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ORIGINAL ARTICLE

Cardio-facio-cutaneous and Noonan syndromes due to mutations in the RAS/MAPK signalling pathway: genotype–phenotype relationships and overlap with Costello syndrome

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Cardio-facio-cutaneous (CFC) syndrome, Noonan syndrome (NS), and Costello syndrome (CS) are clinically related developmental disorders that have been recently linked to mutations in the RAS/MEK/ERK signalling pathway. This study was a mutation analysis of the *KRAS, BRAF, MEK1* and *MEK2* genes in a total of 130 patients (40 patients with a clinical diagnosis of CFC, 20 patients without *HRAS* mutations from the French Costello family support group, and 70 patients with NS without *PTPN11* or *SOS1* mutations). *BRAF* mutations were found in 14/40 (35%) patients with CFC and 8/20 (40%) *HRAS*-negative patients with CS. *KRAS* mutations were found in 1/40 (2.5%) patients with CFC, 2/20 (10%) *HRAS*-negative patients with CS and 4/70 patients with NS (5.7%). *MEK1* mutations were found in 4/40 patients with CFC (10%), 4/20 (20%) *HRAS*-negative patients with CS and 3/70 (4.3%) patients with NS, and *MEK2* mutations in 4/40 (10%) patients with CFC. Analysis of the major phenotypic features suggests significant clinical overlap between CS and CFC. The phenotype associated with *MEK* mutations seems less severe, and is compatible with normal mental development. Features considered distinctive for CS were also found to be associated with *BRAF* or *MEK* mutations. Because of its particular cancer risk, the term "Costello syndrome" should only be used for patients with proven *HRAS* mutation. These results confirm that *KRAS* is a minor contributor to NS and show that *MEK* is involved in some cases of NS, demonstrating a phenotypic continuum between the clinical entities. Although some associated features appear to be characteristic of a specific gene, no simple rule exists to distinguish NS from CFC easily.

Since its original description by Reynolds *et al*,[1] cardio-facio-cutaneous (CFC) syndrome has been reported in about 60 patients, allowing precise elucidation of its phenotype.[2–5] The developmental anomalies in CFC include congenital heart defects (CHD), ectodermal anomalies, and short stature. The degree of mental retardation is variable, usually moderate to severe. Affected individuals also present a characteristic facial appearance with a high forehead, bi-temporal constriction, down-slanting palpebral fissures, short nose with depressed nasal bridge, and relative macrocephaly. The CFC phenotype is reminiscent of Noonan syndrome (NS) and Costello syndrome (CS) and differential diagnosis can be difficult, particularly in infancy. Diagnostic indexes have been proposed by Grebe and Clericuzio[6] and Kavamura *et al*.[7] Some clinical signs are useful to differentiate the three entities clinically. Sparse hair and eyebrows, follicular hyperkeratosis and palmoplantar hyperkeratosis characterise CFC, whereas cutis laxa, diffuse skin hyperpigmentation, papillomata, ulnar deviation of the hands and nail dystrophy are hallmarks of CS. Qualitatively, facial dysmorphology is similar in NS, CS and CFC, but compared with patients with NS, the face of patients with CFC is wider. The mouth of patients with CS is also wider with thick lips, and coarsening of face is typical in both CS and CFC. CHD in CFC are remarkably similar to those noted in NS and CS. The incidence of CHD and hypertrophic cardiomyopathy are comparable in CFC and CS. Severe cardiac arrhythmias occur in a third of patients with CS, whereas they are rare in NS and CFC. In infancy, feeding problems and failure to thrive are more frequent and severe in CFC and CS than in NS. CS and CFC are associated with more severe developmental delay than NS. CS is associated with a greatly increased risk of malignancy, notably rhabdomyosarcoma. The incidence of CFC is unknown. All cases reported to date have been sporadic.

In 2001, Tartaglia *et al* discovered that activating mutations of *PTPN11* cause 40–50% of cases of NS.[8] As *PTPN11* encodes SHP2, a non-receptor tyrosine phosphatase involved in RAS pathway activation, genes encoding RAS/MAPK components have systematically been screened. Activating mutations of *HRAS* were found in roughly 85% of patients with a clinical diagnosis of CS.[9–12] Patients with CFC harbour activating missense mutations in *KRAS*,[13] *BRAF*,[13 14] *MEK1* and *MEK2*.[14] *KRAS* was also shown to cause a small subset of NS cases.[15 16] About 10% of patients with NS carry activating mutations in *SOS1*, a RAS-activating molecule of the guanosine exchange factor (GEF) family.[17 18] These proteins are part of the RAS/MAPK signalling pathway, which is involved in many biological processes and plays crucial roles during embryonic development.[19] Somatic mutation and/or increased transcription of the genes encoding these proteins are a common feature in tumour progression.

The aim of this study was to screen the genes causing CFC syndrome in three cohorts of patients referred with (1) a clinical diagnosis of CFC, (2) a clinical diagnosis of CS but no

Abbreviations: CFC, cardio-facio-cutaneous; CHD, congenital heart defects; CS, Costello syndrome; GEF, guanosine exchange factor; JMML, juvenile myelomonocytic leukaemia; NF1, neurofibromatosis type 1; NS, Noonan syndrome

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

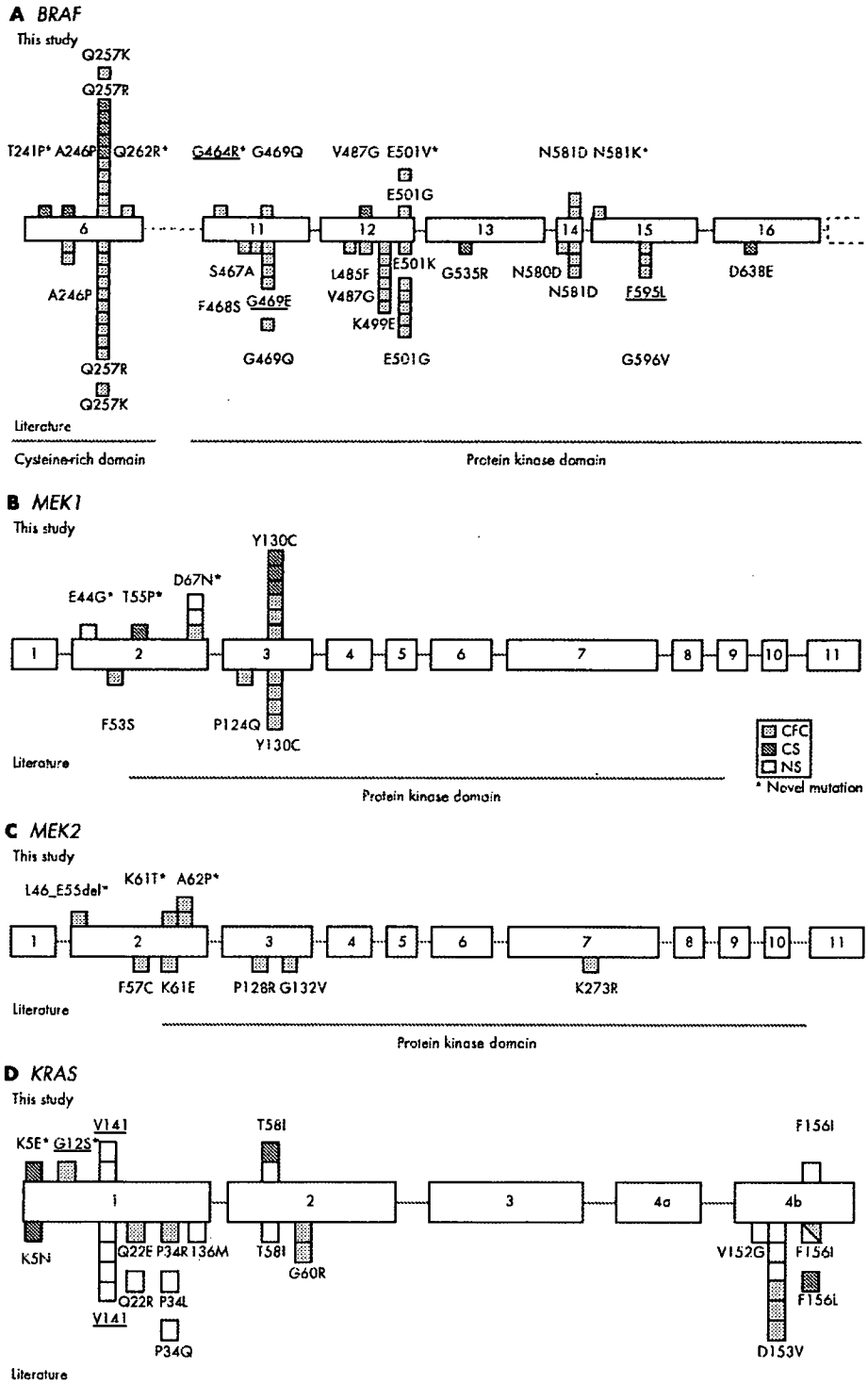

Figure 1 Mutations in (A) *BRAF*, (B) *MEK1*, (C) *MEK2*, and (D) *KRAS* found in patients with cardio-facio-cutaneous (CFC) syndrome (blue squares), Costello syndrome (CS; grey squares) and Noonan syndrome (NS; white squares) in this study and in the literature. Exons are represented by bars and numbered; 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

	Niihori *et al*,[12] Narumi *et al*[20]	Rodriguez-Viciana *et al*[24]	Schubbert *et al*[9]		Carta *et al*[6]		Raven *et al*[25]	Zenker *et al*[11]			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 Niihori *et al*[12] Narumi *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 *Caenorhabditis elegans*.

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%
Hypotonia	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*	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 Naruni *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

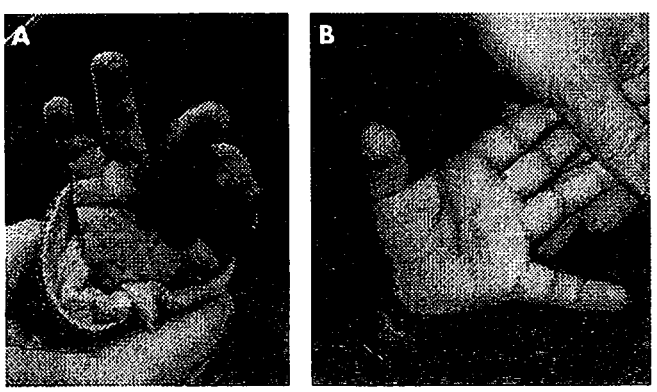

Figure 2 Close-up of hand creases in patients with (A) *BRAF* A246P and (B) G469D mutations: note thick fingers with wide, squared tips, redundancy of skin with deep palmar creases. Parental/guardian informed consent was obtained for publication of this figure.

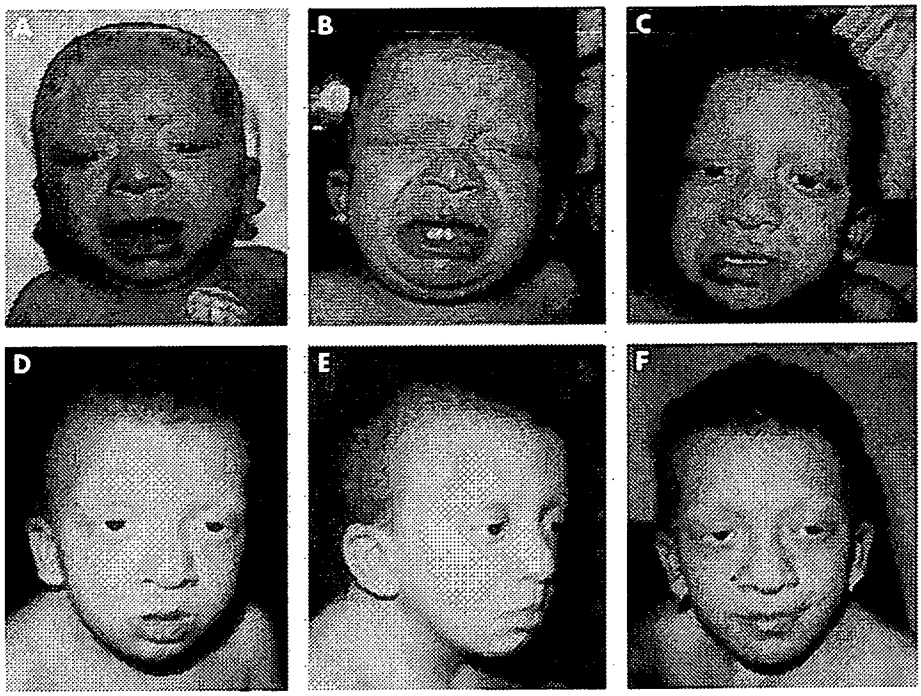

Figure 3 Changing facial phenotype of a patient with *MEK2* A62P mutation, depicted at various ages. (A, B) Facial dysmorphism at (A) 4 months and (B) 12 months is clearly NS-like, with mild ptosis, deep philtrum with prominent ridges and uplifted ear lobules. (C) At 3.5 years of age, thick lips and 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.

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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*[18] 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.

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

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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

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

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

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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

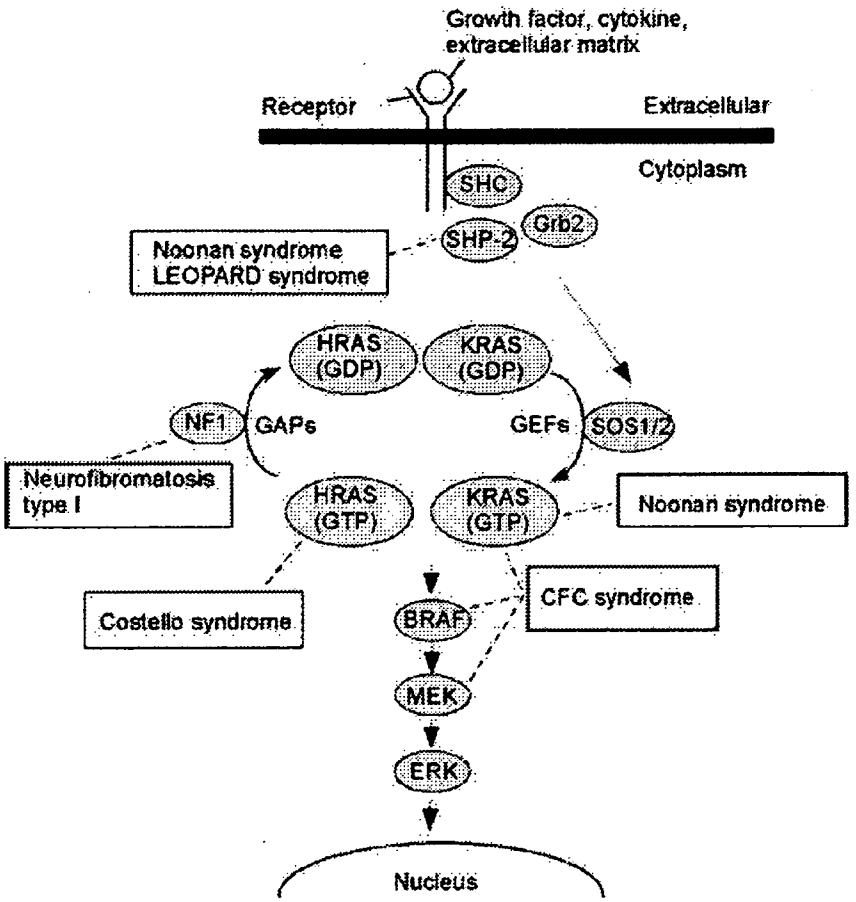

FIG. 1. A: The RAS-RAF-MEK-ERK signaling pathway and associated disorders. Mutations with enhanced catalytic activity of tyrosine phosphatase SHP-2 have been identified in patients with Noonan syndrome [Tartaglia et al., 2001]. In contrast, loss-of-function mutations in SHP-2 have been identified in 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

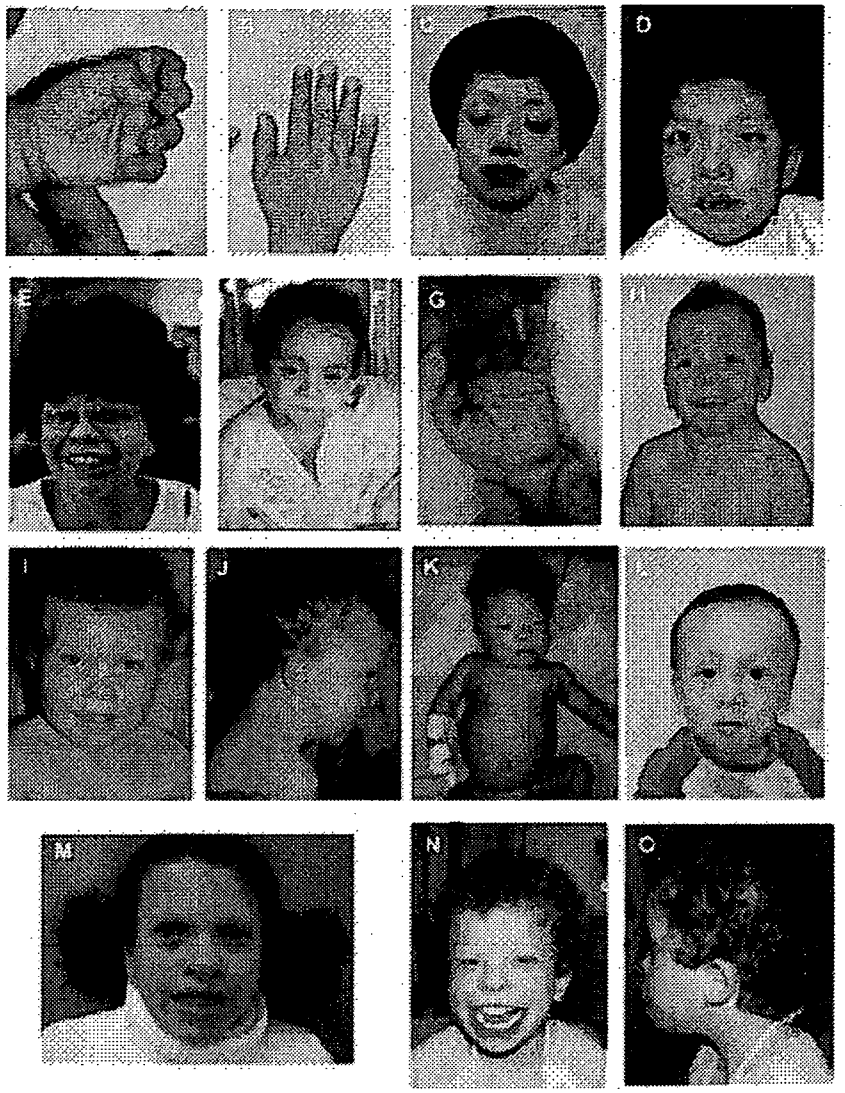

FIG. 2. Typical facial appearances and hands of mutation-positive patients. **A**: Wrinkled palm of CFC73 with *KRAS* D153V mutation. **B**: Hand with deep wrinkles in CFC 8 with *KRAS* 153V mutation. **C**: CFC7 with *BRAF* Q257K mutation. This patient has been contracted with intractable epilepsy. **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.

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ORIGINAL ARTICLE

Mutation and haplotype analyses of the *MUT* gene in Japanese patients with methylmalonic acidemia

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Abstract Methylmalonic acidemia (MMA) is caused by a deficiency in the activity of L-methylmalonyl-CoA mutase (MCM), a vitamin B12 (or cobalamin, Cbl)-dependent enzyme. Apoenzyme-deficient MMA (*mut* MMA) results from mutations in the nuclear gene *MUT*. Most of the *MUT* mutations are thought to be private or restricted to only a few pedigrees. Our group elucidated the spectrum of mutations of Japanese *mut* MMA patients by performing mutation and haplotype analyses in 29 patients with *mut* MMA. A sequence analysis identified mutations in 95% (55/58) of the disease alleles. Five mutations were relatively frequent (p.E117X, c.385 + 5G > A, p.R369H, p.L494X, and p.R727X) and four were novel (p.M1V, c.753_753 + 5delGGTATA, c.1560G > C, and c.2098_2099delAT). Haplotype analysis suggested that all of the frequent mutations, with the exception of p.R369H, were spread by the founder effect. Among 46 Japanese patients investigated in the present and previous studies, 76% (70/92) of the mutations were located in exons 2, 6, 8, and 13. This finding – that a limited number of mutations account for most of the mutations in Japanese *mut* MMA patients – is in contrast with results of a previous study in Caucasian patients.

Keywords Methylmalonic academia · L-Methylmalonyl-CoA mutase

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Introduction

Methylmalonic acidemia (MMA) is an autosomal-recessive disorder of propionate metabolism caused by a defect in the isomerization of L-methylmalonyl-CoA to succinyl-CoA. The reaction is catalyzed by L-methylmalonyl-CoA mutase (MCM, EC 5.4.99.2), an enzyme which requires adenosylcobalamin (AdoCbl) as a cofactor (Fenton et al. 2001). MMA is classified into two forms: one resulting from a defect in the MCM apoenzyme (*mut* MMA or vitamin B_{12}-unresponsive MMA; MIM 251000) and another resulting from a defect in the steps leading to AdoCbl synthesis (*cbl* MMA or vitamin B_{12}-responsive MMA) (Rosenblatt and Fenton 2001). Typical MMA is characterized clinically by lethargy, vomiting, and hypertonia with abnormal movements, and biochemically by an accumulation of methylmalonic acid in the tissues and body fluid associated with hyperammonemia and ketoacidosis.

MCM is encoded by a single gene, *MUT*, which has been located to 6p21. *MUT* consists of 13 exons spanning 35 kb and it produces a 2.7-kb mRNA. To date, more than 100 disease-causing mutations in the human *MUT* gene have been reported (Ledley and Rosenblatt 1997; Acquaviva et al. 2005; Martinez et al. 2005), most of which seem to be unique or restricted to