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A Novel *HRAS* Substitution (c.266C>G; p.S89C) Resulting in Decreased Downstream Signaling Suggests a New Dimension of RAS Pathway Dysregulation in Human Development

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Abstract

Costello syndrome is caused by *HRAS* germline mutations affecting Gly¹² or Gly¹³ in >90% of cases and these are associated with a relatively homogeneous phenotype. Rarer mutations in other *HRAS* codons were reported in patients with an attenuated or mild phenotype. Disease-associated *HRAS* missense mutations result in constitutive *HRAS* activation and increased RAFMEK-ERK and PI3K-AKT signal flow. Here we report on a novel heterozygous *HRAS* germline alteration, c. 266C>G (p.S89C), in a girl presenting with severe fetal hydrops and pleural effusion, followed by a more benign postnatal course. A sibling with the same mutation and fetal polyhydramnios showed a Dandy–Walker malformation; his postnatal course was complicated by severe feeding difficulties. Their apparently asymptomatic father is heterozygous for the c.266C>G change. By functional analyses we identified reduced levels of active *HRAS*^{S89C} and diminished MEK, ERK and AKT phosphorylation in cells over-expressing *HRAS*^{S89C}, which represent novel consequences of disease-associated *HRAS* mutations. Given our patients' difficult neonatal course and presence of this change in their asymptomatic father, we hypothesize that its harmful consequences may be time limited, with the late fetal stage being most sensitive. Alternatively, the phenotype may develop only in the presence of an additional as-yet-unknown genetic modifier.

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Additional supporting information may be found in the online version of this article.

The authors declare that they have no conflict of interest. All experiments in this study comply with the current laws of the country in which they were performed.

GenBank reference sequences: *HRAS* (*HRAS*, H-Ras) NM_005343.2, NM_176795.3; *MAP2K1/MAP2K2* (*MEK1/2*) NM_002755.3/ NM_030662.2; *MAPK3/MAPK1* (*ERK1/2*) NM_002745.4/ NM_001040056.1; *AKT1/ AKT2/AKT3* (*AKT1-3*) NM_001014431.1/ NM_001626.3/ NM_005465.3; *GAPDH* (*GAPDH*) NM_002046.3; *RAF1* (*CRAF*, c-Raf) NM_002880.3; *RALGDS* (*RalGDS*, *RalGEF*) NM_006266.2; *PIK3CA* (*PI3Ka*, p110-alpha) NM_006218.2; protein names are denoted in parentheses.

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While the pathogenicity of the *HRAS* c.266C>G change remains unproven, our data may illustrate wide functional and phenotypic variability of germline *HRAS* mutations.

Keywords

RASopathy; RAS-pathway disorder; Costello syndrome; *HRAS* germline mutation; phenotypic variability; functional variability

INTRODUCTION

Costello syndrome (OMIM #218040) is a very rare congenital cancer predisposition syndrome encompassing effects on multiple organ systems, including cognitive disability and postnatal cerebellar overgrowth, coarse facial features, hypertrophic cardiomyopathy, and cardiac rhythm disturbance, severe feeding difficulties resulting in failure-to-thrive and skeletal abnormalities [Gripp and Lin, 2009]. Individuals with Costello syndrome have a 15% risk to develop malignant tumors, most commonly rhabdomyosarcoma [Gripp, 2005]. Similarities in the facial features, cardiac involvement, skin abnormalities, and short stature with relative macro-cephaly in Costello, cardio-facio-cutaneous (CFC; OMIM #115150) and the more common Noonan (OMIM #163950) syndromes suggested related disease causing gene changes. After identification of the first Noonan syndrome gene, *PTPN11* [Tartaglia et al., 2001], the RAS-MAPK signaling pathway became the focus of further investigations, and pathogenic changes resulting in CFC and Costello syndrome were discovered. Germline mutations in the proto-oncogene *HRAS*, resulting in a gain-of-function of the abnormal protein product, underlie Costello syndrome [Aoki et al., 2005].

Specific amino acid substitutions encoded by somatic *HRAS* changes were extensively studied due to their prominent role in sporadic malignancies, and their respective transforming potential is documented [Fasano et al., 1984; Gideon et al., 1992; Scheffzek et al., 1997; Seeburg et al., 1984]. *HRAS* serves as signal transducer by alternating between its active GTP-bound and inactive GDP-bound state. The oncogenic *HRAS* mutations affect its hydrolytic activity, and thus maintain the active form which binds to effector proteins, such as serine/threonine RAF kinases, the catalytic subunits of phosphoinositide 3-kinase (PI3K), and RAL guanine nucleotide dissociation stimulator (RALGDS) [Karnoub and Weinberg, 2008]. As a result, signal flow via these *HRAS* effector proteins is increased.

HRAS missense mutations in sporadic tumors and Costello syndrome most often affect the glycine in position 12. The substitution for a valine has the highest transforming activity and is the most common in tumors. In contrast, p.G12V is very rare in individuals with Costello syndrome, likely because it results in an extremely severe, neonatal lethal phenotype [van der Burgt et al., 2007; Lo et al., 2008]. The *HRAS* p.G12S occurs in >80% of Costello syndrome individuals, and consequently is associated with the typical presentation [Gripp et al., 2006; Sol-Church and Gripp, 2009]. Rarer *HRAS* changes have been reported in individuals with an attenuated phenotype [Kerr et al., 2006; Zampino et al., 2007; Gripp et al., 2008] and less characteristic facial and clinical features [Denayer et al., 2008].

With the delineation of germline mutations affecting proteins acting within the RAS-MAPK signaling pathway and resulting in Noonan, CFC, Costello syndrome and other disorders, we now appreciate this pathway's primary role in embryologic development and postnatal growth, in addition to its secondary oncogenic potential. Since dysregulation of the complex RAS-dependent signal transduction cascades can result in variable phenotypic effects, the characterization of functional consequences of specific mutations is ongoing. Here we report on a novel *HRAS* mutation, its functional and phenotypic consequences.

MATERIALS AND METHODS

Patient Information

The female Patient 1 was the second child born to a 32-year-old mother with pre-gestational type II diabetes. The diabetes was well controlled with insulin. At 31 weeks gestation severe polyhydramnios and fetal hydrops necessitated an emergency caesarean. Apgar scores were 1, 3, and 4 at 1, 5, and 10 min, respectively, and resuscitation and intubation were required. Her birth weight was 3.05 kg (>90th centile for gestational age); length 52 cm (>90th centile) and head circumference 33 cm (>90th centile). Bilateral chest tubes were placed due to pleural effusions and ascites was noted. Postnatal hypoglycemia occurred. She required a feeding tube for 6 weeks. Subsequently, she was able to eat by mouth, but required medication for gastro-esophageal reflux. On renal ultra-sounds mild hydronephrosis, unilateral pelvicalectasis and a contralateral cortical renal cyst measuring 1.6 cm in diameter were noted. At age 11 months her weight was at the 10–25th centile, length at the 25–50th centile and head circumference at the 75th centile. She required glasses for hyperopia and bilateral intermittent esotropia. She received physical therapy for mild gross motor delay and hypotonia. Physical examination showed the deep philtrum (Fig. 1B), a vascular lesion on her left external ear, persistent fetal finger pads and scars from the chest tubes. The previously noted redundancy of tissue in the back of her neck had resolved. Two left renal arteries were noted on imaging studies monitoring the stable renal cyst. The patient's presentation of severe hydrops without other identifiable cause led to clinical molecular testing for Noonan syndrome associated gene changes, and a testing panel combining resequencing for several genes on a single chip resulted in the identification of the novel *HRAS* change. Her father, who carries the same mutation in two different tissues, has no obvious dysmorphism or cognitive impairment.

A younger brother (Patient 2) was born after a pregnancy complicated by limited prenatal care because the pregnancy was recognized in the late third trimester. Prenatal ultrasound revealed polyhydramnios and suggested a Dandy–Walker malformation in the fetus. Delivery was by caesarean at estimated 35 weeks gestation. Apgar scores were 8 at 1 min and 9 at 5 min. His birth weight of 3.5 kg and length of 52 cm were at or above the 90th centile; his head circumference of 34 cm was at the 75th to 90th centile. Hyperbilirubinemia and hypoglycemia required treatment. Respiratory problems included stridor, laryngomalacia, and unilateral vocal cord paralysis. Severe feeding difficulties with recurrent emesis necessitated intravenous nutrition. The patient has not taken oral feeds to his current age of 8 weeks. No structural gastrointestinal tract anomalies were identified and a rectal suction biopsy showed presence of ganglion cells, thus a congenital aganglionic

megacolon (Hirschsprung disease) could be excluded. Brain imaging studies confirmed the Dandy–Walker malformation (Fig. 1C) without overt hydro-cephalus. Echocardiogram, eye examination, and renal ultra-sound had essentially normal results. Laboratory studies are notable for presence of the familial *HRAS* c.266C>G change. A comparative genomic hybridization using an Agilent oligonucleotide array was performed as a clinical test and had a normal male result.

Both patients and their parents enrolled in the IRB (institutional review board) approved research study (AIDHC IRB# 2005-051) as part of our Costello research. However, they have not been open to further familial segregation analysis and they have not consented to exome or genome sequencing.

Mutation Analysis

We collected blood samples and buccal cells from Patient 1 and her parents and isolated genomic DNA using standard methods. Parent–child biologic relationships were confirmed using the AmpFISTR® Profiler Plus kit™ (Applied Biosystems, Foster City, CA). We amplified the coding regions including the flanking intronic sequences of *BRAF*, *HRAS*, *KRAS*, *MAP2K1*, *MAP2K2*, *NRAS* (exons 2, 3 and 4), *PTPN11*, *RAF1*, and *SOS1*. Mutation analysis of *SHOC2* was performed by PCR amplification of a discrete portion of *SHOC2* exon 2 containing amino acid 2. Primer sequences are described elsewhere [Sol-Church et al., 2009] or are available on request. All PCR products were purified and sequenced in both directions using the ABI BigDye Terminator Cycle Sequencing Ready Reaction kit v 3.1 and analyzed on an ABI3130XL Genetic Analyzer. All sequences were verified by direct observation of the electropherogram and using Sequencher 4.8 and 5.0 (Gene Codes Corporation, Ann Harbor, MI). For Patient 2, targeted analysis for the familial mutation was performed in a clinical diagnostic laboratory.

Plasmid Construction

We amplified the coding region of wild-type *HRAS* for the generation of an expression construct by using *HRAS*-specific PCR primers and *HRAS* cDNA as a template. Mutated *HRAS* cDNA inserts [c.34G>A (p.G12S), c.35G>T (p.G12V), c.50G>A (p.S17N) and c.266C>G (p.S89C)] were established by PCR-mediated mutagenesis. Purified PCR products were cloned into pENTR/D-TOPO (Invitrogen, Karlsruhe, Germany) according to the protocol provided. Constructs were sequenced for integrity and used for subcloning wild-type and mutated *HRAS* coding regions into plasmid pMT2SM-HA-DEST (N-terminal HA epitope).

Cell Culture and Transfection

COS-7 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) containing 10% serum (Invitrogen) and penicillin–streptomycin (100 U/ml and 100 mg/ml, respectively) (Invitrogen) at 37° C and 5% CO₂. Transfections were performed using Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer's protocol. For EGF stimulation, cells were blocked overnight by serum starvation (0.1% serum), followed by incubation in DMEM containing 10 ng/ml EGF (Sigma, Taufkirchen, Germany).

RAS Activation Assay

The RAS-binding domain (RBD) of RAF1 (amino acids 51–131), the RBD of PI3K (PIK3CA) (amino acids 127–314), and the RAS-association (RA) domain of RALGDS (amino acids 777–872) were used to specifically precipitate GTP-bound RAS proteins from cell extracts [de Rooij and Bos, 1997]. Preparation of GST-RBD/RA beads, cell lysis, and precipitation of GTP-bound RAS has been described elsewhere [Rosenberger et al., 2009]. Subsequent SDS PAGE and transfer to PVDF membranes, total and precipitated (active) HA-tagged HRAS was detected using peroxidaseconjugated rat monoclonal anti-HA (hemagglutinin) antibody (Roche, Mannheim, Germany; clone 3F10; 1:5,000 dilution). For loading control, membranes were incubated with mouse anti-GAPDH (Abcam, Cambridge, UK; no. ab8245, 1:5,000 dilution) followed by peroxidase-coupled secondary anti-mouse antibody (Amersham Pharmacia Biotech, Freiburg, Germany; no. NA9310; 1:8,000 dilution).

Immunoblotting

Twenty-four hours after transfection, cells were cultured as specified, then washed with PBS and scraped off in modified RIPA buffer [50 mM Tris-HCl, pH 8.0; 150 mM NaCl; 1% Nonidet P40; 0.5% sodium deoxycholate; 0.1% SDS; 1 mM PMSF; 1 mM Na₃VO₄; 10 mM NaF; 1 Complete Mini protein inhibitor cocktail tablet (Roche) per 10 ml]. Cellular biochemical reactions were stopped by freezing lysates in liquid nitrogen. After thawing on ice, cell debris was removed and protein concentration was determined by using the BCA Protein Assay Kit (Pierce, Bonn, Germany). Proteins were separated on SDS–polyacrylamide gels and transferred to PVDF membranes. Following blocking (20 mM Tris-HCl, pH 7.4; 150 mM NaCl; 0.1% Tween-20; 4% non-fat dry milk) and washing (20 mM Tris-HCl, pH 7.4; 150 mM NaCl; 0.1% Tween-20) membranes were incubated in primary antibody solution (20 mM Tris-HCl, pH 7.4; 150 mM NaCl; 0.1% Tween-20; 5% BSA or 0.5% non-fat dry milk) containing the appropriate antibodies. Rabbit polyclonal antibodies against MEK1/2 (Cell Signaling Tech., Danvers, MA; no. 9122; 1:1,000 dilution), phospho-MEK1/2 (Ser217/221) (Cell Signaling Tech.; no. 9121; 1:1,000 dilution), p44/42 MAP kinase (ERK1/2) (Cell Signaling Tech.; no. 9102, 1:1,000 dilution), phospho-p44/42 MAP kinase (ERK1/2) (Thr202/Tyr204) (Cell Signaling Tech.; no. 9101, 1:1,000 dilution), Akt (Cell Signaling Tech.; no. 9272; 1:1,000 dilution) and phospho-Akt (Ser473) (Cell Signaling Tech.; no. 9271; 1:1,000 dilution) were used. Next, membranes were washed and incubated with peroxidase-coupled secondary anti-rabbit antibody (Amersham Pharmacia Biotech; no. NA9340V; 1:8,000 dilution). After final washing, immunoreactive proteins were visualized using the Immobilon Western Chemiluminescent HRP Substrate (Millipore, Schwalbach, Germany).

Statistical Analysis

Signals on autoradiographs from three to eight independent experiments were quantified by densitometric analysis using the ImageJ software (NIH; <http://rsb.info.nih.gov/ij/index.html>). MEK1/2-, ERK1/2- and AKT phosphorylation as well as HRAS activation levels were assessed as described in the figure legends (Figs. 4 and 5). Two-tailed paired Student's *t*-tests were used to determine the significance of the average difference between

HRAS^{S89C} and HRAS^{WT} overexpressing cells in Figure 4D, whereas two-tailed unpaired Student's *t*-tests were used to determine the significance of the difference of the mean values in Figure 5B. Values were considered significant at *P* (*P*-value) <0.05.

Molecular Modeling

Molecular models for inactive and active HRAS are based on the crystal structure of GDP-bound HRAS (Protein Data Bank [PDB] ID 4Q21) [Milburn et al., 1990] and GppNHp-bound HRAS (PDB ID 5P21) [Pai et al., 1990], respectively. The structure of HRAS p.S89C was solved by molecular replacement using the automatic modeling mode of the SWISS-MODEL homology-modeling server [Peitsch, 1995; Arnold et al., 2006; Kiefer et al., 2009] (<http://swissmodel.expasy.org/>) with wild-type HRAS (PDB IDs 4Q21 and 5P21) as search model. HRAS p.S89C structure was analyzed and superposed on wild-type HRAS by using DeepView/Swiss PDBViewer [Guex and Peitsch, 1997] (<http://www.expasy.org/spdbv/>). Before calculation of hydrogen bonds energy minimization was performed for all models using the energy minimization tool (steepest descent) installed in the DeepView/Swiss PDBViewer software. Figures were visualized by using either POLYVIEW-3D [Porollo and Meller, 2007] (<http://polyview.cchmc.org/polyview3d.html>) or Deep-View/Swiss PDBViewer.

RESULTS

Identification of a Novel Heterozygous *HRAS* Alteration in Siblings with Clinical Characteristics of RAS-MAPK Pathway Disorders

Patient's 1 prenatal presentation of severe hydrops without other identifiable cause led to molecular testing for gene changes associated with Noonan syndrome or related disorders, but no mutation could be detected in *BRAF*, *KRAS*, *MAP2K1*, *MAP2K2*, *NRAS*, *PTPN11*, *RAF1*, *SHOC2*, and *SOS1*. However, we identified the novel heterozygous alteration c.266C>G in the second coding exon (exon 3) of *HRAS*. This change was predicted to cause the p.S89C substitution of serine at position 89 for a cysteine (Fig. 1A), a change that has not been reported in malignancies (www.sanger.ac.uk/genetics/CGP/cosmic). Following her complicated newborn period, Patient 1 showed a more benign course including strabismus, epicanthal folds, full nasal tip and deep philtrum, persistent fetal pads on the fingers and a small vascular lesion behind the left ear (Fig. 1B). Her brother, Patient 2, presented prenatally with polyhydramnios, and similar to his sister had postnatal respiratory and feeding difficulties. In addition, he had a Dandy-Walker malformation (Fig. 1C). He also carries the *HRAS* c.266C>G mutation. We did not detect this alteration in 488 control alleles or in the proband's mother. However, the same *HRAS* mutation was discovered in two different tissues of the patients' father (Fig. 1A), who has no obvious dysmorphia or cognitive impairment and no documented clinical history. Given the benign postnatal course of Patient 1 and her asymptomatic father, we speculated that harmful consequences of c.266C>G alteration might be time limited during embryonal/fetal development. To validate this hypothesis we examined the functional consequences of the HRAS p.S89C change.

Structural Considerations for the p.S89C Change

Amino acid serine 89 in HRAS is highly conserved among RAS paralogs and HRAS orthologs (Fig. 2A). It is part of the α 3-helix of HRAS (Fig. 2B), a secondary structure motif implicated in binding to HRAS regulating proteins including guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) [Parrini et al., 1996; Scheffzek et al., 1997; Boriack-Sjodin et al., 1998]. We extrapolated the structural environment and spatial orientation of residue 89 from the structures of inactive, GDP-bound HRAS and active, GTP-bound HRAS (Fig. 2C). Unlike Costello syndrome-critical amino acids glycine 12, glycine 13, lysine 117 and alanine 146, serine 89 is not predicted to be directly involved in the formation of the GTP/GDP binding pocket (Fig. 2C). However, detailed structural analysis may indicate that p.S89C destabilizes the tertiary structure of both HRAS conformations, which could affect its functional properties (see Supplemental Fig. 1 in supporting information online).

The p.S89C Change Results in Decreased GTP-Bound HRAS in Response to Epidermal Growth Factor Induction

To explore the molecular consequences of the p.S89C alteration, we precipitated activated HA-tagged HRAS protein variants from COS-7 cell extracts by using GST-fusion proteins of interacting motifs from various RAS effectors (Fig. 3). We could efficiently pull down active HA-HRAS^{G12V} and HA-HRAS^{G12S}, but not HA-HRAS^{S17N} from cell extracts with all tested effectors upon serum starvation (0.1% serum; Fig. 3A, precipitates) and under steady-state cell culture conditions (10% serum; Fig. 3B, precipitates). The amount of active HA-HRAS^{S89C} was similar to or slightly lower than that of active HRAS^{WT} under these culture conditions (Fig. 3A,B, precipitates). A wide variety of extracellular stimuli promote the activation of RAS proteins [Karnoub and Weinberg, 2008]. Accordingly, stimulation of serum-deprived cells with specific growth factors can uncover cryptic alterations in RAS-dependent signaling pathways [Rosenberger et al., 2009]. We stimulated serum-starved COS-7 cells with epidermal growth factor (EGF), the classical activator of the RAS-MAPK cascade [Buday and Downward, 1993], and assessed levels of active HRAS. Again, HA-HRAS^{G12V} and HA-HRAS^{G12S} exhibited high levels of activation, but not HA-HRAS^{S17N} (Fig. 3C, precipitates). Most interestingly, HA-HRAS^{S89C} did not accumulate in the GTP-bound form; in the contrary, EGF-stimulated activation of HA-HRAS^{S89C} was clearly decreased compared to HA-HRAS^{WT} (Fig. 3C, precipitates). Taken together, the active form of HA-HRAS^{S89C} was normal or moderately reduced under serum-starved and basal conditions, respectively; however HA-HRAS^{S89C} activation was strongly decreased in EGF-induced cells.

p.S89C Moderately Decreases Activation of RAS-Dependent Effector Pathways

To gain insight into the effects of the p.S89C change on HRAS downstream signal flux, we measured levels of phosphorylated MEK1/2, ERK1/2 and AKT in COS-7 cells ectopically expressing HA-tagged HRAS protein variants. HA-HRAS^{G12V} and HA-HRAS^{G12S} were found to promote enhanced MEK1/2, ERK1/2 and AKT phosphorylation under serum-starved (Fig. 4A) and basal (Fig. 4B) conditions as well as upon EGF stimulation (Fig. 4C). Expectedly, the dominant negative mutant HA-HRAS^{S17N} [Farnsworth and Feig, 1991;

Stacey et al., 1991] induced only a very weak signaling response (Fig. 4A–C). Cells expressing HAHRAS^{S89C} showed apparently decreased MEK1/2, ERK1/2 and AKT phosphorylation levels under starved condition and upon EGF induction, compared with HA-HRAS^{WT} (Fig. 4A,C). Indeed, densitometric measurement of autoradiographs followed by statistical analysis revealed significantly reduced phosphorylation under these culture conditions (Fig. 4D). Together, the data suggest that p.S89C negatively influences HRAS-dependent downstream signaling.

The p.S89C Change Results in Reduced Epidermal Growth Factor Sensitivity

Next we compared the intensity of EGF-induced HRAS activation and downstream signaling over time between cells expressing HA-HRAS^{S89C} and HA-HRAS^{WT}. Following EGF treatment, we detected activation of both HRAS^{WT} and HRAS^{S89C} (Fig. 5A, panels 1 and 2). However, GTP-loading was significantly reduced for HRAS^{S89C} compared to HRAS^{WT}; for example, 5 min EGF stimulation resulted in 2.25-fold HRAS^{WT} activation relative to untreated cells (0 min EGF), whereas we observed only 1.62-fold activation of HRAS^{S89C} (Fig. 5B). The phosphorylation levels of HRAS downstream effectors MEK1/2, ERK1/2, and AKT correlated well with the amounts of active HRAS (Fig. 5A). EGF stimulation induced a strong phosphorylation response in HRAS^{WT} cells; in contrast, this effect was rather moderate in HRAS^{S89C} cells (Fig. 5A, panels 3–8). For example, in the former we observed up to 5.1-fold increased MEK1/2 phosphorylation levels (relative to untreated cells; 0 min EGF), whereas in the latter this increase was maximal 3.4-fold (Fig. 5B). Together, our data demonstrate limited GTP loading and signaling capabilities of HRAS^{S89C}. We propose that reduced growth factor sensitivity is a pathogenic consequence of the p.S89C alteration.

DISCUSSION

With the identification of the inherited c.266C>G alteration we add novel aspects to the knowledge of disease-associated *HRAS* mutations. First, the clinical presentation of the subjects with the c.266C>G change shares limited findings with the typical presentation of Costello syndrome and therefore broadens the phenotypic spectrum associated with germline *HRAS* mutations. And second, the functional consequences of the p.S89C change in HRAS are the inverse of all other known pathogenic HRAS amino acid alterations.

Phenotypic Variability

Germline mutations in *HRAS* result in Costello syndrome, a disorder characterized by severe failure to thrive, skin and musculo-skeletal defects, coarse facial features, cardiac abnormalities, mental retardation, and a high risk for malignancies. Shared physical and developmental findings in patients with Costello syndrome, cardio-facio-cutaneous syndrome, and Noonan syndrome complicate the clinical diagnosis in infancy. The typical Costello syndrome phenotype is easily recognizable in older individuals, and it is most often due to a mutation affecting Gly at position 12 [Gripp and Lin, 2009]. Rarer *HRAS* germline changes can be associated with a more severe presentation, including neonatal death [van der Burgt et al., 2007; Lo et al., 2008; Lin et al., 2009], or milder findings [Zampino et al., 2007; Gripp et al., 2008], widening the phenotypic spectrum of Costello syndrome. Our

understanding of the full phenotypic spectrum associated with germline mutations in *HRAS* is incomplete, as it will likely expand in the future. Parallels may be drawn to the *KRAS* mutation associated phenotype, which shows a striking variability with a wider mutational spectrum [Zenker et al., 2007].

It is noteworthy that Patient 2 has a cerebellar malformation, while individuals with the more typical *HRAS* mutations show postnatal overgrowth of the cerebellum [Gripp et al., 2010]. This may suggest qualitatively different effects of these mutations on the same developing organ. Specific mutations can have cell type specific effects, as exemplified by the distinctive dolichocilia requiring cutting of eye lashes noted exclusively in individuals with *HRAS* p.G13C [Gripp et al., 2011]. This mutation is currently the only *HRAS* change associated with loose anagen hair syndrome [Gripp et al., 2011], a condition more characteristic of a *SHOC2* change [Cordeddu et al., 2009].

The consequence of *HRAS* mutations may also be time sensitive, exerting the strongest effect during late fetal development, as suggested by our Patients' 1 difficult perinatal phase followed by a more benign postnatal course. Unfortunately we lack detailed information on the father's early medical history. A hypothesized time dependent effect is supported by the unusually difficult early childhood of individuals with *HRAS* p.G12S, complicated by severe feeding difficulties and extreme irritability [Gripp et al., 2006; Kerr et al., 2006]. Similarly, individuals with the presumably attenuated phenotype caused by *HRAS* changes p.T58I and p.A146V were identified due to significant medical problems in early childhood, and had few medical issues thereafter [Gripp et al., 2008]. Importantly, father-to-child transmission of the *HRAS* p.T58I change has been demonstrated recently [Gripp et al., 2012]. Because of the RAS-MAPK pathway's important role in embryological development [Schubbert et al., 2007a; Aoki et al., 2008], it is biologically plausible that dysregulation exerts its most serious effect in early childhood. Identification of the same *HRAS* c.266C>G germline mutation in the apparently unaffected father may thus be less surprising. Noteworthy, the common autosomal dominant RAS-opathy Neurofibromatosis 1 (NF1) is characterized by extreme clinical variability, not only between unrelated individuals and among affected individuals within a single family but even within a single person with NF1 at different times in life [Friedman, 2009]. Furthermore, genotyping of parents and relatives of severely affected patients with Noonan syndrome and *PTPN11* mutations, revealed the same *PTPN11* alterations, though these persons exhibited mild phenotypes that did not fulfill the Noonan syndrome diagnostic criteria [Tartaglia et al., 2001; Bertola et al., 2004; Zenker et al., 2004; Kitsiou-Tzeli et al., 2006].

Alternatively, the presence of the c.266C>G change in the patient's unaffected father may imply that this heterozygous substitution alone is insufficient to cause obvious phenotypic changes. This may suggest a second factor in the affected subjects, such as a common polymorphism functioning as a genetic modifier of the *HRAS* c.266C>G mutation. Many Mendelian disorders are associated with substantial clinical variability ranging from a very severe form of disease to no apparent symptoms. Modifier genes may explain these differences in disease expression [Nadeau, 2001; Slavotinek and Biesecker, 2003; Genin et al., 2008]. Indeed, genetic modifiers have been identified or proposed for several Mendelian disorders such as ectrodactyly disorders [Sidow et al., 1999; van Bokhoven et al., 2001; van

Bokhoven and McKeon, 2002], spinal muscular atrophy [Oprea et al., 2008], cystic fibrosis [Cutting, 2010], deafness—autosomal recessive 26 [Riazuddin et al., 2000], and thrombocytopenia-absent radius syndrome [Klopocki et al., 2007]. A similar situation may exist for the phenotype of the subjects described in our study: the c.266C>G change could represent the prerequisite for the phenotype, but an additional modifier may be required to elicit the clinical presentation. This hypothetical modifier could affect a protein within RAS-dependent signaling pathways, thereby contributing to the molecular consequences of the p.S89C change.

Taken together, parental studies should be considered in individuals with rare *HRAS* mutations, not only to test for parental mosaicism as previously reported [Sol-Church et al., 2009], but also to identify heterozygous parents and the inherent recurrence risk, as exemplified by our Patient 2.

Functional Consequences

Functional analyses of Costello syndrome-associated amino acid changes in *HRAS* including the frequently observed substitutions at residues 12 and 13 and the rare variant p.K117R, consistently revealed increased *HRAS* activation and enhanced RAF-MAPK and/or PI3K-AKT signal flow [Schubbert et al., 2007b; Denayer et al., 2008; Karnoub and Weinberg, 2008]. Based on structural considerations or by inference of information derived from *KRAS*, similar consequences on *HRAS* activation and downstream signaling have been proposed for the disease-associated *HRAS* amino acid substitution p.Q22K, p.T58I, p.E63K, p.A146V, and p.A146T [van der Burgt et al., 2007; Zampino et al., 2007; Gripp et al., 2008]. In contrast, here we report for the first time a germline mutation that reduces *HRAS* activation and RAF-MAPK and PI3K-AKT signal transduction, in particular upon EGF stimulation. This indicates that the p.S89C change interferes with the finely tuned balance of *HRAS* regulation. Interestingly, levels of active *HRAS*^{S89C} were normal in serum-deprived cells, thus it is unlikely that the intrinsic hydrolytic activity of *HRAS*^{S89C} is generally enhanced. A cell's primary response to growth factor induction is recruitment of GEF proteins to *HRAS*, followed by GEF-stimulated *HRAS* activation [Karnoub and Weinberg, 2008]. Therefore, we speculate that impaired GEF-catalyzed nucleotide exchange due to subtle structural modifications may account for the observed consequences on *HRAS*^{S89C} activation. However, since the molecular surface of *HRAS*^{S89C} is only minimally altered, interaction with regulatory proteins including GEFs and GAPs may not be affected. For the same reasons binding to effector proteins might not be impaired. Consequently, we suggest that the negative consequences of *HRAS*^{S89C} on downstream signaling are qualitatively similar to *HRAS*^{S17N}, which exerts its dominant negative effect on signaling by competitive sequestration of GEFs and effector proteins independent of growth factors and under any cell culture condition [Farnsworth and Feig, 1991]. While several studies showed that multiple RAS residues can critically influence its activation by GEFs, the function of serine 89 in this process has not yet been documented [Willumsen et al., 1986; Fasano et al., 1988; Mistou et al., 1992; Verrotti et al., 1992; Howe and Marshall, 1993; Segal et al., 1993; Mosteller et al., 1994; Quilliam et al., 1994].

Taken together, we suggest that reduced growth factor sensitivity is a major biochemical consequence of the p.S89C alteration, thereby representing a novel pathomechanism for disease associated HRAS mutations.

Lessons from *Caenorhabditis elegans* let-60

Most interestingly, our findings are in line with data from the genetic model organism *C. elegans*. The worm RAS homologue *let-60* is essential for vulval induction and heterozygous activating gain-of-function mutations, such as p.G13E, result in multivulval organisms [Sternberg and Han, 1998]. In contrast, substitution of the evolutionary conserved residue serine 89 with phenylalanine (p.S89F) and heterozygous expression of this *let-60* mutant resulted in a vulvaless phenotype [Han and Sternberg, 1990]. Moreover, the *let-60* p.S89F acted as intragenic suppressor mutation of the p.G13E-induced multivulval phenotype [Beitel et al., 1990]. It has been proposed that the dominant-negative effect of the *let-60* p.S89F mutation arise from altered guanine nucleotide binding properties and depletion of the pool of available *let-60* GEFs [Han and Sternberg, 1991].

In this context it should be noted that orthologs of several human RASopathy-associated genes (<http://ras-pathway-syndromes.com/>) such as *RAF1*, *MAP2K2*, *CBL*, or *SHOC2* are implicated in the vulval induction pathway in *C. elegans*.

Analogies

Different functional consequences of germline mutations in *PTPN11* underlying two related RAS-pathway-disorders with shared features—Noonan syndrome with multiple lentigines/LEOPARD syndrome (OMIM #151100) and Noonan syndrome— have been reported previously [Hanna et al., 2006; Tartaglia et al., 2006]. *PTPN11* encodes the protein-tyrosine phosphatase SHP2 which is a positive modulator of RAS signaling [Neel et al., 2003]. Whereas gain-of-function SHP2 amino acid substitutions enhancing RAF-MAPK signal transduction are responsible for Noonan syndrome [Fragale et al., 2004], Noonan syndrome with multiple lentigines-associated mutations act in a dominant-negative manner and interfere with MAPK signaling in transient transfection assays [Kontaridis et al., 2006].

BRAF mutations in CFC syndrome introduce amino acid substitutions that are widely distributed across the BRAF protein [Niihori et al., 2006; Rodriguez-Viciano et al., 2006; Rauen, 2010]. As a direct downstream effector of RAS and activator of MEK1/2 the serine/threonine kinase BRAF participates in signal transduction from the cell surface to the nucleus [Wellbrock et al., 2004]. Most CFC-associated BRAF proteins such as BRAF^{Q257R}, the most common CFC causing variant, are strongly activated due to increased kinase activity and result in hyperphosphorylation of ERK; individuals with this BRAF change have many phenotypic features in common. In contrast, some BRAF mutants identified in individuals with a milder phenotype show normal or impaired BRAF kinase activity (BRAF^{E501G} and BRAF^{G596V}, respectively) and consequently did not induce increased ERK activation [Niihori et al., 2006; Rodriguez-Viciano et al., 2006].

CONCLUSION

Our available data and the circumstantial evidence from the literature provide a reasonable basis for the hypothesis that the patients' phenotype is related to the *HRAS* c.266C>G substitution. On the other hand, the apparently unaffected father and the incomplete phenotypic concordance in the siblings remain challenging for this hypothesis. Indeed, the identified variant could also be an incidental finding with no phenotypical consequences, and variants elsewhere in the genome, possibly in a gene influencing RAS pathways, may underlie the physical manifestations in the siblings. Regrettably, genome or exome sequencing is not possible in this family; thus, the final interpretation of our findings will depend on the identification of similar changes in other individuals.

In conclusion, it is obvious that the functional consequences of different mutations in a specific protein from the RASopathy-associated signaling module are variable and result in a broad phenotypic spectrum. In line with this, our data demonstrate that the *HRAS* p.S89C germline mutation exerts a negative effect on *HRAS* activation and downstream signaling; this contrasts with a gain in signal flow, the main consequence of all previously tested disease-associated *HRAS* mutations. Accordingly, the p.S89C change may have different impact on human development resulting in substantial variation of the clinical outcome.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIG. 1.

Substitution c.266C>G (p.S89C) associated with a clinical presentation resembling other RAS-MAPK pathway disorders. A: Details of sequence chromatograms of HRAS exon 3. Patient 1, Patient 2, and their father are heterozygous for the c.266C>G substitution in HRAS predicting the amino acid change of serine 89 for a cysteine. Sequence of the wild-type and mutated allele is given; codons and encoded amino acids are indicated below the father's sequence. The patients' mother is homozygous for the wild-type allele. B: Photographs of Patient 1 at age 2 months (left) and 11 months (right). Note the epicanthal folds, full nasal tip and deep philtrum in the facial photographs, and persistent fetal pads on the fingers. C: Photograph and magnetic resonance images of Patient 2 at age 5 weeks. Note the prominent philtrum and a nasogastric tube. The sagittal and axial T₂ weighted magnetic resonance images obtained at age 5 days show a typical Dandy–Walker malformation with absence of the lower posterior cerebellar vermis and upward rotation of the remaining vermis (arrowhead). This results in the appearance of cystic dilatation of the 4th ventricle (cross). The cerebellar hemispheres are small (asterisks).

FIG. 2. Conservation and position of residue serine 89. A: Evolutionary (orthologous) and paralogous conservation of amino acid serine 89 in human HRAS. Species, species-specific gene names, protein database accession numbers (www.ncbi.nlm.nih.gov/protein) and partial amino acids sequences are given. Orthologous and paralogous residues identical to human HRAS serine 89 are indicated in green letters. The amino acid sequence alignment demonstrates a high evolutionary conservation of serine 89. B: Schematic representation of HRAS with the distribution of germline disease-causing HRAS amino acid

changes. Secondary structural elements of HRAS are shown as green arrows and blue cylinders representing β -sheets and α -helices, respectively. Affected residues are indicated above the HRAS structure. Motifs representing the P-loop and switch I and II regions are indicated below the protein structure; amino acids (aa) constituting these motifs are given. The C-terminus of HRAS contains the membrane-targeting CVLS motif (orange cubes). C: HRAS accessible surfaces (left) and ribbon structures (right) are shown in the inactive (HRAS::GDP) and active (HRAS::GTP) conformations. HRAS amino acids altered in patients with Costello syndrome are highlighted in green and side chains are shown. Residue S89 with side chain is highlighted in red. Complexed GDP and the non-hydrolysable GTP analogue GppNHp are depicted as sticks.

FIG. 3. Reduced cellular levels of active, GTP-bound HRAS^{S89C}. COS-7 cells transiently expressing HA-HRAS^{WT} (wild type), HA-HRAS^{S89C}, HA-HRAS^{G12V}, HA-HRAS^{G12S} or HA-HRAS^{S17N} were cultured under serum-starved condition (0.1% serum) (A), steady-state condition (10% serum) (B), or serum-starved condition followed by 20 min stimulation with 10 ng/ml EGF (C). GTP-bound HA-tagged HRAS protein variants were precipitated from cell extracts using GST-RAF1::RBD, GST-PIK3CA::RBD, or GST-RALGDS::RA fusion proteins as indicated. Precipitated HA-HRAS (precipitates) and total amounts of HA-HRAS proteins in the cellular extracts (tcl) were detected by immunoblotting using anti-HA antibody. Cellular extracts were probed with anti-GAPDH antibody to control for equal loading. tcl, total cell lysates.

FIG. 4.

Expression of HRAS^{S89C} leads to reduced phosphorylation of MEK1/2, ERK1/2 and AKT. COS-7 cells were transfected with constructs expressing HA-HRAS^{WT}, HA-HRAS^{S89C}, HA-HRAS^{G12V}, HA-HRAS^{G12S} or HA-HRAS^{S17N} as indicated. Cells were cultured under serum-starved condition (0.1% serum) (A), steady-state condition (10% serum) (B), or serum-starved condition followed by 20 min stimulation with 10 ng/ml EGF (C). Cell extracts (tcl) were analyzed by immunoblotting using specific antibodies. The representative autoradiographs show levels of total MEK1/2, phospho-MEK1/2, total ERK1/2, phospho-ERK1/2, total AKT and phospho-AKT. tcl, total cell lysates. D: Based on densitometric quantification of autoradiographic signals the graphs show levels of phosphorylated (p) MEK1/2, ERK1/2 and AKT normalized with total MEK1/2, ERK1/2 and AKT, respectively, in cells expressing HA-HRAS^{WT} (gray circles) or HA-HRAS^{S89C} (gray squares) and under culture conditions as indicated. The strong variance results from different exposures of autoradiographs in individual experiments, which leads to variable signal densities on the blots. To determine the significance of the average difference between HA-HRAS^{WT} and HA-HRAS^{S89C} paired Student's *t*-tests were applied. The scaling on Y-axes is arbitrary and data pairs (HA-HRAS^{WT} and HA-HRAS^{S89C}) are connected with lines for each experiment. The number of independent experiments (n) is indicated and P-values are given (NS, statistically not significant).

FIG. 5.

HRAS^{S89C} shows decreased GTP loading and signaling capabilities. A: COS-7 cells transiently expressing HA-HRAS^{WT} or HA-HRAS^{S89C} were serum-starved (0.1% serum) and subsequently stimulated with 10 ng/ml EGF for various times (5, 15, 30 min EGF) or left untreated (0 min EGF) as indicated. GTP-bound HRAS was precipitated from cell extracts using GST-RAF1::RBD fusion protein. Total amounts of HA-HRAS in the total cell lysates (tcl) and precipitated HA-HRAS (precipitates) was detected by using anti-HA antibody. Cellular extracts (tcl) were also analyzed by immunoblotting using specific antibodies against total MEK1/2, phospho-MEK1/2, total ERK1/2, phospho-ERK1/2, total AKT, and phospho-AKT. Equal loading was confirmed by immunoblotting for GAPDH. B: After densitometric quantification of autoradiographic signals, levels of active HA-HRAS, phosphorylated MEK1/2, ERK1/2, and AKT were normalized relative to levels of total HA-HRAS, MEK1/2, ERK1/2, and AKT, respectively. Subsequently, HA-HRAS activation levels as well as MEK1/2, ERK1/2, and AKT phosphorylation levels in the untreated parallel cell cultures (0 min EGF) were considered as basal levels (=1) in each experiment. Thus, the graphs show activation and phosphorylation levels upon 5, 15, and 30 min EGF stimulation relative to the respective basal levels (0 min EGF) both in cells expressing HRAS^{WT} (black) and HRAS^{S89C} (gray). The number of independent experiments (n) is indicated and the data represent the mean of n independent experiments SD. [*P < 0.05 (unpaired Student's t-test); NS, statistically not significant].