250	G/A	0.381	0.318	4.73	1.32 (1.02-1.69)	0.030	0.33	ND		
IL1R2-SNP8	21335	intron3	rs2110562	C/T	0.179	0.193	0.32	0.91 (0.67-1.25)	0.57	1	ND		
IL1R2-SNP9	40304	3′-downstream	rs4851531	T/C	0.462	0.408	3.16	1.25 (0.98-1.59)	0.075	0.83	ND		
IL1R2-SNP10	42202	3′-downstream	rs11688145	C/A	0.244	0.330	9.23	0.66 (0.50-0.86)	0.0024	0.026	0.296	3.22	0.073
IL1R2-SNP11	54346	3′-downstream	rs7588933	A/G	0.186	0.206	0.70	0.88 (0.65-1.19)	0.40	1	ND		

*Numbers indicate the nucleotide position from the first nucleotide of exon 1.
†M and m denote major and minor alleles, respectively, at each SNP site.
‡Corrected *P* values were obtained using Bonferroni's correction.
MAF, minor allele frequency; AIA, aspirin intolerant asthma; CTR, non-asthmatic control; ATA, aspirin tolerant asthma; ND, not determined

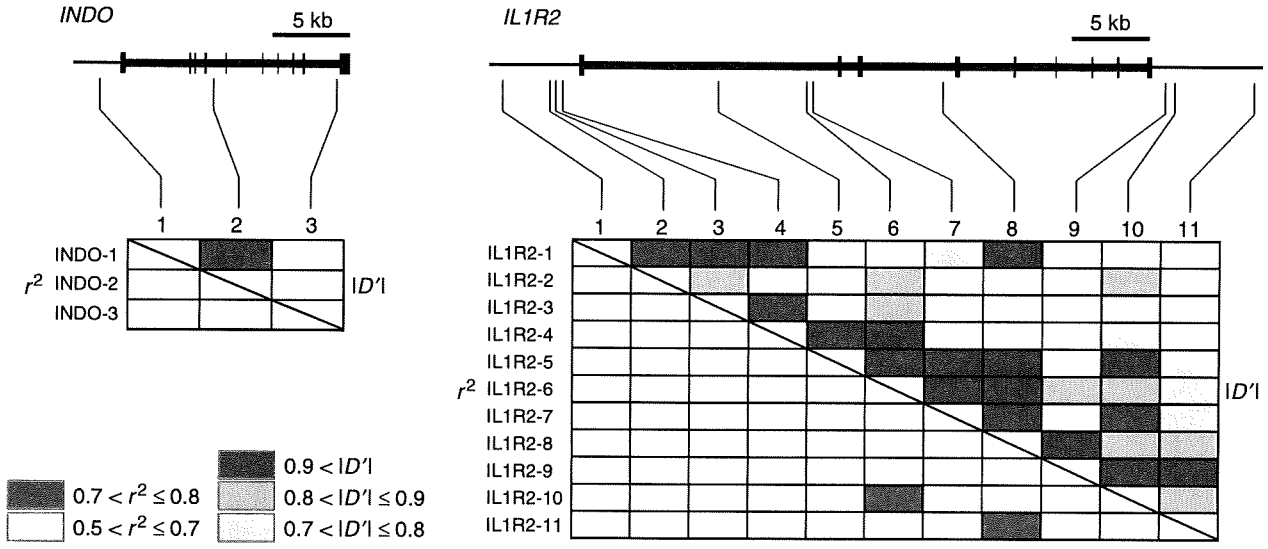

Fig. 3. Linkage disequilibrium pattern of *INDO* and *IL1R2*. The gene structures of *INDO* (left) and *IL1R2* (right), together with positions of the 14 single nucleotide polymorphisms (SNPs) examined, are shown. Pairwise LD coefficients, D' and r^2, were determined and expressed as a block structure. In the schematic block, red boxes indicate a pairwise LD of $|D'|>0.9$, pink $0.9\geqslant|D'|>0.8$, and orange $0.8\geqslant|D'|>0.7$; blue boxes indicate a pairwise LD of $0.8\geqslant r^2>0.7$ and light blue $0.7\geqslant r^2>0.5$. Blank boxes represent $|D'|\leqslant 0.7$ or $r^2\leqslant 0.5$.

metabolism is the usual pathogenesis proposed for AIA, the elevated level of IL1R2 that inhibits the inflammatory effects of IL-1α in the respiratory tract might well be involved in the pathogenesis of AIA and the formation of nasal polyps. Although IL1R2-SNP10 showed the strongest allelic association with AIA (Table 5), the functional impact of an SNP locating 3′-downstream of the gene is currently unclear despite its up-regulated expression in the AIA polyp (Table 3). There is also the possibility that an unidentified functional SNP in LD with the IL1R2-SNP10 could be a causality. Further genetic fine mapping in *IL1R2* will be required to fully understand which genetic variant contributes to the risk of AIA. The functional impacts of the SNP and haplotype also require further investigation.

In conclusion, DNA microarray technology was used to monitor global gene expression patterns specific to AIA nasal polyp tissues to clarify the pathophysiology of AIA. From the gene expression profile, candidate genes underlying AIA were selected and subjected to an association study. We identified SNPs in *INDO* and *IL1R2* that may represent genetic susceptibility to AIA. This genetic study

Table 6. Haplotype-based association of *INDO* with AIA

	Haplotype frequency					
Haplotype (INDO-SNP1/2)*	Total	AIA	CTR	χ^2	*P*	Corrected *P*†
M/M (T/C)	0.552	0.591	0.531	3.75	0.053	0.21
m/M (C/C)	0.306	0.304	0.307	0.01	0.94	1
m/m (C/T)	0.141	0.105	0.161	6.74	0.0094	0.038
			Global comparison	8.05 (df = 3)	0.045	0.18

*M and m denote major and minor alleles, respectively, at each SNP site. The corresponding nucleotides at the respective sites are shown in parentheses.
†Corrected *P* values were obtained using Bonferroni's correction.
AIA, aspirin intolerant asthma; CTR, non-asthmatic control; SNP, single nucleotide polymorphism.

Table 7. Haplotype-based association of *IL1R2* with AIA

	Haplotype frequency					
Haplotype (IL1R2-SNP6/SNP10/SNP11)*	Total	AIA	CTR	χ^2	*P*	Corrected *P*†
M/M/M (A/C/A)	0.494	0.552	0.458	8.94	0.0028	0.011
m/m/M (G/A/A)	0.277	0.243	0.299	4.03	0.045	0.18
M/M/m (A/C/G)	0.182	0.170	0.189	0.59	0.44	1
			Global comparison	8.72 (df = 4)	0.069	0.28

*M and m denote major and minor alleles, respectively, at each SNP site. The corresponding nucleotides at the respective sites are shown in parentheses.
†Corrected *P* values were obtained using Bonferroni's correction.
AIA, aspirin intolerant asthma; CTR, non-asthmatic control; SNP, single nucleotide polymorphism.

represents only first-stage evidence of the association because only Japanese individuals were included, and so further replication in independent case–control samples is required to confirm the role of *INDO* and *IL1R2* genotypes in the genetic risk for AIA. A pathophysiological link between the two gene products is unclear and further investigation is evidently needed. In addition, further studies including functional analyses of the SNPs with respect to how genetic variants are responsible for the risk of AIA are also required for a full understanding of the pathogenesis of AIA.

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