HRAS mutation, or (3) with a diagnosis of NS without *PTPN11* or *SOS1* mutation, to establish the pattern and frequency of mutations in these diseases, to delineate the overlap between these clinically related syndromes and to investigate possible genotype–phenotype correlations.

PATIENTS AND METHODS

Patients

Our original cohort comprised 53 patients with CFC syndrome; 13 patients, previously reported,[13 20] are not included in this paper. The study thus comprised 40 new patients with a clinical diagnosis of CFC, 20 patients with a clinical diagnosis of CS but no *HRAS* mutation, and 70 patients with NS but no *PTPN11* or *SOS1* mutation.

Patients with CFC and NS were referred for molecular testing to our laboratory by a network of geneticists from France, Belgium and Switzerland. Patients with CS were found through the French Costello Syndrome Association. A diagnosis of CS had been proposed at some time in all these patients by a clinical geneticist. It was usually based on severe developmental delay, failure to thrive and/or skin anomalies, and was the referral diagnosis for all patients within this group. This group is clinically more heterogeneous, mixing patients with truly convincing CS and patients who would probably have been diagnosed as CFC by trained dysmorphologists, but who were still carrying a diagnosis of CS and remained in the Costello Support Group. As these uncertainties in diagnosis may reflect a general difficulty in clinical differentiation between CS and CFC, we decided to keep the diagnoses of referral. Pictures of the patients were collected, and a questionnaire containing 72 clinical items about neonatal data, characteristic facial features, heart defects, skin abnormalities, growth retardation, developmental delay or mental retardation, and occurrence of solid tumour or leukaemia was used to collect clinical data. Informed signed consent for genetic investigation was obtained from all patients or their parents.

All cases of CFC and CS were apparently sporadic, with clinically and developmentally normal parents. The same statement applied to patients with NS, although it is known in this syndrome that expressivity of a mutation in a carrier may be sufficiently mild to remain clinically unsuspected (at least for patients carrying mutations in *PTPN11*).

Molecular analysis

DNA samples were obtained from peripheral leucocytes. In one patient, DNA from cultured fibroblasts was also tested. Mutation screening was performed by direct bidirectional sequencing of exons and their flanking intron–exon boundaries. The entire coding region of *KRAS*, *BRAF*, *MEK1*, *MEK2*, *PTPN11* and *HRAS* was tested in all patients. Primers and PCR conditions are available on request.

The PCR products were sequenced (Big Dye Terminator Cycle Sequencing Ready Reaction Kit; (Applied Biosystems, Foster City, California, USA), and reaction products run on an automated capillary sequencer (ABI 3100 Genetic Analyzer, Applied Biosystems). Sequences were aligned using Seqscape analysis software (Applied Biosystems) and compared with the reference sequences for genomic DNA and mRNA. GenBank accession number for genomic and mRNA reference sequences, respectively, are as follows: *KRAS* NC_000012 and NM_033360 (isoform a) or NM_004985 (isoform b), *BRAF* NC_000007 and NM_004333, *MEK1* NC_000015 and NM_002755, *MEK2* NC_000019 and NM_030662, *PTPN11* NC_000012 and NM_002834, *HRAS* NC_000011 and NM_176795.

The *Catalogue for somatic mutations in cancer* (http://www.sanger.ac.uk/genetics/CGP/cosmic) was used to check for previous implication of the mutations in cancer. Presence of single-nucleotide polymorphisms was ascertained using the Ensembl genome browser (http://www.ensembl.genome.org). Interspecies alignments and prediction of functional effects of amino acid substitutions on the function and structure of proteins were achieved using PolyPhen. (http://genetics.bwh.harvard.edu/).

RESULTS

Mutation screening

In total, 12 *BRAF* mutations including 5 unreported mutations (T241P, Q262R, G464R, E501V, N581K) were identified in 22 patients (fig 1A). All patients had CFC (n = 14) or CS (n = 8). There were 14/22 (64%) patients with a mutation in exon 6, with a hot spot on Q257. A mutation of exon 6 was found in seven of the eight patients with CS, whereas mutations associated with CFC tended to be more evenly distributed (fig 1A). All mutations occurred in exons previously shown to harbour CFC mutations. No mutation was found in exons 13 or 16.

Four *MEK1* and 4 *MEK2* mutations, including 3 novel mutations for *MEK1* (E44G, T55P, D67N), and 3 novel mutations for *MEK2* (L46_E55del, K61T, A62P) were identified in 15 patients with CFC (n = 8), CS (n = 4), or NS (n = 3) (fig 1B, 1C). Three patients with NS had a novel mutation in the exon 2 of *MEK1*. All mutations were found in exons already identified as mutational hot spots.

Five *KRAS* mutations, including two unreported mutations (K5E, G12S) were identified in seven patients with CFC (n = 1), CS (n = 2) or NS (n = 4) (fig 1D). All mutations occurred in exons 1, 2 and 4b. No *PTPN11* mutation was found in patients with CFC or a CS, and none of the patients referred with CFC had a *HRAS* mutation.

Altogether, a mutation of one of the tested genes was found in 23/40 (57%) patients with CFC syndrome, in 14/20 (70%) patients with CS and in 7/70 (10%) patients referred for NS and who were negative for *PTPN11* and *SOS1* mutation (table 1). All identified mutations except one were missense mutations, and all kept the reading frame open.

All cases with a mutation were considered by the referring clinician to be sporadic. The presence of the mutation could be investigated in both parents for 25 cases (12 with a *BRAF* mutation, 6 with a *MEK1* mutation, 4 with a *MEK2* mutation, and 3 with a *KRAS* mutation) and in the mother only for 4 cases (3 with a *BRAF* mutation, 1 with a *KRAS* mutation). The mutation was not found in the parents, with exception of one patients with NS, who had a novel *MEK1* mutation (E44G) inherited from her asymptomatic mother. No *BRAF* mutation was found in patients with NS.

Overall, 14 novel mutations were found in 17 patients. De novo occurrence could be confirmed for six mutations (eight patients), by testing the parents' DNA (table 2). This favours a causative effect of these mutations. Pathogenicity of the *MEK1* alteration found in a NS patient and her clinically unaffected mother cannot be solved so easily. The substitution has not been previously reported and we did not find it in a series of 200 normal subjects with similar ethnic background. This substitution may represent a rare polymorphism or an incompletely penetrant mutation. In the cases for which parental DNA was not available, pathogenicity was considered likely, as these alterations were not identified in 200 controls and have not been reported as polymorphisms. In most cases, the affected amino acids were highly evolutionarily conserved and predicted to be deleterious (table 2).

Most germline mutations identified in our patients are distinct from the somatic mutations present in cancers. Four patients (aged 1.5, 4.5, 8.7 and 14.3 years at the last examination) carry mutations previously reported in tumours

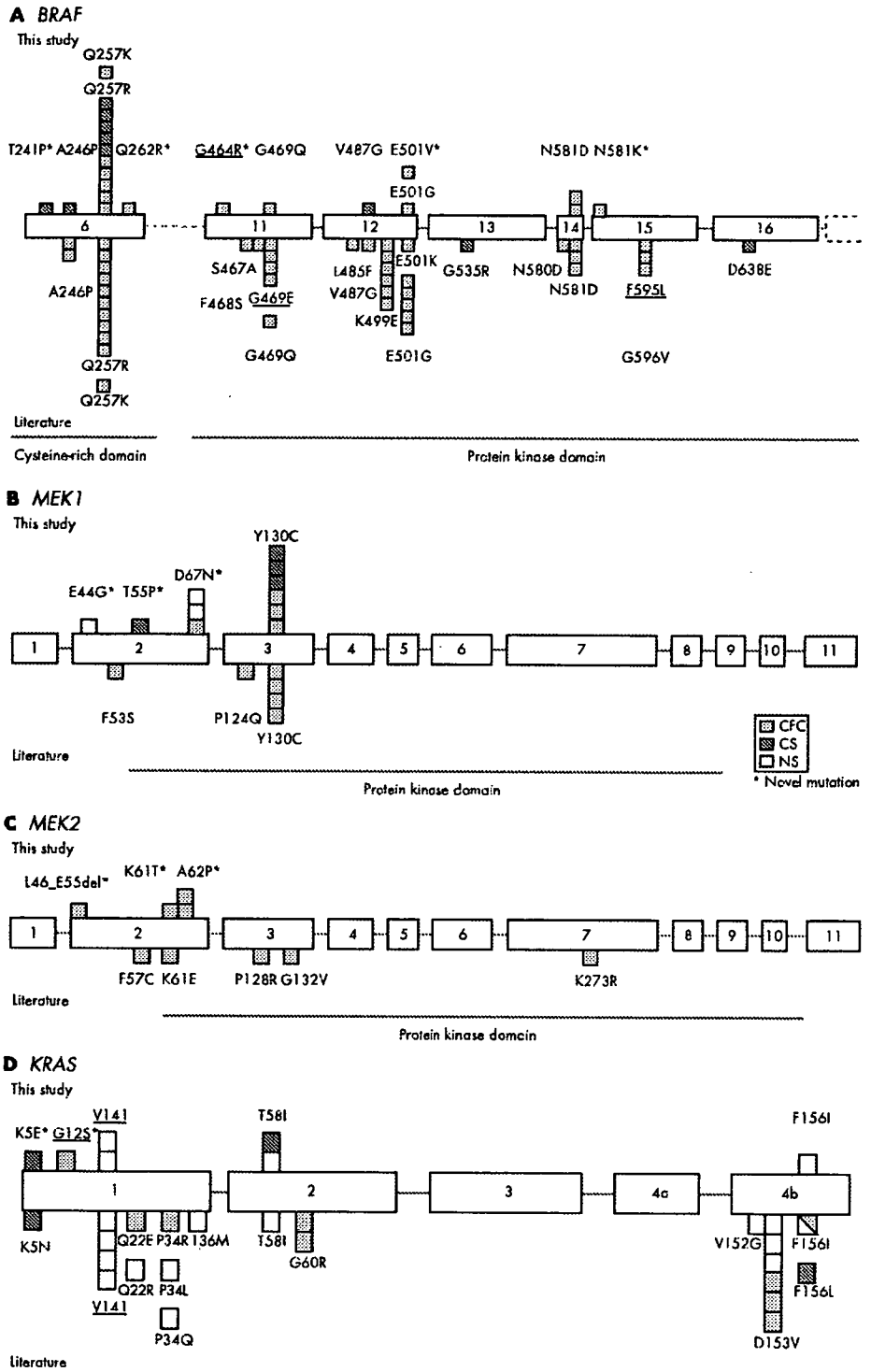

Figure 1 Mutations in (A) *BRAF*, (B) *MEK1*, (C) *MEK2*, and (D) *KRAS* found in patients with cardio-facio-cutaneous (CFC) syndrome (blue squares), Costello syndrome (CS; grey squares) and Noonan syndrome (NS; white squares) in this study and in the literature. Exons are represented by bars and numbered; for more clarity, only *BRAF* exons harbouring mutations are represented. *Novel mutations identified in this study. Mutations previously described as somatic mutations in cancer are underlined. Mutations identified in this study are positioned on top, and those already reported in the literature[13–16 20 21] are underneath.

(*BRAF* G464R, *KRAS* G12S and *KRAS* V14I in two patients) (fig 1). The G12S mutation in *KRAS* was also present in fibroblasts of the second child (now aged 8.7 years). The median age at clinical diagnosis was 1, 1.7, and 2 years and the median age at molecular diagnosis was 4.7, 7.7 and 8.7 years for the patients with *BRAF*, *MEK* and *KRAS* mutations, respectively. None of these children has developed cancer to date.

Clinical description

Because of the probable genetic heterogeneity of patients with no identified mutations, we did not perform comparisons of patients with and without mutations. We compared the phenotypes of patients according to the mutated gene and the initial clinical diagnosis (CFC or CS). Clinical data of patients with CFC were then compared with those of the series of Kavamura *et al*,[7] which was a study of 54 patients with CFC before molecular diagnosis. Finally, patients with CS without *HRAS* mutation were compared with the patients with CS with *HRAS* mutations described by Kerr *et al*[10] (table 3).

All our patients with CFC have the classic dysmorphism (hypertelorism, downslanting palpebral fissures, ptosis, high forehead with bitemporal constriction, short neck). Hair

Table 1 Proportion of patients with *KRAS*, *BRAF*, and *MEK1/2* mutations in our study and a review of the literature

	Nithori *et al*,[13] Narumi *et al*[20]	Rodriguez-Viciana *et al*[14]	Schubbert *et al*[11]		Carta *et al*[16]		Rauen *et al*[15]	Zenker *et al*[21]			This study		
	CFC	CFC	CFC	NS *PTPN11*	CFC	NS *PTPN11*	CS *HRAS*-	CFC	CS *HRAS*	NS *PTPN11*	CFC	CS *HRAS*-	NS *PTPN11*
Patients, n	56	23	12	175	8	87	3	21	3	236	40	20	70
KRAS, n (%)	3 (5.5)	–	1 (8.3)	5 (3)	0	2 (2.3)	–	2+1CFC/NS (14.3)	2 (66.7)	7 (3)	1 (2.5)	2 (10)	4 (5.7)
BRAF, n (%)	24 (43)	18 (78)	–	–	–	–	2 (66.7)	–	–	–	14 (35)	8 (40)	0
MEK1, n (%)	4 (7)	2 (9)	–	–	–	–	–	–	–	–	4 (10)	4 (20)	3 (4.3)
MEK2, n (%)	4 (7)	1 (4.3)	–	–	–	–	–	–	–	–	4 (10)	0	0
Patients with a mutation, n (%)	35 (62.5)	21 (91.3)	1 (8.3)	5 (3)	0	2 (2.3)	2 (66.7)	3 (14.3)	2 (66.7)	7 (3)	23 (57.5)	14 (70)	7 (10)

CFC, cardio-facio cutaneous syndrome; CS, Costello syndrome; NS, Noonan syndrome.

anomalies were found in 95%, and sparse or absent eyebrows in 78%. CHD was recorded in 77%. These features are in agreement with the series of Kavamura *et al* (table 3). However, in contrast with that study, our patients have a more severe neurological presentation, with hypotonia in 68% (vs 28%, $p \leqslant 0.01$), speech delay in 95% (vs 46%, $p \leqslant 0.001$), and mental retardation in 100% (vs 91%, NS). In our series, growth retardation was postnatal, with a median birth weight of 3110 g for a mean gestational age of 37 weeks. Short stature (<−2SD) was less frequent in our patients than in those reported by Kavamura *et al* (56% vs 78%) although this difference was not significant.

The dysmorphic features observed in patients with CS are those usually considered typical for this CFC syndrome also. These patients show a similar incidence of heart defects and failure to thrive to French and British patients with CS with *HRAS* mutation.[10] However, our patients with CS are younger than those reported by Kerr *et al*[10] (median 6 years vs 9 years) and six patients were diagnosed with CS before the age of 2 years. They present features overlapping with CFC, notably sparse or absent eyebrows in 92% of cases, in contrast to patients with CS and *HRAS* mutations, who have normal eyebrows. Moreover, none of our patients with CS presents papillomata, one of the more distinctive features of CS. Therefore, it is likely that some patients are actually misdiagnosed CFC cases. However, our patients with CS have a more severe phenotype than those with CFC. They present more hypotonia, failure to thrive and growth retardation are more marked in infancy, and large mouth, thick lips and coarse facies are more frequent. Developmental delay is more marked; age at first steps was 3.0 years versus 2.1 years for patients with CFC. Most of them present deep palmar creases and skin hyperlaxity, which were often considered characteristic of CS, and probably have contributed to their clinical diagnosis (fig 2).

In general, patients with a *MEK1* or *MEK2* mutation present with a milder phenotype than those with a *BRAF* mutation. Heart defect is less frequent (43% vs 90%, $p \leqslant 0.001$) (table 3). Motor delay tends to be milder (median age of walking 2 years vs 2.5 years for *BRAF* and 2.7 years for *KRAS*) and two patients have no mental retardation. Dysmorphism less commonly includes hypertelorism ($p \leqslant 0.05$) or sparse hair ($p \leqslant 0.01$). Skin anomalies are similar to those reported with CS: coarse facies (9/12), deep palmar/plantar creases (7/10), redundant skin folds on hands and feet (5/11) and hyperextensible joints (8/11). A recurrent novel mutation (D67N) was found in three patients (proven to be de novo in two). One of these patients has CFC syndrome. He has relative macrocephaly, wide face, temporal constriction, curly hair, sparse brows and lashes, pulmonary valve stenosis, failure to thrive and developmental delay. The second, aged 12 years, has typical NS: short stature, triangular face without temporal constriction, non-curly hair, ptosis, almost absent eyebrows and borderline intelligence with

Table 2 Description of novel mutations

Gene	Substitution	Number of affected people	Parental analysis	Number of negative controls tested: This study	Number of negative controls tested: Literature*	Pathogenic mutation affecting the same residue	Interspecies conservation†	PolyPhen prediction	Conclusion
BRAF	T241P	1	De novo	200	155		Yes	Probably damaging	Mutation
	Q262R	1	Absent in mother	200	155		S in *Drosophila*	Benign	Mutation (probable)
	G464R	1	–	200			Yes	Probably damaging	Mutation (probable)
	E501V	1	–	200	105	E501G, E501K	Yes	Probably damaging	Mutation (probable)
	N581K	1	De novo	–	–		Yes	Probably damaging	Mutation
MEK1	E44G	1	Mutated in asymptomatic mother	200	–		T in *Drosophila*	Possibly damaging	Possible rare polymorphism
	T55P	1	–	200	–		S in *Drosophila*	Possibly damaging	Mutation (probable)
	D67N	3	De novo (2 patients)	200	–		Yes	Benign	Mutation
MEK2	L46_E55del	1	De novo	200	50		–	–	Mutation
	K61T	1	De novo	200	50	K61E	Yes	Benign	Mutation
	A62P	2	De novo (2 patients)	200	50		E in *C elegans*	Benign	Mutation
KRAS	K5E	1	–	200	>500		Yes	Probably damaging	Mutation
	G12S	1	Absent in mother	200	>500	Somatic G12S	Yes	Benign	Mutation

*_BRAF_, _MEK1_ and _MEK2_ negative controls were tested in Nithori *et al*[13] Norumi *et al*.[20]
†When orthologous genes were present, the human sequence was compared with that of *Mus musculus*, *Rattus norvegicus*, *Danio rerio*, *Drosophila melanogaster* and *Caenorhabditiselegans*.

Table 3 Frequencies of clinical abnormalities according to the gene mutated (*BRAF*, *KRAS*, or *MEK*) and according to the clinical diagnosis (CFC or CS with RAS-pathway mutations)

Characteristic	*BRAF*	*KRAS*	*MEK*	CFC	Kavamura index*	CS with *BRAF*, *MEK* or *KRAS* mutation	CS with *HRAS* mutation**
Patients, n	22	7	15	23	54	14	37
Median age, years	4.7	8.7	7.7	5		6	9
Age at clinical diagnosis	1	2	1.7	1		1.6	
Median age of mother, years	32	31	31	31		33	
Median age of father, years	33	35	32	32		36	
Antenatal							
Birth weight >90th centile	9/18 (50)	3/6 (50)	5/13 (38)	9/19 (47)		6/12 (50)	
Polyhydramnios	11/20 (55)	3/7 (43)	10/15 (67)	12/22 (54)		9/13 (69)	
Nuchal lucency	4/19 (21)	2/6 (33)	1/7 (14)	14/19 (21)		2/10 (20)	
Caesarean	5/20 (25)	3/5 (60)	2/12 (17)	3/18 (17)		5/12 (42)	
Hypoglycaemia	2/17 (12)	0/6 (0)	0/9 (0)	1/19 (5)		1/10 (10)	9%
Hypotonic	16/19 (84)	6/6 (100)	7/10 (70)	13/19 (68)	28%	13/13 (100)	
Failure to thrive	19/20 (95)	6/7 (86)	10/14 (71)	17/21 (81)		14/14 (100)	100%
Postnatal growth retardation	14/19 (74)	5/7 (71)	9/13 (69)	13/20 (65)		12/14 (86)	
Splenomegaly	2/18 (11)	2/7 (29)	2/13 (15)	4/21 (19)	15%	1/11 (9)	
Hepatomegaly	4/20 (20)	3/6 (50)	2/13 (15)	6/21 (29)	9%	2/12 (17)	
Growth							
Short stature, <−2SD	13/21 (62)	7/7 (100)	11/15 (73)	13/23 (56)	78%	12/13 (92)	
Median stature, SD	−2.3	−3.2	−2	−2		−2.8	
Heart							
Pulmonic valve stenosis	11/22 (50)	3/7 (43)	3/14 (21)	6/22 (27)		7/14 (50)	
Atrial septal defect	5/22 (23)	2/7 (29)	3/14 (21)	6/22 (27)		3/14 (21)	
Hypertrophic cardiomyopathy	9/22 (41)	3/7 (43)	3/14 (21)	9/22 (41)		4/14 (29)	51%
Arrhythmia	0/20 (0)	0/7 (0)	0/14 (0)	0/21 (0)		0/13 (0)	31%
Total heart defect	19/22 (86)	7/7 (100)	6/14 (43)	17/22 (77)	78%	9/14 (64)	63%*
Oncology							
Leukaemia	0/22 (0)	0/6 (0)	0/12 (0)	0/21 (0)		0/12 (0)	
Solid tumour	0/22 (0)	0/6 (0)	0/12 (0)	0/21 (0)		0/12 (0)	13.5%
Dysmorphism							
Relative macrocephaly	17/22 (77)	7/7 (100)	11/15 (73)	14/23 (61)	78%	14/14 (100)	91%
Microcephaly	0/22 (0)	0/7 (0)	1/15 (7)	1/23 (4)		0/13 (0)	
Triangular facies	8/22 (36)	3/7 (43)	3/13 (23)	7/21 (33)		4/14 (29)	
Hypertelorism	20/22 (91)	7/7 (100)	11/15 (73)	21/23 (91)	46%	11/14 (79)	
Downslanting palpebral fissures	13/22 (59)	6/7 (86)	9/14 (64)	14/22 (64)	61%	8/14 (57)	
Ptosis	9/19 (47)	6/7 (86)	8/13 (61)	12/19 (63)	52%	7/13 (54)	
Epicanthal folds	10/19 (53)	5/5 (100)	6/12 (50)	12/20 (60)	59%	5/11 (45)	
Posteriorly angulated ears	19/22 (86)	5/7 (71)	12/14 (86)	20/22 (91)	76%	12/14 (86)	
Thick ears	19/22 (86)	5/7 (71)	9/12 (75)	19/21 (90)	30%	10/13 (77)	
Large earlobes	17/22 (77)	2/5 (40)	10/12 (83)	17/20 (85)		10/13 (77)	
Low-set ears	17/21 (81)	6/7 (86)	13/15 (87)	20/22 (91)		10/14 (71)	
Anteverted nostrils	10/22 (45)	3/5 (60)	8/12 (67)	12/22 (55)		6/12 (50)	
High cranial vault	16/21 (76)	4/6 (67)	10/13 (77)	18/21 (86)		7/12 (58)	
Bitemporal constriction	13/22 (59)	3/5 (60)	7/11 (64)	16/20 (80)	81%	5/13 (38)	
Large mouth	9/22 (41)	1/6 (17)	4/13 (31)	6/21 (29)		8/13 (61)	
Thick lips	9/22 (41)	2/7 (29)	7/13 (54)	10/21 (48)		8/14 (57)	
Micrognathia	4/19 (21)	2/7 (29)	4/11 (36)	5/18 (28)	24%	2/12 (17)	
Prominent philtrum	10/29 (50)	3/7 (43)	9/12 (75)	13/20 (65)		6/12 (50)	
Short neck	20/22 (91)	6/7 (86)	11/12 (92)	20/21 (95)	50%	11/13 (85)	
Webbed neck	13/20 (65)	6/7 (86)	6/11 (54)	13/20 (65)	41%	8/12 (67)	
Pterygium colli	6/22 (27)	3/7 (43)	3/12 (25)	14/20 (70)		4/13 (31)	
Coarse face	14/21 (67)	4/7 (57)	9/12 (75)	14/20 (70)		12/14 (86)	
Low posterior hairline	7/20 (35)	2/5 (40)	5/9 (55)	8/17 (47)		4/12 (33)	
Malformations							
Hyperextensible fingers	10/19 (53)	4/6 (67)	8/11 (73)	10/17 (59)	13%	10/14 (71)	100%
Pectus excavatum/carinatum	10/16 (63)	4/6 (67)	11/14 (79)	11/17 (65)		9/12 (75)	
Skin characteristics							
Curly hairs	19/22 (86)	2/7 (29)	13/15 (87)	21/23 (91)	72%	11/14 (79)	
Sparse hairs	21/22 (95)	5/7 (71)	7/13 (54)	20/21 (95)	85%	10/14 (71)	
Sparse or absent eyebrows	17/22 (77)	4/7 (57)	12/14 (86)	18/23 (78)	63%	12/13 (92)	
Sparse or absent eyelashes	12/21 (57)	3/7 (43)	9/13 (69)	14/21 (67)	67%	9/13 (69)	
Palmoplantar hyperkeratosis	4/21 (19)	0/7 (0)	3/14 (21)	5/21 (24)	13%	2/14 (14)	
General hyperkeratosis	3/20 (15)	1/6 (17)	0/11 (0)	3/20 (15)	37%	1/13 (8)	
Eczema	1/19 (5)	0/6 (0)	2/10 (20)	1/19 (5)		2/13 (15)	
Deep palmar/plantar creases	15/21 (71)	2/5 (40)	7/10 (70)	12/19 (63)		11/14 (79)	
Hyperpigmentation	2/18 (11)	1/6 (17)	4/11 (36)	5/20 (25)	6%	2/11 (18)	
Hyperelastic skin	13/20 (65)	2/6 (33)	6/11 (54)	10/21 (48)	22%	9/13 (69)	
Dry skin	10/19 (53)	1/6 (17)	4/10 (40)	6/19 (32)		7/12 (58)	
Excess skin hands/foot	9/20 (45)	2/6 (33)	5/11 (36)	6/19 (32)	9%	9/14 (64)	100%
Ichytosis	1/18 (6)	1/6 (17)	1/10 (10)	2/20 (10)	31%	1/11 (9)	
Café-au-lait patches	4/20 (20)	1/6 (17)	2/13 (15)	3/20 (15)	9%	2/13 (15)	
Nevi >10	4/21 (19)	0/7 (0)	2/14 (14)	4/22 (18)		1/13 (8)	
Lentigines >100	3/22 (14)	0/7 (0)	1/14 (7)	1/22 (4)		2/14 (14)	
Papillomata	0/20 (0)	0/6 (0)	0/10 (0)	0/19 (0)		0/13 (0)	39%
Haemangioma	5/20 (25)	2/6 (33)	1/10 (10)	4/19 (21)	24%	3/13 (23)	

Table 3 Continued

Characteristic	*BRAF*	*KRAS*	*MEK*	CFC	Kavamura index[7]	CS with *BRAF*, *MEK* or *KRAS* mutation	CS with *HRAS* mutation[19]
Neurological							
Motor delay	21/21 (100)	7/7 (100)	13/14 (93)	21/21 (100)		14/14 (100)	
Age of walk (median)	2.5	2.7	2.0	2.1		3.0	
Speech delay	20/21 (95)	7/7 (100)	11/13 (85)	19/20 (95)	46%	14/14 (100)	
First words (median)	3.0	–	2.3	3.0		2.9	
Mental retardation	21/21 (100)	6/7 (86)	11/13 (85)	21/21 (100)	91%	14/14 (100)	
Autistic features	3/15 (20)	2/6 (33)	5/8 (62)	3/14 (21)		5/11 (45)	
Seizures	3/18 (17)	0/6 (0)	4/13 (31)	3/19 (16)	15%	4/12 (33)	11%
Nystagmus	4/18 (22)	3/6 (50)	4/7 (57)	5/17 (29)	30%	4/11 (36)	
Neurosensory							
Strabismus	9/20 (45)	3/5 (60)	6/12 (50)	8/19 (42)	33%	8/12 (67)	
Myopia	5/13 (38)	1/4 (25)	3/9 (33)	5/11 (45)		3/10 (30)	
Deafness	3/12 (25)	0/3 (0)	2/12 (17)	1/13 (8)		3/8 (38)	

CFC, cardio-facio-cutaneous syndrome; CS, Costello syndrome.
*Without hypertrophic cardiomyopathy.
All data are number affected/total number (%) unless otherwise indicated.
Kavamura index[7] and *HRAS*-positive patients with CS patients reported by Kerr *et al*[19] are also indicated for comparison.

hyperactivity–attention deficit disorders. He is able to have normal schooling with extra help. The third, diagnosed as mild NS, has short stature, hypertelorism, wide face without temporal constriction, normal brows and non-curly hair, no failure to thrive, pulmonary valve stenosis, and normal psychomotor development at 6 years of age. The evolution of the phenotype with age must be taken into account, as illustrated by one of our patients with CFC who had a NS phenotype in infancy (fig 3).

The four patients having NS with a *KRAS* mutation were considered to have the typical NS gestalt, notably the triangular shape of the face, and absence of major skin involvement. They are nevertheless at the severe end of the NS spectrum: marked developmental delay, short stature, heart defects (two pulmonary valve stenosis, one mitral valve defect associated with hypertrophic cardiomyopathy, one hypertrophic cardiomyopathy). Three of the four have failure to thrive. Sparse hair (2/4) and eyebrows (1/4) indicate a clinical overlap with CFC in two of these patients.

DISCUSSION

Our results confirm the high proportion of patients with *BRAF* mutations in CFC, illustrate the clinical overlap between the phenotype of patients with *HRAS* mutations compared with *KRAS* and their downstream effectors, and suggest, to our knowledge for the first time, the implication of *MEK1* in NS.

The mutation frequency observed in our series of 40 patients with CFC (57%) is in accordance with the data from Narumi *et al*[20] (35/56; 62%), but is clearly lower than the mutation rate reported by Rodriguez-Viciana[14] (21/23; 91%). This difference is mainly due to a higher mutation rate of *BRAF* in the latter series (78% vs 35%) and is probably caused by more stringent clinical criteria, as patients with a *BRAF* mutation are, as a whole, more typical than those with mutations in the other genes.

A mutation in *BRAF*, *KRAS* or *MEK1* was found in 70% of patients clinically diagnosed as CS but without *HRAS* mutation, whereas *HRAS* mutation was not found in patients with a clinical diagnosis of CFC. This observation, together with the clinical presentation of these patients, suggest that CFC is clinically closer to CS than previously appreciated, to a point that distinction in a single individual may be impossible, at least in infants and young children. Indeed, early manifestations (such as deep palmar creases or severe failure to thrive), which were once thought to be "specific" for CS, are in fact present with or without *HRAS* mutation. As patients with *HRAS* mutations age, some clinical features (arrhythmia, multiple papillomas, facial coarseness, preservation of eyebrows) allow easier distinction between CFC and CS. Our data suggest that mutations within the cysteine-rich domain of *BRAF* could be associated with a phenotype closer to CS, whereas mutations in the protein kinase domain result in a phenotype more typical for CFC. However, the small number of patients meant this did not reach significance.

Patients with *KRAS* mutations presented the most variable phenotype, confirming the experience of Zenker *et al*.[21] One of these was diagnosed with CFC, four with NS, and two with CS. The phenotype was generally severe, with hypotonia, short stature, and heart defect in all cases and failure to thrive in 6/7 patients. One of our patients (with V14I mutation) has no mental retardation. He presented developmental delay in infancy, with first steps at 2.1 years and first words at 2.3 years. He now has normal schooling at 14 years of age. This confirms a recent observation[22] of high intelligence in a patient with *KRAS*-associated familial NS. However, this latter patient had a mutation restricted to isoform a, which is not the case in our patient. We confirm that patients with *KRAS* mutation may have hypotrichosis but not hyperkeratosis.

We also confirm the implication of *KRAS* in NS. We identified *KRAS* mutation in 5% of *PTPN11*-negative and *SOS1*-negative

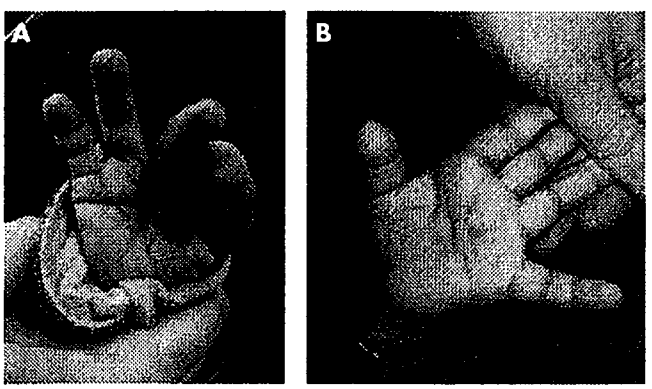

Figure 2 Close-up of hand creases in patients with (A) *BRAF* A246P and (B) G469D mutations: note thick fingers with wide, squared tips, redundancy of skin with deep palmar creases. Parental/guardian informed consent was obtained for publication of this figure.

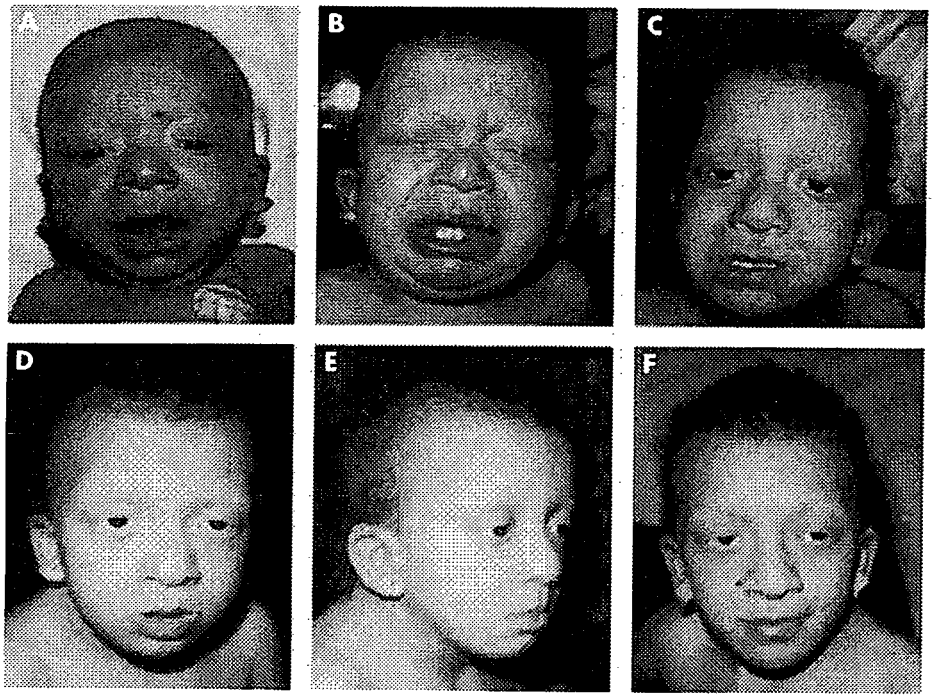

Figure 3 Changing facial phenotype of a patient with *MEK2* A62P mutation, depicted at various ages. (A, B) Facial dysmorphism at (A) 4 months and (B) 12 months is clearly NS-like, with mild ptosis, deep philtrum with prominent ridges and uplifted ear lobules. (C) At 3.5 years of age, thick lips and some coarsening of the traits may be evocative of CS. At (D,E) 5 years and (F) 7 years of age, the facial dysmorphism becomes clearly CFC-like. Parental/guardian informed consent was obtained for publication of this figure.

patients (4/70), a proportion similar to the findings of Schubbert *et al* (5/175 *PTPN11*-negative patients with NS).[15] Mutation V14I is recurrently associated with NS, indicating a possible genotype–phenotype correlation. We also show, for the first time to our knowledge, mutations in *MEK1* in patients with NS. Interestingly, three of our patients harbour the same D67N mutation but different phenotypes, emphasising intrinsic phenotypical variability of the mutation.

Somatic mutations in *KRAS* and *BRAF* have been identified in 7% and 15% of tumours, respectively. CS is associated with a high malignancy rate, mainly rhabdomyosarcoma, usually occurring before 6 years of age.[23] Malignancies are reported in 13% of *HRAS*-mutated CS; risk may vary with the mutation.[10] NS is associated with juvenile myelomonocytic leukaemia (JMML) in about 1–2% of cases, and possibly with an excess of childhood acute lymphoid and myeloid leukaemias. At least two patients with CFC and a *BRAF* mutation developed an acute lymphoblastoid leukaemia.[13 24] Cancer has only been reported in two patients with CFC: one rhabdomyosarcoma in a patient with no molecular confirmation[25] and hepatoblastoma in a patient with *MEK1* mutation.[26] Although some of our patients harbour mutations that have been reported in tumours, none has developed malignancies to date, including the patient with *KRAS* G12S, who is now close to 9 years old. This sporadic *KRAS* mutation is frequently associated with tumours and leukaemias, and has recently be reported in association with spontaneously improving JMML.[27] G12S could thus induce a milder tumorigenic phenotype than other *KRAS* G12 mutations. Because of their young age, these children remain at a theoretical high risk of developing some malignancies. As all are sporadic cases, we cannot exclude mosaicism in these patients; however, they all display the classic phenotypic features, and the presence of the mutation was confirmed in fibroblasts in the patient harbouring G12S.

Based on current knowledge of the genotype–phenotype correlations, three clusters of genes can be classified. The first group comprises genes ouside the *RAS–RAF–MEK* backbone, which encompasses those upstream of *RAS* and those that could interact with the mainstream cascade. Most, if not all patients with *PTPN11* mutations have NS or LEOPARD syndrome. Neurofibromatosis type 1 (NF1) is a neurocutaneous syndrome due to mutation in neurofibromin, a GTPase activating protein promoting RAS inactivation. When patients with NF1 have dysmorphism, they disclose a mild NS gestalt. The initial data about *SOS1* seem comparable with those obtained for *PTPN11*, leading to the hypothesis that mutations in this group usually lead to an NS phenotype, with a low rate of mental impairment and a low rate of keratinisation disorder, but a tendency to patchy skin hyperpigmentation, and, at least for *NF1* and *PTPN11*, a slightly increased risk of leukaemias, biased towards JMML.

The second group comprises *KRAS* and the cascading genes downstream. Mutations in these genes usually affect the cognitive functions, have more influence on somatic growth, skin redundancy and looseness, keratinisation (except for *KRAS*) and hair development, but they rarely affect pigmentation and usually result in a CFC phenotype. Malignancy risk appears to be low, but could include the commoner leukaemias rather than JMML.

The third group is restricted to *HRAS*. Diffuse hyperpigmentation, ulnar deviation of the wrists, papillomata, chaotic atrial fibrillation and tendency to soft-tissue tumours are the most distinguishing endophenotypes in this group.

Unravelling the molecular bases of CS, NS and CFC raises nosological problems. Do we have to base a diagnosis on clinical criteria, and accept genetic heterogeneity as a "curiosity", or should we change to a molecular-based definition of the three entities? A molecular definition implies that a molecular diagnosis is possible (which is not the case for the 50% of patients for whom no mutation can be detected) and available (which is not the case for most patients worldwide, for practical reasons). Clinicians would have to accept that two patients with the same clinical phenotype could have two different diagnoses and that each gene-based syndrome is highly variable in its expression and shows wide overlap with the others. Obviously, a molecular-based definition can be confusing for parents, caregivers not accustomed to the subtleties of molecular dysmorphology, and even geneticists. For the NS–CFC continuum, there is to date no obvious reason to abandon

clinically based diagnosis, although we probably need to redefine the border between both disorders. On the other hand, a molecular definition is appropriate when prognosis and risks for some complications (with implication for the daily care) depend upon the genotype more than on the phenotype. This is typically the case for CS, for which cancer risk and the risk for arrhythmia or vascular anomalies is clearly genotype-dependent. For that reason, we strongly recommend limiting the diagnosis of CS exclusively to patients carrying *HRAS* mutation. Patients with *BRAF, KRAS, MEK1* or *MEK2* mutations should be diagnosed as NS or CFC, whatever their phenotype. The term "severe CFC" could be used for those clinically resembling CS. Based on this, we decided to modify the diagnosis of patients with *HRAS*-negative CS from CS to CFC. Most parents accepted this change easily, as we could use the fact that the reclassification of their child was based on the newly acquired molecular data and was not a correction of an erroneous diagnosis. Interestingly, after the disclosure of our results, the French CS support group decided to change its name to "CS and CFC support group".

We will progressively have to think of disorders in terms of mutation-specific complications, and not only in term of gene-specific phenotype, as illustrated by LEOPARD syndrome. Kratz *et al*[12] showed that 8/19 patients with NS and myelodysplasia or JMML carried a single T73I substitution, a mutation that confers a much high risk of leukaemia than other alterations of *PTPN11*, even though the developmental anomalies are similar to those observed with other mutations.

ACKNOWLEDGEMENTS

We wish to thank the patients and their families who participated in this study and physicians who referred the patients reported in this paper: Drs MC Addor, A Afenjar, J Amiel, JM André, E Barouk, C Bellesme, D Bonneau, A Bottany, L Burglen, V Cormier-Daire, A David, S de Almeida, MA Delrue, B Doray, V Drouin-Garraud, T Edouard, B Gilbert, F Guiliano, R Hennekam, A Hovnanian, H Journel, M Kassis, M Le Merrer, S Lyonnet, S Manouvrier, G Morin, G Mortier, M Moutard, S Odent, L Pasquier, G Plessis, A Pennerah, N Phillip, P Sarda, Y Sznajer, J Vigneron, and C Vincent-Delorme. We also sincerely thank Michelle Rallet for secretary work and Céline Liger for technical assistance.

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Funding: The work was supported in part by the Programme Hospitalier de Recherche Clinique (grant PHRC-AOM02 004).

Competing interests: none declared.

The first three authors contributed equally to this work

Parental/guardian informed consent was obtained for publication of figures 2 and 3.

Note added in proof: Since submission of this manuscript, Gripp *et al*[28] has reported a series of eight patients with *BRAF* and five with *MEK1* mutations, for which the clinical diagnosis was felt to be CS. Comparison with *HRAS*-mutated showed similar trends to our own observations. They also favoured a molecular definition of CS.

REFERENCES

1 **Reynolds JF,** Neri G, Hermann JP, Blumberg B, Caldwell JG, Miles PV, Opitz JM. New multiple congenital anomalies/mental retardation syndrome with cardio-facio-cutaneous involvement. *Am J Med Genet* 1986;**23**:413–27.
2 **Verloes A,** Le Merrer M, Soyeur D, Kaplan J, Pangalos C, Rigo J. CFC syndrome: a syndrome distinct from Noonan syndrome. *Ann Génét* 1988;**31**:230–4.
3 **Wieczorek D,** Majewski F, Gillessen-Kaesbach G. Cardio-facio-cutaneous (CFC) syndrome—a distinct entity? Report of three patients demonstrating the diagnostic difficulties in delineation of CFC syndrome. *Clin Genet*, 1997 Jul, **52**:37–46.
4 **Neri G,** Kavamura MI, Zollino M, Opitz JM. CFC syndrome. *Am J Med Genet A* 2003 1;**116**:410.
5 **Roberts A,** Allanson J, Jadico SK, Kavamura MI, Noonan J, Opitz JM, Young T, Neri G. The Cardio-Facio-Cutaneous (CFC) syndrome: a review. *J Med Genet* 2006;**43**:833–42.
6 **Grebe TA,** Clericuzio C. Neurologic and gastrointestinal dysfunction in cardio-facio-cutaneous syndrome: identification of a severe phenotype. *Am J Med Genet*, 2000 Nov 13, **95**:135–43.
7 **Kavamura MI,** Peres CA, Alchorne MM, Brunoni D. CFC index for the diagnosis of cardiofaciocutaneous syndrome. *Am J Med Genet*, 2002 Sep 15, **112**:12–16.
8 **Tartaglia M,** Mehler EL, Goldberg R, Zampino G, Brunner HG, Kremer H, van dB I, Crosby AH, Ion A, Jeffery S, Kalidas K, Patton MA, Kucherlapati RS, Gelb BD. Mutations in PTPN11, encoding the protein tyrosine phosphatase SHP-2, cause Noonan syndrome. *Nat Genet* 2001;**29**:465–8.
9 **Aoki Y,** Niihori T, Kawame H, Kurosawa K, Ohashi H, Tanaka Y, Filocamo M, Kato K, Suzuki Y, Kure S, Matsubara Y. Germline mutations in HRAS proto-oncogene cause Costello syndrome. *Nat Genet* 2005;**37**:1038–40.
10 **Kerr B,** Delrue MA, Sigaudy S, Perveen R, Marche M, Burgelin I, Stef M, Tang B, Eden T, O'sullivan J, De Sandre-Giovannoli A, Reardon W, Brewer C, Bennett C, Quarrell O, McCann E, Donnai D, Stewart F, Hennekam R, Cave H, Verloes A, Philip N, Lacombe D, Levy N, Arveiler B, Black G. Genotype-phenotype correlation in Costello syndrome; HRAS mutation analysis in 43 cases. *J Med Genet* 2006;**43**:401–5.
11 **Gripp KW,** Lin AE, Stabley DL, Nicholson L, Scott CI Jr, Doyle D, Aoki Y, Matsubara Y, Zackai EH, Lapunzina P, Gonzalez-Meneses A, Holbrook J, Agresta CA, Gonzalez IL, Sol-Church K. HRAS mutation analysis in Costello syndrome: genotype and phenotype correlation. *Am J Med Genet A* 2006;**140**:1–7.
12 **Kratz CP,** Niemeyer CM, Castleberry RP, Cetin M, Bergstrasser E, Emanuel PD, Hasle H, Kardos G, Klein C, Kojima S, Stary J, Trebo M, Zecca M, Gelb BD, Tartaglia M, Loh ML. The mutational spectrum of PTPN11 in juvenile myelomonocytic leukemia and Noonan syndrome/myeloproliferative disease. *Blood*, 2005 Sep 15, **106**:2183–5.
13 **Niihori T,** Aoki Y, Narumi Y, Neri G, Cave H, Verloes A, Okamoto N, Hennekam RC, Gillessen-Kaesbach G, Wieczorek D, Kavamura MI, Kurosawa K, Ohashi H, Wilson L, Heron D, Bonneau D, Corona G, Kaname T, Naritomi K, Baumann C, Matsumoto N, Kato K, Kure S, Matsubara Y. Germline KRAS and BRAF mutations in cardio-facio-cutaneous syndrome. *Nat Genet* 2006;**38**:294–6.
14 **Rodriguez-Viciana P,** Tetsu O, Tidyman WE, Estep AL, Conger BA, Cruz MS, McCormick F, Rauen KA. Germline mutations in genes within the MAPK pathway cause cardio-facio-cutaneous syndrome. *Science*, 2006 Mar 3, **311**:1287–90.
15 **Schubbert S,** Zenker M, Rowe SL, Boll S, Klein C, Bollag G, van dB I, Musante L, Kalscheuer V, Wehner LE, Nguyen H, West B, Zhang KY, Sistermans E, Rauch A, Niemeyer CM, Shannon K, Kratz CP. Germline KRAS mutations cause Noonan syndrome. *Nat Genet* 2006;**38**:331–6.
16 **Carta C,** Pantaleoni F, Bocchinfuso G, Stella L, Vasta I, Sarkozy A, Digilio C, Palleschi A, Pizzuti A, Grammatico P, Zampino G, Dallapiccola B, Gelb BD, Tartaglia M. Germline missense mutations affecting KRAS Isoform B are associated with a severe Noonan syndrome phenotype. *Am J Hum Genet* 2006;**79**:129–35.
17 **Roberts AE,** Araki T, Swanson KD, Montgomery KT, Schiripo TA, Joshi VA, Li L, Yassin Y, Tamburino AM, Neel BG, Kucherlapati RS. Germline gain-of-function mutations in SOS1 cause Noonan syndrome. *Nat Genet* 2006;**39**:70–4.
18 **Tartaglia M,** Pennacchio LA, Zhao C, Yadav KK, Fodale V, Sarkozy A, Pandit B, Oishi K, Martinelli S, Schackwitz W, Ustaszewska A, Martin J, Bristow J, Carta C, Lepri F, Neri C, Vasta I, Gibson K, Curry CJ, Siguero JP, Digilio MC, Zampino G, Dallapiccola B, Bar-Sagi D, Gelb BD. Gain-of-function SOS1 mutations cause a distinctive form of Noonan syndrome. *Nat Genet* 2007;**39**:276.
19 **Schubbert S,** Bollag G, Shannon K. Deregulated Ras signaling in developmental disorders: new tricks for an old dog. *Curr Opin Genet Dev* 2007;**17**:15–22.
20 **Narumi Y,** Aoki Y, Niihori T, Neri G, Cave H, Verloes A, Nava C, Kavamura MI, Okamoto N, Kurosawa K, Hennekam RC, Wilson LC, Gillessen-Kaesbach G, Wieczorek D, Lapunzina P, Ohashi H, Makita Y, Kondo I, Tsuchiya S, Ito E, Sameshima K, Kato K, Kure S, Matsubara Y. Molecular and clinical characterization of cardio-facio-cutaneous (CFC) syndrome: Overlapping clinical manifestations with Costello syndrome. *Am J Med Genet A* 2007;**143**:799–807.
21 **Zenker M,** Lehmann K, Schulz AL, Barth H, Hansmann D, Koenig R, Korinthenberg R, Kreiss-Nachtsheim M, Meinecke P, Morlot S, Mundlos S, Quante AS, Raskin S, Schnabel D, Wehner LE, Kratz CP, Horn D, Kutsche K. Expansion of the genotypic and phenotypic spectrum in patients with KRAS germline mutations. *J Med Genet* 2007;**44**:131–5.

22 **Unger S**, Horn K, Lehmann K, Zenker M. Familial Noonan syndrome with high intelligence associated with an isoform-specific KRAS mutation. *Am J Hum Genet* 2006;**79**(suppl):A1839.
23 **Gripp KW**, Scott CI Jr, Nicholson L, Donald-McGinn DM, Ozeran JD, Jones MC, Lin AE, Zackai EH. Five additional Costello syndrome patients with rhabdomyosarcoma: proposal for a tumor screening protocol. *Am J Med Genet* 2002;**108**:80–7.
24 **Makita Y**, Narumi Y, Yoshida M, Niihori T, Kure S, Fujieda K, Matsubara Y, Aoki Y. Leukemia in Cardio-facio-cutaneous (CFC) syndrome: a patient with a germline mutation in BRAF proto-oncogene. *J Pediatr Hematol Oncol* 2007;**29**:287–90.
25 **Bisogno G**, Murgia A, Mammi I, Strafella MS, Carli M. Rhabdomyosarcoma in a patient with cardio-facio-cutaneous syndrome. *J Pediatr Hematol Oncol* 1999;**21**:424–7.
26 **Al-Rahawan MM**, Chute DJ, Sol-Church K, Gripp KW, Stabley DL, McDaniel NL, Wilson WG, Waldron PE. Hepatoblastoma and heart transplantation in a patient with cardio-facio-cutaneous syndrome. *Am J Med Genet A* 2007;**143**:1481–8.
27 **Matsuda K**, Shimada A, Yoshida N, Ogawa A, Watanabe A, Yajima S, Iizuka S, Koike K, Yanai F, Kawasaki K, Yanagimachi M, Kikuchi A, Ohtsuka Y, Hidaka E, Yamauchi K, Tanaka M, Yanagisawa R, Nakazawa Y, Shiohara M, Manabe A, Kojima S, Koike K. Spontaneous improvement of hematologic abnormalities in patients having juvenile myelomonocytic leukemia with specific RAS mutations. *Blood* 2007;**109**:5477–80.
28 **Gripp KW**, Lin AE, Nicholson L, Allen W, Cramer A, Jones KL, Kutz W, Peck D, Rebolledo MA, Wheeler PG, Wilson W, Al-Rahawan MM, Stabley DL, Sol-Church K. Further delineation of the phenotype resulting from BRAF or MEK1 germline mutations helps differentiate cardio-facio-cutaneous syndrome from Costello syndrome. *Am J Med Genet A* 2007;**143**:1472–80.
29 **Raven KA**. Distinguishing Costello versus cardio-facio-cutaneous syndrome: BRAF mutations in patients with a Costello phenotype. *Am J Med Genet A* 2006;**140**:1681–3.

American Journal of Medical Genetics Part A 143A:799–807 (2007)

Molecular and Clinical Characterization of Cardio-Facio-Cutaneous (CFC) Syndrome: Overlapping Clinical Manifestations With Costello Syndrome

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Received 12 July 2006; Accepted 17 November 2006

Cardio-facio-cutaneous (CFC) syndrome is a multiple congenital anomaly/mental retardation syndrome characterized by heart defects, a distinctive facial appearance, ectodermal abnormalities and mental retardation. Clinically, it overlaps with both Noonan syndrome and Costello syndrome, which are caused by mutations in two genes, *PTPN11* and *HRAS*, respectively. Recently, we identified mutations in *KRAS* and *BRAF* in 19 of 43 individuals with CFC syndrome, suggesting that dysregulation of the RAS/RAF/MEK/ERK pathway is a molecular basis for CFC syndrome. The purpose of this study was to perform comprehensive mutation analysis in 56 patients with CFC syndrome and to investigate genotype–phenotype correlation. We analyzed *KRAS*, *BRAF*, and *MAP2K1/2* (MEK1/2) in 13 new CFC patients and identified five *BRAF* and one *MAP2K1* mutations in nine patients. We detected one *MAP2K1* mutation in three patients and four new *MAP2K2* mutations in four patients out of 24 patients without *KRAS* or *BRAF* mutations in the previous study [Niihori et al., 2006]. No mutations were identified in *MAPK3/1* (ERK1/2) in 21 patients without any mutations. In total, 35 of 56 (62.5%) patients with CFC syndrome had mutations (3 in *KRAS*, 24 in *BRAF*, and 8 in *MAP2K1/2*). No significant differences in clinical manifestations were found among 3 *KRAS*-positive patients, 16 *BRAF*-positive patients, and 6 *MAP2K1/2*-positive patients. Wrinkled palms and soles, hyperpigmentation and joint hyperextension, which have been commonly reported in Costello syndrome but not in CFC

This article contains supplementary material, which may be viewed at the American Journal of Medical Genetics website at http://www.interscience.wiley.com/jpages/1552-4825/suppmat/index.html.

Grant sponsor: Ministry of Education, Culture, Sports, Science and Technology of Japan; Grant sponsor: Ministry of Health, Labor, and Welfare of Japan; Grant sponsor: Takeda Science Foundation.

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DOI 10.1002/ajmg.a.31658

syndrome, were observed in 30–40% of the mutation-positive CFC patients, suggesting a significant clinical overlap between these two syndromes. © 2007 Wiley-Liss, Inc.

Key words: multiple congenital anomaly; cardio-facio-cutaneous syndrome; RAF; RAS; MEK; ERK; Costello syndrome; Noonan syndrome

How to cite this article: Narumi Y, Aoki Y, Niihori T, Neri G, Cavé H, Verloes A, Nava C, Kavamura MI, Okamoto N, Kurosawa K, Hennekam RCM, Wilson LC, Gillessen-Kaesbach G, Wieczorek D, Lapunzina P, Ohashi H, Makita Y, Kondo I, Tsuchiya S, Ito E, Sameshima K, Kato K, Kure S, Matsubara Y. 2007. Molecular and clinical characterization of cardio-facio-cutaneous (CFC) syndrome: Overlapping clinical manifestations with Costello syndrome. Am J Med Genet Part A 143A:799–807.

INTRODUCTION

Cardio-facio-cutaneous (CFC; OMIM 115150) syndrome was first described in 1986 as a syndrome showing congenital heart defects, mental retardation, ectodermal abnormalities, and a characteristic facial appearance [Reynolds et al., 1986]. Typical facial characteristics include a high forehead with bitemporal constriction, hypoplastic supraorbital ridges, downslanting palpebral fissures, a depressed nasal bridge and posteriorly angulated ears with prominent helices. Affected individuals present with heart defects, including pulmonic stenosis (PS), atrial septal defects and hypertrophic cardiomyopathy, and ectodermal abnormalities such as sparse, friable hair, and hyperkeratotic skin lesions. There are phenotypic similarities between this syndrome, Noonan syndrome (OMIM 163950) and Costello syndrome (OMIM 218040) [Wieczorek et al., 1997; van Eeghen et al., 1999; Grebe and Clericuzio, 2000; Kavamura et al., 2002].

The RAS/MAPK (mitogen-activated protein kinase) pathway is a signaling pathway implicated in growth factor-mediated cell proliferation, differentiation or cell death [Malumbres and Barbacid, 2003]. RAS is a member of a large family of approximately 21-kDa membrane-associated monomeric GTPases, which cycles between a GTP-bound active and a GDP-bound inactive state [Malumbres and Barbacid, 2003]. RAS activates RAF serine-threonine kinases including BRAF. Activated RAF activates mitogen-activated protein kinase kinase 1/2 (MAP2K1/2 or MEK1/2). MEK1 and MEK2 then phosphorylate their two known substrates, ERK1 and ERK2, products of MAPK3 and MAPK1 genes, respectively (Fig. 1) [Zheng and Guan, 1993].

Gain-of-function mutations in protein tyrosine phosphatase SHP-2 (*PTPN11*) have been identified in approximately 50% of individuals with clinically diagnosed Noonan syndrome [Tartaglia et al., 2001; Musante et al., 2003; Niihori et al., 2005]. We recently identified mutations in *HRAS* in 12 of 13 individuals with Costello syndrome [Aoki et al., 2005] and mutations in *KRAS* and *BRAF* in 19 of 43 patients with CFC syndrome [Niihori et al., 2006]. Rodriguez-Viciana et al. [2006] also reported *BRAF* and *MAP2K1/2* mutations in 21 of 23 patients with CFC syndrome (Fig. 1). These findings suggest that the dysregulation of the RAS/MAPK pathway is the common underlying mechanism of the three related syndromes, that is, Noonan syndrome, Costello syndrome, and CFC syndrome [Bentires-Alj et al., 2006; Niihori et al., 2006]. In our previous report, mutations were identified in 44% of patients with CFC syndrome [Niihori et al., 2006]. The aim of the present study was to characterize molecular defects in total 56 patients with CFC syndrome and to investigate genotype–phenotype correlation.

MATERIALS AND METHODS

Patients

The original study population consisted of 56 patients with the clinical diagnosis of CFC syndrome. The diagnosis of CFC syndrome was evaluated by clinical geneticists based on typical facial appearance, heart defects, skin findings and developmental delay or mental retardation. *KRAS* and *BRAF* have been analyzed in 43 of 56 patients and *KRAS* or *BRAF* mutations were identified in 3 and 16 patients, respectively [Niihori et al., 2006]. We obtained genomic DNA from blood leukocytes, lymphoblasts from 13 previously unanalyzed individuals with CFC syndrome (8 patients from Japan, 3 from Spain, 1 from France, and 1 from England) and blood leukocytes from their parents. Control DNA was obtained from 105 healthy Japanese individuals. Control DNA from 105 healthy Caucasian individuals was purchased from Coriell Cell Repositories. This study was approved by the Ethics Committee of Tohoku University School of Medicine. We obtained informed consent from all subjects involved in the study and specific consent for photographs from 12 patients. Pictures from mutation-positive CFC patients were shown in Figure 2. Eighty-one clinical manifestations, extracted from the description of 54 CFC patients in the literature [Kavamura et al., 2002], were obtained from 25 mutation-positive patients with CFC syndrome (CFC8, 73, and 91 with *KRAS* mutations [Niihori et al., 2006]; CFC16, 24, 96, 76, 81, 94, 83, 143, 79, 77, 90, 95, 116, 118, 141, and 148 with *BRAF* mutations [Niihori et al., 2006]; CFC112, 75, 87, 111, 80, and 85 with *MAP2K1/2* mutations) (see the online

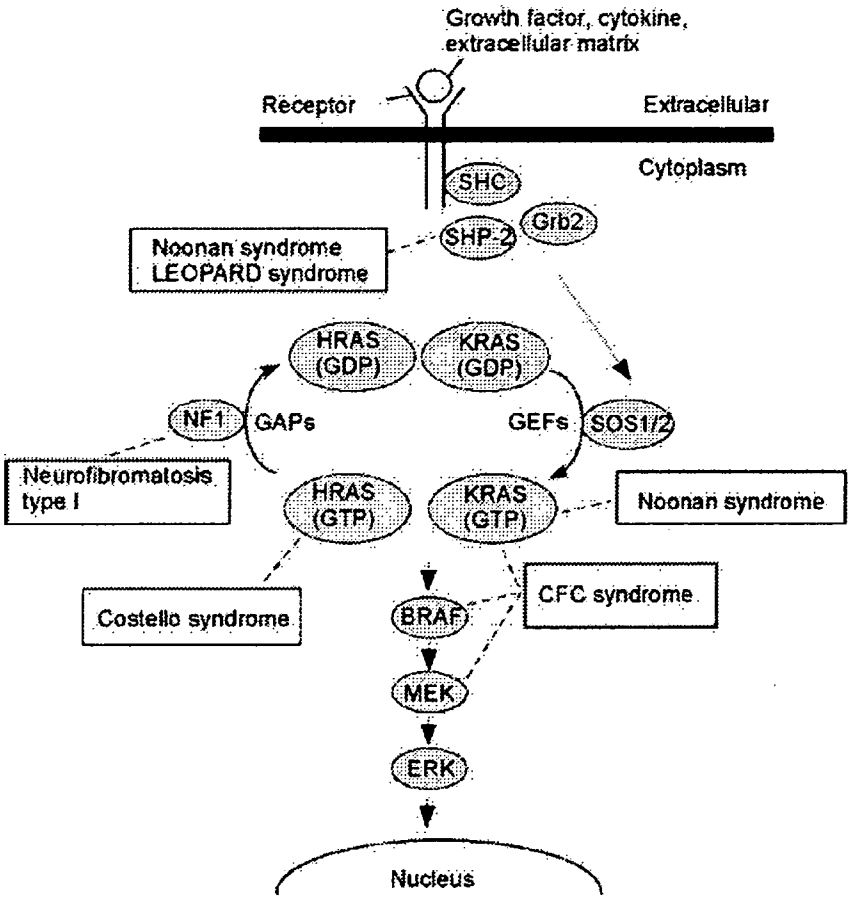

FIG. 1. A: The RAS-RAF-MEK-ERK signaling pathway and associated disorders. Mutations with enhanced catalytic activity of tyrosine phosphatase SHP-2 have been identified in patients with Noonan syndrome [Tartaglia et al., 2001]. In contrast, loss-of-function mutations in SHP-2 have been identified in patients with LEOPARD (multiple *l*entigines, *e*lectrocardiographic conduction abnormalities, *o*cular hypertelorism, *p*ulmonary stenosis, *a*bnormal genitalia, *r*etardation of growth, and sensorineural *d*eafness) syndrome [Hanna et al., 2006; Kontaridis et al., 2006; Tartaglia et al., 2006]. Oncogenic mutations in *HRAS* cause Costello syndrome [Aoki et al., 2005]. Mutations in *KRAS*, *BRAF*, or *MAP2K1/2* have been identified in patients with cardio-facio-cutaneous (CFC) syndrome [Niihori et al., 2006; Rodriguez-Viciana et al., 2006]. Loss-of-function mutations in *NF1* cause neurofibromatosis type I. *KRAS* mutations have also been identified in a few patients with Noonan syndrome [Schubbert et al., 2006; Carta et al., 2006].

Supplementary Table I at http://www.interscience.wiley.com/jpages/1552-4825/suppmat/index.html). The CFC index was calculated as previously described [Kavamura et al., 2002].

Sequencing and Mutation Analysis

We isolated genomic DNA by a standard protocol. PCR primers amplifying the entire coding region of *MAP2K1*, *MAP2K2*, *MAPK3*, and *MAPK1* were designed (see the online Supplementary Table II at http://www.interscience.wiley.com/jpages/1552-4825/suppmat/index.html). The M13 reverse or forward sequence was added to the 5′ end of the PCR primers for use as sequencing primers. PCR was performed in 30 ml of a solution containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 10% (v/v) DMSO, 0.4 pmol of each primer, 100 ng genomic DNA and 2.5 units of Taq DNA polymerase. The reaction condition consisted of 35 cycles of denaturation at 94°C for 15 sec, annealing at the indicated temperature for 15 sec and extension at 72°C for 30 sec. The products were gel-purified and sequenced on an ABI PRISM 310 automated DNA sequencer (Applied Biosystems, Foster City, CA).

RESULTS

Mutation Analysis

The entire coding regions of *KRAS*, *BRAF*, and *MAP2K1/2* were analyzed in 13 new CFC patients (Table I). Five different mutations in *BRAF* were identified in eight patients, including three novel mutations: a 769C→A mutation (Q257K), a 1460T→G mutation (V487G), and a 1738A→G mutation (N580D). Q257R and E501G mutations were identified in five patients. E501G mutation was identified in a 9-year-old patient who developed acute lymphoblastic leukemia at the age of 1 year and

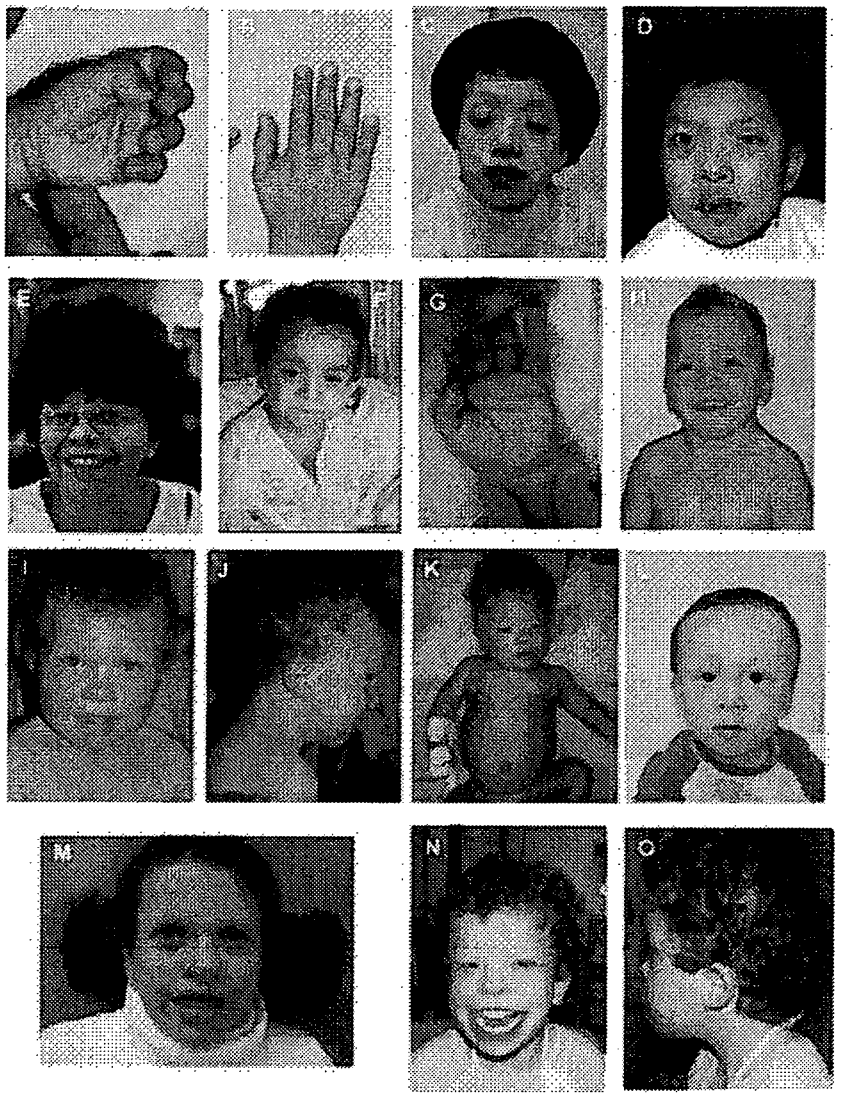

FIG. 2. Typical facial appearances and hands of mutation-positive patients. **A**: Wrinkled palm of CFC73 with *KRAS* D153V mutation. **B**: Hand with deep wrinkles in CFC 8 with *KRAS* 153V mutation. **C**: CFC7 with *BRAF* Q257K mutation. This patient has been contracted with intractable epilepsy. **D**: CFC149 with *BRAF* Q257R mutation. **E**: CFC94 with *BRAF* G469E mutation. This patient developed acute lymphoblastic leukemia [van Den Berg and Hennekam, 1999]. Face (**F**) and wrinkled palm (**G**) in CFC143 with *BRAF* V487 mutation. (**H**) CFC90 with *BRAF* E501G mutation. **I,J**: CFC116 with *BRAF* E501G mutation. **K**: CFC141 with *BRAF* E501G mutation. Hyperpigmentation is noted in his face and forearm. This patient developed acute lymphoblastic leukemia [Makita et al., submitted] (**L**) CFC148 with *BRAF* N580D mutation. Heart defects and skin abnormalities were not observed in this patient. **M**: CFC95 with *BRAF* N581D mutation. **N,O**: CFC75 with MEK1 Y130C mutation.

9 months [Makita et al., submitted for publication]. A novel P124L mutation in *MAP2K1* was identified in CFC 154. We then analyzed *MAP2K1/2* in 24 patients who have been negative for *KRAS* and *BRAF* in the previous study [Niihori et al., 2006]. The entire coding sequencing of *MAP2K1* revealed a 389A→G mutation, resulting in a Y130C mutation in three patients. The Y130C mutation has been detected in a CFC patient and shown to enhance the phosphorylation of ERK [Rodriguez-Viciana et al., 2006]. We identified four novel *MAP2K2* mutations in four patients: K61E (181A→G), P128R (383C→G), G132V (395G→T), and K273R (818A→G). No mutations in *MAPK3/1* were identified in 21 patients whose mutations were not identified in *KRAS*, *BRAF*, and *MAP2K1/2*.

None of the newly identified mutations were observed in the control DNA of ethnically matched 105 healthy subjects. Parental samples were obtained in four patients (CFC87, CFC 111, CFC112, and CFC141). No mutations were identified in parents, suggesting these mutations occurred de novo.

Genotype–Phenotype Correlations

We obtained detailed clinical manifestations [Kavamura et al., 2002] in 25 mutation-positive CFC

TABLE I. Mutations Identified in This Study

Gene	Individual	Origin	Exon	Nucleotide substitution	Amino acid change	Genotype of father/mother
Mutations identified in 13 new CFC patients						
BRAF	CFC7	Japan	6	769C→A	Q257K[a,b]	
	CFC149	Japan	6	770A→G	Q257R	
	CFC152	Japan	6	770A→G	Q257R	
	CFC143	Spain	12	1460T→G	V487G[a,c]	
	CFC116	England	12	1502A→G	E501G	
	CFC118	France	12	1502A→G	E501G	
	CFC141	Japan	12	1502A→G	E501G[d]	WT/WT
	CFC148	Japan	14	1738A→G	N580D[a]	
MAP2K1	CFC154	Japan	3	371C→T	P124L[a,e]	
Mutations identified in 24 CFC patients without *KRAS* or *BRAF* mutations in the previous study [Niihori et al., 2006]						
MAP2K1	CFC75	England	3	389A→G	Y130	
	CFC87	France	3	389A→G	Y130	WT/WT
	CFC112	Italy	3	389A→G	Y130	WT/WT
MAP2K2	CFC80	France	2	181A→G	K61E[a]	
	CFC111	Italy	3	383C→G	P128R[a,e]	WT/WT
	CFC85	France	3	395G→T	G132V[a]	
	CFC104	Italy	7	818A→G	K273R[a,f]	

[a]Novel mutation.
[b]The Q257K mutation is located at residue 257, the site of Q257R, most common mutations.
[c]The V487G is located between the glycine-rich loop and activation segment [Garnett and Marais, 2004].
[d]This patient developed acute lymphoblastic leukemia at the age of 1 year and 9 months.
[e]Proline at amino acid 124 in MEK1 and proline at amino acid 128 in MEK2 are homologous residues.
[f]K273 is located near the proline-rich domain (residues 276–305) in the kinase domain, which is an important regulatory domain in MEK1/2 [Ohren et al., 2004.]

patients (3 patients with *KRAS* mutations, 16 patients with *BRAF* mutations, and 6 patients with *MAP2K1/2* mutations) (see the online Supplementary Table I at http://www.interscience.wiley.com/jpages/1552-4825/suppmat/index.html). No significant differences were observed in manifestations among patients with mutations in *KRAS*, *BRAF*, or *MAP2K1/2*. In the previous study, we reasoned that patients with *KRAS* mutations had no skin problems such as ichthyosis, hemangioma, or hyperkeratosis [Niihori et al., 2006]. However, detailed analysis showed that patients with *KRAS* mutations also had skin abnormalities, including follicular keratosis, eczema, or palmoplantar hyperkeratosis (Table II). The CFC indices were 16.7, 16.0, and 16.8 in patients with mutations of *KRAS*, *BRAF*, and *MAP2K1/2*, respectively. These results suggest that CFC patients with *KRAS*, *BRAF*, and *MAP2K1/2* mutations did not show significant differences in clinical manifestations.

Clinical manifestations were classified into three groups with regard to the frequencies seen in 25 mutation-positive CFC patients (Table II). The frequency of each clinical manifestation was compared with values used for the CFC index or with frequencies reported in patients with Costello syndrome [Hennekam, 2003]. There were 24 manifestations observed in 60–100% of mutation-positive CFC patients, such manifestations being important for clinical diagnosis of CFC syndrome. Mental retardation was found in all patients: severe, severe to moderate or moderate mental retardation was observed in 23 of 24 patients (96%), which is in contrast with patients with Noonan syndrome, in which there are lower frequencies of mental retardation (24–35%) [Wieczorek et al., 1997]. Congenital heart defects were found in 84% of the patients. In a previous study, PS, atrial septal defects, and cardiomyopathy showed equal frequencies (38.1%, 28.6%, and 23.8%, respectively) in patients with CFC syndrome [Wieczorek et al., 1997]. Our results suggest that atrial septal defects are less frequent in mutation-positive CFC patients. Regarding the skin, follicular keratosis was seen in 60% of the patients. Eczema, hyperkeratosis, palmoplantar hyperkeratosis, hyperpigmentation or wrinkled palms and soles were observed in 32–56% of the patients. Webbed neck, delayed bone age, and cryptorchidism were observed in 20–24% of the patients, with CFC index values of more than 0.4 [Kavamura et al., 2002]. No patients showed exophthalmos, wide palate, scarring follicular keratosis or comedones.

DISCUSSION

We performed comprehensive molecular analysis by sequencing *KRAS*, *BRAF* and *MAP2K1/2* and *MAPK3/1* on total 56 CFC patients including 43 patients analyzed with *KRAS* and *BRAF* before [Niihori et al., 2006]. Mutations were found to exist in 35 of 56 (62.5%) patients with CFC syndrome: 3 in *KRAS*, 24 in *BRAF*, and 8 in *MAP2K1/2*. *BRAF* mutations were most frequently identified in 68.6% (24 of 35) of mutation-positive CFC patients. Rodriguez-Viciana et al. [2006] reported that patients

TABLE II. Frequencies of Clinical Manifestations in Mutation-Positive CFC Patients, Those Used for Calculation of CFC Index and Those in Patients With Costello Syndrome

Group	Category	Clinical manifestation	KRAS (3 patients)	BRAF (16 patients)	MEK1/2 (6 patients)	Total in 25 mutation-positive patients (%)	Frequency used for CFC index [Kavamura et al., 2002]	Frequency in Costello syndrome [Hennekam, 2003]
60–100%	Hair	Dry	1	10	6	17 (68)	0.148	
		Sparse	2	16	6	24 (96)	0.852	82
		Thin	1	11	5	17 (68)	0.463	
		Curly	3	15	6	24 (96)	0.722	82
	Eyelashes	Sparse	2	10	5	17 (68)	0.5	
	Eyebrows	Sparse	3	8	5	16 (64)	0.426	
	Eyes	Hypertelorism	1	12	4	17 (68)	0.463	
		Downslanting palpebral fissures	2	15	3	20 (80)	0.611	82
	Ears	Low implantation	2	13	5	20 (80)	0.741	85
		Posterior angulation	3	12	5	20 (80)	0.759	
		Thick	3	12	6	21 (84)	0.296	
	Nose	Anteverted nostrils	3	13	3	19 (76)	0.852	
		Depressed nasal bridge	3	15	4	22 (88)	0.889	90
	Craniofacial	Relative macrocephaly	3	14	6	23 (92)	0.778	84[a]
		Bitemporal constriction	3	10	4	17 (68)	0.815	
		High cranial vault	3	9	5	17 (68)	0.944	
		Hypoplasia of supraorbital ridges	3	11	5	19 (76)	0.667	
	Neck	Short	2	14	6	22 (88)	0.5	88
	Skin	Follicular keratosis	2	8	5	15 (60)	0.333	
	Other	Mental retardation	3	16	6	25 (100)	0.907	100[b]
		Severe	1	9	1	11		
		Severe–moderate	0	1	1	2		
		Moderate	1	5	4	10		
		Mild	0	1	0	1		
		Delayed speech	3	15	6	24 (96)	0.463	
		Developmental disability	3	15	5	23 (92)	0.815	100
		Short stature	3	11	5	19 (76)	0.778	
		Congenital heart defect	3	13	5	21 (84)	0.778	75[b]
		Pulmonic stenosis	0	7	2	9		
		Atrial septal defect	0	1	1	2		
		Cardiomyopathy	3	5	3	11		
		Arrhythmia	1	2	0	3		
30–59%	Hair	Low posterior implantation	1	7	4	12 (48)	0.259	
		Slow growth	2	7	0	9 (36)	0.167	
	Eyebrows	Absence	0	8	1	9 (36)	0.241	
	Eyes	Photophobia	0	5	3	8 (32)	0.019	
		Ptosis	2	7	3	12 (48)	0.519	
		Epicanthal folds	1	8	4	13 (52)	0.593	82
		Strabismus	2	7	5	14 (56)	0.333	55
	Ears	Large	2	6	2	10 (40)	0.185	89
	Nose	Short	1	9	4	14 (56)	0.87	77
	Palate	High	2	8	2	12 (48)	0.537	60
	Craniofacial	Long philtrum	2	6	2	10 (40)	0.389	
		Prominent philtrum	2	5	2	9 (36)	0.013	
		Micrognathia	1	4	4	9 (36)	0.241	
	Skin	Eczema	1	5	2	8 (32)	0.259	

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	Hyperkeratosis	0	11	3	14 (56)	0.37	68
	Palmoplantar hyperkeratosis	1	5	2	8 (32)	0.13	
	Wrinkled palms and soles	2	6	0	8 (32)	0.093	100
	Hyperpigmentation	2	8	0	10 (40)	0.056	76
Other	Seizures	0	6	3	9 (36)	0.148	
	Joint hyperextention	1	7	2	10 (40)	0.13	87
	Pectus excavatum	0	4	4	8 (32)	0.278	
	Hypotonic	1	8	5	14 (56)	0.278	
	CFC index	16.7	16.0	16.8			

[a]Frequency of absolute and relative macrocephaly.
[b]van Eeghen et al. [1999].

with *BRAF* mutations accounted for 85.7% of mutation-positive patients. Mutations in *BRAF* were clustered in exons 6, 11, 12, 14, and 15, indicating that these five exons should be sequenced first when CFC patients are analyzed. Our results showed that the frequency of *MAP2K1/2* mutations was 22.9 % (8 of 35 patients), which is in contrast with a report showing that patients with *MAP2K1/2* mutations were few in number (3 of 21 mutation-positive patients (14.3%)) [Rodriguez-Viciana et al., 2006]. Mutations were identified in exons 2 and 3 of *MAP2K1* and exons 2, 3, and 7 of *MAP2K2*. Screening of these five exons should be considered after sequencing the five exons in *BRAF*. *KRAS* mutations were less frequent in our CFC patients (8.6%). *KRAS* mutations have also been identified in a few patients with Noonan syndrome [Schubbert et al., 2006; Carta et al., 2006].

Twenty-one patients were finally negative for *PTPN11*, *HRAS*, *KRAS*, *BRAF*, *MAP2K1/2* and *MAP2K1/2*. These patients have been initially diagnosed with CFC syndrome. Ten *bona fide* CFC patients described by [Kavamura et al., 2003] were included in our study and only five patients were mutation-positive [Roberts et al., 2006]. We collected detailed clinical manifestations in 4 mutation-negative patients of 13 new patients. Their manifestations were similar to those with mutation-positive CFC syndrome (CFC index: 14.0, 18.5, 14.2, 14.2 mean; 15.2). These results suggest that new genes encoding molecules upstream of RAS or parallel regulators of RAS, RAF, and MEK1/2 cause mutation-negative patients. Alternatively, mutations in the promoter region or introns in the known genes might be responsible for the pathogenesis in CFC patients.

Genotype–phenotype analysis showed that there was no obvious difference among patients with mutations in *KRAS*, *BRAF*, or *MAP2K1/2*. The CFC index [Kavamura et al., 2002] also showed no significant differences among patients with mutations in different genes. CFC syndrome was initially designated as manifesting abnormalities in heart, face, and skin [Reynolds et al., 1986]. However, there were two patients who did not have any skin abnormalities (CFC91: D153V in *KRAS* and CFC148: N580D in *BRAF*) and three patients who did not have any heart defects. It is of note that patient CFC148 with *BRAF* N508D mutation (Fig. 2L) did not have any skin or heart symptoms. This patient is still 1 year of age. Further observation will be necessary to see if this patient develops skin problems or not.

The frequency of wrinkled palms and soles (Fig. 2A,B,G), hyperpigmentation (Fig. 2K) and joint hyperextension was 32%, 40%, and 40% in patients with the mutation-positive CFC syndrome, respectively. In previous clinical reports, these manifestations were not regarded as important clinical features in CFC syndrome (0.093, 0.056, and 0.13 in CFC index) [Kavamura et al., 2002]. In contrast, these

clinical manifestations were frequently observed in patients with Costello syndrome (100%, 76%, and 87%, respectively) [Hennekam, 2003]. Two of our patients, CFC149 with *BRAF* Q257R mutation (Fig. 2D) and CFC143 with *BRAF* V487G mutation (Fig 2F,G), had been diagnosed as having Costello syndrome in their infantile periods. Careful clinical evaluation revealed that they had CFC syndrome. Furthermore, *BRAF* mutations were identified in patients who exhibited a phenotype of Costello syndrome rather than CFC syndrome [Rauen, 2006; Aoki et al., unpublished observation]. These results suggest the significant overlap in clinical manifestations between CFC syndrome and Costello syndrome.

In conclusion, we identified *KRAS*, *BRAF*, or *MAP2K1/2* mutations in 35 of 56 (62.5 %) patients with CFC syndrome. Detailed analysis of clinical manifestations in mutation-positive patients revealed the high frequencies of wrinkled palms and soles, hyperpigmentation and joint hyperextension, which are frequently seen in Costello syndrome. These results suggest a significant clinical overlap between these two syndromes.

ACKNOWLEDGMENTS

We wish to thank the individuals and their families who participated in this study and the doctors who referred the patients. We thank Dr. S. Manouvrier, Dr. B. Gilbert, and Dr. T. Edouard for referral of patients. This work was supported by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan, Grants-in-Aid from the Ministry of Health, Labor, and Welfare of Japan and a grant from Takeda Science Foundation.

REFERENCES

Aoki Y, Niihori T, Kawame H, Kurosawa K, Ohashi H, Tanaka Y, Filocamo M, Kato K, Suzuki Y, Kure S, Matsubara Y. 2005. Germline mutations in HRAS proto-oncogene cause Costello syndrome. Nat Genet 37:1038–1040.

Bentires-Alj M, Kontaridis MI, Neel BG. 2006. Stops along the RAS pathway in human genetic disease. Nat Med 12:283–285.

Carta C, Pantaleoni F, Bocchinfuso G, Stella L, Vasta I, Sarkozy A, Digilio C, Palleschi A, Pizzuti A, Grammatico P, Zampino G, Dallapiccola B, Gelb BD, Tartaglia M. 2006. Germline missense mutations affecting KRAS isoform B are associated with a severe Noonan syndrome phenotype. Am J Hum Genet 79:129–135.

Garnett MJ, Marais R. 2004. Guilty as charged: B-RAF is a human oncogene. Cancer Cell 6:313–319.

Grebe TA, Clericuzio C. 2000. Neurologic and gastrointestinal dysfunction in cardio-facio-cutaneous syndrome: Identification of a severe phenotype. Am J Med Genet 95:135–143.

Hanna N, Montagner A, Lee WH, Miteva M, Vidal M, Vidaud M, Parfait B, Raynal P. 2006. Reduced phosphatase activity of SHP-2 in LEOPARD syndrome: Consequences for PI3K binding on Gab1. FEBS Lett 580:2477–2482.

Hennekam RC. 2003. Costello syndrome: An overview. Am J Med Genet Part C Semin Med Genet 117C:42–48.

Kavamura MI, Peres CA, Alchorne MM, Brunoni D. 2002. CFC index for the diagnosis of cardiofaciocutaneous syndrome. Am J Med Genet 112:12–16.

Kavamura MI, Pomponi MG, Zollino M, Lecce R, Murdolo M, Brunoni D, Alchorne MM, Opitz JM, Neri G. 2003. PTPN11 mutations are not responsible for the Cardiofaciocutaneous (CFC) syndrome. Eur J Hum Genet 11:64–68.

Kontaridis MI, Swanson KD, David FS, Barford D, Neel BG. 2006. PTPN11 (Shp2) mutations in LEOPARD syndrome have dominant negative, not activating, effects. J Biol Chem 281: 6785–6792.

Malumbres M, Barbacid M. 2003. RAS oncogenes: The first 30 years. Nat Rev Cancer 3:459–465.

Musante L, Kehl HG, Majewski F, Meinecke P, Schweiger S, Gillessen-Kaesbach G, Wieczorek D, Hinkel GK, Tinschert S, Hoeltzenbein M, Ropers HH, Kalscheuer VM. 2003. Spectrum of mutations in PTPN11 and genotype-phenotype correlation in 96 patients with Noonan syndrome and five patients with cardio-facio-cutaneous syndrome. Eur J Hum Genet 11:201–206.

Niihori T, Aoki Y, Ohashi H, Kurosawa K, Kondoh T, Ishikiriyama S, Kawame H, Kamasaki H, Yamanaka T, Takada F, Nishio K, Sakurai M, Tamai H, Nagashima T, Suzuki Y, Kure S, Fujii K, Imaizumi M, Matsubara Y. 2005. Functional analysis of PTPN11/SHP-2 mutants identified in Noonan syndrome and childhood leukemia. J Hum Genet 50:192–202.

Niihori T, Aoki Y, Narumi Y, Neri G, Cave H, Verloes A, Okamoto N, Hennekam RC, Gillessen-Kaesbach G, Wieczorek D, Kavamura MI, Kurosawa K, Ohashi H, Wilson L, Heron D, Bonneau D, Corona G, Kaname T, Naritomi K, Baumann C, Matsumoto N, Kato K, Kure S, Matsubara Y. 2006. Germline KRAS and BRAF mutations in cardio-facio-cutaneous syndrome. Nat Genet 38:294–296.

Ohren JF, Chen H, Pavlovsky A, Whitehead C, Zhang E, Kuffa P, Yan C, McConnell P, Spessard C, Banotai C, Mueller WT, Delaney A, Omer C, Sebolt-Leopold J, Dudley DT, Leung IK, Flamme C, Warmus J, Kaufman M, Barrett S, Tecle H, Hasemann CA. 2004. Structures of human MAP kinase kinase 1 (MEK1) and MEK2 describe novel noncompetitive kinase inhibition. Nat Struct Mol Biol 11:1192–1197.

Rauen KA. 2006. Distinguishing Costello versus cardio-facio-cutaneous syndrome: BRAF mutations in patients with a Costello phenotype. Am J Med Genet Part A 140A:1681–1683.

Reynolds JF, Neri G, Herrmann JP, Blumberg B, Coldwell JG, Miles PV, Opitz JM. 1986. New multiple congenital anomalies/mental retardation syndrome with cardio-facio-cutaneous involvement—the CFC syndrome. Am J Med Genet 25:413–427.

Roberts A, Allanson J, Jadico SK, Kavamura MI, Noonan J, Opitz JM, Young T, Neri G. 2006. The cardiofaciocutaneous syndrome. J Med Genet 43:833–842.

Rodriguez-Viciana P, Tetsu O, Tidyman WE, Estep AL, Conger BA, Cruz MS, McCormick F, Rauen KA. 2006. Germline mutations in genes within the MAPK pathway cause cardio-facio-cutaneous syndrome. Science 311:1287–1290.

Schubbert S, Zenker M, Rowe SL, Boll S, Klein C, Bollag G, van der Burgt I, Musante L, Kalscheuer V, Wehner LE, Nguyen H, West B, Zhang KY, Sistermans E, Rauch A, Niemeyer CM, Shannon K, Kratz CP. 2006. Germline KRAS mutations cause Noonan syndrome. Nat Genet 38:331–336.

Tartaglia M, Mehler EL, Goldberg R, Zampino G, Brunner HG, Kremer H, van der Burgt I, Crosby AH, Ion A, Jeffery S, Kalidas K, Patton MA, Kucherlapati RS, Gelb BD. 2001. Mutations in PTPN11, encoding the protein tyrosine phosphatase SHP-2, cause Noonan syndrome. Nat Genet 29:465–468.

Tartaglia M, Martinelli S, Stella L, Bocchinfuso G, Flex E, Cordeddu V, Zampino G, Burgt I, Palleschi A, Petrucci TC, Sorcini M, Schoch C, Foa R, Emanuel PD, Gelb BD. 2006. Diversity and functional consequences of germline and

somatic PTPN11 mutations in human disease. Am J Hum Genet 78:279–290.

van Den Berg H, Hennekam RC. 1999. Acute lymphoblastic leukaemia in a patient with cardiofaciocutaneous syndrome. J Med Genet 36:799–800.

van Eeghen AM, van Gelderen I, Hennekam RC. 1999. Costello syndrome: Report and review. Am J Med Genet 82:187–193.

Wieczorek D, Majewski F, Gillessen-Kaesbach G. 1997. Cardio-facio-cutaneous (CFC) syndrome—a distinct entity? Report of three patients demonstrating the diagnostic difficulties in delineation of CFC syndrome. Clin Genet 52:37–46.

Zheng CF, Guan KL. 1993. Cloning and characterization of two distinct human extracellular signal-regulated kinase activator kinases, MEK1 and MEK2. J Biol Chem 268:11435–11439.

Drug Metab. Pharmacokinet. 22 (2): *SNP29* (136)–*SNP33* (140) (2007).

SNP Communication

Three Novel Single Nucleotide Polymorphisms (SNPs) of CYP2S1 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: We analyzed all nine exons and exon-intron junctions of the *CYP2S1* gene in 200 Japanese individuals and identified the following three novel single nucleotide polymorphisms (SNPs): 4612G>A (Glu147Glu) in exon 3, 5478C>T (Leu230Leu) and 5479T>G (Leu230Arg, *CYP2S1*5A*) in exon 5. The allele frequencies were 0.013 for 4612G>A, 0.058 for 5478C>T, and 0.003 for 5479T>G. In addition, a known SNP 1324C>G (Pro74Pro) was detected at a frequency of 0.300.

Key words: CYP2S1; genetic polymorphism; SNP

Introduction

The cytochrome P450s (CYPs) constitute a large and complex gene superfamily. Currently, 57 active *CYP* genes and 58 pseudogenes are known to present in the human genome. Most of the *CYP* genes have the highest expression in the liver, which plays a dominant role in the clearance of foreign compounds. CYP enzymes also metabolize many endogenous compounds important for the maintenance of cellular homeostasis, such as steroids, retinoids, bile acids, fatty acids, and eicosanoids.

Recently, a novel *CYP* gene, *CYP2S1*, has been identified. The *CYP2S1* gene is localized in the *CYP2* gene cluster on chromosome 19q.13.2. Several studies that investigated the tissue distribution of human CYP2S1 mRNA demonstrated that it has low expression levels in the liver but is detectable in extrahepatic tissues such as those of the respiratory and digestive systems.[1-3] Furthermore, CYP2S1 mRNA and protein were detected in human skin, where CYP2S1 was shown to be induced by ultraviolet radiation, coal tar, and *all-trans* retinoic acid.[3,4] In the same study, CYP2S1 was observed to metabolize *all-trans* retinoic acid, indicating that CYP2S1 may be involved in the biotransformation of endogenous substrates important for cell proliferation and differentiation.[5]

The human *CYP2S1* gene has recently been shown to be polymorphic; two amino acid-changing allelic variants *CYP2S1*2* (10347C>T; 13255A>G) and *CYP2S1*3* (13106C>T; 13255A>G) have been detected in Caucasians.[6] *CYP2S1* has been genetically analyzed in Caucasians but not in Japanese individuals. In the present study, nine exons and exon-intron junctions of the *CYP2S1* gene from 200 Japanese individuals were screened for genetic polymorphisms by using denaturing HPLC (DHPLC). We identified three novel single nucleotide polymorphisms of the *CYP2S1* gene, including a nonsynonymous polymorphism, 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 the blood donors, and the study was approved by the Local Ethics Committee of Tohoku University Hospital and Tohoku Pharmaceuti-

As of December 1, 2006, 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/). The *CYP2S1* haplotype with 5479T>G (Leu230Arg) was assigned as *CYP2S1*5A* by the Human CYP Allele Nomenclature Committee (http://www.imm.ki.se/CYPalleles/).

Received; December 19, 2006, Accepted; February 13, 2007
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Table 1. Amplification and DHPLC conditions for *CYP2S1* 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)
1	247	gccgcgcggagcgcctggga	ccaggacgtccccagagccc	Slowdown[a] 70.0–55.0	63	65.8, 68.5
2	376	cttggatcgaagaggtcacagc	ttgggatttcaggcactagcc	60.0	35	64.3
3	303	caacagagcgagattccgtctc	agttttcccttttcactcggctg	65.0	35	62.9
4	367	ctctctccctgcgctgtcc	gagaagggcagctagttctcatgg	60.0	35	62.6
5	257	tcccatgagaactagctgcc	caccatgcccattcagagag	60.0	30	64.7
6	336	taacttgtgtttccgaccccag	ctcagcctcccaaagtgctg	65.0	35	59.2
7	436	acaagatgtgtggtctttgggc	agaaaaagtcagggagacactgacag	60.0	35	63.0
8	485	tctctcacctcagcctcccac	tcagtattcctcacacccaggc	60.0	35	60.5
9	342	tgaggaatactgactcagccctctc	acactctggagacattaaccctgtcc	60.0	35	62.4

[a]Slowdown protocol: The annealing temperature was decreased after cycle 3 by 1.0°C every 3 cycles, beginning at 70°C and decreased to a "slowdown" annealing temperature of 55°C, followed by 15 additional cycles with an annealing temperature of 60°C. The PCR was used at a ramp rate of 2.5°C/s and reached annealing temperature at 1.5°C/s.

cal University. DNA was isolated from K_2EDTA-anticoagulated peripheral blood by using QIAamp DNA Mini Kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Table 1 lists the primer pairs used to amplify nine exons and exon-intron junctions of the *CYP2S1* gene. These primers were designed based on the genomic sequence reported in GenBank (NG_000008.7). The amplicons for exon 1 were generated using AmpliTaq Gold PCR Master Mix (Applied Biosystems, Foster City, CA, USA). PCR reactions were performed using an iCycler (Bio-Rad, Hercules, CA, USA). Moreover, the method relied on a combination of the slowdown method and the addition of betaine (Sigma-Aldrich, St.Louis, MO) for this region with high GC content (>70%).[7] The PCR condition comprised denaturation at 95°C for 5 minutes, followed by 48 cycles of denaturation at 95°C for 30 seconds, annealing for 30 seconds, extension at 72°C for 40 seconds, and finally, 15 additional cycles at an annealing temperature of 60°C. The amplicons for each exon from 2 to 9 were generated using the AmpliTaq Gold PCR Master Mix. The PCR conditions comprised denaturation at 95°C for 10 minutes, followed by 30 or 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 annealing temperatures and PCR cycles for the screening of *CYP2S1* variants are summarized in **Table 1**. Heteroduplexes were generated by performing 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).[8-12] Amplified 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 of a heterozygous *CYP2S1* 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 detected due to a slight shift in the elution time as compared with that of the reference. The addition of an approximately equal amount of wild-type DNA to the samples (1:1) at the denaturation step enabled the reliable detection of homozygous alterations in exon 2. This was performed for all samples in order to identify homozygous sequence variations. Therefore, all samples were analyzed as follows. First, equal amounts of four samples were mixed to identify the heterozygotes, and then, each sample was mixed with wild-type DNA to detect the homozygous variants. The resultant chromatograms were compared with those of the wild-type DNA.

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

Table 2. The location of SNPs and frequencies of the *CYP2S1* gene in 200 DNA samples of Japanese individuals

Location	Variant	Amino acid change	SNP ID dbSNP (NCBI)	The number of each genotype	Observed Frequency (%) (95% CI)	Frequency (%) predicted by Hardy-Weinberg law
Exon 2	1324C>G	Pro74Pro	rs338599	C/C: 99	49.5 (42.6–56.4)	49.0
				C/G: 82	41.0 (34.2–47.8)	42.0
				G/G: 19	9.5 (5.4–13.6)	9.0
Exon 3	4612G>A	Glu147Glu	—	G/G: 195	97.5 (95.3–99.7)	97.5
				G/A: 5	2.5 (0.3–4.7)	2.5
				A/A: 0	0.0 (0.0)	0.0
Exon 5	5478C>T	Leu230Leu	—	C/C: 177	88.5 (84.1–92.5)	88.8
				C/T: 23	11.5 (7.1–15.9)	10.8
				T/T: 0	0.0 (0.0)	0.3
Exon 5	5479T>G	Leu230Arg	—	T/T: 199	99.5 (98.5–100)	99.5
				T/G: 1	0.5 (0–1.5)	0.5
				G/G: 0	0.0 (0.0)	0.0

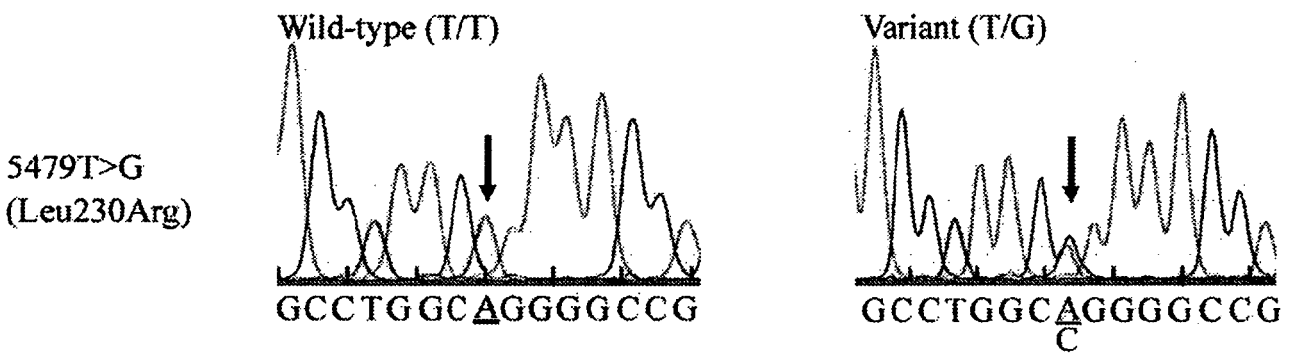

Fig. 1. The nucleotide sequences of the *CYP2S1* gene in exon 5.
Although sequences are shown for anti-sense strands, both strands were sequenced. Arrows indicate the positions of the variant nucleotide.

Results and Discussion

We found the following three novel SNPs:

1) SNP: 061023Hiratsuka10; GENE NAME: *CYP2S1*;
ACCESSION NUMBER: NG_000008; LENGTH: 25 bases;
5′-AGAAGGCGAGGAG/ACTGATCCAGGCG-3′.
2) SNP: 061023Hiratsuka11; GENE NAME: *CYP2S1*;
ACCESSION NUMBER: NG_000008; LENGTH: 25 bases;
5′-TTCCTGCGGCCCC/TTGCCAGGCCCCC-3′.
3) SNP: 061023Hiratsuka12; GENE NAME: *CYP2S1*;
ACCESSION NUMBER: NG_000008; LENGTH: 25 bases;
5′-TCCTGCGGCCCCT/GGCCAGGCCCCCA-3′.

DHPLC analysis of the *CYP2S1* gene in the 200 DNA samples obtained from Japanese individuals revealed chromatographic profiles that were distinct from that of the wild-type in exons 2, 3, and 5. We tested the specificity of DHPLC in detecting the variant allele in these exons by comparing the results with those of direct sequencing. Four SNPs including three novel and one known SNP (rs338599) were detected (**Table 2**). The electrophoretograms of the novel nonsynonymous SNP are shown in **Fig. 1**. The SNP in exon 5 was 5479T>G and resulted in an amino acid change of Leu230Arg. Among the 200 individuals, one was heterozygous for the 5479T>G SNP, suggesting that the allele frequency was 0.003 in the Japanese population. The other novel SNPs 4612G>A and 5478C>T were detected at frequencies of 0.013 and 0.058, respectively. The sequences for each sample were obtained from at least two different PCR amplifications.

The novel SNP 5479T>G is located in exon 5 of the *CYP2S1* gene and results in an amino acid substitution. Homology modeling of the human CYP2 family enzymes based on the CYP2C5 crystal structure lead to speculation that Leu230 is located at the start of the