92.1% in the IM, and 97.8% in the PM [73]. The factors responsible for the eradication failure include the presence of clarithromycin-resistant *H. pylori* strains, and in the future it will be necessary to test not only CYP2C19 polymorphism, but also genotypes for clarithromycin resistance in *H. pylori*.

4.3. TPMT genotyping for 6-MP treatment

Phenotypes of TPMT activity are often evaluated by the less invasive measurement of the activity in the red blood cells of the patients. It was reported that 92% exhibited high activity while 7.7% showed intermediate activity, and 1 in 300 revealed no TPMT activity in Caucasians [74]. In Caucasian infant patients with acute myeloid leukemia, the concentrations of the active metabolite of 6-MP were significantly higher in the red blood cells in those carrying TPMT*2, *3A, *3B, or *3C alleles, and dose reduction or termination of the administration was required in all patients [21]. Evans et al. [75] reported that when 6-MP was administered at 500 mg/m^2/week in the EM for TPMT, the dose should be reduced to half in the IM and 20 mg/m^2/week or 1/25-fold in the PM. Indeed, the evaluated results of phenotypes and genotypes for TPMT were applied to determining doses of 6-MP to patients at St. Jude Children's Hospital.

4.4. mtDNA A1555G genotyping for aminoglycoside treatment

It has been reported that the administration of aminoglycoside antibiotics is likely to cause irreversible perceptive deafness in humans with the SNP at the 1555th base (A to G) in the 12S rRNA gene in mtDNA [76]. Aminoglycosides basically inhibit the synthesis of bacterial protein, but in cases with the alteration at the 1555th base from A to G in the mtDNA in normal humans, the sequence shares a similar three-dimensional structure with bacterial 16S rRNA, and the site that originally does not have the affinity becomes the target for aminoglycosides and protein synthesis involved in the mitochondrial electron transfer system and oxidative phosphorylation is suppressed. As a result, ATP production decreases and hair cells in the internal ear show dysfunction [77].

We performed genetic testing for mtDNA A1555G in patients with perceptive deafness using real-time PCR and point-of-care testing [70]. Recently, following the request by a patient (Mother) with perceptive deafness carrying the mtDNA A1555G alteration, we performed genetic testing for the same polymorphism in her children (a 13-year-old boy and an 11-year-old girl) without the symptom of perceptive deafness. As a result, both children carried the genotype of 1555G and were recommended to carry an adverse effect avoidance card reported by Usami et al. [78] so that aminoglycosides would not be given (personal communications). Perceptive deafness is irreversible and decreases the QOL of the patients remarkably. It is necessary to perform genetic testing routinely before aminoglycoside administration and promote counseling for mtDNA A1555G subjects to avoid side effects.

5. Cost effectiveness

Few reports have investigated the benefit for medical economy by testing the genes involved in drug responses prior to drug therapy. Tavadia et al. [79] calculated that on the assumption that azathioprine caused myelosuppression in 100% of the PM and 30% of the IM for TPMT, genetic testing would provide about $200 in cost benefit. Simulating eradication therapy in 100 cases with omeprazole and amoxicillin for 3 months according to the eradication regimen for *H. pylori* reported by Furata et al. [73], Desta et al. [80] calculated the cost per patient for the testing of CYP2C19 polymorphisms (*1, *2, and *3) with the conventional method in the laboratories and the drug cost for omeprazole, and concluded that genetic testing for CYP2C19 in advance could save Asians $5680 in medical costs.

6. Conclusions

Today it is possible to predict the responder and non-responder, and the emergence of adverse effects for some drugs by polymorphism testing of the genes involved in drug responses. However, there are markedly few institutes in the world where genetic testing is routinely performed and applied to tailor-made drug therapy. Recently, the Food and Drug Administration (FDA) in the US declared guidance on using individual information on genetic polymorphisms in the approval of drugs on clinical trials. It is anticipated that the development of simple, rapid, accurate, and low-cost genetic testing will promote research on pharmacogenetics and lead to the development of new drugs and therapy.

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Competitive allele-specific short oligonucleotide hybridization (CASSOH) with enzyme-linked immunosorbent assay (ELISA) for the detection of pharmacogenetic single nucleotide polymorphisms (SNPs)

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Abstract

Individualization of drug therapy through genetic testing would maximize the effectiveness of medication and minimize its risks. Recent progress in genetic testing technologies has been remarkable, and they have been applied for the analysis of genetic polymorphisms that regulate drug responses. Clinical application of genetic information to individual health care requires simple and rapid identification of nucleotide changes in clinical settings. We previously reported a novel DNA diagnostic method for detecting single nucleotide polymorphisms (SNPs) using competitive allele-specific short oligonucleotide hybridization (CASSOH) with an immunochromatographic strip. We have developed the method further in order to incorporate an enzyme-linked immunosorbent assay (ELISA) into the final detection step; this

Abbreviations: CASSOH, competitive allele-specific short oligonucleotide hybridization; ELISA, enzyme-linked immunosorbent assay; SNP, single nucleotide polymorphism; mtDNA, mitochondrial DNA; NAT2, *N*-acetyltransferase 2; PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism; PCR-SSCP, polymerase chain reaction-single strand conformational polymorphism; FITC, fluorescein isothiocianate; HRP, horseradish peroxidase; TMB, 3,3′,5,5′-tetramethylbenzidine.

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enables multiple SNP detection. Special ELISA chips have been fabricated so that disposal of buffer waste is not required and handling procedures are minimized. This method (CASSOH-ELISA) has been successfully applied for the detection of clinically important SNPs in drug metabolism, such as *N*-acetyltransferase 2, *NAT2*6* (590G>A) and *NAT*7* (857G>A), and mitochondrial DNA (1555A>G). It would also facilitate point-of-care genetic testing for potentially diverse clinical applications.

Keywords: Genotyping; Single nucleotide polymorphism; Pharmacogenetics

1. Introduction

Single nucleotide polymorphisms (SNPs) are the most common type of genetic variations found in individuals. Clinical application of pharmacogenetic SNPs information is important in customizing the species of the drug and providing optimal dosage and schedule for individual patients. Pharmacogenetics involves determination of genetic polymorphisms that influence drug exposure levels. Specifically, increased toxicity or altered efficacy can result from variations in a gene that encodes an important drug-metabolizing enzyme. Alterations in genes that are known to influence the drug response and/or toxicity include SNPs at the loci for *N*-acetyltransferase 2 (*NAT2*) [1–3] and mitochondrial DNA (mtDNA) [4].

N-Acetyltransferase 2 (NAT2) exhibits hereditary determined polymorphism, and the individual phenotypes can be classified as rapid, intermediate, or slow acetylators according to their acetylation activity [1]. NAT2 metabolizes many drugs, including isoniazid, procainamide, and polycyclic amines such as several sulfonamides and hydralazines [5]. The relationship between polymorphic acetylation of isoniazid and procainamide by NAT2 and idiosyncratic drug toxicity has been well-documented [6]. This polymorphism shows racial differences; in the Caucasian population, nearly 50% are slow acetylators, whereas this frequency is only 10% in the Japanese population [7–9]. Among all the NAT2 allelic variants that have been identified [10], 3 variants (*NAT2*5* (341T>C), *NAT2*6* (590G>A), and *NAT2*7* (857G>A)) were shown to account for the majority of the slow acetylator genotype in Japanese subjects [9,11].

It has been reported that the administration of aminoglycoside antibiotics is likely to cause irreversible perceptive deafness in humans with an SNP at the 1555th base (A to G) in the 12S rRNA gene in mtDNA [4]. Aminoglycosides basically inhibit the synthesis of bacterial protein: however, in cases where the 1555th base is altered from A to G in the mtDNA in normal humans, the sequence shares a similar three-dimensional structure with bacterial 16S rRNA. Further, the site that originally does not have affinity becomes a target for aminoglycosides and protein synthesis involved in the mitochondrial electron transfer system, and oxidative phosphorylation is suppressed. As a result, ATP production decreases and hair cells in the internal ear show dysfunction [12]. Thus, it is necessary to perform routine genetic testing prior to aminoglycoside administration and promote counseling for mtDNA (A1555G) subjects to avoid side effects.

Prior to drug treatment, it is extremely desirable to use bedside genotyping methods to identify drug responders or nonresponders as well as patients with increased risk of toxicity. Numerous SNP detection methods have been developed, including PCR-RFLP, allele-specific PCR [13], PCR-SSCP [14], oligonucleotide ligation assay [15], TaqMan PCR [16–18], Invader assay [19,20], pyrosequencing [21], microarrays [22], and matrix-assisted laser desorption/ ionization-time of flight mass spectrometry [23]. However, these methods require either

cumbersome laboratory procedures or high-tech instrumentation for high-throughput analysis. None of these procedures are readily performed in local clinical laboratories in which molecular biology expertise is unavailable.

Matsubara and Kure [24] and our group [25] have recently developed a novel DNA diagnostic method for detecting SNPs using competitive allele-specific short oligonucleotide hybridization (CASSOH) with an immunochromatographic strip. The discrimination of a one-base mismatch is achieved by employing unusually short oligonucleotide probes (11–17 mer), which would reduce the reassociation of mismatched hybrids by a factor of two, thereby providing unsurpassed reliability and reproducibility. However, at present, the production of immunochromatographic strips (DNA detection test strips™ (Roche)) has been discontinued. In this study, we have devised a method for incorporating an enzyme-linked immunosorbent assay (ELISA) into the final detection step; this enables multiple SNP detection.

2. Materials and methods

2.1. Isolation of DNA from human blood

The local ethics committee of Tohoku Pharmaceutical University and Tohoku University Hospital approved the study, and all blood donors provided written, informed consent. DNA was isolated from K_2EDTA-treated anticoagulated peripheral blood using a DNA Extractor WB-Rapid kit (Wako Pure Chemical Industries, Osaka, Japan) or a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

2.2. Oligonucleotides

The nonlabeled primers were synthesized by Fasmac, Inc. (Atsugi, Japan). The fluorescein isothiocyanate (FITC)-labeled primer and biotin-labeled probe were synthesized by Nihon Gene Research Laboratories, Inc. (Sendai, Japan).

2.3. CASSOH assay

The CASSOH assay for the detection of polymorphisms of *NAT2* (*NAT2*6* (590G>A) and *NAT2*7* (857G>A)) and mitochondrial DNA (1555A>G) was carried out according to the method described by Matsubara and Kure [24] and our group [25] with minor modifications. The principle of the method is illustrated in Fig. 1. A target sequence containing an SNP site was amplified by PCR using a pair of PCR primers, one of which was labeled with FITC at its 5′-end. The PCR reaction mixture also contained two sets of hybridization probes. One set was used for the detection of the variant type nucleotide sequence and consisted of an oligonucleotide containing the variant type sequence labeled with biotin at its 3′-end and an unlabeled oligonucleotide containing the wild sequence (reaction 1). The second set consisted of an oligonucleotide containing the wild sequence labeled with biotin at its 3′-end and an unlabeled oligonucleotide containing the variant type sequence (reaction 2). All hybridization probes were designed on the strand opposite to the FITC-labeled PCR primer. Table 1 presents a summary of the primers and probes used.

The PCR reaction was carried out in a mixture containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 250 μM each dNTP, 1 μM PCR forward primer, 1 μM PCR reverse primer, 600 nM biotinylated probe, 3 μM unlabeled competing probe, 1.25 U Ex Taq DNA polymerase

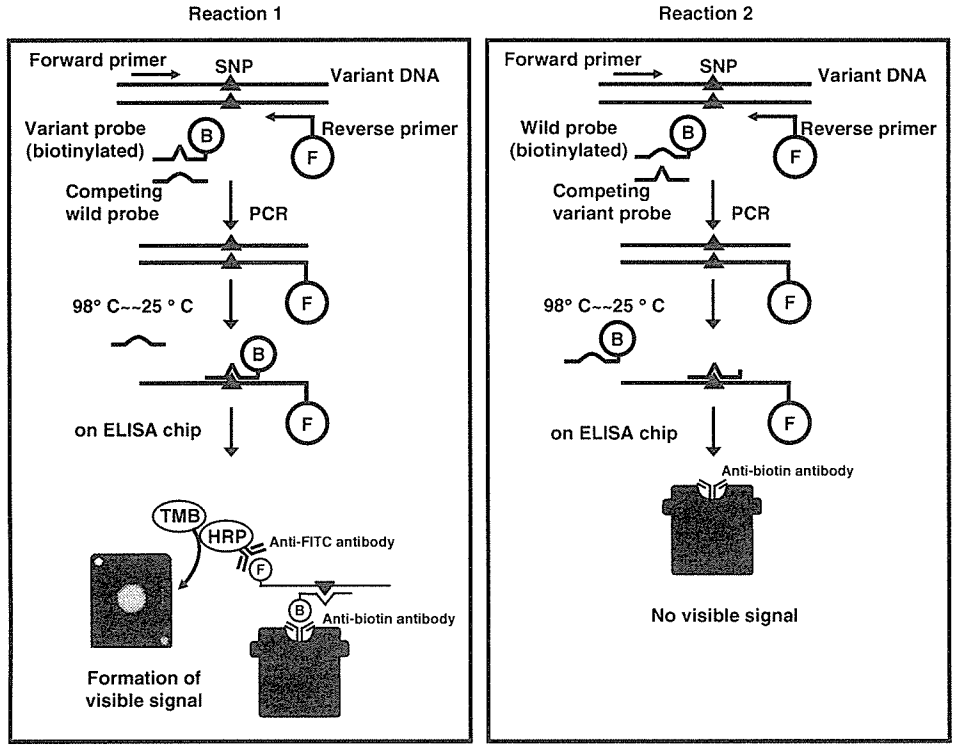

Fig. 1. CASSOH assay with the ELISA chip. Analysis of variant DNA using reaction 1 (left) for the detection of a variant sequence or reaction 2 (right) for the detection of a wild-type sequence. Closed triangles indicate an SNP site. F, fluorescein isothiocyanate (FITC) labeling; B, biotin labeling; HRP, horseradish peroxidase; TMB, 3,3′,5,5′-tetramethylbenzidine.

(Takara, Otsu, Japan), and 0.04–100 ng of genomic DNA in a total volume of 25 μL. The PCR reactions were performed in a BIO-RAD iCycler (Hercules, CA, USA) under the following cycling conditions: denaturation at 94 °C for 3 min followed by 35 cycles of denaturation at 98 °C for 10 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s, and, finally, successive incubations at 72 °C for 3 min, 98 °C for 3 min, 65 °C for 1 min, 55 °C for 1 min, 45 °C for 1 min, 35 °C for 1 min, and 25 °C for 1 min.

2.4. ELISA chip detection

As shown in Fig. 2, 50 μL of the block solution (1% casein in phosphate buffer (pH 7.2)) was pipetted onto the ELISA chip (Toyobo Co., Fukui, Japan). The ELISA chip uses a porous membrane filter (glass-fiber, 8 mm in diameter) immobilized anti-biotin antibody (IgG) on the top of the chip. The inside of the chip is filled with the absorbent pad which can hold a solution to approximately 400 μL. A mixture of 20 μL of the PCR reaction mixture plus 20 μL of the horseradish peroxidase (HRP)-labeled anti-FITC antibody solutions (DAKO, Glostrup, Denmark) was pipetted onto the ELISA chip. Following incubation for 4 min, 80 μL of the wash solution (10 mM phosphate buffer, 0.2% Tween 20) was pipetted onto the chip. After incubation for 1 min, 80 μL of the wash solution was again pipetted onto the chip. Incubation for 1 min was

Table 1
Sequences of PCR primers and hybridization probes

Gene (allele)		Forward primer		Reverse primer	
PCR primers					
*NAT2 (*6)*		5′-ttggaaacattaactgacattcttgag-3′		5′-FITC-tgtggttataaatgaagatgttggagac-3′	
*NAT2 (*7)*		5′-agggtatttttacatccctccagtt-3′		5′-FITC-ggtagagaggatatctgatagcacataagt-3′	
mtDNA		5′-cccaaactgggattagataccc-3′		5′-FITC-ttagctcagagcggtcaagttaag-3′	
Gene (allele)	Substitution	Detection of wild-type sequence		Detection of variant sequence	
		Biotinylated wild probe	Competing variant probe	Biotinylated variant probe	Competing wild probe
Hybridization probes					
*NAT2 (*6)*	590G>A	5′-aacctcGaacaa-Bio-3′	5′-gaacctcAaacaa-3′	5′-gaacctcAaacaa-Bio-3′	5′-aacctcGaacaa-3′
*NAT2 (*7)*	857G>A	5′-tgatgGatccct-Bio-3′	5′-gtgatgAatccct-3′	5′-gtgatgAatccct-Bio-3′	5′-tgatgGatccct-3′
mtDNA	1555A>G	5′-aggagAcaagtcg-Bio-3′	5′-aggagGcaagtc-3′	5′-aggagGcaagtc-Bio-3′	5′-aggagAcaagtcg-3′

Underlined nucleotides indicate targeted substitutions.
FITC, fluorescein isothiocyanate; Bio, biotin.

followed by pipetting 40μL of a substrate solution containing 3,3′,5,5′-tetramethylbenzidine (TMB) onto the chip. The HRP-labeled anti-FITC antibody, FITC-labeled PCR products, and biotin-labeled probe complexes were further trapped by the anti-biotin antibody on the ELISA chip, resulting in a visible blue signal after 10min. The genotype of the specimen was determined by the presence or absence of the blue signal in reactions 1 and 2.

3. Results

Fig. 3 shows representative results for *NAT2*6* (590G>A), *NAT2*7* (857G>A), and mtDNA (1555A>G) using the CASSOH-ELISA. False positive or false negative signals were not

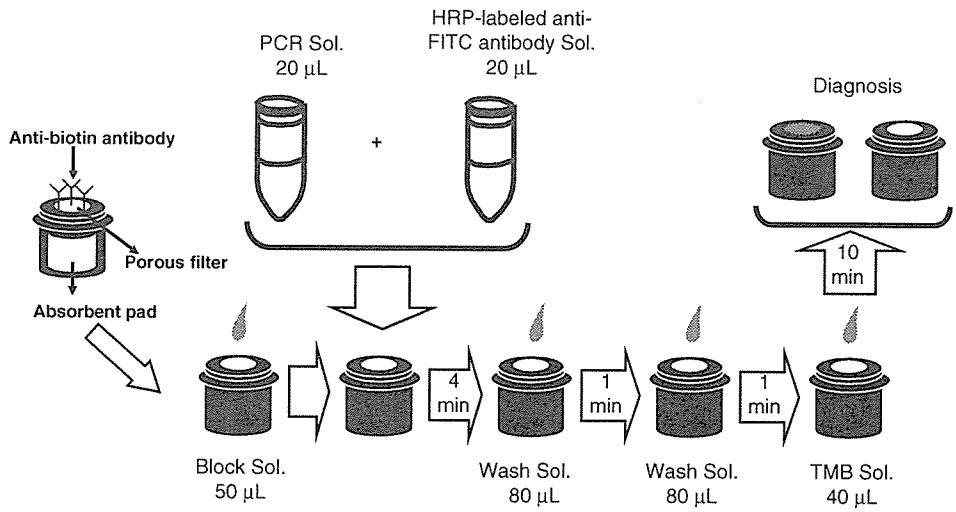

Fig. 2. Procedure for CASSOH with ELISA chips.

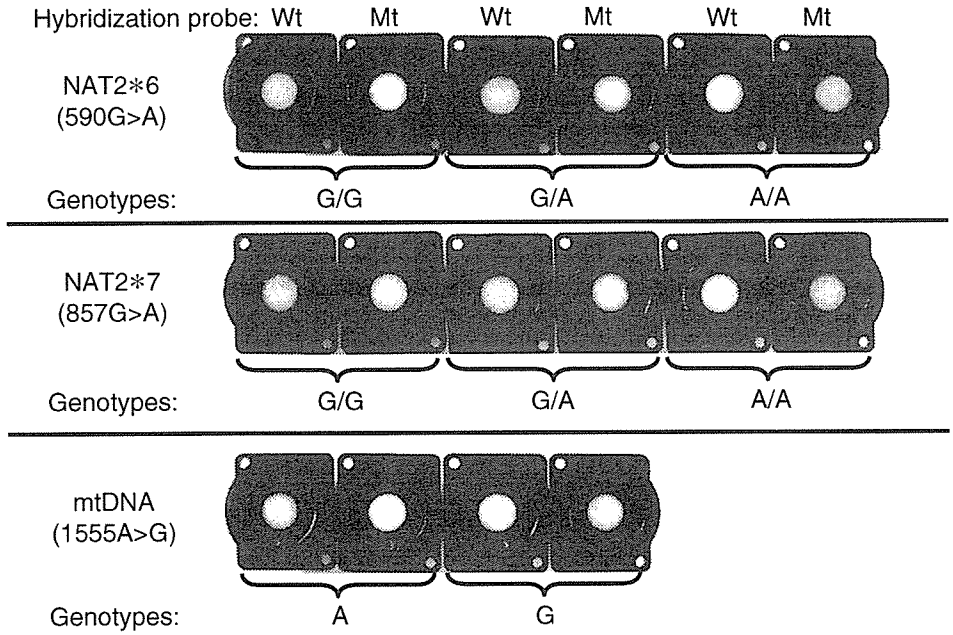

Fig. 3. Genotyping by CASSOH assay with ELISA chips. Detection of the *NAT2*6* (590G>A), *NAT2*7* (857G>A), and mtDNA (1555A>G) polymorphisms. Wt, detection probe for wild type; Mt, detection probe for variant type.

observed. A 100% match was observed in the genotyping results of the 590G-homozygotes, heterozygotes, and 590A-homozygotes of *NAT2*; the 857G-homozygotes, heterozygotes, and 857A-homozygotes of *NAT2*; and the 1555A-homoplasmy and 1555G-homoplasmy of mtDNA. Although signal intensities of the wild-type probes and variant probes in a heterozygous sample of *NAT2*6* were slightly different, no false negative signals were observed under the conditions. To estimate the precision and reproducibility of the assay, selected samples (n=3–17) of a known genotype were analyzed in duplicate. The genotype of all samples was tested by sequencing and CASSOH-ELISA, and identical results were obtained by the two methods (data not shown). Test results on the ELISA chip were stable for at least 60 min at room temperature (data not shown).

4. Discussion

The employment of the ELISA chip enabled low-tech detection of SNP, which is suitable for point-of-care DNA diagnosis in clinical settings. Advantages of the CASSOH-ELISA assay are that electrophoresis and disposal of buffer waste are not required and handling procedures are minimized. Although the ELISA chip cannot be purchased from other company except for the Toyobo Co., the ELISA part of this method can be carried out using a conventional ELISA plate. If the ELISA chip or palate is applied with samples, washed and detected blue signals automatically, SNP detection should be easier from the present way. One disadvantage of the ELISA chips in comparison with the immunochromatographic strip [24,25] is the number of steps required to complete the assay, which could increase the likelihood of carryover contamination. Sensitivity of the ELISA chip method was as high as that of the immunochromatographic strip method.

Primer design and reaction conditions were remarkably similar for the three different diagnostic tests. Therefore, the method may be readily applied to the detection of any SNP of interest, although empirical optimization of probe is required for each application. Especially, the length of an optimal wild-type probe may be different from that of an optimal mutant probe due to different base compositions.

The CASSOH-ELISA assay may be used for point-of-care genetic diagnosis for potentially diverse clinical application. For example, there is increasing evidence for the role of genetic polymorphisms in determining drug disposition and drug response and, thus, susceptibility to adverse drug reactions. Several SNPs that alter the rate of drug metabolism have been described in the genes of various drug-metabolizing enzymes, including CYP2C9, CYP2C19, CYP2D6, *N*-acetyltransferase 2, dihydropyrimidine dehydrogenase, and thiopurine methyltransferase [26]. Prospective bedside genotyping to identify poor drug metabolizers would allow individualization of drug therapy and thereby maximize efficacy and minimize toxicity.

5. Simplified description of the method and its future applications

In this research, we have developed a CASSOH-ELISA genotyping method for SNPs that are particularly important for the identification of drug responders or nonresponders and patients with increased risk of drug toxicity. This method is rapid and simple and should be suitable for routine clinical genotyping.

Acknowledgements

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Germline *KRAS* and *BRAF* mutations in cardio-facio-cutaneous syndrome

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Cardio-facio-cutaneous (CFC) syndrome is characterized by a distinctive facial appearance, heart defects and mental retardation. It phenotypically overlaps with Noonan and Costello syndrome, which are caused by mutations in *PTPN11* and *HRAS*, respectively. In 43 individuals with CFC, we identified two heterozygous *KRAS* mutations in three individuals and eight *BRAF* mutations in 16 individuals, suggesting that dysregulation of the RAS-RAF-ERK pathway is a common molecular basis for the three related disorders.

Cardio-facio-cutaneous (CFC) syndrome (OMIM 115150) was first described in 1986 (ref. 1). Affected individuals present with heart defects, including pulmonic stenosis, atrial septal defects and hypertrophic cardiomyopathy, and ectodermal abnormalities such as sparse, friable hair, hyperkeratotic skin lesions and a generalized ichthyosis-like condition. Typical facial characteristics include high forehead with bitemporal constriction, hypoplastic supraorbital ridges, downslanting palpebral fissures, a depressed nasal bridge and posteriorly angulated ears with prominent helices. The molecular basis of CFC syndrome has remained unknown. There are phenotypic similarities between this syndrome, Noonan syndrome (OMIM 163950) and Costello syndrome (OMIM 218040)[2,3]. Gain-of-function mutations in protein tyrosine phosphatase SHP-2 (*PTPN11*) have been identified in approximately 40% of individuals with clinically diagnosed Noonan syndrome[4]. No *PTPN11* mutations have been found in individuals with CFC syndrome[5–7]. Recently, we identified *HRAS* mutations in 12 of 13 individuals with Costello syndrome[8]. These findings suggest that the activation of the RAS-MAPK pathway is the common underlying mechanism of Noonan syndrome and Costello syndrome and, hence, possibly of CFC syndrome.

To elucidate the molecular basis of CFC syndrome, we first sequenced the entire coding regions of three RAS genes, *HRAS* (NC_000011), *KRAS* (NC_000012) and *NRAS* (NC_000001), in genomic DNA from 43 individuals with CFC syndrome (**Supplementary Methods** online). We identified two *KRAS* mutations: G60R (178G→C) in CFC73 and D153V (458A→T) in CFC8 and CFC91 (**Fig. 1a** and **Table 1**). Neither mutation has been previously identified in individuals with cancer (Sanger Institute Catalogue of Somatic Mutations in Cancer (COSMIC); http://www.sanger.ac.uk/cosmic). Gly60 and Asp154 are evolutionally conserved or chemically similar (**Supplementary Fig. 1** online). Neither of the two mutations was observed in 100 control chromosomes (data not shown). Their parents did not carry the mutations (**Supplementary Fig. 1**). The D153V mutation was identified in DNA extracted from both blood and buccal cells of individual CFC91. These results suggest that these germline mutations occurred *de novo*. No mutations in *KRAS*, *NRAS* or *HRAS* were found in the other 40 individuals with CFC syndrome.

Next, we examined the downstream molecules of RAS in the signaling pathway. The *RAF* proto-oncogene family consists of three isoforms, *CRAF*, *BRAF* and *ARAF*, and encodes for cytoplasmic serine/threonine kinases that are activated by binding RAS. Among these RAF molecules, BRAF is expressed at high levels in the brain and mutations in *BRAF* have been identified in 7% of all cancers[9]. We sequenced the entire 18 coding exons of *BRAF* (NC_000007) in 40 individuals with CFC syndrome and identified eight mutations in sixteen individuals (**Table 1**). Six mutations were located in the kinase domain (**Fig. 1b**). A G469E (1406G→A) mutation, which resides in the glycine-rich loop where somatic mutations are clustered in cancer, was identified in four individuals (CFC76, CFC81, CFC94 and CFC114). N581D (1741A→G), located in the catalytic loop, was identified in CFC95 and CFC110. Four mutations in the kinase domain between the glycine-rich loop and the catalytic loop were identified in five affected individuals: L485F (1455G→C) in CFC83, K499E (1495A→G) in CFC79, E501K (1501G→A) in CFC77 and E501G (1502A→G) in CFC90 and CFC105. A246P (736G→C) and

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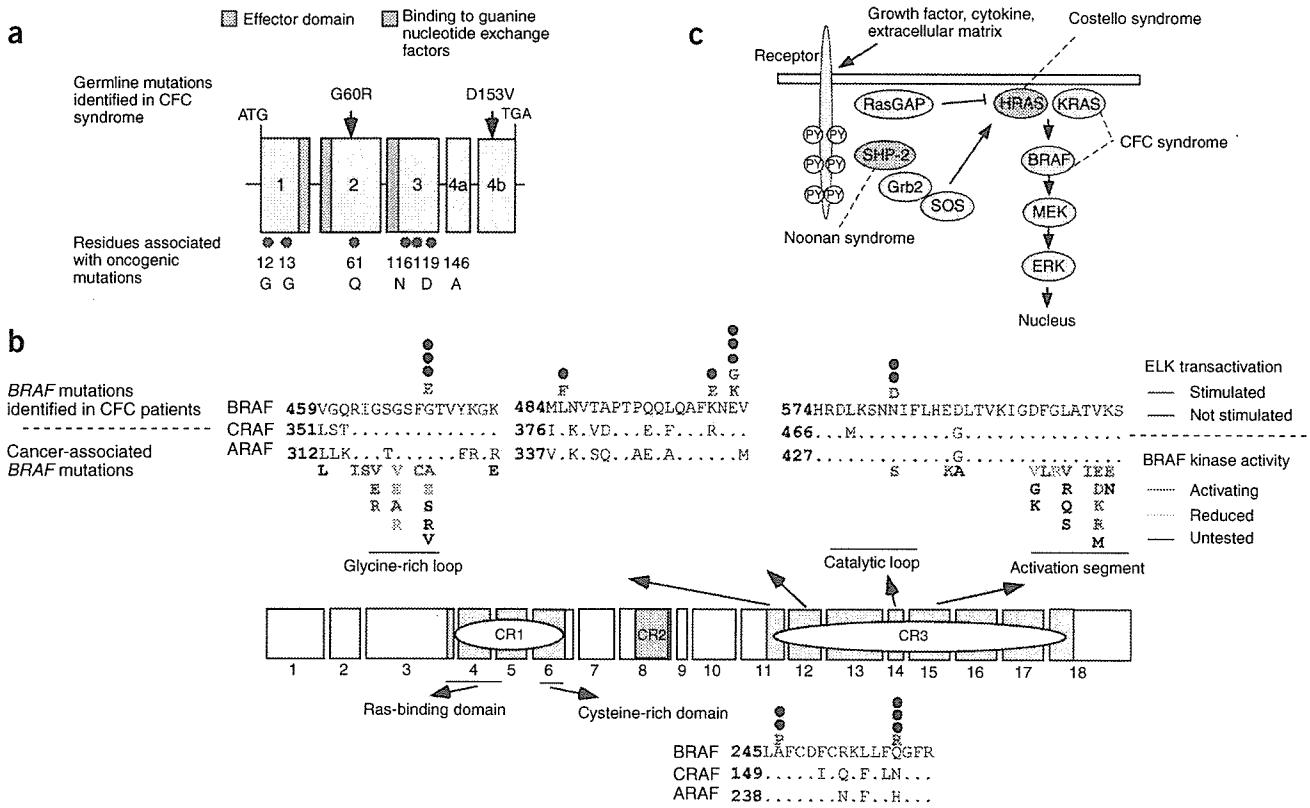

Figure 1 Mutations in *KRAS* and *BRAF* were identified in individuals with CFC syndrome. (**a**) Domain organization and genomic structure of the *KRAS* gene. Coding exons are numbered. In 98% of the transcripts, exon 4a is spliced out and only exon 4b is available for translation into protein. (**b**) *BRAF* consists of 18 exons. The three regions conserved in all RAF proteins (conserved region (CR) 1, CR2, and CR3) are shown in blue, green and yellow, respectively. The kinase domain is located in the CR3 domain. Six substitutions identified in CR3 are shown above. Filled circles indicate number of individuals having the substitution. Cancer-associated *BRAF* mutations are shown below the alignment of three RAF proteins[9,12]. Mutations detected in cancer are clustered in the glycine-rich loop and the activation segment of CR3 domain. The V600E mutation accounts for over 90% of the mutations in melanoma and thyroid cancer. Two mutations in the cysteine-rich domain were identified in five CFC individuals. Amino acids in CRAF and ARAF that are conserved in BRAF are shown by dots[13]. (**c**) RAS-ERK signaling pathway and associated disorders. RAS binds and stimulates RAF activation, which then activates MEK, which in turn activates ERK. ERK regulates gene expression and cytoskeletal rearrangements to coordinate the response to extracellular signals and regulate proliferation, differentiation, senescence and apoptosis[8,9]. Substitutions in *PTPN11*, *HRAS*, *KRAS* or *BRAF*, which potentially dysregulate the RAS-ERK signaling pathway, account for similar developmental disorders.

Q257R (770A→G), located in the cysteine-rich domain, were identified in five patients (**Fig. 1b** and **Table 1**). The identified eight substitutions were not found in 100 control chromosomes (data not shown). Mutation analysis in parents of five individuals (CFC76, CFC77, CFC96, CFC103 and CFC114) showed that these mutations occurred *de novo* (**Supplementary Fig. 2** online). The identified *BRAF* mutations were located in exons 6, 11, 12 and 14, and these domains were highly conserved in *CRAF* and *BRAF*. Sequencing of four corresponding exons in *CRAF*, ubiquitously expressed RAF, did not show any mutations in 24 individuals (data not shown).

KRAS and BRAF molecules are the key regulators of the RAS-RAF-MEK-ERK pathway, which is important for proliferation, growth and death of cells[9]. To elucidate critical steps, we examined the effect of the identified mutations on the RAS-ERK pathway by studying the activation of the ELK transcription factor. We transfected expression constructs (*KRAS* cDNA, NM_004985; *BRAF* cDNA, NM_00433) with a pFR-luc *trans*-reporter vector, a pFA2-ELK1 vector and a phRLnull-luc vector in NIH3T3 cells and determined their relative luciferase activity (RLA). We observed a significant increase in RLA in cells transfected with *KRAS* D153V but not in cells transfected with *KRAS* G60R (**Supplementary Fig. 3** online). We observed a two- to fourfold increase in RLA in cells transfected with two *BRAF* mutations (A246P and Q257R) in the cysteine-rich domain as well as in cells transfected with two *BRAF* mutations (L485S and K499E) in the kinase domain. We did not observe any significant increase in RLA in the other four mutations. Protein blotting showed that the wild-type and mutant proteins of *KRAS* and *BRAF* were equally expressed (data not shown). These results suggest that one *KRAS* and four *BRAF* mutants identified in CFC syndrome stimulated a common signaling pathway.

We identified substitutions of two proto-oncogenes, *KRAS* and *BRAF*, in 44% of individuals with CFC syndrome, suggesting that *KRAS* and *BRAF* have similar roles in human development. Controversy has existed as to whether CFC and Noonan syndromes are distinct disorders or different phenotypes of the same condition[2,10]. The clinical data of the 19 mutation-positive CFC individuals showed a high frequency of growth failure (78.9%), mental retardation (100%), relative macrocephaly (78.9%), characteristic facial appearance, including bitemporal constriction (84.2%) and downslanting palpebral fissures (94.7%), curly sparse hair (100%), heart defects (84.2%) and skin abnormalities (68.4%) (**Supplementary Table 1** online). This is in contrast with Noonan syndrome, in which there are lower frequencies of mental retardation (24–35%), heart defects (50–67%) and skin

Table 1 Mutations in 19 individuals with CFC syndrome

Individual	Gene	Exon	Nucleotide substitution	Amino acid change
CFC73	KRAS	2	178G→C	G60R
CFC8	KRAS	4b	458A→T	D153V
CFC91	KRAS	4b	458A→T	D153V
CFC100	BRAF	6	736G→C	A246P
CFC103	BRAF	6	736G→C	A246P
CFC16	BRAF	6	770A→G	Q257R
CFC24	BRAF	6	770A→G	Q257R
CFC96	BRAF	6	770A→G	Q257R
CFC76	BRAF	11	1406G→A	G469E
CFC81	BRAF	11	1406G→A	G469E
CFC94	BRAF	11	1406G→A	G469E
CFC114	BRAF	11	1406G→A	G469E
CFC83	BRAF	12	1455G→C	L485F
CFC79	BRAF	12	1495A→G	K499E
CFC77	BRAF	12	1501G→A	E501K
CFC90	BRAF	12	1502A→G	E501G
CFC105	BRAF	12	1502A→G	E501G
CFC95	BRAF	14	1741A→G	N581D
CFC110	BRAF	14	1741A→G	N581D

abnormalities (2–27%)[2]. Mutation analysis of *PTPN11* was negative in 43 CFC individuals. We did not identify any mutations in any exons of *KRAS* or in exons 6, 11, 12 and 14 of *BRAF* in 26 individuals with *PTPN11*-negative Noonan syndrome (data not shown), suggesting that Noonan syndrome and CFC syndrome are distinct clinical entities.

Comparison of manifestations between *KRAS*-positive and *BRAF*-positive individuals showed similar frequencies of growth and mental retardation, craniofacial appearance, abnormal hair and heart defects (**Supplementary Tables 2** and **3** online). However, we did observe a difference between the two groups in manifestations of skin abnormality, including ichthyosis, hyperkeratosis and hemangioma, which were observed in 13 *BRAF*-positive individuals. In contrast, no *KRAS*-positive individuals had these skin problems ($P < 0.05$). Somatic mutations in *BRAF* were identified in 60% of malignant melanoma or nevi[9], suggesting that BRAF has an important role in the skin. Comparison of manifestations between individuals with mutations that induced ELK transactivation and those with mutations that did not induce ELK transactivation showed no significant differences. Further analysis in a larger cohort would clarify the genotype-phenotype relationship in affected individuals.

The crystal structure of the BRAF kinase domain showed that the six *BRAF* mutations identified in this study are located in the interface of the ATP binding cleft, suggesting that these mutations may alter the catalytic activity of kinase domain (**Supplementary Fig. 4** online). Luciferase assays showed that two mutations (L485F and K499E) stimulated ELK-dependent transcription, suggesting that these mutants activated the ERK pathway. Missense mutations of *BRAF* were identified in approximately 7% of cancers, including human malignant melanoma and colorectal cancer[9]. The most frequent (>90%) V600E mutant showed elevated kinase activity, resulting in the activation of ERK and increased transformation activity[11]. Other less frequent mutations identified in cancer had either elevated or reduced kinase activity[9]. The four mutations identified in the kinase domain in our study did not enhance ELK-dependent transcription. This is in agreement with recent studies reporting that the activation of ERK or ELK transcription was not observed in cancer-associated mutations, including G469E (ref. 12). In cancer, *BRAF* mutations other than V600E are sometimes coincident with *RAS* mutations[9]. Other genetic background may contribute to the pathogenesis of CFC syndrome, although we did not detect any mutations in *KRAS*, *HRAS* or *NRAS* in *BRAF*-positive individuals. Further functional analysis of *BRAF* mutations will help elucidate the effects of these mutations on cell signaling.

The A246P and Q257R mutations are the first to be identified in the cysteine-rich domain in *BRAF*. This cysteine-rich domain is adjacent to the RAS-binding domain in conserved region 1 (ref. 13). A past study has suggested that the cysteine-rich domain of CRAF not only binds activated small GTPase RAS, but also inhibits basal catalytic RAF activity by direct or indirect interaction with the catalytic domain[14]. Our luciferase assay showed that these two mutations significantly activated ELK-dependent transcription, suggesting that they contribute to the activation of *BRAF*, leading to stimulation of the RAS-ERK pathway.

Previous clinical reports have shown that the association with cancers is rare in CFC syndrome[15]. This is in contrast with individuals with Costello syndrome, who have a higher risk of cancer, including rhabdomyosarcoma, ganglioneuroblastoma and bladder carcinoma[8]. It is of note that individual CFC94 with a *BRAF* G469E mutation had acute lymphoblastoid leukemia[15]. Careful observation of affected individuals would clarify the possible predisposition to hematopoietic malignancy in CFC syndrome as described in Noonan syndrome[4].

To the best of our knowledge, this is the first report of germline mutations in *KRAS* and *BRAF*. Our results suggest that mutations in human oncogenes (*HRAS*, *KRAS*, *BRAF* and *PTPN11*) that potentially dysregulate the RAS-MAPK pathway represent a common fundamental mechanism of related developmental disorders, namely, Noonan syndrome, Costello syndrome and CFC syndrome (**Fig. 1c**).

GenBank accession numbers. *KRAS* coding region, NC_000012; *HRAS* coding region, NC_000011; *NRAS* coding region, NC_000001; *BRAF*, NC_000007; *KRAS* cDNA, NM_004985; *BRAF* cDNA, NM_004333.

Note: Supplementary information is available on the Nature Genetics website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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SNP Communications

Three Novel Single Nucleotide Polymorphisms of the Human Thiopurine S-Methyltransferase Gene in Japanese Individuals

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Full text of this paper is available at http://www.jstage.jst.go.jp/browse/dmpk

Summary: In this study, the entire coding sequence and the exon-intron junctions of the *thiopurine S-methyltransferase* (*TPMT*) gene from 200 Japanese individuals were screened for mutation. Three novel single nucleotide polymorphisms (SNPs) were identified −106G>A in exon 3 (Gly36Ser, **20* allele), 967A>G in 3′-untranslated region, and −87C>T in intron 8. The allele frequencies were 0.003 for 106G>A, 0.003 for 967A>G, and 0.010 for IVS8 −87C>T. In addition, the three known SNPs, 474T>C (Ile158Ile), 719A>G (Tyr240Cys, **3C* allele), and IVS4 +35C>T were detected at frequencies of 0.299, 0.010, and 0.421, respectively.

Key words: thiopurine S-methyltransferase; genetic polymorphism; Japanese

Introduction

Thiopurine S-methyltransferase (TPMT) catalyses the S-methylation of thiopurine drugs such as 6-mercaptopurine and azathioprine[1,2]; these drugs are used to treat childhood leukemia patients and organ transplant recipients.[3] Recent studies have shown that the TPMT activity in human tissues is regulated by common genetic polymorphisms.[4-7] Genetic polymorphisms of the *TPMT* gene are thought to be mainly responsible for the large individual variations in toxicity and therapeutic effects.[8-10] To date, 21 variant *TPMT* alleles have been identified; these are, or may be, associated with decreased enzyme activity.[11] Four variant *TPMT* alleles, *TPMT*2* (238G>C), *TPMT*3A* (460G>A, 719A>G), *TPMT*3B* (460G>A), and *TPMT*3C* (719A>G), were detected in over 80% of Caucasian individuals with intermediate metabolizers or poor metabolizers.[12] On the other hand, we have reported that *TPMT*3C* is the most common allele in the Japanese population.[13]

In the present study, the entire coding sequence and the exon-intron junctions of the *TPMT* gene from 200 Japanese individuals were screened for mutation by using denaturing HPLC (DHPLC). Three novel single nucleotide polymorphisms (SNPs) of the *TPMT* gene, including a nonsynonymous polymorphism, were identified in Japanese individuals.

Materials and Methods

Venous blood was obtained from 200 unrelated healthy Japanese volunteers and patients admitted to Tohoku University Hospital. Written informed consent was obtained from all blood donors, and the study was approved by the Local Ethics Committee of Tohoku University Hospital and Tohoku Pharmaceutical University. DNA was isolated from anticoagulated (with K_2EDTA) peripheral blood by using QIAamp DNA Mini Kits (Qiagen, Hilden, Germany).

Table 1 lists the primer pairs that were used to amplify the entire coding sequence and exon-intron junctions of the *TPMT* gene. These primers were designed based on the genomic sequence reported in GenBank (AB045146). The amplicons for exons 3 and 6–10 were generated using the AmpliTaq Gold PCR Master Mix (Applied Biosystems, Foster City, CA, USA). PCR reactions were performed using an iCycler

On January 16, 2006, these SNPs were not found in dbSNP in National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/SNP/), GeneSNPs at the University of Utah Genome Center (http://www.genome.utah.edu/genesnps/). According to the allele nomenclature for *TPMT*, we named the novel nonsynonymous SNP (106G>A, Gly36Ser) as *TPMT*20*.

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Table 1. Amplification and DHPLC conditions for *TPMT* SNP analysis of genomic DNA

Exon	Size (bp)	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Annealing Temp. (°C)	PCR cycles	DHPLC Temp. (°C)
3	424	actgctaagaataataggttttcatttagtt	gccacagatgcactgtgactcgggag	50.0	35	54.8, 58.0
4[a]	479	taccactgactgggtgtgtgtctga	ctcaatccagaaagacttcatacctgtt	50.0	35	—
4[b]	177	cctcttcaggctattaaaga	ctcacatcctgttaaatcac	55.0	35	55.0
5[a]	379	cctgcatgttctttgaaaccctatgaa	taaataggaaccatcggacac	50.0	35	—
5[b]	185	gtatgattttatgcaggtttg	atatggatacaattatttac	50.0	35	54.6
6	179	cattatttcattacagagttcttcg	gtggatgttacacaggaggaagagag	Touchdown[c] 55.0–47.5	40	57.4
7	365	ataacagagtggggaggctgc	ctagaacccagaaaaagtatag	Touchdown[c] 55.0–47.5	40	55.7, 60.5
8	298	cccagcttaggcaggggccataa	ccaacaactttacctggatg	Touchdown[c] 55.0–47.5	40	56.1
9	330	agctaaagatggatatgatggggatgtaac	gaaatacaggcatgagccagcacgccaggc	50.0	35	56.0
10	401	aatccctgatgtcattcttcatagtattt	cacatcataatctcctctcc	50.0	35	53.5

[a]First-round PCR. [b]Second-round PCR.
[c]Touchdown protocol: The annealing temperature was decreased after cycle 10 by 0.5°C every 2 cycles beginning at 55°C and decreased to a "touchdown" annealing temperature of 47.5°C, which was then used for 30 cycles.

(Bio-Rad, Hercules, CA, USA). The PCR condition consisted of denaturation at 95°C for 10 minutes, followed by 35 or 40 cycles of denaturation at 95°C for 30 seconds, annealing for 30 seconds, and extension at 72°C for 30 seconds. The amplicons for exons 4 and 5 were generated with the AmpliTaq Gold PCR Master Mix. The first-round PCR conditions consisted of denaturation at 95°C for 10 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing for 30 seconds, extension at 72°C for 30 seconds, and a final extension at 72°C for 7 minutes. The PCR products amplified in the first round, diluted 1:10000 in water, were used as a DNA template for the second-round PCR. The second-round PCR conditions were the same as the first-round PCR conditions. The annealing temperatures and PCR cycles for the screening of *TPMT* variants are summarized in **Table 1**. Heteroduplexes were generated by thermal cycling as follows: 95°C for 1 minute, followed by a reduction in temperature from 95°C by 45 increments of 1.5°C per minute.

The PCR products were analyzed using the DHPLC system (WAVE®; Transgenomic Inc., Omaha, NE, USA). Unpurified PCR samples (5 μL) were separated on a heated C18 reverse-phase column (DNASep®) by using 0.1 M triethylammonium acetate (TEAA) in water and 0.1 M TEAA in 25% acetonitrile at a flow rate of 0.9 mL/min. The software provided with the instrument selected the temperature for the heteroduplex separation in the heterozygous *TPMT* fragment. **Table 1** summarizes the DHPLC running conditions for each amplicon. The linear acetonitrile gradient was adjusted to the retention time of the DNA peak at 4–5 minutes. Homozygous nucleotide exchanges can occasionally be distinguished due to a slight shift in the elution time when compared with the reference. The addition of an approximately equal amount of wild-type DNA to the samples (1:1) denaturation step enabled reliable detection of homozygous alterations in exons 4 and 7. This was performed for all samples in order to identify homozygous sequence variations. Therefore, all samples were first analyzed without mixing them with an equal amount of wild-type DNA; subsequently, wild-type DNA was mixed with each sample to detect homozygous variants. The resultant chromatograms were compared with the chromatograms of the wild-type DNA.

Both strands of the samples with variants that were detected using DHPLC were analyzed with a CEQ8000® automated DNA sequencer (Beckman-Coulter Inc., Fullerton, CA, USA). We sequenced the PCR products by the fluorescent dideoxy termination sequencing using the DTCS DNA Sequencing Kit (Beckman-Coulter Inc.).

Results and Discussion

We found the following three novel SNPs:

1) SNP: 050920Hiratsuka07; GENE NAME: *TPMT*; ACCESSION NUMBER: AB045146; LENGTH: 25 bases; 5′-AAGTGGGTGAACG/AGCAAGACTGC-TT-3′.

2) SNP: 050913Hiratsuka08; GENE NAME: *TPMT*; ACCESSION NUMBER: AB045146; LENGTH: 25 bases; 5′-TACTTTTTCTAAA/GAAAGTTTTAGAA-3′.

3) SNP: 050606Hiratsuka09; GENE NAME: *TPMT*; ACCESSION NUMBER: AB045146; LENGTH: 25

Table 2. Detected SNPs of the *TPMT* gene in 200 DNA samples of Japanese subjects

Location	Variant	Amino acid change	SNP ID dbSNP (NCBI)	N[a]	The number of each genotype	Observed frequency (%) (95%CI)	Frequency (%) predicted by Hardy-Weinberg law
Exon 3[b]	106G>A	Gly36Ser	—	192	G/G: 191	99.5 (98.5–100)	99.5
					G/A: 1	0.5 (0.0–1.5)	0.5
					A/A: 0	0.0 (0.0)	0.0
Exon 7	474T>C[c]	Ile158Ile	rs2842934	192	T/T: 90	46.9 (39.8–53.9)	49.1
					T/C: 89	46.4 (39.3–53.4)	42.0
					C/C: 13	6.8 (3.2–10.3)	9.0
Exon 10	719A>G	Tyr240Cys	rs1142345	193	A/A: 189	97.9 (95.9–99.9)	97.9
					A/G: 4	2.1 (0.1–4.1)	2.1
					G/G: 0	0.0 (0.0)	0.0
Exon 10[b]	967A>G	—	—	193	A/A: 192	99.5 (98.5–100)	99.5
					A/G: 1	0.5 (0.0–1.5)	0.5
					G/G: 0	0.0 (0.0)	0.0
Intron 4	IVS4 +35C>T[c]	—	rs4449636	197	C/C: 67	34.0 (27.4–40.6)	33.5
					C/T: 94	47.7 (40.7–54.7)	48.8
					T/T: 36	18.3 (12.9–23.7)	17.8
Intron 8[b]	IVS8 −87C>T	—	—	193	C/C: 189	97.9 (95.9–99.9)	97.9
					C/T: 4	2.1 (0.1–4.1)	2.1
					T/T: 0	0.0 (0.0)	0.0

[a]N: Number of samples actually analyzed among 200 samples. [b]Novel variations detected in this study.
[c]The exon 7 and intron 4 of variant *TPMT* alleles described in the reference sequence was T/C and C/T transition, respectively. However, these SNP informations obtained from NCBI SNP database was C/T and T/C transition.

bases; 5′-ATGCCACATCATC/TACCTATTTGGAT-3′.

The DHPLC analysis of the *TPMT* gene in the 200 DNA samples obtained from Japanese individuals revealed chromatographic profiles that were distinct from the wild-type in exons 3, 7, and 10 and the exon-intron junctions. We tested the specificity of DHPLC in detecting the variant allele in these exons and exon-intron junctions by comparing the results with those of direct sequencing. Six SNPs, including three novel SNPs, were detected (**Table 2**). The DHPLC chromatograms and the electrophoretograms of the novel nonsynonymous SNP are shown in **Figs. 1** and **2**, respectively. The SNP in exon 3 was 106G>A resulting in an amino acid change of Gly36Ser. According to the allele nomenclature for *TPMT*, we named the new sequence variant as *TPMT*20*. Among the 200 individuals, one was heterozygous for the 106G>A SNP, suggesting that the allele frequency was 0.003 in the Japanese population. The other novel SNPs, 967A>G and IVS8 −87C>T, were detected at frequencies of 0.003 and 0.01, respectively. The sequences for each sample were obtained from at least two different PCR amplifications.

The novel SNP 106G>A is located in exon 3 of the *TPMT* gene and results in the amino acid substitution (Gly36Ser) of N-terminal residues. The cleaved N-terminal residues as compared to full-length TPMT cause a dramatic reduction in enzyme activity.[14]

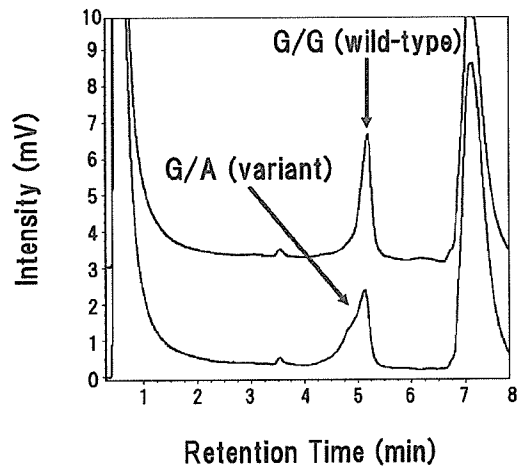

Fig. 1. DHPLC chromatograms of exon 3 of the human *TPMT* gene.
The elution profiles of heterozygous sequence variants are compared with a reference wild-type DNA chromatogram.

Recently, two variant alleles located in exon 3 of the *TPMT* gene, *TPMT*13* (83A>T, Glu28Val) and **17* (124C>G, Gln42Glu), have been clearly identified as being responsible for decreased enzyme activity.[15,16] *TPMT*13* reduced the level of protein expression in COS-1 cells as compared to the wild-type protein level.[15] *TPMT*17* was associated with decreased enzyme activity leading to an intermediate TPMT

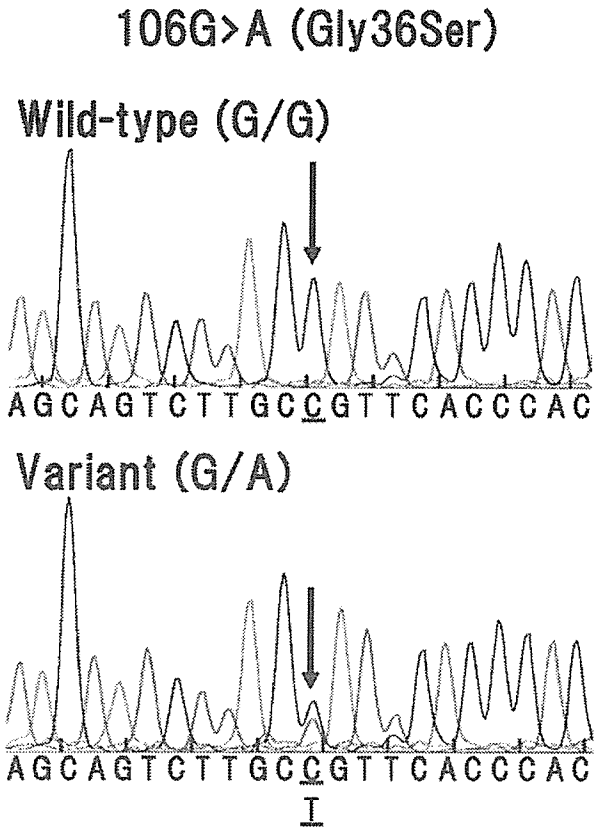

Fig. 2. The nucleotide sequences of the *TPMT* gene in exon 3.
Although the sequences are shown for the antisense strands, both strands were sequenced. Arrows indicate the variant nucleotide positions.

phenotype in heterozygous individuals.[16] The N-terminal residues of TPMT could be critical for the activity. The Gly36Ser substitution is closely located in the methyl donor S-adenosylmethionine binding site of N-terminal side.[17] Thus, it is expected to alter the catalytic properties of TPMT.

The other two novel SNPs were located in the intron or 3′-untranslated region. The biological significance of these two SNPs also remains to be evaluated. Further, the known nonsynonymous SNP 719A>G (Tyr240Cys, **3C* allele) was detected in 200 samples of Japanese subjects. For 719A>G, the frequency (0.01) in our study was comparable to that in Japanese individuals in previous studies.[13,18-21]

In conclusion, we found three novel SNPs of the *TPMT* gene, including a nonsynonymous polymorphism, in Japanese individuals. The nonsynonymous SNP was 106G>A in exon 3 resulting in an amino acid change of Gly36Ser. To date, many functional effects of human *TPMT* variant alleles have been found to alter the encoded amino acid sequence of the enzyme. Although we could not determine whether the SNP (106G>A) found in this study caused an allelic polymorphism associating with decreased enzyme activity and increased risk of thiopurine drugs toxicity, further studies on this are being conducted in our laboratory.

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Genetic polymorphisms and haplotype structures of the *CYP4A22* gene in a Japanese population☆

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Abstract

The CYP4A fatty acid monooxygenases oxidize endogenous arachidonic acid to 20-hydroxyeicosatetraenoic acid that acts as a regulator of blood pressure. Among the isoforms of the *CYP4A* subfamily, the human *CYP4A22* was recently identified. In this study, we report the comprehensive investigation of polymorphisms in the *CYP4A22* gene. To investigate genetic variation in *CYP4A22* in 191 Japanese subjects, we used denaturing HPLC (DHPLC) and direct sequencing. Our investigation has enabled the identification of 13 sequence variations in the *CYP4A22* coding region, thereby demonstrating for the first time that this gene is subject to polymorphism. Two of these sequence variations correspond to silent mutations located in exons 8 (His323His) and 9 (Gly390Gly). Nine of these sequence variations correspond to missense mutations located in exons 1 (Arg11Cys), 3 (Arg126Trp), 4 (Gly130Ser and Asn152Tyr), 5 (Val185Phe), 6 (Cys231Arg), 7 (Lys276Thr), 10 (Leu428Pro), and 12 (Leu509Phe). One of these sequence variations corresponds to nonsense mutations located in exon 9 (Gln368stop). The 13th mutation corresponds to a nucleotide deletion (G7067del) that causes a frameshift and consequently results in a stop codon 80 nucleotides downstream. In addition to the wild-type *CYP4A22*1* allele, 20 variants, namely *CYP4A22*2–15*, were characterized by haplotype analysis. Based on these data, we concluded that allelic variants of the human *CYP4A22* gene exist and speculated that some of these variants may be functionally relevant.

Keywords: *CYP4A22*; Genetic polymorphism; Haplotype; Denaturing HPLC

1. Introduction

Cytochrome P450s (CYPs) are heme-binding proteins that play an important role in the biotransformation of endogenous compounds and the detoxication of xenobiotics such as drugs and environmental contaminants. The endogenous substrates include numerous substances that are important for the maintenance of cellular homeostasis, such as steroids, retinoids, bile acids, fatty acid, and eicosanoids (e.g., prostaglandins and leukotrienes). Currently, 57 active *CYP* genes and 58 pseudogenes

☆ On September 12, 2005, these SNPs detected in this study were not present in dbSNP in the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/SNP/), GeneSNPs at the Utah Genome Center (http://www.genome.utah.edu/genesnps/), or the Human CYP Allele Nomenclature Committee database (http://www.imm.ki.se/CYPalleles/).

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