

SNP Communications

Two Novel Single Nucleotide Polymorphisms (SNPs) of the CYP2D6 Gene in Japanese Individuals

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Summary: We analyzed all the exons and exon-intron junctions of the *CYP2D6* gene from 286 Japanese individuals. We detected two novel single nucleotide polymorphisms (SNPs) 2556C>T in exon 5 (Thr261Ile) and 3835A>C in exon 8 (Lys404Gln). Both these SNPs showed a frequency of 0.002.

Key words: CYP2D6; genetic polymorphism; Japanese

Introduction

CYP2D6 metabolizes more than 50 clinically important drugs including some tricyclic antidepressants, neuroleptics, and β -adrenergic blockers.¹⁾ The *CYP2D6* gene locus is extremely polymorphic, with more than 80 allelic variants (<http://www.imm.ki.se/CYPalleles/cyp2d6.htm>). The homozygous of defective *CYP2D6* alleles, which result in the absence of CYP2D6 enzyme activity, are classified as poor metabolizer (PM) phenotypes. The frequency of PMs is 5%–10% in Caucasian population and less than 1% in Asian population.^{1–6)} Among the variant alleles reported to date, three alleles, *CYP2D6**3, *CYP2D6**4, and *CYP2D6**5, have been reported to account for approximately 95% of the alleles of PMs in Caucasian population.^{6,7)} However, as yet, PMs associated with CYP2D6 function in the

Japanese population could not be accounted for by the known variant alleles of *CYP2D6*.⁸⁾

In the present study, we analyzed all the exons and exon-intron junctions of the *CYP2D6* gene from 286 Japanese individuals by using denaturing HPLC (DHPLC). Additionally, we identified two novel SNPs of the *CYP2D6* gene in Japanese individuals.

Materials and Methods

Venous blood was obtained from 286 unrelated healthy Japanese volunteers and patients admitted to Tohoku University Hospital. Written informed consent was obtained from all the 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 K₂EDTA) peripheral blood by using QIAamp DNA Mini Kits (Qiagen, Hilden, Germany) accordance with the manufacturer's instructions.

Long PCR was performed in order to amplify the entire *CYP2D6* gene and to detect the *CYP2D6**5 allele using primer pairs (Table 1), as described by Johansson *et al.*⁹⁾ Genomic DNA (10–50 ng) was amplified using LA-Taq DNA polymerase (TaKaRa Co., Kyoto, Japan). The amplification was performed on an iCycler (Bio-Rad, Hercules, CA, USA). The resultant PCR products were a 5-kb fragment contained the entire *CYP2D6* gene and a 6-kb fragment that indicated the presence of the *CYP2D6**5 allele. The thermal profile consisted of denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for

On March 1, 2005, these SNPs were not found 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/>). *CYP2D6**53 is consisted of two variants which have already found in dbSNP in the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/SNP/>). However, this variant allele has not been registered found in the Human CYP Allele Nomenclature Committee database (<http://www.imm.ki.se/CYPalleles/>). The two *CYP2D6* haplotypes that possess each of 2556C>T (T261I) and 3835A>C (K404Q) were assigned as *CYP2D6**54 and *CYP2D6**55, respectively, by the Human CYP Allele Nomenclature Committee (<http://www.imm.ki.se/CYPalleles/>).

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Table 1. Primers used for the amplification of the entire *CYP2D6* gene and identification of the *CYP2D6*5* allele

	5' Primer	3' Primer	Annealing Temp. (°C)
entire <i>CYP2D6</i> gene	ccagaaggccttgaggcttca	actgagccctgggagtaggta	65.0
<i>CYP2D6*5</i> allele	gccactctcgtgtcgtcagcttt	ggcatgagctaaaggcacc	61.2

Table 2. Amplification and DHPLC conditions for *CYP2D6* SNP analysis of genomic DNA

Exon	Size (bp)	5' Primer	3' Primer	Predicted Temp. (°C)	DHPLC Temp. (°C)
1	280	gtgggggtgccagggtgtccagaggagcc	ggtaggggagcctcagcacctctgccgcc	63.4	63.4, 66.0
2	272	agtctggggtgatcctggcttgacaagagg	caccaccgggtcccacggaatctgtct	64.7	64.7, 67.0
3	253	gtggggctaatagccttcagccacgcgca	gtccccgcctcccagttcccgtttgtg	65.4	65.4
4	261	aaggcgggggacggggaaggcgaccctta	acctctcgggagctcgcctgcagagactc	65.4	64.4
5	277	ggtgaacgcagagcacaggaggattgaga	gggacgctcaaccaccaccttgcccc	63.2	62.2
6	242	atttggggctacccgttctgtcccaggt	cctgtacccttctcctcctggccccctgcac	63.6	62.6
7	287	gccggacccccctgggtgctgaccattgtg	tatcaccagggtgctgtgctgagctggggt	63.6	62.6
8	242	ccagcatcctagatccagtcaccactctc	cctgcaagactccacggaaggggacagggga	63.4	63.4
9	277	gggtatcaccaggagccaggctcactga	cattagacctctggctaggagcaggtctg	63.0	63.0

1 minute, annealing for 1 minute, extension at 68°C for 5 minutes, and a final extension at 72°C for 7 minutes. The annealing temperatures for long PCR summarized in **Table 1**.

All the *CYP2D6* specific products, diluted 1:10 in water, were used as a DNA template for a second round PCR of all the *CYP2D6* exons. **Table 2** lists the primer pairs that were used to amplify *CYP2D6* exons. These primers were designed based on the genomic sequence reported in GenBank (M33388). Amplicons were generated with the AmpliTaq Gold PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The thermal profile consisted of denaturation at 95°C for 10 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 30 seconds, and a final extension at 72°C for 7 minutes. 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®) 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 *CYP2D6* fragment.

Table 2 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 sometimes 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) prior to the denaturation step enabled homozygous alterations to be detected reliably. This was done routinely for all the samples in order to identify homozygous sequence variations. Therefore, all the samples were first analyzed without mixing 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 wild-type DNA.

Both strands of samples with variants detected using DHPLC were analyzed using a CEQ8000® automated DNA sequencer (Beckman-Coulter Inc., Fullerton, CA, USA). We also sequenced all the samples with chromatographic findings that differed from the wild-type DNA in order to establish links between mutations and specific profiles. We sequenced the PCR products by the fluorescent dideoxy termination sequencing using the DTCS DNA Sequencing Kit (Beckman-Coulter Inc.) accordance with the manufacturer's instructions.

For the haplotype analysis of *CYP2D6* variant alleles, the PCR products of entire *CYP2D6* genes were

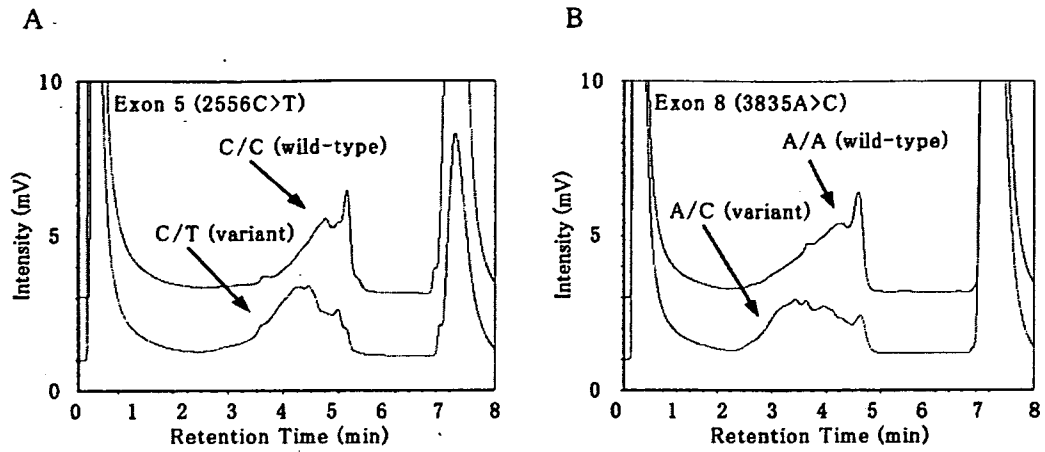
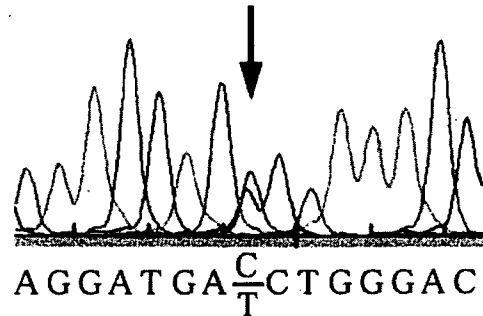
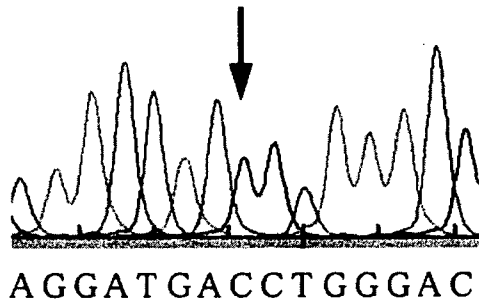


Fig. 1. DHPLC chromatograms of exon 5 (A) and exon 8 (B) of human *CYP2D6* gene. The elution profiles of heterozygous sequence variants are compared with a reference wild-type DNA chromatogram.

2556C>T
(Thr²⁶¹Ile)



3835A>C
(Lys⁴⁰⁴Gln)

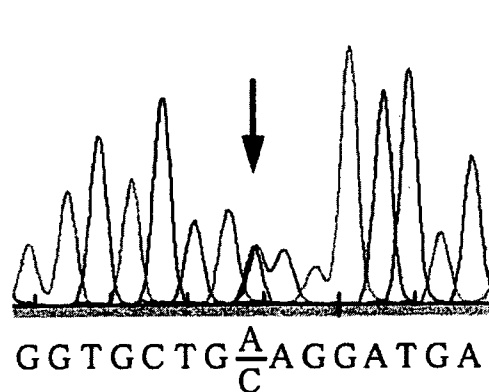
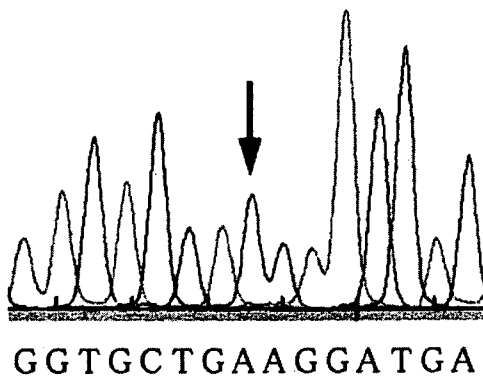


Fig. 2. The nucleotide sequences of the *CYP2D6* gene in exon 5 and exon 8. Although sequences are shown for sense strands, both strands were sequenced. Arrows indicate the variant nucleotide positions.

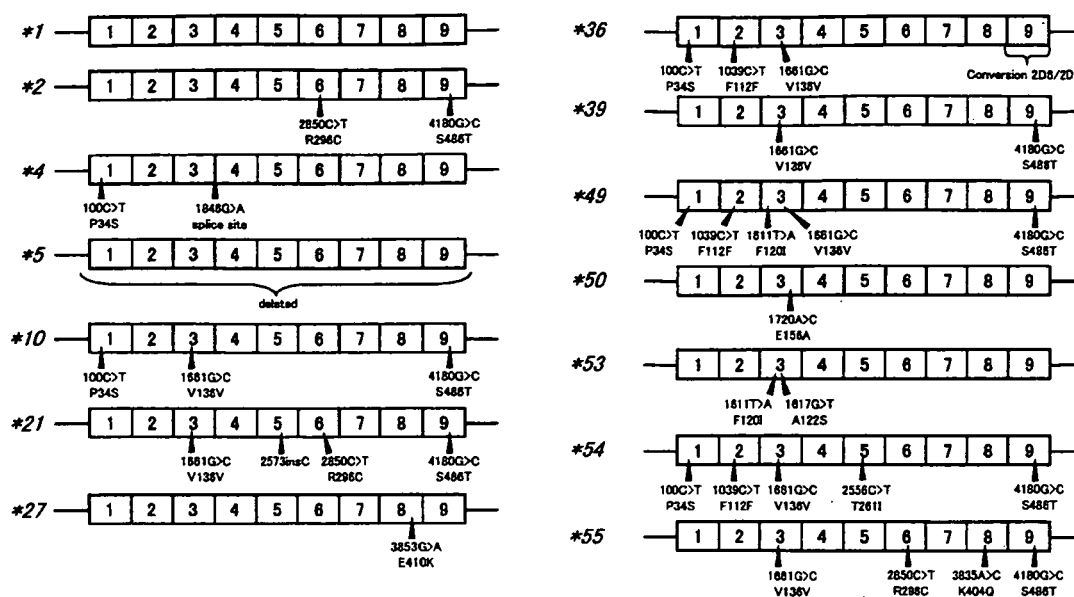


Fig. 3. Structure of *CYP2D6* alleles isolated from the Japanese individuals. The 9 exons of *CYP2D6* are indicated by numbered boxes. The positions of the various polymorphisms associated with each allele are indicated.

subcloned into a pCR[®]-XL-TOPO[®] vector (Invitrogen Co., CA, USA). The clones inserted into the *CYP2D6* fragments were sequenced using a CEQ8000[®] automated DNA sequencer.

Results and Discussion

We found two novel SNPs as follows:

- 1) SNP: 050301Hiratsuka04; GENE-NAME: *CYP2D6*; ACCESSION NUMBER: M33388; LENGTH: 25 bases; 5'-AGCACAGGATGAC/TCTGGGACCCAGC-3'.
- 2) SNP: 050301Hiratsuka05; GENE-NAME: *CYP2D6*; ACCESSION NUMBER: M33388; LENGTH: 25 bases; 5'-TCATCGGTGCTGA/CAGGATGAGGCCG-3'.

The DHPLC chromatograms and the electrophoretograms of the novel SNPs are shown in Figs. 1 and 2, respectively. The first SNP was 2556C>T in exon 5 resulting in an amino acid change of Thr261Ile. Haplotype analysis indicated that 100C>T, 1039C>T, 1661G>C, and 4180G>C existed in the same allele of the *CYP2D6* gene (Fig. 3). Of the 286 individuals, one was heterozygous for the 2556C>T SNP, suggesting that the allele frequency was 0.002 in the Japanese population. The second SNP was 3835A>C in exon 8 resulting in an amino acid change of Lys404Gln. Haplotype analysis indicated that 1661G>C, 2850C>T, and 4180G>C existed in the same allele of the *CYP2D6* gene (Fig. 3). Of the 286 individuals, one was heterozygous for the 3835A>C SNP, suggesting that the allele frequency was 0.002 in the Japanese population. The sequences for each sample were obtained from at least two different PCR amplifications.

These novel SNPs are located in the exons of the

CYP2D6 gene and result in amino acid substitutions. The Thr261 and Lys404 in *CYP2D6* are located in G-helix and Kⁿ-helix, respectively.¹⁰⁻¹² These amino acid residues are not mapped in substrate recognition site, but are conserved in the *CYP2D* subfamily in mammals.¹³ Thus, these amino acid substitutions, Thr261Ile and Lys404Gln, are expected to alter the catalytic properties of the *CYP2D6*. Further studies are required to elucidate the functional characteristics of these novel variant alleles of the *CYP2D6* gene.

In the present study, fourteen *CYP2D6* alleles were detected in all the 286 Japanese individuals. The most frequent variant allele was *CYP2D6**10, followed by *2, *5, and *21, and their frequencies were observed to be 0.362, 0.112, 0.072, and 0.007, respectively. The most frequent defective allele in the Japanese population is *CYP2D6**5, which is associated with the PM phenotype. To date, the non-functional alleles of *CYP2D6* that have been observed in Japanese population are *CYP2D6**4, *5, *14, *18, *21, and *44. However, none of the 286 individuals analyzed at least by DHPLC method in this study showed the presence of *CYP2D6**14, *18, or *44. This discrepancy may be caused by differences in sample sizes among these studies. Soyama *et al.*¹⁴ have recently reported the detection of five novel alleles, *CYP2D6**47, *48, *49, *50, and *51. In the present study, *CYP2D6**49 and *50 alleles were also found, with frequencies of 0.003 and 0.002, respectively.

The 23 different genotypes found in this study are listed together with their respective frequencies in Table 3. The most frequent genotypes were *CYP2D6**1/*10, *1/

Table 3. *CYP2D6* genotypes among the Japanese individuals

<i>CYP2D6</i> genotype	No. of subjects (n = 286)	Observed frequency (%) (95%CI)	Frequency (%) predicted by Hardy-Weinberg law
<i>CYP2D6</i> *1/*1	51	17.8 (13.4–22.3)	18.2
<i>CYP2D6</i> *1/*2	32	11.2 (7.5–14.8)	9.7
<i>CYP2D6</i> *1/*4	1	0.3 (0–1.0)	0.1
<i>CYP2D6</i> *1/*5	18	6.3 (3.5–9.1)	6.1
<i>CYP2D6</i> *1/*10	85	29.7 (24.4–35.0)	30.9
<i>CYP2D6</i> *1/*21	2	0.7 (0–1.7)	0.6
<i>CYP2D6</i> *1/*27	1	0.3 (0–1.0)	0.1
<i>CYP2D6</i> *1/*36	1	0.3 (0–1.0)	0.1
<i>CYP2D6</i> *1/*49	1	0.3 (0–1.0)	0.3
<i>CYP2D6</i> *1/*53	1	0.3 (0–1.0)	0.1
<i>CYP2D6</i> *2/*2	3	1.0 (0–2.2)	1.3
<i>CYP2D6</i> *2/*5	3	1.0 (0–2.2)	1.6
<i>CYP2D6</i> *2/*10	22	7.7 (4.6–10.8)	8.2
<i>CYP2D6</i> *2/*21	1	0.3 (0–1.0)	0.2
<i>CYP2D6</i> *2/*54	1	0.3 (0–1.0)	0.04
<i>CYP2D6</i> *5/*5	2	0.7 (0–1.7)	0.5
<i>CYP2D6</i> *5/*10	15	5.2 (2.7–7.8)	5.2
<i>CYP2D6</i> *5/*39	1	0.3 (0–1.0)	0.05
<i>CYP2D6</i> *10/*10	41	14.3 (10.3–18.4)	13.1
<i>CYP2D6</i> *10/*21	1	0.3 (0–1.0)	0.5
<i>CYP2D6</i> *10/*39	1	0.3 (0–1.0)	0.3
<i>CYP2D6</i> *10/*49	1	0.3 (0–1.0)	0.3
<i>CYP2D6</i> *50/*55	1	0.3 (0–1.0)	0.0006

*1, *10/*10 and *1/*2, which were present in 29.7%, 17.8%, 14.3%, and 11.2% of the Japanese individuals, respectively. According to previous reports, the incidence of the *CYP2D6* PM in the Japanese population has been estimated to be 0.87%.⁸⁾ We estimated the frequencies of *CYP2D6**4, *5, *14, *18, *21 and *44 to be 0.52%, calculated from the sum of frequencies obtained from six reports.^{8,14–18)} If the two novel SNPs cause PM, by adding the frequencies of these SNPs to the previous data, the PM frequency increased from 0.52% to 0.58%, which accounted for approximately 67% of PMs.

In conclusion, we found two novel nonsynonymous SNPs of *CYP2D6* in Japanese individuals. Further studies are being conducted in our laboratory to establish whether the newly identified SNPs (Thr261Ile and Lys404Gln) affect the *CYP2D6* function.

References

- Zanger, U. M., Raimundo, S. and Eichelbaum, M.: Cytochrome P450 2D6: overview and update on pharmacology, genetics, biochemistry. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **369**: 23–37 (2004).
- Nakamura, K., Goto, F., Ray, W. A., McAllister, C. B., Jacqz, E., Wilkinson, G. R. and Branch, R. A.: Interethnic differences in genetic polymorphism of debrisoquin and mephenytoin hydroxylation between Japanese and Caucasian population. *Clin. Pharmacol. Ther.*, **38**: 402–408 (1985).
- Ishizaki, T., Eichelbaum, M., Horai, Y., Hashimoto, K., Chiba, K. and Dengler, H. J.: Evidence for polymorphic oxidation of sparteine in Japanese subjects. *Br. J. Clin. Pharmacol.*, **23**: 482–485 (1987).
- Horai, Y., Nakano, M., Ishizaki, T., Ishikawa, K., Zhou, H. H., Zhou, B. J., Liao, C. L. and Zhang, L. M.: Metoprolol and mephenytoin oxidation polymorphisms in Far Eastern Oriental subjects: Japanese versus mainland Chinese. *Clin. Pharmacol. Ther.*, **46**: 198–207 (1989).
- Sohn, D. R., Shin, A. G., Park, C. W., Kusaka, M., Chiba, K. and Ishizaki, T.: Metoprolol oxidation polymorphism in a Korean population: comparison with native Japanese and Chinese populations. *Br. J. Clin. Pharmacol.*, **32**: 504–507 (1991).
- Broly, F., Gaedigk, A., Heim, M., Eichelbaum, M., Morike, K. and Meyer, U. A.: Debrisoquine/sparteine hydroxylation genotype and phenotype: analysis of common mutations and alleles of *CYP2D6* in a European population. *DNA Cell Biol.*, **8**: 545–558 (1991).
- Dahl, M. L., Johansson, I., Palmertz, M. P., Ingelman-Sundberg, M. and Sjoqvist, F.: Analysis of the *CYP2D6* gene in relation to debrisoquin and desipramine hydroxylation in Swedish population. *Clin. Pharmacol. Ther.*, **51**: 12–17 (1992).
- Chida, M., Yokoi, T., Nemoto, N., Inaba, M., Kinoshita, M. and Kamataki, T.: A new variant *CYP2D6* allele (*CYP2D6**21) with a single base insertion in exon 5 in a Japanese population associated with a poor metabolizer phenotype. *Pharmacogenetics*, **9**: 287–293 (1999).
- Johansson, I., Lundqvist, E., Dahl, M. L. and Ingelman-Sundberg, M.: PCR-based genotyping for duplicated and deleted *CYP2D6* genes. *Pharmacogenetics*, **6**: 351–355 (1996).

- 10) Gotoh, O.: Substrate recognition sites in cytochrome P450 Family 2 (CYP2) proteins inferred from comparative analysis of amino acid and coding nucleotide sequences. *J. Biol. Chem.*, **267**: 83-90 (1992).
- 11) Lewis, D. F. V.: The CYP2 family: models, mutants and interactions. *Xenobiotica*, **28**: 617-661 (1998).
- 12) Smith, G., Modi, S., Pillai, I., Lian, L.Y., Sutcliffe, M. J., Pritchard, M. P., Friedberg, T., Roberts, G. C. K. and Wolf, C. R.: Determinants of the substrate specificity of human cytochrome P-450 CYP2D6: design and construction of a mutant with testosterone hydroxylase activity. *Biochem. J.*, **331**: 783-792 (1998).
- 13) Lewis, D. F. V., Eddershaw, P. J., Goldfarb, P. S. and Tarbit, M. H.: Molecular modeling of cytochrome P4502D6 (CYP2D6) based on an alignment with CYP102 structural studies on specific CYP2D6 substrate metabolism. *Xenobiotica*, **27**: 319-340 (1997).
- 14) Soyama, A., Kubo, T., Miyajima, A., Saito, Y., Shiseki, K., Komamura, K., Ueno, K., Kamakura, S., Kitakaze, M., Tomoike, H., Ozawa, S. and Sawada, J.: Novel nonsynonymous single nucleotide polymorphisms in the *CYP2D6* gene. *Drug Metab. Pharmacokin.*, **19**: 313-319 (2004).
- 15) Yokoi, T., Kosaka, Y., Chida, M., Chiba, K., Nakamura, H., Ishizaki, T., Kinoshita, M., Sato, K., Gonzalez, F. J. and Kamataki, T.: A new *CYP2D6* allele with a nine base insertion in exon 9 in a Japanese population associated with poor metabolizer phenotype. *Pharmacogenetics*, **6**: 395-401 (1996).
- 16) Hiratsuka, M., Agatsuma, Y., Omori, F., Narahara, K., Inoue, T., Kishikawa, Y. and Mizugaki, M.: High throughput detection of drug-metabolizing enzyme polymorphisms by allele-specific fluorogenic 5' nuclease chain reaction assay. *Biol. Pharm. Bull.*, **23**: 1131-1135 (2000).
- 17) Kubota, T., Yamaura, Y., Ohkawa, N., Hara, H. and Chiba, K.: Frequencies of *CYP2D6* mutant alleles in a normal Japanese population and metabolic activity of dextromethorphan *O*-demethylation in different *CYP2D6* genotypes. *Br. J. Clin. Pharmacol.*, **50**: 31-34 (2000).
- 18) Yamazaki, H., Kiyotani, K., Tsubuko, S., Matsunaga, M., Fujieda, M., Saito, T., Miura, J., Kobayashi, S. and Kamataki, T.: Two novel haplotypes of *CYP2D6* gene in a Japanese population. *Drug Metab. Pharmacokin.*, **18**: 269-271 (2003).

SNP Communications

A Novel Single Nucleotide Polymorphism of the Human Methylenetetrahydrofolate Reductase 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: The genetic polymorphisms of methylenetetrahydrofolate reductase (MTHFR) have been associated with increased toxicity of methotrexate (MTX), a folic acid antagonist that is widely used to treat cancer and immunosuppressive disorders such as rheumatoid arthritis. In this study, we analyzed all the exons and exon/intron junctions of the MTHFR gene from 200 Japanese individuals. We detected a novel single nucleotide polymorphism (SNP) 148C>T (Arg46Trp) in exon 1. The allele frequency of this polymorphism in the Japanese population appears to be extremely low (0.25%).

Key words: methylenetetrahydrofolate reductase; MTHFR; genetic polymorphism; pharmacogenetics; Japanese

Introduction

Methylenetetrahydrofolate reductase (MTHFR) is involved in maintaining folate and homocysteine homeostasis, and deficiencies of MTHFR are implicated in neurological and vascular diseases. To date, many severe mutations and polymorphisms have been identified in MTHFR gene.^{1–12} Severe MTHFR deficiency results in marked hyperhomocysteinemia and homocystinuria. Milder deficiencies of MTHFR are more common in the general population. Two common genetic polymorphisms in MTHFR, 677C>T (Ala222Val) and 1298A>C (Glu429Ala), are non-synonymous amino acid changes that have been associated with a decreased activity of MTHFR and increased levels of homocysteine.^{3,6,13} The MTHFR 677C>T variant allele has also been associated with increased toxicity of methotrexate (MTX),^{14–17} a folic acid antagonist that is widely used to treat cancer and immunosuppressive disorders such as rheumatoid arthritis. Individuals with homozygous 677TT or the heterozygous 677CT genotype present increased risk of

side effects following MTX therapy as compared with those having the wild type 677CC genotype.^{16,18–20} Studies on genetic variations in MTHFR would be useful to reduce the trial-and-error dosing and the risk of adverse drug reaction.

In the present study, we analyzed all the exons and exon/intron junctions of the MTHFR gene from 200 Japanese individuals by using denaturing HPLC (DHPLC). Additionally, we identified a novel non-synonymous SNP of the MTHFR gene located in exon 1.

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 the blood donors, and the study was approved by the Local Ethics Committee of Tohoku University Hospital and Tohoku Pharmaceutical University. DNA was isolated from K₂EDTA-anticoagulated peripheral blood by using QIAamp DNA Mini Kits (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions.

Table 1 lists the primer pairs that were used to amplify all exons and exon/intron boundaries of the MTHFR. These primers were designed based on the genomic sequence reported in GenBank (AY338232).

On June 11, 2005, these SNPs were not found in dbSNP in the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/SNP/>) or GeneSNPs at the Utah Genome Center (<http://www.genome.utah.edu/genesnps/>).

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

Exon	Amplified length (bp)	Forward primer (5' to 3')	Reverse primer (5' to 3')	DHPLC Temp. (°C)
1	350	GTGGCTGCCTGCCCCCTGATGCTCC	AGTTTGCTCCCCAGGCACCACCACT	61.5
2	353	CAGTGACGGATGGTATTTCTCCTGG	TACCAAGTGGCCTCCGGGAAAGCCAG	62.7
3	230	AGAAAGGGTCTCTGGAGGTTGGGTG	TCTGGGCATCTCAGCCTCCCTAGCT	62.2
4	310	TCGCCTTGAACAGGTGGAGGCCAGC	GTGCGAGGACGGTGCGGTGAGAGTG	62.3
5	360	AGGGTGGGAGACGGGCTGGCCAGCA	CAGCCGGGGCTGCTCTTGACCCTC	63.4
6	250	GCTTCCGGCTCCCTCTAGCCAATCC	CCCTCCCGCTCCCAAGAACAAAGAT	61.6
7	316	TGGCACTGCCCTCTGTCAGGAGTGT	ACAGCCCCGCAGCCTGGCCTGCAGCT	61.8
8	290	ACTCAGGGTGCCAAACCTGATGGTC	GAACCCACGGGTGCCGGTCAAGAGA	64.7
9	278	GGCTGCCAGTAGTCTGATACCTTAG	CTTGACAATGCCTAGCCCAGGCTAG	62.4
10	231	AGTGGGACTCCAGTTGTTCTTGGCC	TCCCTCCCCACGGTTTTCCAGGTGG	62.7
11	330	TTGCCTCTGTGTGTGTGCATGTG	TGTGGAGGAGGAAGCGGGACAGGA	62.0

Amplicons were generated with the AmpliTaq Gold PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The thermal profile consisted of denaturation at 95°C for 10 min, followed by 35–40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 7 min. Heteroduplexes were then generated by means of a thermal cycler as follows: 95°C for 1 min; 95°C, reducing at 1.5°C per 1 min, for 47 cycles.

The PCR products were denatured 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®) 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 MTHFR fragment. Table 1 summarizes the DHPLC running conditions for each amplicon. The linear acetonitrile gradient was adjusted so that the retention time of the DNA peaks was 4–5 minutes.

Both the strands of 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.) in accordance with the manufacturer's instructions.

For the haplotype analysis of MTHFR variant alleles, the PCR products including fragments from exon 1 to exon 4 of the MTHFR genes were subcloned into a pCR®XL-TOPO® vector (Invitrogen Co., CA, USA). The clones inserted into the MTHFR fragments were

sequenced using a CEQ8000® automated DNA sequencer.

Results and Discussion

We found the following novel SNP:

SNP: 050611Hiratsuka06; GENE NAME: MTHFR; ACCESSION NUMBER: AY338232; LENGTH: 25 bases; 5'-CATGAGAGACTCC/TGGGAGAAGATGA-3'.

The DHPLC analysis of the MTHFR gene (11 exons) in the 200 DNA samples from Japanese individuals revealed chromatographic profiles that were distinct from the wild type in exon 1. We tested the specificity of DHPLC in detecting the variant allele in the exon by comparing the results with those of direct sequencing. The DHPLC chromatograms and the electrophoretograms of the novel SNPs are shown in Figs. 1 and 2, respectively. The SNP in exon 1 was 148C>T resulting in an amino acid change of Arg46Trp. Haplotype analysis indicated that other SNPs did not exist in the same allele of the MTHFR gene (data not shown). Among the 200 individuals, one was heterozygous for the 148C>T SNP, suggesting that the allele frequency was 0.0025 in the Japanese population. The sequences for each sample were obtained from at least two different PCR amplifications.

The novel SNP 148C>T is located in exon 1 of the MTHFR gene and results in amino acid substitution. The N-terminal domain of MTHFR contains the flavin binding site and residues necessary to bind a folate substrate and catalyze its reduction. Furthermore, Arg46 is conserved in the MTHFR gene in humans, *Escherichia coli*, and *Salmonella typhimurium*.¹⁾ Thus,

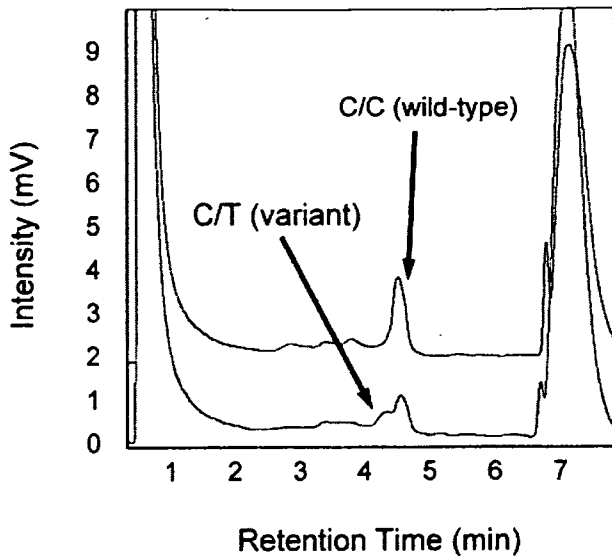
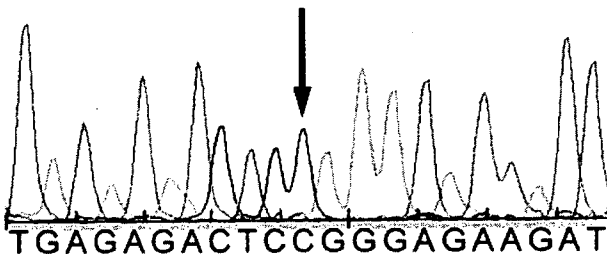


Fig. 1. DHPLC chromatograms of exon 1 of human MTHFR gene. The elution profiles of heterozygous sequence variants are compared with a reference wild-type DNA chromatogram.

148C>T, Arg46Trp

Wild-type (C/C)



Variant (C/T)

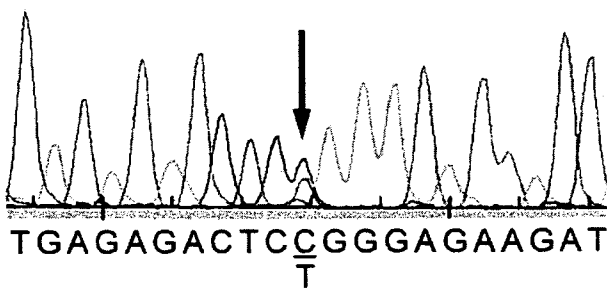


Fig. 2. The nucleotide sequences of the MTHFR gene in exon 1. Although sequences are shown for sense strands, both strands were sequenced. Arrows indicate the variant nucleotide positions.

the amino acid substitution is expected to alter the catalytic properties of the MTHFR gene. To date, many severe mutations and polymorphisms have been identified in the MTHFR gene.¹⁻¹²⁾ Although we could not determine whether the SNP (148C>T) found in this study caused a severe mutation leading to hyperhomocysteinemia or a polymorphism which in turn resulted in mild enzyme activity, further studies are being conducted in our laboratory to establish whether the newly identified SNP (148C>T, Arg46Trp) affect the MTHFR function.

In conclusion, we found a novel nonsynonymous SNP (148C>T) located in exon 1 of MTHFR gene in Japanese individuals. In this mutation, a substitution of Arg to Trp occurs at position 46 in the catalytic domain of MTHFR. The frequency of this mutation in the Japanese population appears to be extremely low (0.25%).

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References

- 1) Goyette, P., Sumner, J. S., Milos, R., Duncan, A. M., Rosenblatt, D. S., Matthews, R. G. and Rozen, R.: Human methylenetetrahydrofolate reductase: isolation of cDNA, mapping and mutation identification. *Nat. Genet.*, 7: 195-200 (1994).
- 2) Goyette, P., Frosst, P., Rosenblatt, D. S. and Rozen, R.: Seven novel mutations in the methylenetetrahydrofolate reductase gene and genotype/phenotype correlations in severe methylenetetrahydrofolate reductase deficiency. *Am. J. Hum. Genet.*, 56: 1052-1059 (1995).
- 3) Frosst, P., Blom, H. J., Milos, R., Goyette, P., Sheppard, C. A., Matthews, R. G., Boers, G. J., den Heijer, M., Kluijtmans, L. A., van den Heuvel, L. P. and Rozen, R.: A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. *Nat. Genet.*, 10: 111-113 (1995).
- 4) Goyette, P., Christensen, B., Rosenblatt, D. S. and Rozen, R.: Severe and mild mutations in cis for the methylenetetrahydrofolate reductase (MTHFR) gene, and description of five novel mutations in MTHFR. *Am. J. Hum. Genet.*, 59: 1268-1275 (1996).
- 5) Kluijtmans, L. A., Wendel, U., Stevens, E. M., van den Heuvel, L. P., Trijbels, F. J. and Blom, H. J.: Identification of four novel mutations in severe methylenetetrahydrofolate reductase deficiency. *Eur. J. Hum. Genet.*, 6: 257-265 (1998).
- 6) Weisberg, I., Tran, P., Christensen, B., Sibani, S. and Rozen, R.: A second genetic polymorphism in methylenetetrahydrofolate reductase (MTHFR) associated with decreased enzyme activity. *Mol. Genet. Metab.*, 64: 169-172 (1998).
- 7) Sibani, S., Christensen, B., O'Ferrall, E., Saadi, I., Hiou-Tim, F., Rosenblatt, D. S. and Rozen, R.: Charac-

- terization of six novel mutations in the methylenetetrahydrofolate reductase (MTHFR) gene in patients with homocystinuria. *Hum. Mutat.*, **15**: 280-287 (2000).
- 8) Homberger, A., Linnebank, M., Winter, C., Willenbring, H., Marquardt, T., Harms, E. and Koch, H. G.: Genomic structure and transcript variants of the human methylenetetrahydrofolate reductase gene. *Eur. J. Hum. Genet.*, **8**: 725-729 (2000).
 - 9) Tonetti, C., Amiel, J., Munnich, A. and Zittoun, J.: Impact of new mutations in the methylenetetrahydrofolate reductase gene assessed on biochemical phenotypes: a familial study. *J. Inher. Metab. Dis.*, **24**: 833-842 (2001).
 - 10) Rady, P. L., Szucs, S., Grady, J., Hudnall, S. D., Kellner, L. H., Nitowsky, H., Tyring, S. K. and Matalon, R. K.: Genetic polymorphisms of methylenetetrahydrofolate reductase (MTHFR) and methionine synthase reductase (MTRR) in ethnic populations in Texas; a report of a novel MTHFR polymorphic site, G1793A. *Am. J. Med. Genet.*, **107**: 162-168 (2002).
 - 11) Sibani, S., Leclerc, D., Weisberg, I. S., O'Ferrall, E., Watkins, D., Artigas, C., Rosenblatt, D. S. and Rozen, R.: Characterization of mutations in severe methylenetetrahydrofolate reductase deficiency reveals an FAD-responsive mutation. *Hum. Mutat.*, **21**: 509-520 (2003).
 - 12) Yano, H., Nakaso, K., Yasui, K., Wakutani, Y., Nakayasu, H., Kowa, H., Adachi, Y. and Nakashima, K.: Mutations of the MTHFR gene (428C>T and [458G>T+459C>T]) markedly decrease MTHFR enzyme activity. *Neurogenetics*, **5**: 135-140 (2004).
 - 13) van der Put, N. M., Gabreels, F., Stevens, E. M., Smeitink, J. A., Trijbels, F. J., Eskes, T. K., van den Heuvel, L. P. and Blom, H. J.: A second common mutation in the methylenetetrahydrofolate reductase gene: an additional risk factor for neural-tube defects? *Am. J. Hum. Genet.*, **62**: 1044-1051 (1998).
 - 14) Ulrich, C. M., Yasui, Y., Storb, R., Schubert, M. M., Wagner, J. L., Bigler, J., Ariail, K. S., Keener, C. L., Li, S., Liu, H., Farin, F. M. and Potter, J. D.: Pharmacogenetics of methotrexate: toxicity among marrow transplantation patients varies with the methylenetetrahydrofolate reductase C677T polymorphism. *Blood*, **98**: 231-234 (2001).
 - 15) Taub, J. W., Matherly, L. H., Ravindranath, Y., Kaspers, G. J., Rots, M. G. and Zantwijk, C. H.: Polymorphisms in methylenetetrahydrofolate reductase and methotrexate sensitivity in childhood acute lymphoblastic leukemia. *Leukemia*, **16**: 764-765 (2002).
 - 16) Chiusolo, P., Reddicono, G., Casorelli, I., Laurenti, L., Sora, F., Mele, L., Annino, L., Leone, G. and Sica, S.: Preponderance of methylenetetrahydrofolate reductase C677T homozygosity among leukemia patients intolerant to methotrexate. *Ann. Oncol.*, **13**: 1915-1918 (2002).
 - 17) Toffoli, G., Russo, A., Innocenti, F., Corona, G., Tumolo, S., Sartor, F., Mini, E. and Boiocchi, M.: Effect of methylenetetrahydrofolate reductase 677C>T polymorphism on toxicity and homocysteine plasma level after chronic methotrexate treatment of ovarian cancer patients. *Int. J. Cancer*, **103**: 294-299 (2003).
 - 18) Robien, K., Schubert, M. M., Bruemmer, B., Lloid, M. E., Potter, J. D. and Ulrich, C. M.: Predictors of oral mucositis in patients receiving hematopoietic cell transplants for chronic myelogenous leukemia. *J. Clin. Oncol.*, **22**: 1268-1275 (2004).
 - 19) Urano, W., Taniguchi, A., Yamanaka, H., Tanaka, E., Nakajima, H., Matsuda, Y., Akama, H., Kitamura, Y. and Kamatani, N.: Polymorphisms in the methylenetetrahydrofolate reductase gene were associated with both the efficacy and the toxicity of methotrexate used for the treatment of rheumatoid arthritis, as evidenced by single locus and haplotype analyses. *Pharmacogenetics*, **12**: 183-190 (2002).
 - 20) van Ede, A. E., Laan, R. F., Blom, H. J., Huizinga, T. W., Haagsma, C. J., Giesendorf, B. A., de Boo, T. M. and van de Putte, L. B.: The C677T mutation in the methylenetetrahydrofolate reductase gene: a genetic risk factor for methotrexate-related elevation of liver enzymes in rheumatoid arthritis patients. *Arthritis Rheum.*, **44**: 2525-2530 (2001).

Germline mutations in *HRAS* proto-oncogene cause Costello syndrome

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Costello syndrome is a multiple congenital anomaly and mental retardation syndrome characterized by coarse face, loose skin, cardiomyopathy and predisposition to tumors. We identified four heterozygous *de novo* mutations of *HRAS* in 12 of 13 affected individuals, all of which were previously reported as somatic and oncogenic mutations in various tumors. Our observations suggest that germline mutations in *HRAS* perturb human development and increase susceptibility to tumors.

Costello syndrome (OMIM 218040) is characterized by mental retardation, high birth weight, neonatal feeding problems, curly hair, coarse face, nasal papillomata and loose integuments of the back of the hands (Fig. 1a,b)^{1,2}. This syndrome phenotypically overlaps with Noonan syndrome (OMIM 163950). Missense mutations in *PTPN11*, encoding tyrosine phosphatase SHP-2, were identified in 50% of affected individuals with Noonan syndrome³ but not in those with Costello syndrome^{4,5}. Mutant SHP-2 proteins identified in Noonan syndrome were gain-of-function mutants with enhanced phosphatase activity, which resulted in activation of a RAS-MAPK (mitogen activated protein kinase) cascade in a cell-specific manner^{6,7}. We hypothesized that genes mutated in Costello syndrome and *PTPN11*-negative Noonan syndrome encode molecules that function upstream or downstream of SHP-2 in signal pathways (Fig. 1c). Among these molecules, we sequenced the entire coding regions of the four RAS genes⁸, *KRAS*, *HRAS*, *NRAS* and the recently identified *ERAS*, in genomic DNA from 13 individuals with Costello syndrome and 28 individuals with *PTPN11*-negative Noonan syndrome (Supplementary Methods online).

We identified four heterozygous mutations in *HRAS* in 12 individuals with Costello syndrome: 38G→A (G13D) in two individuals (COS30 and COS44), 34G→A (G12S) in seven individuals (COS35, COS38, COS64–COS66, COS68 and COS70), 35G→C (G12A) in two individuals (COS62 and 69) and 35GC→TT (G12V) in individual COS37 (Table 1). Individual COS37 died of severe cardiomyopathy (Supplementary Fig. 1 online). These mutations have been identified

somatically in various tumors⁸ (Fig. 1d,e). Mutation analysis of genomic DNA from two different tissues in three affected individuals and genomic DNA from parents in four families indicated that these 'oncogenic' and germline mutations occurred *de novo* (Supplementary Fig. 2 online). None of the above mutations were observed in 100 control chromosomes (data not shown). We observed no mutations in *KRAS*, *NRAS*, *HRAS* or *ERAS* in 28 individuals with Noonan syndrome or in one individual with Costello syndrome.

RAS genes encode 21-kDa proteins that are members of the superfamily of small GTP-binding proteins. Mutations at codons 12 and 13 are in constitutively active GTP-bound conformation and activate downstream effectors such as MAPK, PI-3 kinase and RalGDS⁹. Individuals with Costello syndrome often show hyperkeratosis of the skin, hypertrophic cardiomyopathy or relative macrocephaly, suggesting that the disorder is associated with altered cell growth, proliferation or cell death. Five of the seven affected individuals whom we examined had hypertrophic cardiomyopathy (Supplementary Table 1 and Supplementary Fig. 1 online). Cell proliferation studies showed that fibroblasts from four affected individuals had increased uptake of 5-bromodeoxyuridine compared with three independent controls when treated with 1 or 10 ng ml⁻¹ epidermal growth factor or 10% fetal calf serum (Fig. 1f). These results are indicative of increased growth factor-dependent proliferation, which might partially explain the mechanism of organ hypertrophy in Costello syndrome.

Costello syndrome is often associated with benign tumors, including papillomata, and cancers, including rhabdomyosarcoma, ganglioneuroblastoma and bladder carcinoma^{2,10}. The ganglioneuroblastoma of individual COS62 was surgically removed when she was two years and six months of age (Supplementary Fig. 1 online). We sequenced genomic DNA extracted from the tumor tissue and detected heterozygosity with respect to the wild-type (G) and mutated (C) alleles, excluding the possibility of loss of heterozygosity in this region (Fig. 1g). Sequencing of cDNA transcribed from ganglioneuroblastoma mRNA showed monoallelic expression of the mutated allele (C). In contrast, we observed biallelic expression in cDNA from her fibroblasts. This monoallelic expression in tumor, the mechanism of which remains to be elucidated, might be responsible for the pathogenesis of cancer development in Costello syndrome.

Since an oncogenic *HRAS* mutation was first identified in T24/EJ bladder carcinoma¹¹, mutations of *KRAS*, *HRAS* and *NRAS* have been found in ~30% of human cancers⁸. To the best of our knowledge, Costello syndrome is the first disorder associated with germline mutations in the RAS family of GTPases. Germline mutations in Costello syndrome were detected only in *HRAS*, and not in other RAS

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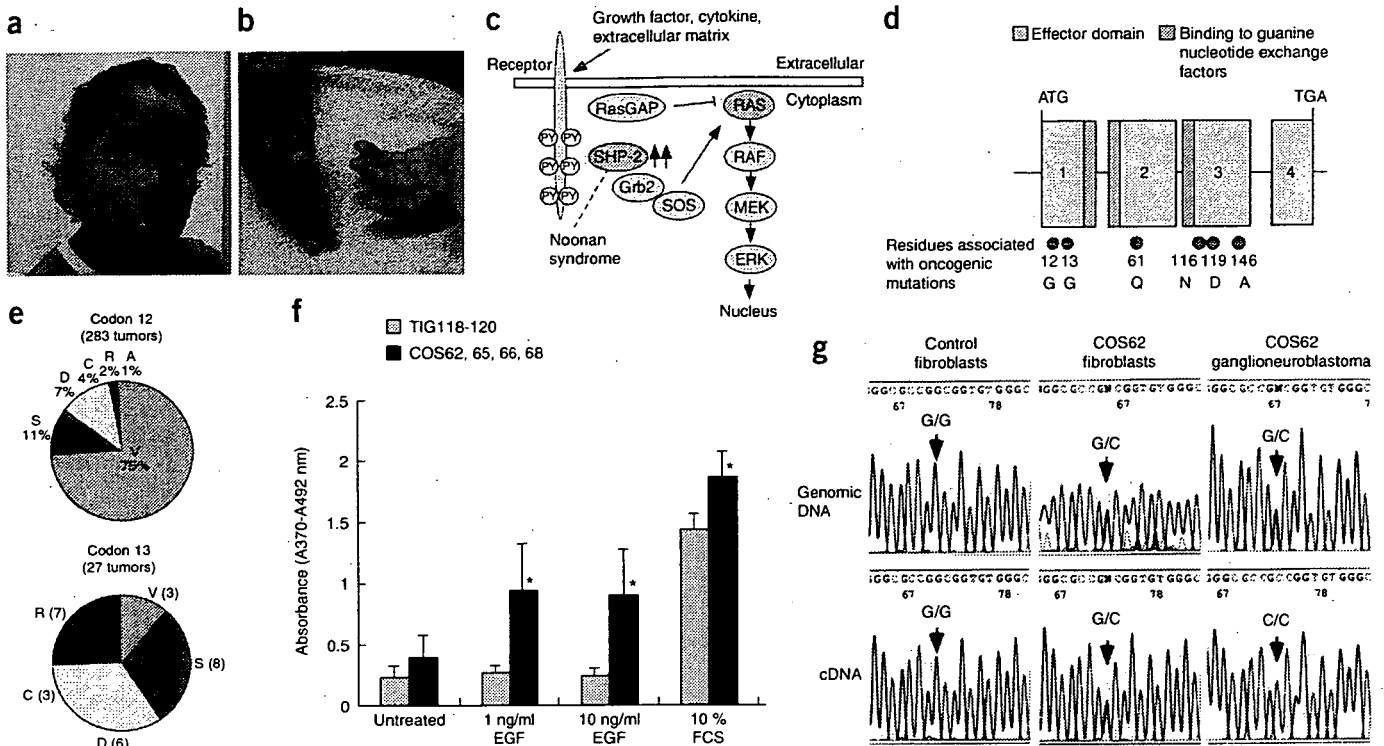


Figure 1 *HRAS* mutations in Costello syndrome. (a) Typical facial appearance and (b) loose skin and increased creases of the hands in individual COS35. (c) Signal transduction pathway including SHP-2 and RAS-MAPK. (d) Domain organization and genomic structure of *HRAS*. (e) The spectrum and relative frequency of amino acid substitutions at codons 12 and 13 of *HRAS* found in somatic tumors, according to the Sanger Institute Catalogue of Somatic Mutations in Cancer website. Parenthesis showed the number of tumors reported. (f) Increased 5-bromodeoxyuridine uptake in fibroblasts derived from affected individuals (black bars) when treated with 1 or 10 ng ml⁻¹ epidermal growth factor (EGF) or 10% fetal calf serum (FCS). 5-bromodeoxyuridine uptake was assayed by immunostaining with antibody to 5-bromodeoxyuridine-POD. Absorbance was read at dual wavelengths of 370 and 492 nm. **P* < 0.05. Results are expressed as the means ± s.d. from each cell line. TIG118-120, control fibroblasts. (g) Monoallelic RNA expression in ganglioneuroblastoma from individual COS62.

family genes, suggesting that *HRAS* has a distinct role from other RAS molecules in human development. This is in accordance with recent studies reporting embryonic lethality in *Kras*-knockout mice but not in *Hras1*- or *Nras*-knockout mice⁹. It is not known whether SHP-2 transmits its activating signals only to *HRAS* or to all RAS homologs. The overlapping phenotype between Noonan syndrome and Costello syndrome suggests that the activation of signals is transmitted from SHP-2 at least to *HRAS*. Increased activation of downstream pathways from *HRAS* may be a common pathogenic mechanism in Noonan syndrome and Costello syndrome.

The G12V mutant of *HRAS* had the lowest GTPase activity among various amino acid substitutions at codon 12 (ref. 12). Biological assays by focus formation in NIH3T3 cells or soft agar growth show that the substitution by valine at codon 12 has the highest transformation potential (G12V > G12S, G12A > G13D)^{13,14}. Individual COS37 with the G12V mutation died of severe cardiomyopathy at the age of 18 months. The most potent mutation, G12V, is predominant in human cancers (Fig. 1e). The germline mutations found in Costello syndrome seem to be less potent in general than the mutations identified in cancer.

Table 1 Mutations in *HRAS* in individuals with Costello syndrome

Individual	Origin	Material used for genotyping	Nucleotide substitution	Amino acid change	Genotype of father/mother
COS30	Japanese	Leukocytes	38G → A	G13D	NA/NA
COS35	Japanese	Leukocytes	34G → A	G12S	NA/NA
COS38	Japanese	Leukocytes, buccal cells	34G → A	G12S	WT/WT
COS62	Japanese	Lymphoblasts, fibroblasts	35G → C	G12A	WT/WT
COS37	Japanese	Leukocytes	35GC → TT	G12V	WT/WT
COS44	Japanese	Leukocytes	38G → A	G13D	NA/NA
COS64	Japanese	Leukocytes, buccal cells	34G → A	G12S	WT/WT
COS65	Italian	Fibroblasts	34G → A	G12S	NA/NA
COS66	Italian	Fibroblasts	34G → A	G12S	NA/NA
COS68	Italian	Fibroblasts	34G → A	G12S	NA/NA
COS69	Italian	Lymphoblasts	35G → C	G12A	NA/NA
COS70	Italian	Lymphoblasts	34G → A	G12S	NA/NA

NA, not available; WT, wild-type.

BRIEF COMMUNICATIONS

A tumor screening protocol for those with Costello syndrome has been proposed¹⁰. *HRAS* mutations alone are not sufficient to transform human cells¹⁵, and oncogenic RAS mutations seem to be one of the genetic events during multistep carcinogenesis. We hope our findings will provide a reliable means of diagnosing Costello syndrome and help to elucidate the precise clinical representations, including cancer development, response to therapy and overall natural history of affected individuals.

URL. The Sanger Institute Catalogue of Somatic Mutations in Cancer website is <http://www.sanger.ac.uk/cosmic/>.

Accession codes. GenBank: *KRAS* coding region, NC_000012; *HRAS* coding region, NC_000011; *NRAS* coding region, NC_000001; *ERAS* coding region, NC_000023; *HRAS* cDNA, NM_005343.

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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1. Costello, J.M. *N. Z. Med. J.* **74**, 397 (1971).
2. Hennekam, R.C. *Am. J. Med. Genet. C Semin. Med. Genet.* **117**, 42–48 (2003).
3. Tartaglia, M. *et al. Nat. Genet.* **29**, 465–468 (2001).
4. Troger, B. *et al. Am. J. Med. Genet. A* **121**, 82–84 (2003).
5. Tartaglia, M., Cotter, P.D., Zampino, G., Gelb, B.D. & Rauen, K.A. *Clin. Genet.* **63**, 423–426 (2003).
6. Niihori, T. *et al. J. Hum. Genet.* **50**, 192–202 (2005).
7. Tartaglia, M. *et al. Nat. Genet.* **34**, 148–150 (2003).
8. Bos, J.L. *Cancer Res.* **49**, 4682–4689 (1989).
9. Oliva, J.L. *et al. J. Biol. Chem.* **279**, 33480–33491 (2004).
10. Gripp, K.W. *et al. Am. J. Med. Genet.* **108**, 80–87 (2002).
11. Land, H. *et al. Science* **222**, 771–778 (1983).
12. Colby, W.W. *et al. Mol. Cell. Biol.* **6**, 730–734 (1986).
13. Seeburg, P.H. *et al. Nature* **312**, 71–75 (1984).
14. Fasano, O. *et al. Proc. Natl. Acad. Sci. USA* **81**, 4008–4012 (1984).
15. Hahn, W.C. *et al. Nature* **400**, 464–468 (1999).

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Functional analysis of PTPN11/SHP-2 mutants identified in Noonan syndrome and childhood leukemia

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Abstract Noonan syndrome (NS) is characterized by short stature, characteristic facial features, and heart defects. Recently, missense mutations of *PTPN11*, the gene encoding protein tyrosine phosphatase (PTP) SHP-2, were identified in patients with NS. Further, somatic mutations in *PTPN11* were detected in childhood leukemia. Recent studies showed that the phosphatase activities of five mutations identified in NS and juvenile myelomonocytic leukemia (JMML) were increased. However, the functional properties of the other mutations remain unidentified. In this study, in order to clarify the differences between the mutations identified

in NS and leukemia, we examined the phosphatase activity of 14 mutants of SHP-2. We identified nine mutations, including a novel F71I mutation, in 16 of 41 NS patients and two mutations, including a novel G503V mutation, in three of 29 patients with leukemia. Immune complex phosphatase assays of individual mutants transfected in COS7 cells showed that ten mutants identified in NS and four mutants in leukemia showed 1.4-fold to 12.7-fold increased activation compared with wild-type SHP-2. These results suggest that the pathogenesis of NS and leukemia is associated with enhanced phosphatase activity of mutant SHP-2. A comparison of

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the phosphatase activity in each mutant and a review of previously reported cases showed that high phosphatase activity observed in mutations at codons 61, 71, 72, and 76 was significantly associated with leukemogenesis.

Keywords Tyrosine phosphatase · SHP-2 · SHP2 · *PTPN11* · Noonan syndrome · Leukemia

Introduction

Noonan syndrome (NS; MIM#163950) is an autosomal dominant developmental disorder characterized by facial dysmorphism including hypertelorism, low-set ears and ptosis, short stature, skeletal abnormalities, and heart defects (Mendez and Opitz 1985; Allanson 1987). Frequently observed features in NS patients are pulmonary stenosis, hypertrophic cardiomyopathy, chest deformities, webbed and short neck, mental retardation, and genitourinary defects including cryptorchidism in males, and bleeding diathesis due to factor XI deficiency (Mendez and Opitz 1985). The incidence of this syndrome is estimated to be 1:1,000 to 1:2,500 live births (Allanson 1987). Tartaglia et al. (2001) have identified missense mutations in *PTPN11*, a gene encoding tyrosine phosphatase SHP-2, in 45% of clinically diagnosed NS patients. Patients with NS have been known to be associated with juvenile myelomonocytic leukemia (JMML), a myeloproliferative disorder characterized by excessive production of myelomonocytic cells (Tartaglia et al. 2003). Interestingly, somatic mutations in *PTPN11* were identified in 34% of JMML cells and in a small percentage of patients with myelodysplastic syndrome (MDS), acute myeloid leukemia (AML), and acute lymphoid leukemia (ALL) from non-NS-leukemic patients (Tartaglia et al. 2003; Loh et al. 2004; Tartaglia et al. 2004).

SHP-2, a widely expressed cytoplasmic tyrosine phosphatase, has been implicated in signal transduction pathways elicited by growth factors, cytokines, hormones, and extracellular matrix (Servidei et al. 1998; Feng 1999; Neel et al. 2003). One of the well-known pathways is the activation of the RAS/MAPK (mitogen-activated protein kinase) cascade induced by epidermal, fibroblast, and hepatocyte growth factors (Feng 1999; Neel et al. 2003). SHP-2 contains a tandem array of two SH2 domains at its N terminus, a catalytic domain in the middle, and a C-terminal domain that contains tyrosine phosphorylation sites. A crystallographic analysis indicates that intramolecular conformational change controls its catalytic activity (Hof et al. 1998). In the inactive state, the N-SH2 domain directly binds the PTP domain and blocks its catalytic activity. Once the N-SH2 domain binds phosphotyrosine peptide, conformational change results in the active state. Previously identified mutations in NS and leukemic patients were primarily restricted in or around the interacting face of the N-SH2 and PTP domains

(Kosaki et al. 2002; Musante et al. 2003; Sarkozy et al. 2003; Zenker et al. 2004), suggesting that they are gain-of-function mutations with enhanced phosphatase activity. Mutations identified in leukemia and NS occurred at the same amino acid residues, but the type of substitutions rarely overlapped.

Recent studies have shown that the phosphatase activities of five mutations were elevated and that the phosphatase activities of two mutations (D61Y and E76K) identified in JMML cells were higher than that of the N308D mutation identified in NS (Tartaglia et al. 2003; Fragale et al. 2004). In this study, we performed phosphatase assays for 14 mutants identified in NS and leukemia to examine the presence of any functional differences between the mutations. Finally, we examined the functional consequences of the mutations in cultured cells.

Materials and methods

Patients

Forty-one patients with NS were recruited. The diagnosis of NS was evaluated by clinical geneticists based on the cardinal criteria delineated by Allanson (1987); characteristic faces including hypertelorism, down-slanting palpebral fissures and ptosis, webbed or short neck, chest deformity, cubitus valgus, short stature, congenital heart defects, developmental delay, and cryptorchidism. Thirty-eight patients were sporadic cases, and three were familial cases. Bone marrow cells, peripheral blood, or cell lines were obtained from 29 leukemia cases without NS (seven ALL, 18 AML, one CML, two MDS, and one JMML). Eighteen AML patients were classified according to French-American-British classification (FAB); two with M0, one with M1, five with M2, three with M4, three with M5, one with M7, and three with unknown classification. This study was approved by the Ethics Committee of the Tohoku University School of Medicine.

Mutation analysis

After obtaining written informed consent, genomic DNA was isolated from the patients' peripheral leukocytes, Epstein-Barr virus-transformed lymphoblastoid cells, or leukemia cells. Fifteen coding exons of *PTPN11* from 41 NS patients and exons 3 and 13 from leukemia patients were sequenced. Each *PTPN11* exon with flanking intronic sequences was amplified with primers based on GeneBank sequences (GeneBank accession no. *NT 009775*). The M13 reverse or forward sequence was added to the 5' end of the polymerase chain reaction (PCR) primers for use as a sequencing primer. The PCR was performed in 30 μ l of a solution containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM dNTP, 10% (v/v) DMSO, 0.4 pmol of each

primer, 100 ng genomic DNA, and 2.5 U of Taq DNA polymerase. The reaction condition consisted of 35 cycles of denaturation at 94°C for 15 s, annealing at 57°C for 15 s, and extension at 72°C for 30 s. The products were gel-purified and sequenced on an ABI PRISM 310 automated DNA sequencer (Applied Biosystems, Fostercity, CA, USA).

Construction of the plasmids

The human SHP-2 cDNA was PCR amplified by using the following primers: the 5' primer included sequences encoding an *EcoRI* site followed by the FLAG M5 epitope (MDYKDDDDK) (Aoki et al. 2000). In addition, the 3' primer had an *EcoRI* site. The amplified fragment was digested with *EcoRI* and subcloned into an *EcoRI* site of pBluescript KSII+ (Stratagene). Mutant constructs were generated using a Quickchange site-directed mutagenesis kit (Stratagene). All mutant and normal constructs were verified by sequencing. The full-length SHP-2 cDNA and mutants were digested with *EcoRI* and subcloned into an *EcoRI* site of the pCAGGS expression vector, a mammalian expression vector that has a cytomegalovirus enhancer, and a chicken β -actin promoter (Niwa et al. 1991).

Immune complex tyrosine phosphatase assay

The COS7 cells were purchased from the American Tissue Culture Collection (ATCC). The COS7 cells were maintained in DMEM containing 10% FCS, 100 U/ml penicillin, and 100 μ g/ml streptomycin. Cells were seeded at 6×10^5 cells/6-cm dish, and 24 h later, 2.0 μ g of pCAGGS vectors encoding one of the wild-type (WT) or mutant SHP-2 cDNAs were transfected using 8 μ l of LipofectAMINE reagent and 12 μ l of PLUS reagent (Invitrogen, Carlsbad, CA, USA). The E76A mutant identified somatically in leukemia cells (Tartaglia et al. 2003) was used as the positive control in each assay because this mutant was proved to have an increased phosphatase activity in a *Xenopus* experiment (O'Reilly et al. 2000). Cells were serum starved for 24 h, scraped, and collected by centrifugation after two washes with phosphate buffered saline (PBS). Lysates were prepared in 1 ml ice-cold RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1:100 protease inhibitor, and 1% Triton X) and incubated on ice for 15 min. The lysates were centrifuged at 14,000 g for 15 min at 4°C, and 1 mg of protein was used for immunoprecipitation. The FLAG-tagged WT SHP-2, and mutants were immunoprecipitated with anti-FLAG M5 antibody (Sigma, St Louis, Mosby, USA) for 2 h at 4°C. Immune complexes were collected by adding 50 μ l of 50% protein G-Sepharose beads slurry (Amersham Biosciences Corp., Piscataway, NJ, USA) for 1 h at 4°C, washed twice with RIPA buffer, and then washed twice with phosphatase assay buffer (20 mM Hepes pH 7.5, 250 mM NaCl, 1 mM EDTA, 1 mM DTT).

Immune complex phosphatase assay was performed at 30°C for 30 min in 25 μ l phosphatase assay buffer supplemented with 500 μ M Src phosphopeptide (pTSTEPQpYQPGENL). The reaction was linear at the range of 0–1,000 μ M Src. After brief centrifugation, supernatants were collected, added to 100 μ l Malachite Green solution (Upstate Biotechnology), and incubated for 15 min at room temperature. The absorbance at 620 nm was measured, and the increase in the phosphatase activity was calculated by subtracting the background blank values (negative Src). Values for phosphate release were then obtained by comparing with the standard curve (linear at 0–2,000 pmol of phosphate). In order to measure the level of immunoprecipitated proteins, 50 μ l 2 \times SDS sample buffer was added to the beads and boiled for 3 min. Twenty μ l was used for SDS-PAGE followed by immunoblotting with anti-FLAG M5 antibody.

ERK (extracellular signal-regulated kinase) phosphorylation

Human embryonic kidney (HEK) 293 cells (ATCC) were maintained in DMEM containing 10% FCS, 100 U/ml penicillin, and 100 μ g/ml of streptomycin. Cells were seeded at 6×10^5 cells/6-cm dish, and 24 h later, 3.6 μ g of pCAGGS vectors encoding one of the WT or mutant SHP-2 cDNAs and 0.9 μ g of pcDNA3 containing HA-tagged extracellular signal-regulated kinase (ERK)2 cDNA were cotransfected using 8 μ l of LipofectAMINE reagent and 12 μ l of PLUS reagent. Two major mutants in NS, D61N and E76D, were used for this study. Three hours later, the medium was changed to DMEM containing 10% FCS. At 48 h after transfection, cells were unstimulated or stimulated with 20 ng/ml of epidermal growth factor (EGF) for 5, 30, 60, 90, and 120 min.

Immunoprecipitation of HA-ERK2 was performed, as described previously (Takeda et al. 1999). The 293 cells were washed twice with PBS, and immediately frozen in liquid nitrogen. The cells were lysed on ice in 0.3 ml of ice-cold RIPA buffer [20 mM Tris-HCl (pH 7.5), 140 mM NaCl, 2.6 mM CaCl₂, 1 mM MgCl₂, 1% (v/v) NP-40 containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 3 μ l phosphatase inhibitor (Sigma)]. The lysates were centrifuged at 14,000 g for 15 min at 4°C. For immunoprecipitation, the resultant supernatants were incubated with anti-HA antibody (clone12CA5, gifted by Dr. Matozaki) for 2 h at 4°C and then incubated with 30 μ l of protein G-Sepharose beads (Amersham) for 1 h on a shaker. The beads were washed twice with 800 μ l wash buffer [20 mM Tris-HCl (pH 7.5), 140 mM NaCl, 0.1% (v/v) Triton X-100] and boiled for 5 min in 2 \times SDS sample buffer. Immunoprecipitants were subjected to SDS-polyacrylamide gel electrophoresis (10% gel), transferred to nitrocellulose membrane, and probed with the indicated antisera. All the membranes were visualized using enhanced chemi-

luminescence (ECL) Western blotting Detection System Kit (Amersham Pharmacia Biotech, Chalfont, UK).

Reporter assay

One day before the transfection, the 293 cells were plated in 6-well plates with a density of 5×10^5 cells per well. Cells were transiently transfected using LipofectAMINE plus reagent with 1 μ g pSREluc, 0.1 μ g of pRLTKluc, and 1 μ g of SHP-2 wild or mutant expression constructs. Four mutants in the N-SH2 domain from NS patients were introduced in 293 cells. Eighteen hours after transfection, the cells were serum-starved in DMEM for 24 h. For EGF stimulation, cells were unstimulated or stimulated with 20 ng/ml of EGF for 5 h. Cells were harvested in passive lysis buffer, and luciferase activity was assayed using a dual luciferase assay kit (Promega, Madison, WI, USA). Renilla luciferase expressed from pRLTKluc was used to normalize the transfection efficiency. The experiments were performed in triplicate. Data are shown as mean \pm SEM. Statistical analysis was performed with the Statview 4.0 package (AVACUS Corporation, Berkeley, CA, USA).

Results

Mutation analysis in patients with NS

Sequencing analysis in 41 affected individuals with NS revealed nine different missense mutations in 16 patients (15 sporadic cases and one index case of familial origin) (Table 1). Ten patients had mutations in the N-SH2 domain (Y63C in three patients; D61N, Q79R, and T73I in two patients each; and F71I in one patient). The novel F71I mutation identified in patient 6 was inherited from his father, who exhibited only ptosis. The F71I mutation was not detected in 83 controls (data not shown), suggesting the mutation was not likely to be a polymorphism. Although the D61N, Y63C, and Q79R mutations were detected in more than two patients, their clinical findings, including facial anomalies and heart defects, varied significantly. Patient 8, who had the T73I mutation, exhibited JMML at 2 months of age. Her leukocytosis persisted for 20 years after remission. Four mutations in the PTP domains were detected in six patients. Interestingly, patient 15 with the S502T mutation had an episode of transient abnormal myelopoiesis at 3 months of age and was associated with neuroblastoma at 6 months, suggesting the extending characteristics of the mutation (Kondoh et al. 2003). The analysis of her parents' DNA indicated the de novo mutation.

Phosphatase activities of mutants identified in NS

Structural analysis suggested that mutant proteins have an altered phosphatase activity. We transfected ten

SHP-2 mutants, including nine mutants identified in NS patients in this study, and the E76A mutant as the positive control. Remarkable activation (six-fold to 12-fold compared with the activity of WT) was observed in the immune complexes derived from cells expressing the constructs carrying the D61N, F71I, or T73I mutation located in the N-SH2 domain (Fig.1). The activities of the D61N and F71I mutants were higher than that of the E76A mutant. Mild activation (two-fold to 3.9-fold) was observed in the immune complexes derived from cells transfected with constructs harboring the Y63C or Q79R mutation in the N-SH2 domain and constructs with the I282V, N308D, S502T, or M504V mutation in the PTP domain. Interestingly, all nine mutations had activated phosphatase activity.

PTPN11 mutation detection and phosphatase assay in childhood leukemia

In order to screen *PTPN11* mutation in leukemia cells, we sequenced exons 3 and 13 in the *PTPN11* gene from genomic DNA extracted from 29 leukemic children. Two mutations were detected in three cases (Fig.2). The A72V mutation was identified in bone marrow from one case with AML (M0 according to FAB classification) and in bone marrow from one patient with MDS (refractory anemia with excess blasts) (Monosomy 7). The novel G503V (1508G > T) mutation was detected in a bone-marrow-derived cell line from a JMML patient. The phosphatase activity in an immune complex precipitated from cells overexpressing the A72V mutant showed a 5.2-fold increase and that from cells overexpressing the G503V mutant showed a 1.4-fold increase compared with the WT cDNA (Fig.2b).

In order to compare the phosphatase activity of mutations found in NS and leukemia at the same residue, we examined the phosphatase activity of two mutants occurring at D61 and E76. The activity of D61N (NS) was equivalent to that of D61Y (leukemia) (Fig.2c). In contrast, the activity of E76A (leukemia) was 4.1-fold that of E76D (NS) (Fig.2d).

Effects of mutants on MAPK pathway

Since SHP-2 has been known essentially as a positive regulator of the RAS/MAPK pathway (Feng 1999), we examined whether mutants activated ERK, a kind of MAPK, in the presence of EGF. In the absence and presence of EGF, the ERK activation was examined using an anti-phospho-ERK antibody that detected phosphorylated ERK. In cells expressing WT SHP-2 and ERK plasmids, the phosphorylation of ERK2 was maximum at 5 min after EGF treatment, and sustained activation persisted for 120 min (Fig.2a). In cells expressing the D61N or the E76D mutant, the maximum activation of ERK2 at 5 min was almost the same as in WT-transfected cells, but the activation decreased more

Table 1 Clinical findings and *PTPNI1* mutations in our Noonan syndrome (NS) patients. *ASD* atrial septal defect, *PDA* patent ductus arteriosus, *PS* pulmonary stenosis, *VSD* ventricular septal defect, *PR* pulmonary valve regurgitation, ? unknown, *JMML* juvenile myelomonocytic leukemia, *NB* neuroblastoma, *TAM* transient abnormal myelopoiesis, *N-SH2* aminoterminal *SH2*, *C-SH2* carboxyl terminal *SH2*, *PTP* protein tyrosine phosphatase

Patient	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Gender	M	M	M	F	F	M	F	F	F	M	M	M	M	F	M	F
Age	12 years	6 years	14 years	1 years 9 Months	2 years	9 Months	11 years	19 years	8 years	22 years	3 years	14 years	7 years	12 Months	8 years	3 years
Short stature (Standard deviation)	+	+ (-3.2)	+ (-2.5)	+	- (-2.0)	- (-1.6)	+	+	- (-1.1)	- (-1.6)	- (-1.6)	+	+ (-2.5)	+ (-3.3)	+ (-2.4)	- (-1.5)
Facial anomalies																
Hypertelorism	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+
Ptosis	+	-	+	-	+	+	-	-	-	-	-	+	?	-	-	-
Down-slanting palpebral fissures	+	+	-	+	+	+	+	-	+	-	-	+	+	+	+	+
Low-set ears	-	+	-	+	-	+	+	-	+	-	-	-	+	+	-	+
Webbed neck	+	+	+	+	-	+	+	-	+	-	+	+	?	+	+	-
Cubitus valgus	+	-	+	+	+	-	+	-	+	-	+	+	?	+	+	-
Cardiovascular anomalies	ASD	-	-	PDA	PS ASD	ASD	PS ASD	-	PS	PS	PS	PS	PS ASD	PS ASD	PS PR	PS ASD HCM
Cryptorchidism?	-	-	+	-	-	Hydrocele	+	-	-	+	+	-	-	-	-	+
Mental retardation	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
Miscellaneous	Bleeding diathesis					Familial	Bleeding diathesis	JMML					Macrocephaly	NB TAM	Skin pigmentation	Large tongue
<i>PTPNI1</i> mutation																
Exon	3	3	3	3	3	3	3	3	3	3	7	7	8	13	13	13
Nucleotide substitution	181G>A	181G>A	188A>G	188A>G	188A>G	211T>A	218T>C	218T>C	236A>G	236A>G	844A>G	844A>G	844A>G	922A>G	1504T>A	1510A>G
Amino acid substitution	D61N	D61N	Y63C	Y63C	Y63C	F71I	T73I	T73I	Q79R	Q79R	I282V	I282V	I282V	N308D	S502T	M504V
Domain	N-SH2	N-SH2	N-SH2	N-SH2	N-SH2	N-SH2	N-SH2	N-SH2	N-SH2	N-SH2	PTP	PTP	PTP	PTP	PTP	PTP

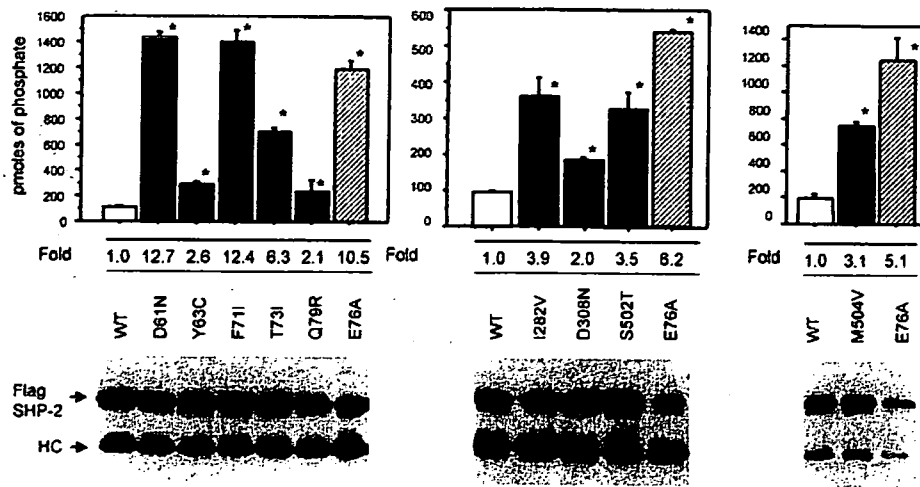


Fig. 1 Immune complex phosphatase assay of wild-type (WT) cDNA and mutants identified in Noonan syndrome (NS). The E76A mutant was used as a positive control in each assay. Results are expressed as mean \pm SD obtained from three independent experiments. Asterisks indicate statistically significant differences ($p < 0.01$) in activity compared with that of WT. Fold activation was expressed as 1 for WT cDNA at each assay (upper panel). Immunoblot analysis of SHP-2 showed the comparative efficiency of immunoprecipitation (lower panel). HC heavy chain

rapidly. These results suggest that these two mutants do not promote the activation of the MAPK pathway in EGF-treated 293 cells.

In order to examine the downstream signaling pathway of ERK, we examined the activation of the serum-responsive element (SRE) within *c-fos* or other immediate early genes in EGF-treated 293 cells. As a key target of MAPK, ELK1 transcription factors phosphorylated by ERK bind to the SRE to initiate transcription (Bennett et al. 1996; Hakak and Martin 1999). Cells expressing the SRE-luciferase reporter gene and a catalytically inactive SHP-2 (C459S) showed a decreased induction of the luciferase gene in the presence of EGF (Fig. 2b). Cotransfection of plasmids harboring mutants detected in NS had neither enhanced SRE activity in the unstimulated state nor enhanced fold induction in the presence of EGF. These results suggest that the SHP-2 mutants did not promote the MAPK pathway in the EGF-treated 293 cells whereas they showed enhanced phosphatase activity in vitro.

Discussion

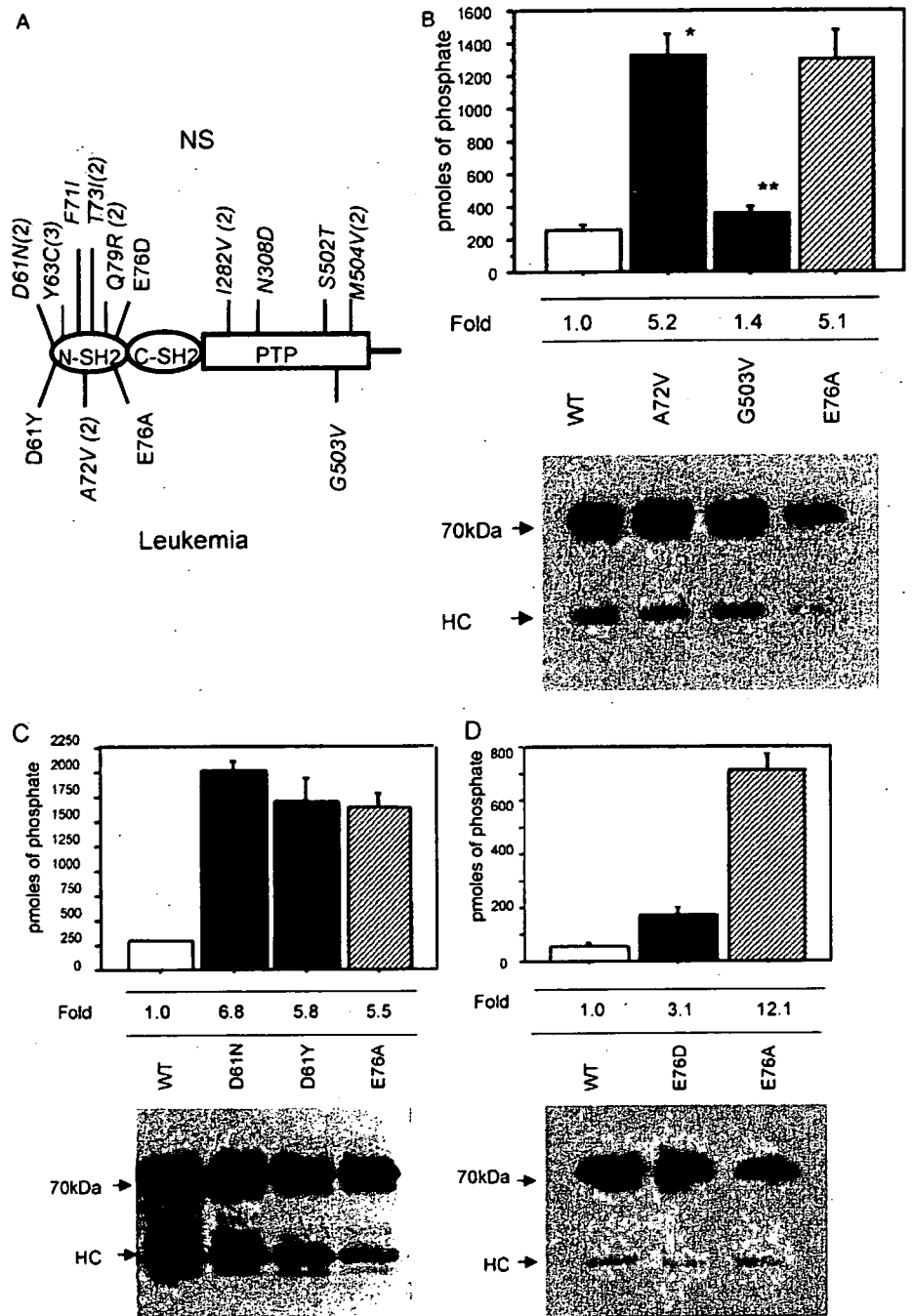
In this study, we identified nine mutations, including a novel F71I mutation, in 16 of 41 Japanese patients with NS and two mutations, including a novel G503V mutation, in three of 29 patients with childhood leukemia. Immune complex phosphatase assays showed that the phosphatase activity in ten SHP-2 mutants detected in NS and four SHP-2 mutants detected in leukemia was elevated to various degrees. Mutations in the *PTPN11* gene were detected in 39% of the patients clinically diagnosed with NS. This result is comparable to that of

previous studies (Tartaglia et al. 2002; Musante et al. 2003). Previous genotype-phenotype correlation studies have shown that pulmonary stenosis was associated with *PTPN11* mutations whereas cardiomyopathy was more common in patients without *PTPN11* mutation (Tartaglia et al. 2002; Zenker et al. 2004). In our study, however, no significant difference was observed (data not shown). Although other abnormalities than exonic mutations in *PTPN11* have not been excluded, further studies to identify the gene(s) associated with NS will pose a formidable challenge in establishing the clinical entity of NS.

The role of *PTPN11* mutations in the pathogenesis of hematologic malignancy remains to be elucidated. In the previous and the present studies, 87% of the mutations found in leukemia cells were identified in the N-SH2 domain, and these were primarily restricted to residues D61, A72, and E76 (Tartaglia et al. 2003; Loh et al. 2004; Tartaglia et al. 2004). To examine whether the degrees of phosphatase activity in mutations at specific residues are associated with leukemia, we compared the phosphatase activity at each affected residue examined in this study, as well as the published phenotype (Table 2a). Variation of each assay was normalized as relative phosphatase activity; activity of WT cDNA was taken as one-fold, and that of E76A was taken as ten-fold. Mutations at codons 61, 71, 72, and 76, in which highest activity at each codon was ten or more, were frequently identified in patients with leukemia, including those with JMML, MDS, AML, and ALL (Table 2a). In NS patients, activities of the most common mutations at N308 and Y63 were two-fold to three-fold. The phosphatase activity of the T73I mutation, which is frequently detected in NS patients associated with JMML, showed an intermediate value of 6.0. Mutations at residues in which activities were below six were rarely detected in leukemia cells. These results suggest that mutations at codons 61, 71, 72, and 76, in which highest activity at each codon was ten or more, were associated with leukemia (Table 2b; $\chi^2 = 97.0$, $p < 0.001$).

Tartaglia et al. (2003) recently showed that the phosphatase activities of two mutants (D61Y and E76K)

Fig. 2 Phosphatase activity of mutants detected in childhood leukemia. **a** Localization of SHP-2 mutations analyzed in this study. *Italic*, mutations identified in this study. *Parenthesis*, number of patients identified. **b** Immune complex phosphatase assay using SHP-2 mutants identified in leukemia. Results are expressed as mean \pm SD obtained from the three independent experiments. *Asterisks* indicate statistically significant differences in activity compared with that of wild type (WT) (* $p < 0.01$, ** $p < 0.05$). **c, d** Comparison of phosphatase activity in mutants derived from either NS or leukemia at amino acid positions 61 and 76. Results are expressed as mean \pm SD obtained from the three independent experiments



identified in JMML patients were higher than that of the N308D mutation identified in NS. They put forth a hypothesis explaining the manner in which the two different phenotypes were determined: somatic mutations identified in leukemia have stronger molecular defects and could cause embryonic lethality if they occur in the germline. In contrast, the defects in mutations found in NS patients were milder and insufficient for the development of leukemia. In our experiments, the relative phosphatase activities of D61N (12.1) and F71I (11.8) associated with NS were higher than those of E76A (10) and G503V (2.7) found in leukemia. A comparison of the different substitutions at the same residues showed

that the phosphatase activity of D61N (NS) was equivalent to that of D61Y (leukemia). Although Src might not be the physiological substrate of SHP-2 at the developmental stage and leukemogenesis, our results suggest that high phosphatase activity in mutants may not necessarily be associated with embryonic lethality. Further biological studies will be required to explore the mechanisms involved in the development of NS or leukemia.

The F71I mutation in a typical NS patient was inherited from the father, who exhibited only ptosis. The F71I mutation was not detected in 83 controls, and a further functional analysis showed an elevated