

Editor's Note

The following paper by Estep and colleagues was submitted to the Journal only a few weeks after the preceding paper by Gripp et al. also published in this issue. Both investigations address in detail the frequency of *HRAS* mutations in large series of patients with Costello syndrome, and both present timely data.

Editorial perusal of this work raised an interesting dilemma that will likely be more commonplace in the future: both groups of investigators had collected samples at the same International Costello Syndrome meetings in 2001, 2003, and 2005. The *AJMG* Editors

considered it important to understand the degree of overlap of patients in each study and requested the respective corresponding authors to provide the blinded identity (initials only) of their patients. Both complied.

From this information, we could determine that there were 20 patients that participated in both investigations. We have resolved that these two studies have investigated at least 56 patients with Costello syndrome. Notably the frequency of mutation-positive patients was high in both studies, and both demonstrate a similar mutation type and location.

Rapid Publication

***HRAS* Mutations in Costello Syndrome: Detection of Constitutional Activating Mutations in Codon 12 and 13 and Loss of Wild-Type Allele in Malignancy**

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Costello syndrome (CS) is a complex developmental disorder involving characteristic craniofacial features, failure to thrive, developmental delay, cardiac and skeletal anomalies, and a predisposition to develop neoplasia. Based on similarities with other cancer syndromes, we previously hypothesized that CS is likely due to activation of signal transduction through the Ras/MAPK pathway [Tartaglia et al., 2003]. In this study, the *HRAS* coding region was sequenced for mutations in a large, well-characterized cohort of 36 CS patients. Heterogeneous missense point mutations predicting an amino acid substitution were identified in 33/36 (92%) patients. The majority (91%) had a 34G → A transition in codon 12. Less frequent mutations included 35G → C (codon 12) and 37G → T (codon 13). Parental samples did not have

an *HRAS* mutation supporting the hypothesis of de novo heterogeneous mutations. There is phenotypic variability among patients with a 34G → A transition. The most consistent features included characteristic facies and skin, failure to thrive, developmental delay, musculoskeletal abnormalities, visual impairment, cardiac abnormalities, and generalized hyperpigmentation. The two patients with 35G → C had cardiac arrhythmias whereas one patient with a 37G → T transversion had an enlarged aortic root. Of the patients with a clinical diagnosis of CS, neoplasia was the most consistent phenotypic feature for predicating an *HRAS* mutation. To gain an understanding of the relationship between constitutional *HRAS* mutations and malignancy, *HRAS* was sequenced in an advanced biphasic rhabdomyo-

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sarcoma/fibrosarcoma from an individual with a 34G → A mutation. Loss of the wild-type *HRAS* allele was observed, suggesting tumorigenesis in CS patients is accompanied by additional somatic changes affecting *HRAS*. Finally, due to phenotypic overlap between CS and cardio-facio-cutaneous (CFC) syndromes, the *HRAS* coding region was sequenced in a well-characterized CFC cohort. No mutations were found

which support a distinct genetic etiology between CS and CFC syndromes. © 2005 Wiley-Liss, Inc.

Key words: cardio-facio-cutaneous syndrome; Costello syndrome; fibrosarcoma; constitutional mutation; *HRAS* mutations; ras pathway; rhabdomyosarcoma

INTRODUCTION

Costello syndrome (CS; MIM 214080) is a rare multiple congenital anomaly disorder in which individuals have characteristic craniofacial features, failure to thrive, ectodermal and musculoskeletal anomalies, cardiac abnormalities, endocrinopathy, developmental delay and a predisposition to neoplasia, both benign and malignant [Costello, 1971, 1977, 1996; Hennekam, 2003]. Malignancies reported in patients with CS are varied, with the most common including rhabdomyosarcoma (RMS), transitional cell carcinoma, and neuroblastoma [Gripp, 2005]. The clinical diagnosis has relied on the recognition of a characteristic phenotype. Facial features are coarse and typically include macrocephaly with a prominent forehead, epicanthal folds, downslanting palpebral fissures, short nose with a depressed nasal bridge and broad tip, low-set, posteriorly rotated ears with thickened helices and lobes. The cheeks may be full and the mouth large with full lips. Typical ectodermal features consist of curly hair, soft, and redundant skin, deep palmar and plantar creases, hyperpigmentation, and papillomas. Cardiac abnormalities vary, including structural anomalies, hypertrophic cardiomyopathy, or rhythm disturbances [Lin et al., 2002]. Musculoskeletal abnormalities include limited range of motion at the elbows, tight calcaneal tendons, ulnar deviation of the hands, laxity of the small joints, and broad distal phalanges. The developmental delay in patients with CS syndrome varies from mild to severe.

Although now being identified more frequently, CS has been a diagnostic dilemma due to the lack of a molecular genetic diagnosis. While CS has a distinct phenotype and clinical course, there are similarities in phenotypic features with cardio-facio-cutaneous syndrome (CFC; MIM 115150) and Noonan syndrome (NS; MIM 163950). Distinguishing these entities can be challenging, especially in newborns and infants in whom seminal features may not yet be evident. In addition, CS also shares a predisposition to developing certain cancers as part of the phenotype, a feature which is also seen in NS, which in turn overlaps with phenotypic features of neurofibromatosis 1 (NF1; MIM 162200). NS and NF1 are both associated with constitutional mutations that cause activation of the Ras/MAPK pathway by two distinct mechanisms, activation of an oncogene, and deletion of a tumor suppressor gene, respectively. Due to

a similarity among these syndromes in developmental and oncologic spheres, we previously proposed that the CS disease gene(s) may prove to be a protein participating in signal transduction in the Ras/MAPK pathway as is seen in NS and NF1 [Tartaglia et al., 2003]. Recently, constitutional *HRAS* mutations have been found in a cohort of twelve patients with the clinical diagnosis of CS [Aoki et al., 2005].

In this study, we examined a large, well-characterized cohort of patients with the clinical diagnosis of CS. We sequenced the *HRAS* coding region to define *HRAS* mutations in CS and to establish a possible genotype–phenotype correlation. In addition, we sequenced *HRAS* to establish the role it may play in the development of a biphasic rhabdomyosarcoma/fibrosarcoma collected from an individual with CS. Because of the phenotypic overlap of CS and CFC syndrome, we also screened for *HRAS* mutations in a well-characterized cohort of patients with the clinical diagnosis of CFC.

MATERIALS AND METHODS

Patients

A cohort of 36 patients with the clinical diagnosis of CS were consented and participated in the present study. The research protocol was performed under an approved institutional review board from the University of California San Francisco. All patients were examined at either the 2001 Second International Costello Conference (Toronto, Ontario, Canada) or the 2005 Fourth International Costello Conference (St. Louis, MO) or in the Department of Pediatrics at the University of California San Francisco. All patients fulfilled strict diagnostic criteria for CS including characteristic craniofacial features, characteristic ectodermal and musculoskeletal anomalies, failure to thrive, growth deficiency, and developmental delay. The majority of patients had known cardiac abnormalities, brain abnormalities detected by head imaging, visual impairment, and papillomas. In addition, five patients in the cohort had a malignancy: three patients with embryonal rhabdomyosarcoma (ERMS) [Gripp, 2005], one patient with alveolar rhabdomyosarcoma (ARMS) [Gripp, 2005], and one patient with low-grade papillary urothelial carcinoma. Patient age ranged from 16 months to 36 years.

CFC patients were either recruited at the 2001 Second International Costello Conference or through CFC International (www.cfcysndrome.org) under an approved institutional review board from the University of California San Francisco. Controls ($n = 10$) consisted of phenotypically unaffected individuals.

Tumor Tissue

Fresh frozen tumor tissue was available from one individual with CS who was diagnosed with ERMS of the bladder. Cytogenetic analysis of the primary tumor at initial diagnosis demonstrated clonal chromosomal anomalies: 60,XY,+2,+3,+5,+7,+8,+8,t(9;16)(p24;q12.1),+11,+13,+13,+18,+19,+20,+21,+mar[5]/61,XY,+der(2)t(1;2)(q21;q33),+3,+5,+7,+8,8,+8,+11,+13,+13,+18,+18,+19,+20,+21[3][Gripp et al., 2002]. The patient subsequently passed away several years later at 7 years of age. Upon autopsy, a large biphasic abdominal mass was present. Gross pathologic examination of the recurrent malignancy demonstrated total encasement of the abdominal contents by a tumor measuring 52 cm × 34 cm × 23 cm. There were two distinct appearing areas upon gross examination and microscopy: a central, red fleshy area that stained strongly positive with the muscle differentiation markers, myogenin and desmin, consistent with rhabdomyosarcoma and a white, firm fibrous outer rim that lacked muscle marker staining. Histopathology of the fibrous outer rim was consistent with a fibrosarcoma, or alternatively could represent fibrosarcomatoid dedifferentiation from the original rhabdomyosarcoma. Cytogenetic analysis of tumor was non-diagnostic due to no growth.

Sequence Analysis of HRAS

Genomic DNA was isolated from peripheral blood lymphocytes using a QIAamp DNA Blood Midi kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. In addition, genomic DNA was extracted from the recurrent tumor specimen including samples from the rhabdomyosarcomatous and the fibrosarcomatous elements using a DNeasy Tissue kit (Qiagen) according to the manufacturer's instructions.

The entire *HRAS* coding region was sequenced for mutations in both the forward and reverse direction. Exons 1–4 and intronic flanking regions were amplified by nested PCR (Table I). The two-step PCR was performed in a 5 μ l volume containing 1.2 pmol of each primer using the VariantSEQr Resequencing System (Applied Biosystems, Foster City, CA) per the manufacturer's specifications. Genomic DNA (10 ng) was used in the first external amplification. The template for the second internal amplification consisted of a 1 μ l aliquot from the first reaction. The following thermocycling parameters were used for both steps of the nested reaction: 94°C for 4 min; 40 cycles of 94°C for 20 sec, 55°C for 25 sec, 72°C for 60 sec; and 72°C for 7 min. DNA sequencing was performed using a Big Dye v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's recommendations and run on an ABI3730xl or ABI3700 capillary sequencing instrument. Data were extracted and analyzed with Sequencer Analysis Software version 3.7 (Applied Biosystems).

RESULTS

HRAS Mutations

The cohort of patients was comprised of 36 unrelated individuals with the clinical diagnosis of CS and 3 sets of parents, 8 patients with CFC, and 10 unaffected controls. Genomic DNA was screened for *HRAS* mutations in exons 1–4. *HRAS* mutations were identified in 33 out of 36 (92%) CS patients. Mutations were identified in codon 12 and codon 13 (Table II). Two different missense point mutations were identified in codon 12: 34G → A and 35G → C, predicting an amino acid substitution of gly12ser and gly12ala, respectively (Fig. 1). The 34G → A transition mutation was the most common mutation observed in this cohort of patients, being found in 30 of 33 patients (91%). Two patients were found to have a codon 12 35G → C transversion mutation. One patient in the cohort had a codon 13 mutation: a 37G → T transversion, predicting an amino acid substitution of gly13cys. In addition, parental DNA samples from three CS patients with the 34G → A mutation did not show any mutations within the *HRAS* gene.

TABLE I. Primer Pairs (Both External and Internal) Used to Amplify *HRAS* Coding Sequence and Corresponding Amplicon Size

Exon	Primer Sequence (5' to 3')		Product length (bp)
	Forward	Reverse	
1	Ext: AGGTAGGCACG CTGCAGTCC Int: CTCATGCCCCCTCTCTCCT	Ext: CTCATGCCCCCTCTCTCCT Int: AGGAGACAGGGCCACAGCAC	377
2	Ext: CAGGACACAGCCAGGATAGGG Int: GTGCTGTGGCCCTGTCTCCT	Ext: AGCACCATGGGCACGTCAT Int: CCGAGTCCCTTACCCGTTTG	530
3	Ext: CATGCGGGACCAGTACATGC Int: TACAGGTGAACCCCGTGAGG	Ext: CGTCCCGGGAGACTTACAGC Int: GCCGCTGGGTCACATGG	484
4	Ext: GGCACCTCGAGATGGCCAGA Int: TCCAGCTTCCCGTGTGTGTG	Ext: CGGTGGCATTGGGATGTTC Int: CCAGCAGCCCTTCTTCCTT	485

TABLE II. Mutation Analysis of *HRAS* at Codon 12 and Codon 13 in Exon 1

Number of CS Patients	Mutation	Amino acid substitution
30	Codon 12: 34G → A	gly12ser
2	Codon 12: 35G → C	gly12ala
1	Codon 13: 37G → T	gly13cys

Due to the similarity in phenotype between CS and CFC, we screened eight patients with the clinical diagnosis of CFC and found no mutations in the *HRAS* coding region.

HRAS Single Nucleotide Variation

A single nucleotide polymorphism (SNP) in exon 1, at position 81 (codon 27), was identified in 53% of the individuals screened for *HRAS* mutations (Table III). Fifty percent (18/36) of CS individuals had the 81T → C transition and 3/36 (8%) were homozygous for the variation (nt81CC). This is a comparable frequency seen in a control population of patients examined by Johne et al. [2003], although slightly different from the frequency observed in a cohort of healthy volunteers in the same study. In addition, three patients with a 34G → A mutation were also found to have a unique heterozygous C → T substitution at position -10 nt from the ATG start site in the 5' untranslated region. This SNP was not observed in the control of CFC groups. Unfortunately, parental DNA samples from these patients were not available for study.

Loss of Wild-Type *HRAS* in Malignancy

Genomic DNA was isolated from a recurrent ERMS from one patient in the cohort that we established had a constitutional activating 34G → A mutation in *HRAS*. Heterozygosity for SNP 81TC was also observed in exon 1. Sequencing of the *HRAS* gene from the rhabdomyosarcomatous and fibrosarcomatous elements revealed the presence of only 34A and 81T in exon 1, thus demonstrating a loss of heterozygosity of the wild-type allele of *HRAS* (Fig. 2). No other mutations were present in the gene (data not shown).

Genotype-Phenotype Correlation

Although CS has a recognizable pattern of malformations, there is clinical heterogeneity observed among patients, as well as an evolution of phenotype with age. With this, we investigated a possible genotype-phenotype correlation. The frequency of major clinical features of CS, with and without *HRAS* mutations, is summarized in Table IV. All patients in

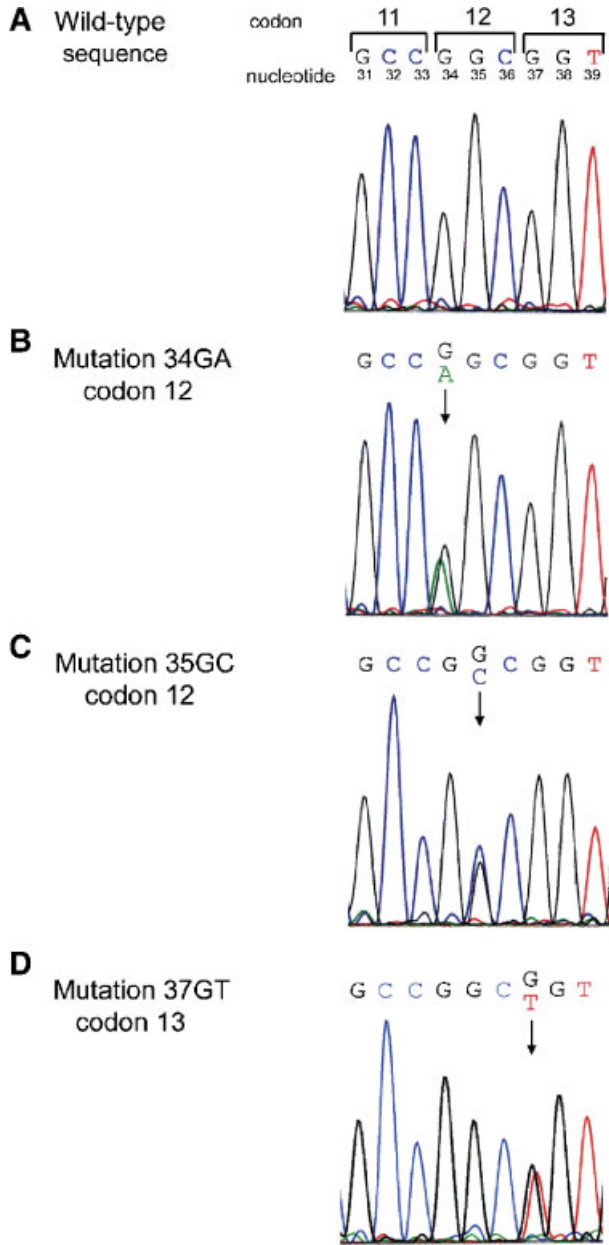


FIG. 1. Electropherogram of three different point mutations observed in patients with CS. Wild-type sequence depicts *HRAS* codons 11, 12, and 13 of

TABLE III. Subgroup Analysis of *HRAS* SNP nt81 T → C Transition (Codon 27) in Individuals With CS, CFC, and Parents Grouped With Controls

	Wildtype (81TT)	Heterozygous (81C/T)	Homozygous (81C/C)
CS	15/36 (42%)	18/36 (50%)	3/36 (8%)
CFC	5/8 (62%)	3/8 (38%)	0/8 (0%)
Parents/controls	6/16 (38%)	7/16 (44%)	3/16 (19%)

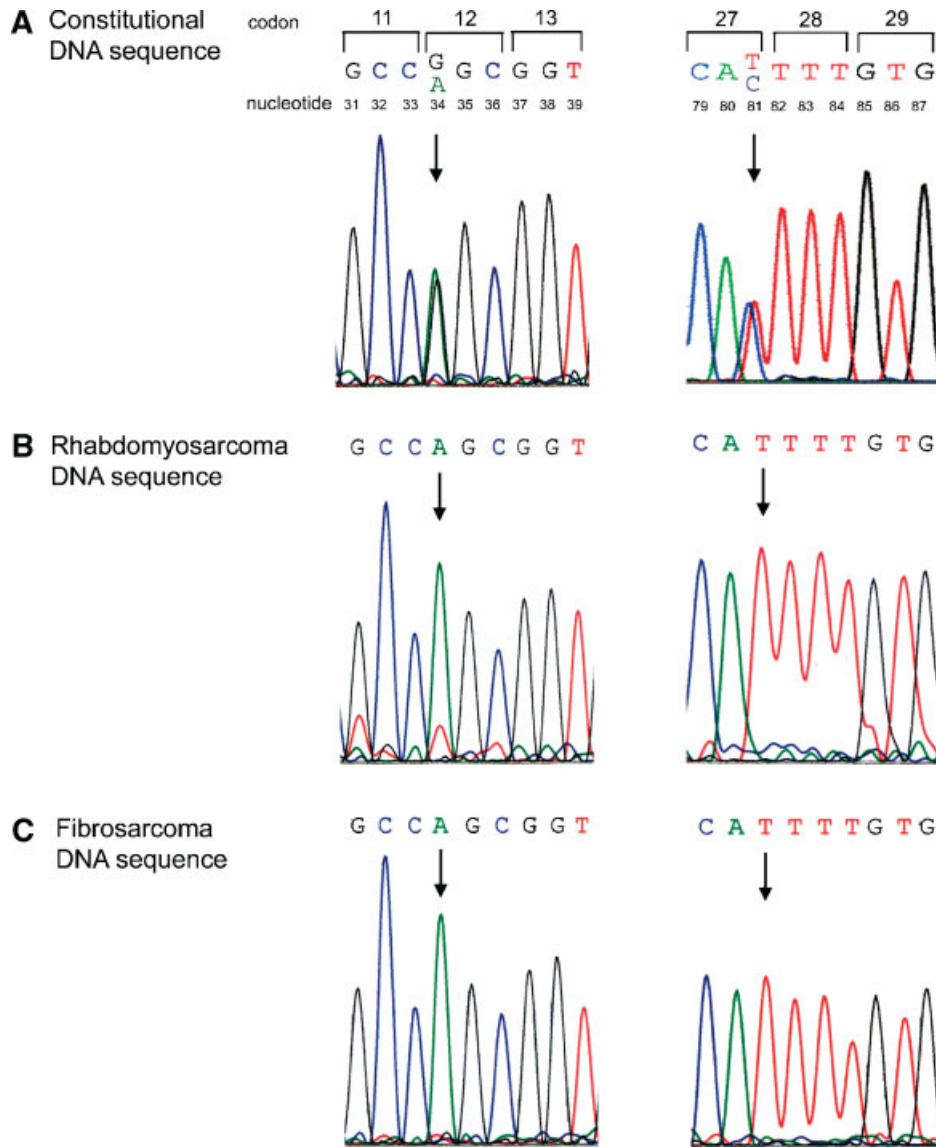


FIG. 2. Electropherogram of *HRAS* from codons 11, 12, and 13 and codons 27, 28, and 29. **A:** Constitutional sequence from a patient who developed an ERMS is depicted. Data demonstrates heterozygosity of 34G → A and 81T → C. **B, C:** *HRAS* sequence of the recurrent biphasic ERMS (see Material and Methods) and fibrosarcoma demonstrated loss of the wild-type allele.

the study had characteristic facial features allowing for minor familial variation observed among individuals. In addition, all patients had characteristic soft, smooth, redundant skin, failure to thrive, and developmental delay. Of patients harboring the 34G → A mutation, 93% had a musculoskeletal abnormality, 87% had visual impairment, 76% had reported cardiac abnormalities, and 63% had generalized hyperpigmentation. All individuals with papillomas and malignancy had a codon 12 mutation. Of all the phenotypic features, neoplasia appeared to be the most consistent for predicating the presence of an *HRAS* mutation.

Notably, the phenotype observed in the two individuals with 35G → C shared a history of cardiac rhythm disturbance, failure to thrive, hypoglycemia,

and musculoskeletal abnormalities. The phenotype observed in the one patient with 37G → T (codon 13) was similar compared to the others in the cohort, however, his facial features were more refined than is typically observed in CS (Table IV). Interestingly, this individual had cardiac hypertrophy that resolved, but then subsequently developed an enlarged aortic root.

Although the sample size is small (3 patients ages 16 months, 6 years, and 13 years of age), individuals with the clinical diagnosis of CS who did not have an *HRAS* mutation demonstrated only minor qualitative difference in phenotypic features as compared to the group of patients with an *HRAS* mutation. Of note, none of the patients had growth hormone deficiency or papillomas, although papilloma development

TABLE IV. Summary of Clinical Feature of Patients With and Without *HRAS* Mutations

Phenotype	Mutations			
	34G → A (n = 30)	35G → C (n = 2)	37G → T (n = 1)	None (n = 3)
Characteristic facies ^d	30/30	2/2	+	3/3
Characteristic skin ^b	30/30	2/2	+	3/3
Developmental delay	30/30	2/2	+	3/3
Failure to thrive	30/30	2/2	+	3/3
Cardiac anomaly: ^c				
Structural	10/30	0/2	–	1/3
Hypertrophy	13/30	0/2	+	0/3
Rhythm disturbance	7/30	2/2	–	0/3
None	7/30			
Hypertension	3/30	0/2	–	0/3
Abnormal brain imaging ^d	13/30	1/2	–	1/3
Hypoglycemia ^e	9/30	2/2	+	1/3
Growth hormone deficiency ^f	14/30	1/2	Not tested	0/3
Musculoskeletal abnormality ^g	28/30	2/2	–	2/3
Ophthalmologic abnormality ^h	26/30	1/2	+	1/3
Papilloma ⁱ	25/30	1/2	–	0/3
Generalized hyperpigmentation	19/30	1/2	–	1/3
Malignancy ^j	5/30	0/2	–	0/3

*This patient had characteristic facies, but features were more refined in that the nose, cheeks, and lips were not as coarse as can sometimes be seen in CS. Patient has had resolution of cardiac hypertrophy, but has developed an enlarged aortic root.

^aCharacteristic facies are coarse with prominent forehead, bitemporal narrowing, epicanthal folds, downslanting palpebral fissures, short nose with a depressed nasal bridge and a broad base, low-set, posteriorly rotated ears with thickened helices and lobes. Cheeks may be full and the mouth large with full lips.

^bCharacteristic skin is soft, velvety, and redundant with deep plantar and palmer creases. Hyperkeratosis may be present.

^cSome patients have more than one cardiac anomaly and may be categorized more than once.

^dBrain imaging may have been done by MRI, CT, or ultrasound. Abnormalities include hydrocephalus, Chiari type I malformation, agenesis of the corpus callosum, or other structural features.

^eA known history of hypoglycemia which may or may not be resolved.

^fMany patients have not been tested for growth hormone deficiency.

^gMusculoskeletal abnormalities include joint laxity, ulnar deviation, kyphosis, scoliosis, and/or asymmetric chest.

^hOphthalmologic abnormalities include strabismus, nystagmus, astigmatism, myopia, and/or hyperopia.

ⁱDevelopment of papillomas is known to be age-dependant. The age of onset range was 1–23 years with the average age of development being 7 years.

^jMalignancies include alveolar rhabdomyosarcoma, embryonal rhabdomyosarcoma, and papillary urothelial carcinoma.

appears to be age-related, and only one individual had a cardiac malformation (VSD) and abnormal brain imaging.

DISCUSSION

We hypothesized, based on similarity of phenotype and the shared increased risk for specific cancers, that the pathogenesis for CS involved activation of the Ras/MAPK pathway as is seen in NS and NF1 [Tartaglia et al., 2003]. *HRAS* mutations were recently reported in a cohort of 12 patients with the clinical diagnosis of CS [Aoki et al., 2005]. In this study, we have analyzed a well-characterized cohort of 36 patients with the clinical diagnosis of CS by sequencing the coding region and flanking intronic regions of the *HRAS* gene in an effort to assess the spectrum of activating mutations in *HRAS*, to establish a genotype–phenotype correlation and to gain insight into the pathogenesis of constitutional *HRAS* activating mutations in the development of malignancies.

All of the mutations in *HRAS* that we identified were missense changes in either codon 12 or codon 13, which predict an amino acid substitution known to increase signaling through the Ras/MAPK pathway. Although mutations were heterogeneous, the

distribution of mutations was very specific and nonrandom. Since no frameshift, nonsense, or splice mutations were detected in either this cohort of patients or the cohort reported by Aoki et al. [2005], *HRAS* haploinsufficiency is not a likely causative mechanism of CS.

In this study, 33 of 36 patients (92%) were found to have single missense mutations in the well-known somatically acquired mutation hotspots in *HRAS* [Bos, 1989]. No mutations were observed in codon 61, another common region for missense point mutations in neoplasia. The majority of patients (91%) had a 34G → A transition in codon 12. Mutations that were less frequent included 35G → C (codon 12) and 37G → T (codon 13). These data were concordant with Aoki et al. [2005] who identified *HRAS* mutations in 12 of 13 patients with the clinical diagnosis of CS. In their study of Japanese or Italian patients, over half of the patients (7/13) had the 34G → A transition in codon 12. Other mutations found at a lesser frequency in codon 12 included 35G → C and 35GC → TT. One mutation, 38G → A, was found in codon 13. In our study, we did not observe patients with 38G → A or 35GC → TT mutations. However, one of our patients had a novel mutation in codon 13: 37G → T. Both studies support the hypothesis that heterogeneous *HRAS* mutations

arise de novo since parental samples sequenced did not have mutations in *HRAS*. However, the possibility of germline mosaicism was not excluded. Although CS has historically been considered sporadic, reported cases of affected siblings in the literature implicate the possibility of germline mosaicism [Zampino et al., 1993; Lurie, 1994].

Two SNPs were identified in *HRAS*. The 81T → C transition in exon 1 is a well-characterized polymorphism that has been associated with a twofold increased risk of urinary bladder cancer in carriers of the homozygous 81CC genotype [Johns et al., 2003]. The allele frequencies of this SNP in the CS cohort presented here was comparable to the frequency seen in a patient control population from Germany. The one patient with bladder cancer reported in this cohort was heterozygous for this SNP. Another SNP, which was observed only in three patients with 34G → A mutations, was a C → T transition found 10 base pairs 5' to the start codon. Unfortunately, parental samples were unavailable to determine if the SNPs were inherited. To our knowledge, this SNP has not been reported and its significance remains unclear.

The major criteria for selecting patients for this study were based on characteristic facial features and skin findings, developmental delay, and failure to thrive. Phenotypic variability among patients with the 34G → A mutation was observed. Musculoskeletal abnormalities, visual impairment, cardiac abnormalities, and generalized hyperpigmentation were all seen in greater than 50% of the patients with mutations. Of note, all patients with either growth hormone deficiency or neoplasia (benign or malignant) had an *HRAS* mutation. These features taken together may assist in the establishment of diagnostic criteria for molecular testing. Interestingly, the two patients with 35G → C both had cardiac rhythm disturbances; whereas, of the patients with the more common 34G → A *HRAS* mutation, only 23% exhibited cardiac arrhythmias. Only one patient in the cohort was reported with an enlarged aortic root; this patient had a codon 13 37G → T transversion. Because only one patient was found to have a unique 37G → T mutation, it is not yet clear whether aortic root involvement is a unique phenotypic feature of this mutation.

Three patients with the clinical diagnoses of CS did not have coding region mutations. Phenotypically, these patients meet criteria for the clinical diagnosis of CS and none had distinguishing features that would identify them as a separate cohort. We specifically examined coding regions and flanking intronic sequence for *HRAS* mutations. The promoter, large intronic and 3' untranslated regions were not evaluated for possible molecular alteration which could cause an increase in Ras activity, and therefore, contribute to CS. Possibilities for lack of mutation identification include an intronic or pro-

motor mutation not detected, or possible tissue-specific mosaicism. An alternate tissue source was not available from these patients to explore this possibility. Further explanation for lack of molecular diagnosis includes a Ras/MAPK pathway mutation yet to be identified, or misdiagnosis of CS, since there is overlap with other syndromes, especially CFC. CFC has many phenotypic features in common with CS and NS, punctuating the diagnostic dilemma. However, these three patients did not have the characteristic ectodermal features CFC syndrome. Because of phenotypic overlap among CS, NS, and CFC, the molecular basis of pathogenesis may be similar. Since NS and CS involve a Ras pathway perturbation affecting both development, and predisposition to malignancy, it may be reasonable to assume that the Ras pathway or a downstream effector is also involved in CFC, albeit without oncogenic consequence.

Ras genes exist as a multigene family that encodes small GTPases: *HRAS*, *NRAS*, and *KRAS* [for review see [Midgley and Kerr, 2002]]. The Ras pathway is a highly controlled, complex intracellular signaling network that involves the transduction of an extracellular stimuli resulting in intracellular effects [Hancock, 2003]. All cells use Ras signaling to some extent, so it is not surprising that perturbation in Ras signaling could have developmental consequences. Although all proteins are widely expressed, *KRAS* is ubiquitously expressed in almost all cell types and shown to be vital for normal mouse development [Johnson et al., 1997]. In contrast, mouse model studies have demonstrated *HRAS* and *NRAS* are not as critical [Malumbres and Barbacid, 2003]. Ras is activated by diverse extracellular stimuli including receptor tyrosine kinases, cytokine receptors, G-protein-coupled receptors, and extracellular matrix receptors (integrins). The Ras pathway, consisting of interlinked networks, activates several downstream cascades including MAP kinase, PI3 kinase, RAL, and phospholipase C ϵ (PLC ϵ). Ras has multiple diverse downstream components that mediate various intracellular effects. The most extensively studied cascade is MAP kinase mediated by Raf. This pathway is important for cell cycle progression and gene expression. The PI3 kinase cascade mediates transcription and anti-apoptotic function through AKT/PKB. In addition, this cascade also modulates cytoskeletal signaling and other transcription factor pathways via RAC, a Rho family protein. Other pathways, though not as well studied, include RAL guanine nucleotide dissociation stimulator (RALGDS) that is involved in transcription, the cell cycle, cell survival, and vesicle transport. The PLC ϵ cascade is reportedly involved in calcium signaling and protein kinase C (PKC) activation. The PKC network exists as a family of proteins that are involved in gene expression, cellular proliferation, and vesicle transport [Downward, 2003].

HRAS is a highly conserved gene located on 11p15.5 with variability of genetic sequence existing in the hypervariable region among other Ras family members [Midgley and Kerr, 2002]. The amino acid substitutions predicted by the missense changes found in codon 12 and 13 in CS are well-known activating alterations in *HRAS*, which affect guanine nucleotide binding and cause reduction of GTP hydrolysis, resulting in a gain of function [Gibbs et al., 1984; McGrath et al., 1984; Sweet et al., 1984]. Substitution of any amino acid other than proline may cause activation, albeit with varying transformation efficiencies [Fasano et al., 1984; Seeburg et al., 1984]. However, activated Ras alone does not cause transformation of primary cells in culture but rather transformation requires an additional, co-operating oncogene such as *myc* [Land et al., 1983; Newbold and Overell, 1983]. This is further supported by the observation that fibroblast cell lines from patients with CS are known to exhibit an abnormal phenotype of increased proliferation, but not transformation [Hinek et al., 2000; Aoki et al., 2005].

Aberrant activation of Ras is frequently found in somatic tumors with oncogenic mutations in codons 12, 13, or 61 occurring in approximately 20% of all tumors [Bos, 1989]. Interestingly, point mutations in *HRAS* account for only a small fraction of Ras mutations observed in sporadic cancers, with bladder cancer being the most common [Levesque et al., 1993]. CS patients have a predisposition to neoplasia including benign papillomas and certain malignancies of which RMS, transitional cell carcinoma (TCC), and neuroblastoma are the most common. RMS and neuroblastoma are common childhood malignancies, whereas TCC is not. The most commonly reported malignancy in CS is embryonal rhabdomyosarcoma (ERMS), a subtype of RMS. ERMS is a small cell tumor, thought to arise from mesenchymal cells, that most commonly occurs in the orbit or the genito-urinary tract in children [Tobar et al., 2000; Gordon et al., 2001]. The genetic etiology of sporadic ERMS is unknown, but in the majority of tumors, loss of heterozygosity (LOH) has been observed at 11p15.5 [Visser et al., 1997; Anderson et al., 1999; Tobar et al., 2000]. LOH of 11p15.5 has also been observed in ERMS from CS patients [Kerr et al., 2003]. We have demonstrated the loss of wild-type *HRAS* with retention of the activated *HRAS* allele in a patient with recurrent biphasic ERMS/fibrosarcoma. Interestingly, *HRAS* mutations are rarely seen in sporadic rhabdomyosarcoma [Yoo and Robinson, 1999; Takahashi et al., 2004] implicating an alternate mechanism of rhabdomyosarcogenesis in patients with CS.

Mouse models have provided evidence that *Hras* can be involved in the initiation of carcinogenesis [Akhurst and Balmain, 1999]. Well-studied mouse models of skin cell tumorigenesis have demonstrated alkylating agents initiate skin tumors by causing the

specific 35G → A transition in codon 12 of *Hras* [Brown et al., 1990]. Subsequent nonrandom duplication of the chromosomal region spanning the *Hras* locus results in an increased copy number of activated Ras [Bianchi et al., 1990], followed by loss of the normal *Hras* allele [Bremner and Balmain, 1990]. We speculate that a similar progression occurred in the ERMS with the primary tumor demonstrating chromosome 11 trisomy, and then subsequent loss of wild-type *HRAS* in the recurrent malignancy.

In conclusion, we have demonstrated in a cohort of patients that sporadic, heterogeneous, constitutional *HRAS* mutations cause CS. Since CS has a progressive, evolving phenotype, the possible use of systemic therapies to reduce *HRAS* activity may be of great benefit to this population of patients. In addition, we demonstrated somatic loss of the wild-type *HRAS* allele in an advanced biphasic ERMS/fibrosarcoma in an individual with a 34G → A mutation. Understanding tumor progression in patients with CS may have therapeutic implications.

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