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Gene-expression profiles in human nasal polyp tissues and identification of genetic susceptibility in aspirin-intolerant asthma

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Summary

Background Aspirin-intolerant asthma (AIA) is a subtype of asthma induced by non-steroidal anti-inflammatory drugs and characterized by an aggressive mucosal inflammation of the lower airway (asthma) and the upper airways (rhinitis and nasal polyp). The lower airway lesion and the nasal polyp in AIA are postulated to have common pathogenic features involving aspirin sensitivity that would be reflected in the gene expression profile of AIA polyps.

Objective This study was conducted to clarify the pathogenesis of AIA using gene expression analysis in nasal polyps, and identify genetic susceptibilities underlying AIA in a case–control association study.

Methods Global gene expression of nasal polyps from nine AIA patients was examined using microarray technology in comparison with nasal polyps from five eosinophilic sinusitis (ES) patients, a related disease lacking aspirin sensitivity. Based on the AIA-specific gene expression profile of nasal polyp, candidate genes for AIA susceptibility were selected and screened by a case–control design of 219 AIA patients, 374 non-asthmatic control (CTR), and 282 aspirin-tolerant asthmatic (ATA) subjects.

Results One hundred and forty-three elevated and three decreased genes were identified as AIA-specific genes that were enriched in immune response according to Gene Ontology analysis. In addition, a *k*-means-based algorithm was applied to cluster the genes, and a subclass characteristic of AIA comprising 18 genes that were also enriched in immune response was identified. By examining the allelic associations of single nucleotide polymorphisms (SNPs) of AIA candidate genes relevant to an immune response with AIA, two SNPs, one each of *INDO* and *IL1R2*, showed significant associations with AIA ($P = 0.011$ and 0.026 after Bonferroni's correction, respectively, in AIA vs. CTR). In AIA–ATA association analysis, modest associations of the two SNPs with AIA were observed.

Conclusion These results indicate that *INDO* and *IL1R2*, which were identified from gene expression analyses of nasal polyps in AIA, represent susceptibility genes for AIA.

Keywords aspirin-intolerant asthma, candidate genes, genetic association, genome-wide gene expression, single nucleotide polymorphism
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Introduction

In some asthmatic patients, aspirin and several other non-steroidal anti-inflammatory drugs (NSAIDs) that inhibit cyclooxygenase enzymes (COXs) induce a severe asthmatic attack, a disease known as aspirin-intolerant asthma (AIA) [1, 2]. Several large surveys have concluded that the incidence of AIA in adult asthmatic patients is 5–15% based on patients' histories alone, but the frequency becomes two to three times higher when adult asthmatic

patients are challenged with aspirin. In women, AIA is overrepresented in a ratio of 2.3 : 1 and is more severe and has an earlier onset. AIA patients have typical clinical features including asthma, aspirin sensitivity, and bilateral nasal polyps, known as Samter's triad. Despite the well-defined pharmacological trigger, the molecular pathogenesis of AIA is still unclear. The usual hypothesis is a disturbance in the metabolism of arachidonic acid, because aspirin and NSAIDs target COXs, key enzymes of the prostaglandin biosynthetic pathway. However, the precise pathogenesis requires further investigation.

There is a moderate genetic background in AIA: the European Network on Aspirin-Induced Asthma found that 5.8% of 500 AIA patients had a family history of aspirin sensitivity [3]. First, a polymorphism in the promoter of leukotriene C_4 synthase, A-444C, was reported to be associated with AIA in Polish patients [4, 5]. A recent report showed that a haplotype of the 5-lipoxygenase gene was weakly associated with AIA in a Korean population [6]. With an extensive candidate gene analysis related to arachidonic acid metabolism, our group reported that single nucleotide polymorphisms (SNPs) in the prostaglandin E_2 receptor subtype 2 gene were significantly associated with AIA, and the functional impact of a promoter variant was further demonstrated [7]. Most recently, SNPs in prostaglandin E_2 receptor subtype 3 gene were associated in Korean population [8].

In the past few years, microarray techniques for gene expression profiling have been applied to a wide range of biological problems and have contributed to the discoveries of complex networks of biochemical processes underlying complex diseases. Microarray techniques have also helped to identify novel biomarkers, disease subtypes, and discrepancies of gene expression in human populations. Despite the advances in microarray techniques, application of the technology to identify susceptibility genes underlying complex diseases appears to be unsuccessful so far, with some exceptions [9, 10].

AIA is characterized by an aggressive mucosal inflammation of the lower airway (asthma) and the upper airways (rhinitis and nasal polyp). Rhinitis symptoms first occur in most AIA patients before the development of asthmatic intolerance to aspirin and other NSAIDs, whereas nasal polyps in AIA patients are first diagnosed at almost the same time aspirin intolerance appears [3]. We postulated that the lower airway lesion and the polyp in AIA have a common pathophysiology of aspirin intolerance, suggesting the nasal polyp as a pleiotropic genetic model of the bronchial inflammation of AIA. Global gene expression of the nasal polyps of AIA patients was examined using microarray technology for comparison with nasal polyps of eosinophilic sinusitis (ES) patients: ES is typically characterized by a nasal polyp with an 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.

Materials and methods

Nasal polyp tissues and Aspirin-Intolerant Asthma Subjects

Nasal polyp tissues for microarray analysis were obtained from nine Japanese patients (aged from 35 to 76 years, five males/four females) with AIA, five (aged from 34 to 73 years, three males/two females) with ES, and two (aged 61 and 71 years, both males) with only chronic sinusitis (CS) (Table 1). These patients had not been exposed to preoperative treatment with steroids for at least 1 year before surgery. According to the definition of rhinosinusitis, CS with nasal polyps with eosinophilic inflammatory features without fungal hyphae includes aspirin-sensitive and aspirin-tolerant types [11]. Thus, three groups of patients with nasal polyps were sequentially defined as follows: first, CS with nasal polyps was diagnosed based on clinical symptoms, such as nasal discharge, postnasal drip, headache, hyposmia, and nasal obstruction, and endonasal findings of muco-purulent secretion and nasal polyps with a paranasal shadow observed by CT examination [12]. Among CS patients with nasal polyps, ES patients were identified histologically by counting the number of eosinophils at ×200 magnification under light microscopy. Five fields were examined for each section,

Table 1. Clinical characteristics of patients with nasal polyps for microarray analysis

		Parameters in peripheral blood				
ID	Age/ gender	WBC (/mm^3)	Eosinophil (%)	Allergic rhinitis	Asthma	AIA episode
AIA#1	76/M	8000	3	−	+	+
AIA#2	48/M	5500	13	−	+	+
AIA#3	73/M	6500	3	−	+	+
AIA#4	59/F	9500	28	−	+	+
AIA#5	50/F	5720	14	−	+	+
AIA#6	40/M	9100	4	−	+	+
AIA#7	35/M	8800	6	−	+	+
AIA#8	50/F	6000	9	+	+	+
AIA#9	66/F	7000	8	−	+	+
ES#1	73/F	7200	2	−	+	−
ES#2	64/F	6400	23	−	+	−
ES#3	69/M	7700	4	+	−	−
ES#4	61/M	4900	5	−	+	−
ES#5	34/M	6300	3	+	+	−
CS#1	61/M	7400	10	−	+	−
CS#2	67/M	9700	10	−	−	−

M, male; F, female; WBC, white blood cell; −, no allergic rhinitis, no asthma, or no AIA episode; AIA, aspirin-intolerant asthma; CS, chronic sinusitis; ES, eosinophilic sinusitis.

and the average was considered to be the number of eosinophils infiltrating the sample. Nasal polyps having more than 100 eosinophils were classified as ES [12]. Among ES patients, those who had had apparent episodes of asthma attacks in response to aspirin and other NSAIDs were classified as AIA patients (AIA#1–9). The remaining five ES patients without AIA episodes (ES#1–5) had no troubles even after taking NSAIDs in postoperative courses during hospitalization. The oral provocation test for AIA patients was not performed in most of the patients due to potential risk, although severe reactions against the provocation were improbable [13], and only verbal history has yielded some false positives [14]. The ethics committees of Kagoshima University approved the study protocols, and each participant gave written informed consent.

DNA samples from 219 unrelated individuals with AIA (age: 55.7±13.5 years; 70 males/149 females) and 374 non-asthmatic controls (CTR) (age: 44.5±23.2 years; 181 males/193 females) were obtained as described previously [7]. For AIA-associated SNPs, 282 unrelated individuals with aspirin-tolerant asthma (ATA) (age: 56.0±16.1 years; 132 males/150 females) [7] were also genotyped, and used as asthmatic controls. The subjects were recruited at Niigata University Hospital, University of Tokyo Hospital, Nagoya University Hospital, Doai Memorial Hospital, and Kyushu University Hospital, with Institutional Review Board approvals. The diagnosis of AIA was based on a self-reported history due to the potential risk of a provocation test. ATA was defined as adult asthma diagnosed by expert physicians according to the American Thoracic Society criteria [15] and no history of aspirin or NSAID-induced asthmatic attack, and comprised of 154 atopic asthmatic (age: 48.0±15.6 years; 80 male/74 female) and 128 non-atopic asthmatic (age: 65.9±10.0 years; 52 male/76 female) subjects. CTR were outpatients with diseases (e.g., hypertension) other than respiratory diseases including asthma, and who self-reported no history of aspirin sensitivity. The patients and controls were all of Japanese ethnicity. Although the Japanese population is thought to be genetically homogenous, nearly identical numbers of patients and controls from the various locations were recruited to avoid geographical differences in allelic frequencies.

RNA extraction

The nasal polyp tissue was removed during endoscopic sinus surgery, submerged in RNA*later* regent (Ambion Inc., Austin, TX, USA) to avoid RNA degradation, and used for RNA extraction within 48 h after resection. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The quality and quantity of the extracted RNA were analysed using the Agilent 2100 bioanalyzer (Agilent Technologies Inc., Palo Alto, CA, USA) with an RNA6000 Nano LabChip Kit (Agilent Technologies). RNAs from two CS patients were equally pooled, and used as a common reference in the two-colour microarray experiments, where a single microarray was used to compare each test sample from an AIA or an ES patient with the reference sample.

cRNA synthesis, labelling, hybridization, and expression profiling

For fluorescent cRNA synthesis, high-quality total RNA (150 ng) was labelled with the Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies) according to the manufacturer's instructions. In this procedure, cyanine 5-CTP (Cy5) and cyanine 3-CTP (Cy3) (PerkinElmer, Boston, MA, USA) were used to generate labelled cRNA from the individual AIA or ES RNA and the pooled CS RNA as a reference, respectively. Labelled cRNAs (0.75 μg each) from the AIA, ES, or CS patients were fragmented in a hybridization mixture with the In Situ Hybridization Kit Plus (Agilent Technologies) according to the manufacturer's instructions. The mixture was hybridized for 17 h at 65 °C to an Agilent Human 1A(v2) Oligo Microarray. After hybridization, the microarray was washed with SSC buffer, and then scanned in Cy3 and Cy5 channels with the Agilent DNA Microarray Scanner, model G2565AA. Signal intensity per spot was generated from the scanned image with Feature Extraction Software ver7.5 (Agilent Technologies) in default settings. Spots that did not pass quality control procedures with the software were flagged and removed for further analysis.

GeneSpring software GX 7.3 (Agilent Technologies) was used for the Lowess (locally weighted linear regression curve fit) normalization of the ratio (Cy5/Cy3) of the signal intensities generated in each microarray and the subsequent data analysis. To determine the AIA-specific expression profile of nasal polyps, ES transcripts with ratios ranging from 0.5 to 2 were extracted, and the AIA transcripts with expression undergoing a twofold change or more were extracted as decreased or elevated genes. Of the transcripts overlapping the two groups, only those with statistically significant differences in expression between the AIA and CS nasal polyps (Benjamini and Hochberg false discovery rate (FDR)<0.01; [16]) were counted as AIA-specific genes. To identify novel expression patterns in nasal polyps from AIA patients, the *k*-means method [17], a well-known unsupervised partitional clustering approach, was applied to the AIA-specific genes. For functional subclassification of the AIA-specific genes, we applied the Gene Ontology (GO) classification for biological processes with DAVID 2.1 (http://david.abcc.ncifcrf.gov/), a web-accessible program [18]. A permutation test with 10 000 iterations was used for multiple test correction when nasal polyps from AIA

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 $D = x_{11} - p_1 q_1$ 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 $D/(p_1 p_2 q_1 q_2)^{1/2}$, 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 $D_{max} = \min(p_1 q_2, p_2 q_1)$ when $D > 0$ or $D_{max} = \min(p_1 q_1, p_2 q_2)$ 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

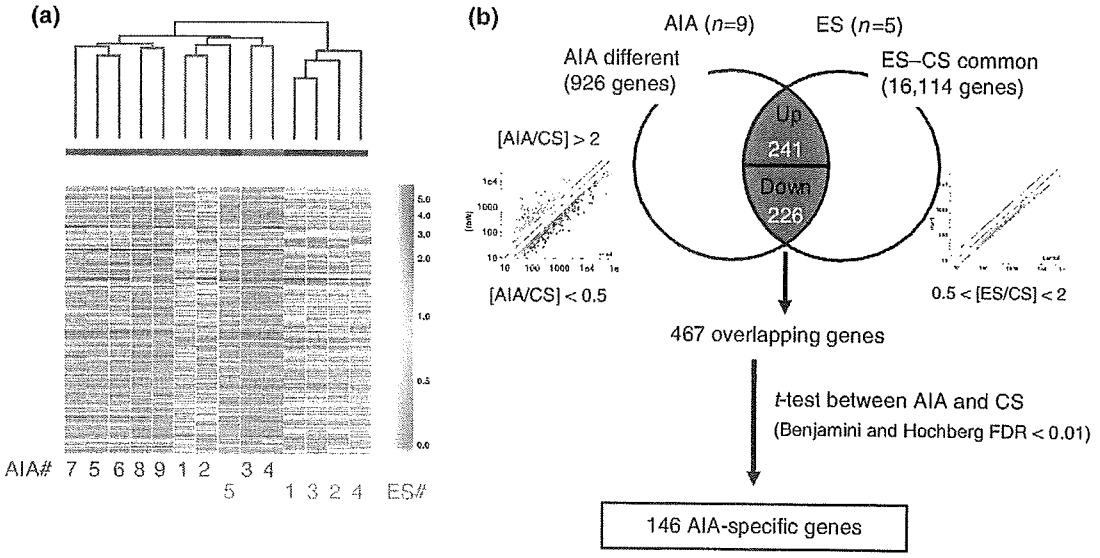

Fig. 1. Experimental design to extract aspirin-intolerant asthma (AIA)-specific genes with microarray analysis. (a) Hierarchical clustering (HC)-based classification of nasal polyps from AIA and eosinophilic sinusitis (ES) patients. Based on the gene expression in nasal polyps, HC clustering shows distinct 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.

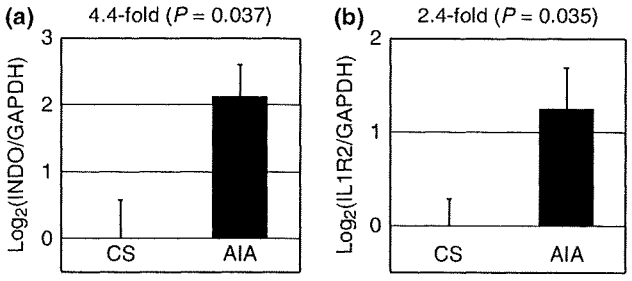

Fig. 2. Real-time reverse transcription polymerase chain reaction (RT-PCR) analysis validates expression differences for two genes, *INDO* and *IL1R2*, in nasal polyps from aspirin-intolerant asthma (AIA) and chronic sinusitis (CS) patients. Relative amounts of the respective genes in nasal polyps (AIA, $n = 10$; 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

					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	P	Corrected P‡	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

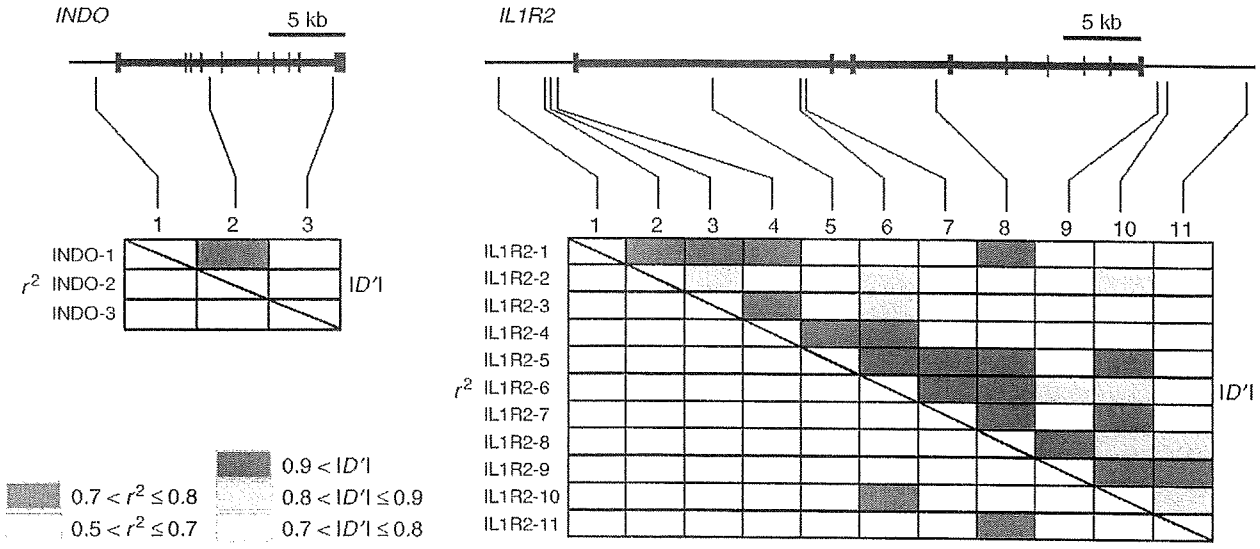

Fig. 3. Linkage disequilibrium pattern of *INDO* and *IL1R2*. The gene structures of *INDO* (left) and *IL1R2* (right), together with positions of the 14 single nucleotide polymorphisms (SNPs) examined, are shown. Pairwise LD coefficients, D' and r^2, were determined and expressed as a block structure. 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 $P^\dagger$
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 $P^\dagger$
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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Association of the growth hormone receptor gene polymorphisms with mandibular height in a Korean population

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ABSTRACT

Growth hormone receptor gene (*GHR*) is one of the likely candidates for determining morphological traits, because GH is a key regulator of bone growth. The genetic association of *GHR* in exon 10 with mandibular ramus height has been found in different populations, Japanese and Chinese. On the other hand, two common isoforms of *GHR*, one full-length (*fl-GHR*) and the other lacking the extracellular domain encoded by exon 3 (*d3-GHR*), are associated with differences in responsiveness to GH. The purpose of this study involving 159 Korean subjects was to study the associations between a *GHR* polymorphism (*d3/fl-GHR*) that results in genomic deletion of exon 3 and craniofacial morphology, and to study the associations between *GHR* genotypes in exon 10 and craniofacial morphology. Moreover, the allelic frequencies in a multi-ethnic population (24 Han Chinese, 24 African-Americans, 24 European-Americans, and 24 Hispanics) in a *GHR* polymorphism (*d3/fl-GHR*) were compared in this study. The five craniofacial linear measurements (cranial base length, maxillary length, overall mandibular length, mandibular corpus length, and mandibular ramus height) obtained from lateral cephalograms were examined as craniofacial morphology. We found that the *d3/fl-GHR* polymorphism had no association for any measurements, and a statistically significant association ($P = 0.024$) between the *GHR* polymorphisms P561T and C422F in exon 10 and mandibular ramus height. Neither SNPs besides P561T and C422F polymorphisms in exon 10 nor the measurements besides mandibular ramus height have statistically significances. Both derived alleles at P561T and C422F SNPs were highly associated with only one haplotype, haplotype-4 in Korean population. As quantitative haplotype association, the results showed a significant difference in mandibular ramus height between individuals having one haplotype-4 and others without haplotype-4 ($P = 0.028$). Moreover, we found that the *d3/fl-GHR* polymorphism showed diverse frequency in different population. Regarding *GHR* genotypes in exon 10, the present study mostly

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reflected the results obtained for a Japanese population, although our current study does not replicate the correlation between the I526L polymorphism of *GHR* and mandibular ramus height as was reported in a previous study of Han Chinese. The results of the present study suggest that the *GHR* exon 10 SNPs, not *d3/fl-GHR*, contribute to changes in the mandibular ramus height of Koreans.

1. Introduction

Craniofacial morphology is a polygenic, quantitative trait that is determined by genetic and environmental factors.[1] Studies on similarities in craniofacial morphology between close relatives have clarified that the genetic factors play an important role in the determination of craniofacial morphology.[2,3] Furthermore, a comparison of monozygous and dizygous twins has revealed a clear genetic influence on craniofacial morphology.[4] Identifying the genetic susceptibility for specific craniofacial phenotypes would enable more effective diagnosis and treatment of cranial malformations such as mandibular prognathism.[5]

Recent advances in clinical genetics have increased the fund of knowledge on genetic susceptibilities for craniofacial phenotypes.[5–7] It has recently been reported that single nucleotide polymorphisms (SNPs) of the growth hormone receptor (*GHR*) gene are associated with mandibular height in Japanese and Chinese populations.[8,9] It has been reported that the *GHR* polymorphism P561T in exon 10 is associated with mandibular height in the Japanese population.[8] Zhou et al. found that the *GHR* polymorphism I526L in exon 10 is associated with mandibular height in the Chinese population,[9] and they speculated that the discordance between Chinese and Japanese populations is due to either a lack of power in their experiments or a real difference between these populations. Considerable similarities have been observed in the linkage disequilibrium (LD) pattern between the East Asian populations (Korean, Japanese, and Han Chinese). The patterns of haplotype structure and the haplotype frequencies in the Korean population are very similar to those in the Japanese and Han Chinese populations. In particular, the LD patterns and haplotype frequencies of the Korean population show high degrees of similarity with those of the Japanese population.[10]

A polymorphism of *GHR* (*d3/fl-GHR*) that results in genomic deletion of exon 3 is associated with increased responsiveness to growth hormone; i.e., children carrying at least one *d3-GHR* allele show 1.7–2 times greater response to growth hormone than do *fl-GHR/fl-GHR* homozygotes.[11] This common polymorphism of human *GHR* that results in genomic deletion of exon 3 has recently been associated with the degree of height increase in response to GH therapy in French short children who were born small for gestational age or with idiopathic short stature[11], as well as in German Turner syndrome patients[12] and Brazilian GH-deficient children[13]. It may be a clue to understanding the ethnic difference of the association between *GHR* and mandibular ramus height to determine the allelic frequencies in a multi-ethnic population.

The purpose of this study is to characterise further the roles of the *d3/fl-GHR* SNP of *GHR* and five SNPs in exon 10 of *GHR* in 159 Korean subjects with regard to craniofacial morphology, and to define the allelic frequencies of *d3/fl-GHR* in a multi-ethnic population, which consisted of Han Chinese, African-Americans, European-Americans, and Hispanics.

2. Material and methods

2.1. Subjects

Genomic DNA samples and lateral cephalograms were obtained for the group of 159 Korean subjects, which comprised 100 men (age range, 20–49 years; mean age, 25.24 years) and 59 women (age range, 18–58 years; mean age, 24.39 years). The subjects were patients at dental hospitals or volunteers from the Pusan area. All the individuals were unrelated. Subjects who had congenital disorders, such as cleft palate or general physical disease, were excluded from the study. None of the subjects had received orthodontic or orthopaedic treatment. The 159 Korean subjects were comprised of 87 Class I, 44 Class II, and 28 Class III subjects.

In addition, DNA samples from 24 Han Chinese, 24 African-Americans, 24 European-Americans, and 24 Hispanics without craniofacial measurement data were obtained from the Coriell Cell Repository (Camden, NJ, USA), and used only as reference populations for the allelic frequencies of the exon 3-deleted/full-length (*d3/fl-GHR*) polymorphism.[14]

The protocol used in this study was approved by the Ethical Committee of Pusan National University, and all patients gave their written informed consent to participate in the study before DNA samples were taken.

2.2. Genotyping and sequencing

For DNA sample collection, the inside of the mouth was scraped with 10 strokes of a brush (MasterAmp™ Buccal Swab DNA Extraction Kit; AR Brown Co. Ltd., Tokyo, Japan). Four samples were collected from each subject. Genomic DNA was obtained from these samples.

We screened the *GHR* coding region for the five known SNPs of exon 10, [9] and for a polymorphic deletion of exon 3.[14] The analysed SNPs were C422F (dbSNP ID; rs6182), S473S (rs6176), P477T (rs6183), I526L (rs6180), P561T (rs6184), and *d3/fl-GHR*. The dbSNP numbers are taken from the dbSNP database at the NCBI (http://www.ncbi.nlm.nih.gov/SNP/).

Polymerase chain reaction (PCR) was performed according to a standard protocol. To determine the *GHR* exon 10

Table 1 – The relationship between six SNPs in *GHR* and six linear measurements of body height and craniofacial morphology in 159 Korean subjects.

	n	Body height (cm)			N-S (mm)			A′-PTM′ (mm)			Gn-Co (mm)			Pog′-Go (mm)			Co-Go (mm)		
		Mean	S.D.	*P*-value	Mean	S.D.	*P*-value	Mean	S.D.	*P*-value	Mean	S.D.	*P*-value	Mean	S.D.	*P*-value	Mean	S.D.	*P*-value
d3/fl-GHR																			
fl/fl	92	171.2	7.2	0.24	75.3	3.8	0.92	52.0	3.2	0.79	139.6	8.3	0.21	84.8	5.5	0.12	72.0	7.6	0.59
fl/d3	24	169.7	6.3		75.6	3.8		52.2	3.4		137.8	6.9		82.8	5.2		70.7	6.3	
d3/d3	9	173.7	6.9		75.4	4.4		51.3	2.5		142.7	10.4		84.5	4.5		72.5	6.6	
C422F/P561T																			
GG/CC	124	170.6	7.1	0.49	75.2	3.8	0.70	52.1	3.3	0.32	138.7	8.0	0.38	83.9	5.5	0.77	71.9	7.1	0.02*
GT/CA	24	169.6	6.7		75.2	3.7		51.3	2.9		137.7	8.6		84.2	5.3		68.5	5.5	
S473S																			
CC	145																		
CT	3																		
P477T																			
CC	145																		
CA	3																		
I526L																			
AA	62	171.5	7.2	0.19	75.8	3.7	0.24	52.6	3.2	0.24	139.1	8.7	0.71	84.3	5.7	0.82	72.0	7.6	0.68
AC	61	170.0	7.2		74.8	3.3		51.7	3.2		137.9	7.8		83.5	5.3		71.2	6.5	
CC	24	169.0	6.3		75.1	4.5		51.7	3.7		139.2	7.9		84.2	5.8		71.1	7.4	

* $P < 0.05$.

genotypes, PCR amplification was performed in the Gene Amp 9700 (Applied Biosystems, Tokyo, Japan) with a 10-μL PCR reaction that contained 30 ng of genomic DNA, 200 μM of each dNTP, 0.25 U of Ex Taq (Takara, Otsu, Japan), and 0.1 μM of each primer. The PCR involved initial denaturation at 95 °C for 1 min, followed by 40 cycles of 94 °C for 30 s and 72 °C for 1 min. After removal of the remaining primers and dNTP using ExoSAP-IT (GE Healthcare Life Science, USA), the products were subjected to BigDye v1.1 sequencing in the ABI PRISM 3700 DNA Analyzer (Applied Biosystems). Polymorphisms were identified using the Sequencer program (Gene Code Co., Ann Arbor, MI, USA). Each polymorphism was confirmed by sequencing the nucleotides from both strands.

For the genotyping of *d3/fl-GHR* in exon 3, PCR was performed using the G1, G2, and G3 primers (GenBank accession no. AF155912) and the published method,[14] with minor modifications. Briefly, we amplified the wild-type allele (*fl-GHR*) using primer pairs G1 and G2, and the deleted allele (*d3-GHR*) using the primer pairs G1 and G3 in separate 50-μl PCR reactions. Amplified PCR products were electrophoretically analysed on a 1% agarose gel and stained with ethidium bromide.

2.3. *Craniofacial measurements*

The lateral cephalograms were traced and measured by a single examiner (EH. K) using the Power Cephalo software (ReazaNet Co., Tokyo, Japan). To determine the errors associated with digitising and measuring, 30 radiographs were selected randomly. The examiner repeated the digitising and measuring 2 weeks later. A paired t test was applied to the first and second measurements, and no error was found. Cranial base length (nasion-sella; N-S), maxillary length (A'-PTM'), overall mandibular length (gnathion-condylion; Gn-Co), mandibular corpus length (pogonion'-gonion; Pog'-Go), and mandibular ramus height (condylion-gonion; Co-Go) were measured.[8] ANB angle have been measured as this angle was used for testing normal distribution. Body height was also measured for all Korean subjects.

The Kolmogorov–Smirnov test was performed to assess the body height and distribution pattern of craniofacial morphology (ANB angle, a cephalometric measurement of the anterior–posterior relationship of the maxilla to the mandible), and the mean, median, standard deviation, skewness, and kurtosis of each measurement were calculated.

2.4. *Haplotyping, LD analysis, and statistical analysis*

Tests for Hardy–Weinberg equilibrium, and the allele and genotype frequencies were all performed. The associations between six *GHR* SNPs and craniofacial measurements were analysed using the Mann–Whitney test. Quantitative haplotype analysis was tested for each Haplotype with One-factor ANOVA or the Mann–Whitney test. All the analyses were performed using the SPSS 10.0 (SPSS Inc., Chicago, IL, USA). A P-value < 0.05 was considered significant.

The LD for all possible two-way combinations of SNPs was tested using the D' and r^2 values.[15,16] Haplotype block analysis was conducted with the Gabriel and the Four Gamete methods.[17,18] SNP haplotypes and their frequencies were estimated by the maximum likelihood method with an expectation-maximisation algorithm.[19] The SNPAlyze 5.1 standard software (DYNACOM, JAPAN) was used to conduct LD and haplotype block.

3. Results

Table 1 shows the frequencies of the six genotypes of *GHR* and the relationships between these genotypes and six linear measurements of body height and craniofacial morphology in 159 Korean subjects. For the distribution pattern and the results of the Kolmogorov–Smirnov test, the heights and ANB angles of the 159 subjects were found to reflect the normal distribution (data not shown). Thus, the subjects of the present study were presumed to represent a normally distributed Korean population in terms of craniofacial morphology and height. The Mann–Whitney U-test was used to identify potential differences in measurements. Heterozygosity for S473S and P477T (genotypes CT and CA, respectively) was found in only three subjects. Therefore, statistical analysis was not performed for S473S and P477T. The genotype-specific association analysis revealed that only Co-Go (mandibular ramus height) was significantly correlated with the P561T (induced by C-to-A transversion) and C422F (induced by G-to-T transversion) variants ($P = 0.024$).

The strength of the LD for each SNP pair was measured using the D' and r^2 values (Fig. 1). The D' coefficient was almost equal to 1 for almost all the pairs of SNPs in the Korean population, which indicates that the gene is located in a single LD block. The r^2 parameter, which measures the correlation between alleles, showed that one of the SNP pairs was tightly

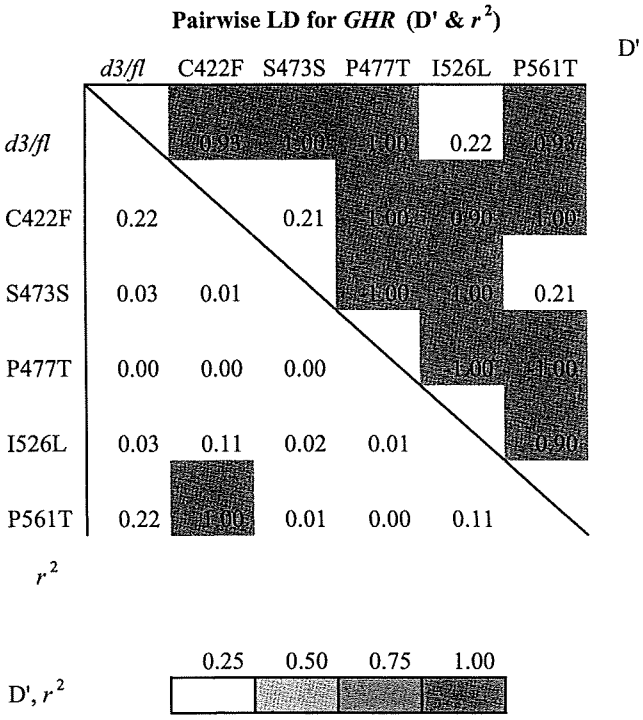

Fig. 1 – Plot of the D' values of the *GHR* polymorphisms in 159 Korean subjects. LD, Linkage disequilibrium.

Table 2 – Estimated haplotype frequencies of six SNPs.

	Haplotype						Estimated common haplotype frequency
	d3/fl	C422F	S473S	P477T	I526L	P561T	
Haplotype-1	*fl*	G	C	C	A	C	0.4785
Haplotype-2	*fl*	G	C	C	C	C	0.2417
Haplotype-3	*d3*	G	C	C	A	C	0.1214
Haplotype-4	*d3*	T	C	C	C	A	0.0738
Haplotype-5	*d3*	G	C	C	C	C	0.0533

Haplotypes constructed on the basis of genotypic data for six SNPs spanning the LD block covering GHR. Frequency of each haplotype within the sample of 159 Korean individuals (nine haplotypes), listed in decreasing order. The three major haplotypes accounted for 85% of the haplotypes in the sample.

correlated. LD analysis revealed that two SNPs, C422F and P561T, were in complete LD.

Haplotype analysis, which was constructed with six SNPs, showed that three major haplotypes accounted for 85% of the estimated haplotypes in the Korean population. All the other haplotypes had frequencies of less than 10% (Table 2).

As shown in Table 2, both derived alleles at P561T and C422F SNPs were highly associated with only one haplotype, haplotype-4 in Korean population. As quantitative haplotype association, the results showed a significant difference in mandibular ramus height between individuals having one haplotype-4 and others without haplotype-4 ($P = 0.028$) (Table 3).

We detected the *d3/fl-GHR* variant in all five populations (Korean, Han Chinese, African-American, European-American, and Hispanic). The *d3/fl-GHR* genotype frequencies varied among the populations (Table 4), with high frequencies *d3-GHR* allele in African-Americans (47.9%) and European-Americans (31.3%). The remaining populations had *d3/fl-GHR* genotype frequencies of 10–20%.

Table 3 – Haplotype association study of mandibular ramus height (CD-GO).

Copy number	*n*	Mean	S.D.	P-value
Haplotype-1				
0	44	71.4	5.8	0.99
1	52	71.5	7.4	
2	29	71.7	8.1	
Haplotype-2				
0	66	71.4	7.5	0.99
1	45	71.6	6.5	
2	14	71.6	7.1	
Haplotype-3				
0	92	71.1	7.3	0.35
1	33	72.5	6.1	
Haplotype-4				
0	104	72.1	7.0	0.03*
1	21	68.4	6.8	
Haplotype-5				
0	119	71.4	7.1	0.54
1	6	72.6	5.3	

* $P < 0.05$.

4. Discussion

Several studies have succeeded in elucidating susceptibility locus-related non-syndromic craniofacial morphology because craniofacial morphology is strongly influenced by genetic background.[5–9] We demonstrate an association between the P56IT and C422F SNPs (not located in exon 3) of the *GHR* locus and mandibular ramus height in a Korean population. In terms of haplotye, we found a significant difference in mandibular ramus height between individuals having one haplotype-4 (*d3*-T-C-C-C-A) and others without haplotype-4 in the Korean population.

Comparisons of the facial morphologies of Korean children with idiopathic short stature and growth hormone deficiency have revealed common characteristics.[20,21] Disproportionate growth of the cranial base structures and jaws results in facial retrognathia, which entails a proportionately smaller posterior than anterior facial height in persons of short stature with growth hormone deficiency.[21] Children who are in receipt of long-term GH replacement therapy show exaggerated growth of the craniofacial skeleton, especially with respect to the height of the mandibular ramus.[22,23] In a comparison of children with Turner syndrome who received recombinant human growth hormone treatment and a large cross-sectional control group, Rongen-Westerlaken et al.[24] have found a statistically significant increase in ramus growth that is associated with mandibular ramus height but not with mandibular body length, maxillary length or anterior cranial base length. Moreover, in *GHR* knockout mice, mandibular ramus height is significantly reduced,[25] and disproportional skeletal growth is reflected by decreased femur: crown-rump and femur:tibia ratios.[26] Therefore, *GHR* is suggested to have a site-, area- or region-specific effects.[27] On the other hand, growth hormone insensitivity syndrome of genetic origin has been linked to many different mutations of the *GHR*, and is associated with a wide range of severities of clinical and biochemical phenotypes. Mandibular growth is also influenced by multiple factors,[28] among which heterozygous *GHR* mutations appear to play a more or less important role, depending on the kind of mutation and on the overall genetic make-up of the individual. Although there is continuing interest in the functional importance of the P56IT and C422F variants, their precise roles remain unknown. The availability of an environmental factor (i.e., orthopaedic treatment) has made it possible to initiate therapeutic trials on children with short ramus height.[27]

Table 4 – Allele distribution of *d3/fl-GHR*.

		Korean ($n = 159$) (%)	Han Chinese ($n = 24$) (%)	African American ($n = 24$) (%)	European Americans ($n = 24$) (%)	Hispanic ($n = 24$) (%)
d3/fl-GHR	*fl*	83.2	84.1	52.1	68.8	81.8
	d3	16.8	15.9	47.9	31.3	18.2

The results of these trials, particularly with respect to the differential effects of such therapy on subjects with or without P561T/C422F will be of great interest.

Dos Santos et al.[11] have recently reported an association between the *d3-GHR* genotype and increased responsiveness to high-dosage recombinant human growth hormone therapy in short children without GH deficiency. In the present study, the prevalence of the *d3-GHR* allele was found to be 15–50%. The prevalence of the *d3-GHR* allele in persons of European origin has been previously reported as 25–32%, with a homozygosity frequency of 9–14%.[11,12,14] It has been speculated that this supposedly very flexible region of the GHR protein plays a role in the conformational changes that occur during transactivation of the GHR dimer by GH.[11] Nonetheless, in the present study, there was no association between *d3/fl-GHR* polymorphism and craniofacial measurements in a Korean population.

On an average, the allele frequencies for populations from different continents differ by 16–19%, and for populations within a continent, such as Koreans and Japanese, they differ by 5–10%.[29] In that report, it was described that these differences may be sufficiently large, even among the closely related Korean, Japanese, and Chinese populations, to cause substructural problems for case–control genetic studies of complex traits. Despite, we analysed five SNPs in exon 10 of the *GHR* gene in Korean subjects, and found an association between the P561T and C422F polymorphisms of *GHR* and mandibular ramus height, reflecting the results obtained for a Japanese population.[8] Although a previous study of 95 Han Chinese reported a correlation between the I526L polymorphism of *GHR* and mandibular ramus height, our current study with 159 unselected Korean subjects does not replicate this finding. The reason of the discordance between Chinese and Japanese/Korean populations remains still unclear. A haplotype-based study based on HapMap data is required to assess a difference among Asian populations. The result of the present study also raises the possibilities for a real difference between these populations.

Information on ethnic differences in allele frequency of disease-associated variants is important for better understanding of the pathologic mechanisms of polymorphisms.[30] We could not obtain craniofacial measurements in other ethnic groups analysed. However, we found that the *d3/fl-GHR* polymorphism showed diverse frequency in different population. Craniofacial morphology has ethnic differences.[31–34] It might imply the need for independent studies of craniofacial morphology for *GHR* in each ethnic group, and our work emphasises the importance of close matching of ethnic groups, especially when craniofacial morphology is examined.

Based on the present data, we conclude that there is a significant association between the P561T and C422F polymorphisms of *GHR* (which are in LD) and mandibular ramus height in a Korean population.

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ORIGINAL ARTICLE

Further evidence for an association between mandibular height and the growth hormone receptor gene in a Japanese population

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Introduction: Craniofacial morphology has a significant genetic component. It has recently been reported that single nucleotide polymorphisms (SNPs) in the growth hormone receptor gene (GHR) are associated with mandibular height. To confirm these findings, we genotyped SNPs in healthy Japanese subjects, about 1.7 times the number analyzed in previous reports. **Methods:** By using 5 SNPs in exon 10 of the GHR, we studied the relationships between genotypes and craniofacial linear measurements. The allelic frequencies in a multiethnic population (Han Chinese, African American, European American, and Hispanic) were also observed. **Results:** There was a significant association between SNPs and mandibular ramus height ($P = 0.029$), confirming our previous report of an association between polymorphism P561T and mandibular ramus height. Moreover, the SNP, C422F, was in linkage disequilibrium with P561T. Subjects with genotype CC of polymorphism P561T and genotype GG of polymorphism C422F had significantly greater mandibular ramus height than those with genotypes CA and GT. Four of the 5 SNPs were found almost exclusively in Asians, with the frequencies in other populations extremely small. **Conclusions:** Our results indicate that the GHR polymorphisms P561T and C422F are associated with mandibular ramus height in Japanese population and suggest that the SNPs of the GHR associated with the Japanese are likely to be different in other ethnic groups. This might partly explain the differing craniofacial morphology among different ethnicities. (Am J Orthod Dentofacial Orthop 2009;136:536-41)

Craniofacial morphology has a strong genetic component, but it is also influenced by environmental factors, making it complex to study. On the other hand, growth hormones play a major role in the growth and development of the craniofacial complex by directly and indirectly modulating the size and the angular relationships of the craniofacial structures,[1] and growth hormone receptors are present in the mandibular condyle with the molecular genetic analysis.[2]

Godowski et al[3] reported that the growth hormone receptor gene (GHR) (*600946) has 9 exons that encode the receptor and several additional exons in the 5-prime untranslated region. The coding exons span at least 87 kilobase of chromosome 5. Exon 2 encodes the signal peptide, exons 3 through 7 the extracellular domain, exon 8 the transmembrane domain, and exon 9 and part of exon 10 the intracellular domain. Reports have shown a relationship between the GHR and idiopathic short stature and Laron syndrome (growth hormone insensitivity syndrome), marked by a characteristic facial appearance. Kaji et al[4] identified compound heterozygous GHR mutations in exon 10, associated with severe growth retardation, Laron syndrome, and an undetectable serum growth hormone-binding protein. In a 53-year-old woman and her 57-year-old brother with growth hormone insensitivity syndrome, Milward et al[5] identified a homozygous 22 base-pair deletion in exon 10 of the GHR. Tiulpakov et al[6] described a GHR mutation comprising a guanine deletion at position 1776 in exon 10 in a patient with Laron

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syndrome. Interestingly, patients with growth hormone receptor deficiency showed significantly decreased vertical facial growth.[7]

We reported first about an association between a single nucleotide polymorphism (SNP) in exon 10 of GHR and mandibular ramus height in Japanese subjects.[8] It has recently been reported that the SNP in exon 10 of GHR is also associated with mandibular height in Chinese people,[9] and GHR is considered a possible genetic marker for mandibular ramus height.[10]

GHR polymorphism I526L is associated with mandibular height in Chinese people, and there might be an ethnic difference in the association of GHR with mandibular ramus height. In patients with Laron syndrome[11,12] or idiopathic short stature,[13-19] the association of GHR is different depending on ethnicity. One hundred Japanese subjects were used in our previous study about the association between the SNP in the GHR and mandibular ramus height; this association was replicated with 95 Han Chinese. However a larger-scale study with distinct ethnicities is required to obtain a conclusive result.[20-23]

Therefore, in this study, we examined the association between 5 SNPs in exon 10 of GHR and craniofacial morphology in 167 normal Japanese subjects, and, to further characterize the importance of the GHR locus, we examined the allelic frequencies of these SNPs in the GHR in a multi-ethnic population of Han Chinese, African Americans, European Americans, and Hispanics.

MATERIAL AND METHODS

Genomic DNA and lateral cephalograms were obtained from 167 Japanese subjects, including 50 men (ages, 20-49 years; average age, 35 years) and 117 women (ages, 18-58 years; average age, 29 years). The men were the same set that we studied previously.[8] The subjects were patients at dental hospitals and volunteers from the Tokyo metropolitan area. They were unrelated. Subjects with congenital disorders such as cleft palate or general physical disease were excluded from the study. None had received orthodontic or orthopedic treatment.

Additionally, DNA samples from 24 Han Chinese, 24 African Americans, 24 European Americans, and 24 Hispanics with no craniofacial measurements were obtained from the Coriell Cell Repository (Camden, NJ) and used only as reference groups for allelic frequencies of the 5 SNPs in exon 10 of the GHR.

The protocol used in this study was approved by the Ethical Committee of Showa University, and all patients gave written informed consent to participate in the study before blood samples were taken.

To collect a sample for DNA analysis, the inside of the mouth was scraped with 10 strokes of a brush (MasterAmp Buccal Swab DNA Extraction Kit, AR Brown, Tokyo, Japan). Four samples were collected from each subject, and genomic DNA was obtained from these samples.

We screened the coding region of the GHR for published polymorphisms and included 5 SNPs: C422F (dbSNP ID; rs6182), S473S (rs6176), P477T (rs6183), I526L (rs6180), and P561T (rs6184).[9] The dbSNP number came from the dbSNP database of National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/SNP/).

Polymerase chain reaction amplifications were performed according to a standard protocol. To determine genotypes at exon 10 of the GHR, polymerase chain reaction amplification was performed in a 10-μL polymerase chain reaction volume containing 30 ng of genomic DNA, 200 μmol/L of each dNTP, 0.25 units EX Taq (Takara, Otsu, Japan), and 0.1 μmol/L of each primer on the Gene Amp 9700 (Applied Biosystems, Tokyo, Japan). An initial denaturation was performed at 95°C for 1 minute, 40 cycles at 94°C for 30 seconds, and 72°C for 1 minute. After removal of the remaining primers and dNTPs by using ExoSAP-IT (USB Corp, Cleveland, Ohio), the products were subjected to sequencing on the ABI PRISM 3700 DNA Analyser (BigDye, version 1.1, Applied Biosystems).

Polymorphisms were identified with the sequencer program (Gene Code Co, Ann Arbor, Mich). Each polymorphism was confirmed by sequencing nucleotides from both strands.

For the craniofacial measurements, the lateral cephalograms were traced and measured by 1 examiner (T.Y.) using the computer software Power Cephalo (ReazaNet, Tokyo, Japan), as previously described.[8] Briefly, we measured cranial base length (nasion-sella), maxillary length, overall mandibular length (gnathion-condylion), mandibular corpus length (pogonion-gonion), and mandibular ramus height (condylion-gonion). Body height was also measured in all subjects.

The Kolmogorov-Smirnov test was used to assess body height and the distribution pattern of craniofacial morphology (ANB angle), and the mean, median, standard deviation, skewness, and kurtosis of each measurement were calculated.

Statistical analysis

The associations between 5 SNPs in exon 10 of the GHR and craniofacial measurements were analyzed by using the Mann-Whitney test with SPSS software (version 10.0, SPSS, Chicago, Ill). Tests for Hardy-Weinberg equilibrium, and the allele and genotype frequencies were all done with this software.

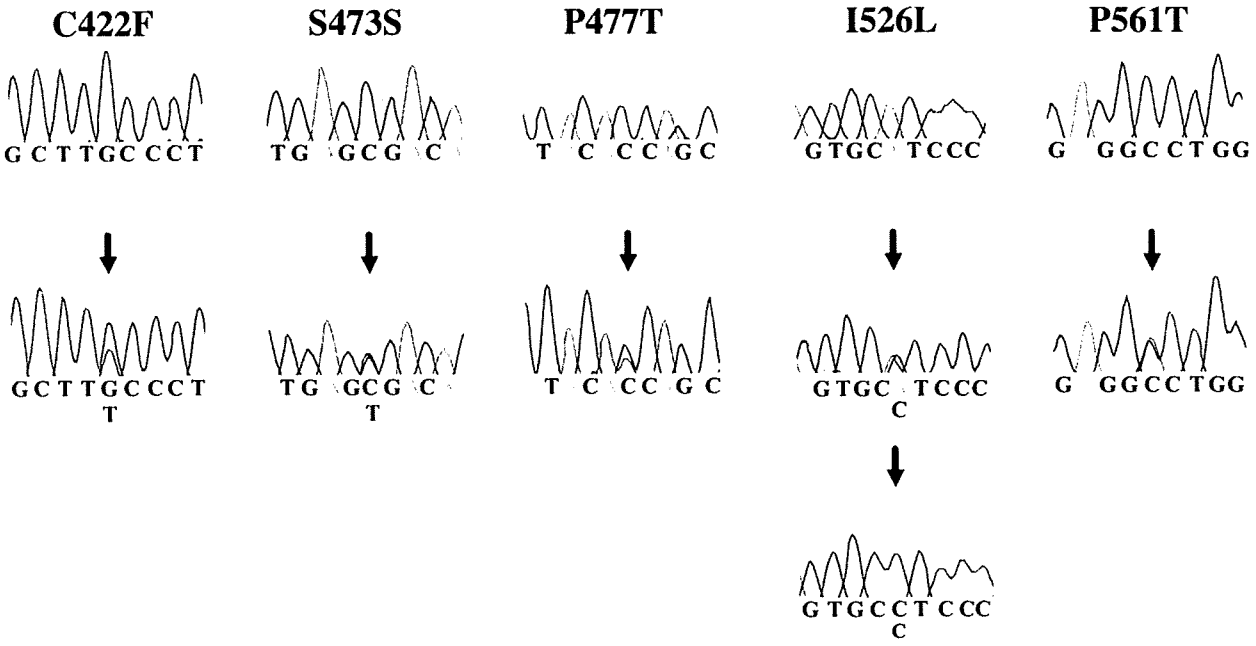

Fig. DNA sequence chromatograms of 5 SNPs in exon 10 of the GHR.

Table I. Allele distribution of 5 SNPs in exon 10 of the GHR

		Japanese (n = 167)	*Han Chinese (n = 24)*	*African American (n = 24)*	*European American (n = 24)*	*Hispanic (n = 24)*
C422F	G	94.1%	79.4%	100.0%	100.0%	100.0%
	T	5.9%	20.6%	0.0%	0.0%	0.0%
S473S	C	96.3%	97.3%	100.0%	97.5%	100.0%
	T	3.7%	2.6%	0.0%	2.5%	0.0%
P477T	C	98.7%	100.0%	100.0%	100.0%	100.0%
	A	1.3%	0.0%	0.0%	0.0%	0.0%
I526L	A	46.7%	38.2%	64.3%	58.3%	62.4%
	C	53.3%	61.8%	35.6%	41.6%	37.5%
P561T	C	94.7%	80.0%	100.0%	100.0%	100.0%
	A	5.2%	19.9%	0.0%	0.0%	0.0%

Haplotypes were inferred, and haplotype frequencies were estimated, by using the expectation-maximization method of haplotype inference in the Arlequin computer program.[24]

RESULTS

For the distribution pattern and the results of the Kolmogorov-Smirnov test, the height and the ANB angle of the 167 subjects reflected normal distribution (data not shown). Thus, these subjects were presumed to represent a normally distributed Japanese population for craniofacial morphology and height.

We detected 5 SNPs in the GHR in all 5 groups: Japanese, Han Chinese, African American, European American, and Hispanic (Fig). The frequencies varied among them, as shown in Table I.

Genotype-specific associations were examined with the Mann-Whitney test and showed that only mandibular ramus height was significantly correlated with the P561T and C422F variants ($P < 0.05$, Table II).

Haplotype analysis, constructed with 5 SNPs, showed 2 major haplotypes accounting for 85% of estimated haplotypes in the Japanese group. All other haplotypes had frequencies less than 10% (Table III).

The strength of linkage disequilibrium for each SNP pair was measured by using D′ and r2 values. The D′ coefficient was equal to or close to 1 for all pairs of SNPs in the Japanese population, indicating that the gene is in single linkage disequilibrium block (Table IV). The r2