SHORT REPORT

Expansion of the genotypic and phenotypic spectrum in patients with *KRAS* germline mutations

Martin Zenker, Katarina Lehmann, Anna Leana Schulz, Helmut Barth, Dagmar Hansmann, Rainer Koenig, Rudolf Korinthenberg, Martina Kreiss-Nachtsheim, Peter Meinecke, Susanne Morlot, Stefan Mundlos, Anne S Quante, Salmo Raskin, Dirk Schnabel, Lars-Erik Wehner, Christian P Kratz, Denise Horn, Kerstin Kutsche

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Background: Noonan syndrome, cardio-facio-cutaneous syndrome (CFC) and Costello syndrome constitute a group of developmental disorders with an overlapping pattern of congenital anomalies. Each of these conditions can be caused by germline mutations in key components of the highly conserved Ras-MAPK pathway, possibly reflecting a similar pathogenesis underlying the three disorders. Germline mutations in *KRAS* have recently been identified in a small number of patients with Noonan syndrome and CFC.

Methods and results: 260 patients were screened for *KRAS* mutations by direct sequencing. Overall, we detected *KRAS* mutations in 12 patients, including three known and eight novel sequence alterations. All mutations are predicted to cause single amino acid substitutions. Remarkably, our cohort of individuals with *KRAS* mutations showed a high clinical variability, ranging from Noonan syndrome to CFC, and also included two patients who met the clinical criteria of Costello syndrome.

Conclusion: Our findings reinforce the picture of a clustered distribution of disease associated *KRAS* germline alterations. We further defined the phenotypic spectrum associated with *KRAS* missense mutations and provided the first evidence of clinical differences in patients with *KRAS* mutations compared with Noonan syndrome affected individuals with heterozygous *PTPN11* mutations and CFC patients carrying a *BRAF*, *MEK1* or *MEK1* alteration, respectively. We speculate that the observed phenotypic variability may be related, at least in part, to specific genotypes and possibly reflects the central role of K-Ras in a number of different signalling pathways.

Noonan syndrome (OMIM 163950), cardio-facio-cutaneous syndrome (CFC; OMIM 115150) and Costello syndrome (OMIM 218040) are distinct entities that share a common pattern of congenital anomalies, including typical heart defects, overlapping craniofacial dysmorphisms, short stature and a variable degree of mental retardation. Discrimination between the three conditions is based mainly on distinct clinical features such as dry hyperkeratotic skin and hair abnormalities in patients with CFC,[1] and redundant and loose skin with deep palmar and plantar creases as well as a coarse facial appearance in those with Costello syndrome.[2] In addition, mental development is more severely impaired in CFC and Costello syndrome whereas Noonan syndrome is usually associated with minor cognitive deficits or even normal intelligence.[3] While patients with Noonan syndrome and CFC have no or only a slightly increased risk of tumour development, the incidence of tumours in Costello syndrome has been estimated to be 7–21%.[4] Although various attempts have been undertaken to develop standardised diagnostic criteria for these entities,[5 6] considerable overlap exists and in some instances a patient's phenotype cannot be clearly assigned to one of these conditions.

Missense mutations in *PTPN11* were first identified in patients with Noonan syndrome[7] and subsequently have been shown to account for almost 50% of cases.[8 9] *PTPN11* encodes the protein tyrosine phosphatase SHP-2 which relays growth signals from activated tyrosine kinase receptors to other signalling molecules, particularly Ras (reviewed by Neel *et al*[10]). Noonan syndrome causing *PTPN11* mutations have been thought to result in gain-of-function of SHP-2 and cause deregulation of Ras dependent signalling cascades.[11] Heterozygous germline mutations in *KRAS* were reported to occur in a minority of patients with Noonan syndrome[12] and CFC,[12 13] shortly after mutations in *HRAS* had been detected in the majority of individuals with Costello syndrome.[14] Moreover, mutations in *BRAF*, *MEK1* and *MEK2*, encoding proteins involved in Ras downstream signalling, were shown to cause CFC syndrome.[13 15] Taken together, the current data suggest that germline missense mutations in the aforementioned genes culminate in deregulated Ras-MAPK signalling that most likely represents the common pathogenetic basis of this group of developmental disorders.[16 17]

Ras isoforms encoded by the three genes *KRAS*, *HRAS* and *NRAS* represent highly conserved signal transduction molecules. They act as molecular switches through cycling between an active GTP bound and an inactive GDP bound state,[18] and in their active form they interact with a variety of downstream effector proteins.[19] *RAS* genes have long been known as proto-oncogenes mutated in various types of human cancers (reviewed by Bos[20]). The majority of these oncogenic *RAS* mutations affect amino acid residues G12, G13 and Q61 and cause Ras to accumulate in the active GTP bound state by impairing intrinsic GTPase activity and conferring resistance to GTPase activating proteins (GAPs).[20] Germline *HRAS* mutations associated with Costello syndrome almost exclusively affect codons 12 and 13 and are identical to somatic alterations identified in cancer,[14] hence explaining the high risk of tumour development in Costello syndrome. In contrast, *KRAS* mutations described to date in patients with Noonan syndrome/CFC are distinct from those found in malignancies. Similar to the concept of activating *PTPN11* mutations in Noonan syndrome and malignancies,[11] *KRAS* mutations associated with Noonan syndrome or CFC might give rise to mutant proteins with a relatively mild gain-of-function which are tolerated in the germline as well as during embryonic development. Specifically, *KRAS* mutations identified in Noonan syndrome patients include V14I and T58I whereas P34R and G60R were found in CFC patients.[12 13] Mutations in *KRAS* exon 6, causing amino acid alterations in the C terminal portion of isoform B, such as D153V and V152G, were found to be

Abbreviations: CFC, cardio-facio-cutaneous syndrome; GAP, GTPase activating protein

associated with a severe Noonan syndrome or CFC phenotype.[12 13 21] It has been proposed that all mutations lead to stabilisation of K-Ras in the active conformation, most likely by different gain-of-function mechanisms.[12 21]

Here we report the results of *KRAS* mutation screening in a large cohort of patients with Noonan syndrome and related disorders.

PATIENTS AND METHODS

The original study population consisted of 333 individuals of predominantly European origin with a clinical diagnosis of Noonan syndrome, 37 with CFC and 30 with Costello syndrome. Patients were clinically assessed by experienced clinical geneticists and classified according to general diagnostic criteria.[3 5 6] Ninety seven patients with Noonan syndrome (29%) were previously tested positive for a *PTPN11* mutation, a proportion that is comparable with that published by Musante *et al*[22] but lower than in other studies.[8 9] These variations probably reflect differences in the selection of the referred cases. Twenty seven of 30 patients with Costello syndrome (90%) were found to have an *HRAS* mutation, a rate that has been consistently reported in previous studies.[14 23–25] In 12 (32%) of the 37 CFC cases, a *BRAF* mutation was detected, a similar proportion to that reported by Niihori *et al*.[13] Two of our patients with CFC (5%) had a mutation of *MEK1* and two (5%) a mutation of *MEK2* (M Zenker and K Kutsche, unpublished data). The remaining 236 *PTPN11* negative patients with Noonan syndrome, 21 individuals with CFC and three patients with Costello syndrome were investigated for mutations in *KRAS*. Informed consent was obtained for genetic analyses from all patients or their legal guardians. Ethics approval for the study was obtained from the ethics committee of the University of Erlangen-Nuremberg. Mutation screening of the entire coding sequence of both *KRAS* isoforms was carried out as described previously.[12] Primer pairs and polymerase chain reaction conditions are available on request. Sequence analysis was performed by bidirectional sequencing using the ABI BigDye Terminator Sequencing Kit (Applied Biosystems, Weiterstadt, Germany) and automated capillary sequencers (Applied Biosystems). Where mutations were shown to have arisen de novo, we verified declared relationships by genotyping of both parents and the patient at 10 microsatellite loci.

RESULTS

Spectrum of *KRAS* mutations

We discovered 11 different heterozygous *KRAS* mutations in 12 of the patients in our study population (table 1). All patients carrying a *KRAS* mutation were sporadic cases. In 10 individuals we could demonstrate that the mutation occurred de novo (including confirmation of paternity). DNA from both parents was not available in the remaining two cases (Nos 4 and 7; table 1).

Three mutations, V14I, G60R and D153V, have been described previously[12 13] whereas eight are novel (table 1). All newly identified mutations are predicted to cause single amino acid substitutions at highly conserved positions of K-Ras (data not shown). All but three of the disease associated amino acid changes affect both isoforms of K-Ras while the nucleotide substitutions leading to the missense mutations D153V, F156I and F156L were found in the alternatively spliced exon 6, thus being present only in isoform B (fig 1).

Five of the novel mutations, P34L, P34Q, I36M, F156I and F156L, are located at or close to the known hotspots for *KRAS* germline mutations whereas missense mutations affecting codons 5 and 22, respectively, represent novel mutation hotspots (K5N, Q22R and Q22E). Notably, mutations at these positions have previously been reported to occur as somatic alterations in cancer (COSMIC database).

Clinical presentation of patients with *KRAS* mutations

Heterozygous *KRAS* mutations were identified in seven patients with Noonan syndrome, two individuals with CFC and one patient with an overlapping phenotype between Noonan syndrome and CFC (table 1), thus confirming that sequence alterations in *KRAS* account for approximately 2% (7/333) of cases with Noonan syndrome and less than 10% (2/37) with CFC.[12 13] Moreover, we also detected mutations in two patients (Nos 1 and 12) with a clinical diagnosis of Costello syndrome who were 7.5 and 14 months old, respectively (table 1).

Clinical details of patients carrying *KRAS* mutations are listed in table 1. All patients with *KRAS* mutations exhibited the typical craniofacial features of the Noonan syndrome–CFC–Costello syndrome spectrum (fig 1). The majority had short stature, relative macrocephaly, short/webbed neck, thorax deformity and typical ophthalmological anomalies. It is noteworthy that all individuals with *KRAS* mutations had mild to moderate mental retardation. Three patients presented with additional cerebral abnormalities which are uncommon in Noonan syndrome and CFC, including mild hydrocephalus, intracranial vascular malformation and Dandy-Walker malformation (table 1). In addition, CFC patients with a *KRAS* mutation had sparse and thin hair but none had hyperkeratotic or ichthyotic skin lesions (table 1).

DISCUSSION

We identified three known and eight novel *KRAS* germline mutations in patients with variable phenotypes of the Noonan syndrome–CFC–Costello syndrome spectrum. The assumption that these novel sequence variations indeed represent pathogenic mutations is justified by the following findings: (i) all amino acid substitutions affect highly conserved residues of K-Ras, (ii) in the majority of cases we demonstrated that the mutation had arisen de novo and (iii) all mutations are located at or near known hotspots for germline and somatic *KRAS* mutations, respectively. Our findings reinforce the picture of clustered germline mutations in *KRAS* that strikingly parallels but hardly overlaps the distribution of somatic mutations found in cancer. However, two exceptions exist, K5N and Q22R, representing germline mutations that have also been reported as somatic events in tumours. K5N was described as the probable causative mutation in gastric cancer[26] while the Q22R mutation was concurrently found with the oncogenic G12S mutation on the same *KRAS* allele, suggesting that the transforming potential of this allele is related to G12S rather than Q22R.[27] None the less, these findings indicate that at least some oncogenic *KRAS* mutations may be compatible with life when occurring in the germline and are associated with Noonan syndrome spectrum disorders. Patients carrying either of these *KRAS* mutations may have an increased tumour risk, similar to patients with Costello syndrome.[4]

Based on data obtained by functional experiments for the mutations T58I and V14I,[12] as well as the structural analysis of both V152G and D153V,[21] we hypothesise that the novel *KRAS* mutations most likely also confer gain-of-function effects. In line with this assumption, previous in vitro mutational studies showed that substitutions of I36 and P34, respectively, caused severe impairment of GAP stimulated GTP hydrolysis, thus causing mutant Ras proteins to accumulate in the active state.[28 29] Moreover, Ras F156L mutant protein exhibited an extremely rapid off rate for both GDP and GTP in vitro and showed increased levels of its GTP bound form in vivo.[30] These findings, together with data from our recent detailed structural analysis, suggest that *KRAS* mutations cause accumulation of Ras mutant proteins in the active GTP bound state, most likely by different gain-of-function mechanisms (R Dvorsky, M Zenker, K Kutsche, MR Ahmadian, personal communication, August 2006).

The phenotypic spectrum in our cohort of patients with *KRAS* mutations was remarkably broad and included two individuals

Table 1 Clinical features of patients with *KRAS* mutations

	Patient No 1	Patient No 2	Patient No 3	Patient No 4	Patient No 5	Patient No 6
KRAS mutation*	**K5N (c.15A→T)**	V14I (c.40G→A)	**Q22E (c.64C→G)**	**Q22R (c.65A→G)**	**P34L (c.101C→T)**	**P34Q (c.101C→A)**
Age at last follow up	7.5 months	16 years	2 years	3.3 years	17 years	3.4 years
Sex	Male	Male	Male	Female	Male	Male
Birth weight (g)†	3195	4000	3400	2820	4470	3060
Congenital heart defect	Pst	No	Pst	Pst	No	Pst
Facial features	Hypertelorism, downslanting palpebral fissures, coarse face, anteverted nostrils, low-set dysplastic ears	Hypertelorism, low-set ears	Hypertelorism, downslanting palpebral fissures, anteverted nostrils, low-set ears	Hypertelorism, downslanting palpebral fissures, low-set ears	Hypertelorism, downslanting palpebral fissures	Hypertelorism, low set ears, flat nasal bridge, posteriorly rotated ears
Stature	<1st centile	1st centile	15th centile	3rd–10th centile	1st centile	<1st centile
Relative macrocephaly	Yes	Yes	No	Yes	Yes	No
Short/webbed neck	Webbed neck	Short neck	Short and webbed neck	Short and webbed neck	Short neck	Webbed neck
Thorax deformity	Pectus carinatum	Mild pectus excavatum	No	Broad chest, mild pectus excavatum	Pectus excavatum (op)	No
Cryptorchidism	No	No	No	–	No	No
Ophthalmological problems	Ptosis, strabismus, nystagmus	Ptosis (op), strabismus	Ptosis	Ptosis, strabismus	Strabismus	Mild ptosis
Developmental delay/ mental retardation	Moderate	Mild	Moderate	Moderate	Mild	Mild
Abnormal hair	Sparse	No, sparse hair in early childhood	Thin hair, sparse eyebrows	No	No	No
Skin abnormalities	Redundant skin, deep palmar and plantar creases	No	Deep palmar and plantar creases	No	No	No
Other	Macroglossia, severe feeding difficulties and failure to thrive	Seizures, mild hydrocephalus internus, cubitus valgus	Unilateral pyelectasia, failure to thrive	–	Received GH treatment	Failure to thrive and feeding problems in infancy
Clinical diagnosis	CS	NS	CFC	Severe NS	NS	NS

	Patient No 7	Patient No 8	Patient No 9	Patient No 10	Patient No 11	Patient No 12
KRAS mutation*	**I36M (c.108A→G)**	G60R (c.178G→C)	D153V (isoform B) (c.460A→T)	D153V (isoform B) (c.460A→T)	**F156I** (isoform B) (c.466T→A)	**F156L** (isoform B) (c.468C→G)
Age at last follow-up	17 years	5 years	20 years	5.5 years	8.5 years	14 months‡
Sex	Female	Female	Male	Male	Male	Male
Birth weight (g)†	3000	3350	3210	2820	4850	3090
Congenital heart defect	ASD	HOCM	No	Pst	No	HCM, Pst, ASD
Facial features	Hypertelorism, downslanting palpebral fissures, posteriorly rotated ears	Hypertelorism, downslanting palpebral fissures, coarse face, low-set ears	Hypertelorism, downslanting palpebral fissures, posteriorly rotated ears	Hypertelorism, anteverted nostrils, low-set ears	Hypertelorism, prominent forehead, bitemporal narrowing, epicanthic folds, downslanting palpebral fissures, ptosis, widely spaced teeth, low-set ears	Hypertelorism, coarse face, anteverted nostrils, low-set dysplastic ears
Stature	3rd centile	<1st centile	10th centile	<1st centile	3rd centile	<3rd centile
Relative macrocephaly	Yes	No	Yes	Yes	Yes	Yes
Short/webbed neck	Short and webbed neck	Short neck	Short and webbed neck	Short neck	Short and webbed neck	Short and webbed neck
Thorax deformity	Pectus excavatum	No	Pectus excavatum	Pectus excavatum	Pectus excavatum	No
Cryptorchidism	–	–	Yes	No	No	Yes
Ophthalmological problems	Mild ptosis	No	Ptosis	Mild ptosis, strabismus	Ptosis, nystagmus, strabismus	Ptosis
Developmental delay/ mental retardation	Mild	Moderate	Mild	Mild	Moderate	Moderate
Abnormal hair	Thin hair	Thin and sparse hair	No	No	Low posterior hair line, sparse hair at early age	Sparse
Skin abnormalities	Loose/redundant skin in early childhood	No	No	No	Wrinkled skin of palms and soles	Loose/redundant and soft skin with deep palmar and plantar creases
Other	Received GH treatment, facial asymmetry, bleeding diathesis	Feeding difficulties and failure to thrive	–	Haemangioma of lower lip, vascular malformation of the brain, brachydactyly, GH treatment	Hearing loss, widely spaced nipples, inguinal hernia, muscular hypotonia	Dandy-Walker malformation, laryngomalacia, conductive hearing deficits, severe feeding difficulties and failure to thrive
Clinical diagnosis	NS	CFC	NS	NS	NS/CFC	CS

ASD, atrial septal defect; CFC, cardio-facio-cutaneous syndrome; CS, Costello syndrome; GH, growth hormone; H(O)CM, hypertrophic (obstructive) cardiomyopathy; MR, mental retardation; nd, not documented; NS, Noonan syndrome; op, operated; Pst, pulmonic stenosis.
*Novel mutations are in bold.
†All children were born at term.
‡Died from unexplained sudden death at 14 months of age.

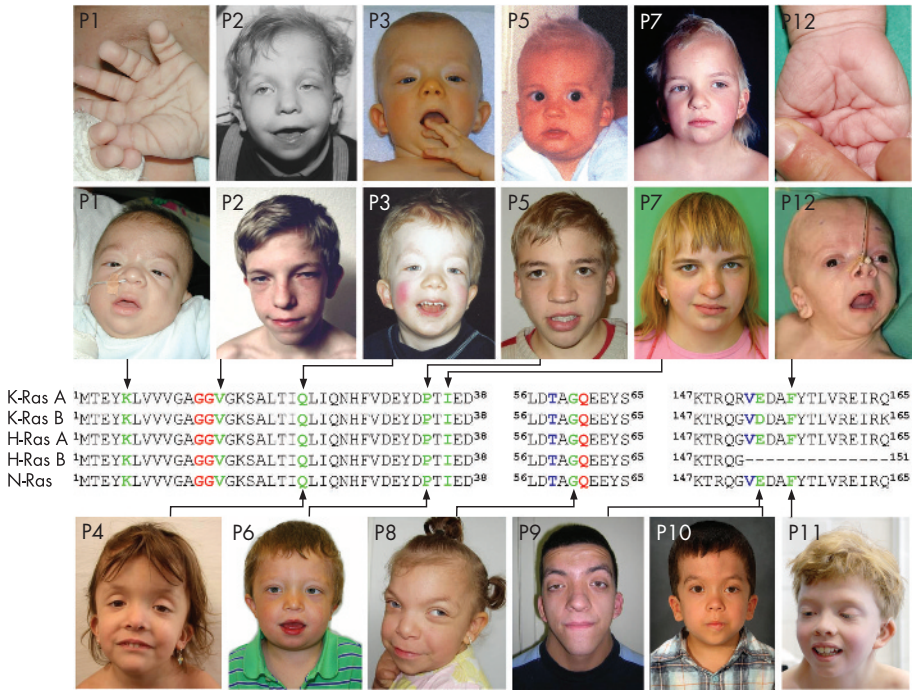

Figure 1 Novel mutations of *KRAS* in patients with Noonan syndrome spectrum disorders. Partial alignment of human K-RasA, K-RasB, N-Ras, H-RasA and H-RasB is shown. Mutated amino acid residues presented in this study are indicated in green, those altered in previously reported patients (but not present in our study cohort) in blue and residues G12, G13 and Q61, which are most commonly found to be mutated in tumours, in red. Clinical photographs document the variable craniofacial phenotype of patients with heterozygous *KRAS* mutations (second panel from top and bottom panel). Arrows point from the patients' photographs to the respective residues mutated in K-Ras. The patient's number (according to table 1) is given in the upper left corner of each photograph. Additional photographs documenting deep palms of patient Nos 1 and 12 as well as earlier photographs of patient Nos 2, 3, 5 and 7 are shown in the top row. We obtained written consent from the patient or his/her legal guardian for publication of the images.

(cases 1 and 12) with a clinical diagnosis of Costello syndrome which was based on their characteristic facial appearance, the presence of loose and redundant skin with deep palmar and plantar creases, heart abnormality, severe feeding difficulties in early infancy and failure to thrive (fig 1, table 1). Admittedly, none of these features is absolutely specific for Costello syndrome, and as both of these patients with Costello syndrome were very young at the time of clinical diagnosis we cannot exclude the fact that the phenotype may emerge to (severe) CFC later in life. None the less, some features of Costello syndrome and CFC have also been described in a recently reported patient with the *KRAS* V152G mutation.[21] Bentires-Alj *et al* proposed that each syndrome should be defined by the specific underlying genetic defect.[16] In fact, it may be reasonable to reserve the diagnosis of Costello syndrome for patients who carry oncogenic *HRAS* mutations and hence must be considered to have an increased risk of tumour development. However, at this point the fact cannot be excluded that specific germline mutations of *KRAS* (this report) or *BRAF*[31] may not only lead to clinical features of Costello syndrome but also have a similar oncogenic potential as activating *HRAS* mutations.

It is important to note that all patients, even those classified as Noonan syndrome, showed mild to moderate mental retardation (table 1) which is generally found in only one third of patients with Noonan syndrome.[3] In patients with a *PTPN11* mutation, this proportion is even lower compared with *PTPN11* negative cases,[9] and in one published cohort only 24% of patients were reported to have needed special education.[8] The level of cognitive function is commonly regarded as one important feature facilitating discrimination between Noonan syndrome and CFC.[1 5] Therefore, the presence of substantial mental retardation in patients with Noonan syndrome-like features may shift the clinical diagnosis towards CFC and lead to classification of a severe Noonan syndrome or a Noonan syndrome–CFC overlapping phenotype in some patients with *KRAS* mutations. Similarly, Carta *et al*[21] had difficulties in finding the correct clinical classification for their patients and suggested that they presented with a severe Noonan syndrome phenotype with some characteristics of CFC and Costello syndrome. The predominance of significant mental retardation in patients with Noonan syndrome and *KRAS* mutations could also explain the lack of familial cases.

Patients with CFC syndrome and *BRAF* mutations frequently show hyperkeratotic or ichthyotic skin lesions,[13] which are typical findings in CFC patients.[32] However, none of the CFC patients carrying a *KRAS* mutation—either previously reported[13 21] or those presented here—showed at least one of these cutaneous features. Taken together, we propose that (i) patients with *KRAS* mutations display a particularly high phenotypic variability and that (ii) *KRAS* mutations are probably responsible for distinct phenotypic subclasses of both Noonan syndrome and CFC. These assumptions raise the question of whether or not the mutant K-Ras proteins display a greater diversity of altered functions compared with other mutated signalling molecules implicated in the same pathway.

In summary, we have presented the largest cohort to date of patients with germline *KRAS* mutations and their association with a remarkably broad spectrum of clinical phenotypes. Clearly, the data underscore the central role of Ras in the pathogenesis of this group of related disorders, including Noonan syndrome, CFC and Costello syndrome. We speculate that the diversity of clinical phenotypes associated with different *KRAS* mutations is caused by the following two factors: (i) K-Ras plays a key role in a number of downstream signalling cascades and (ii) various K-Ras mutant proteins may have quantitatively and/or qualitatively different effects on these Ras dependent pathways. Furthermore, in vitro and

clinical studies are necessary to elucidate the mechanisms leading to deregulated K-Ras signalling by a specific mutation and its association with a defined disease phenotype.

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Authors' affiliations

M Zenker*, Institute of Human Genetics, University of Erlangen-Nuremberg, Germany
K Lehmann*, S Mundlos, D Horn*, Institute of Medical Genetics, Charité, University Medicine of Berlin, Germany
A L Schulz, K Kutsche*, Institut für Humangenetik, Universitätsklinikum Hamburg-Eppendorf, Hamburg, Germany
H Barth, Zentrum für Kinder-und Jugendmedizin, Klinik für Neonatologie und Neuropädiatrie, Marburg, Germany
D Hansmann, Pränatalmedizin und Medizinische Genetik, Meckenheim, Germany
R Koenig, Institute of Human Genetics, University of Frankfurt, Germany
R Korinthenberg, C P Kratz, Department of Paediatrics and Adolescent Medicine, University of Freiburg, Germany
M Kreiss-Nachtsheim, Institute of Human Genetics, Friedrich-Wilhelms-University of Bonn, Germany
P Meinecke, Medizinische Genetik, Altonaer Kinderkrankenhaus, Hamburg, Germany
S Morlot, Praxis für Humangenetik, Ärztliche Partnerschaft Wagner-Stibbe Hannover, Germany
A S Quante, Institute of Human Genetics, Heinrich-Heine-University of Düsseldorf, Germany
S Raskin, Laboratorio Genetika, Alameda Augusto Stellfeld, Curitiba Parana, Brazil
D Schnabel, Institute of Paediatric Endocrinology, University Children's Hospital, University Medicine of Berlin, Germany
L-E Wehner, Institute of Human Genetics, University of Göttingen, Germany

*M Zenker, K Lehmann, D Horn and K Kutsche are equally contributing first and senior authors.

Competing interests: None.

Electronic database information See Gene at http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene for *KRAS* genomic (accession number NC_000012), cDNA (accession numbers NM_004985 and NM_033360) and K-Ras amino acid (accession numbers NP_004976 and NP_203524) sequences; for N-Ras (accession number NP_002515) and H-Ras (accession numbers NP_005334 and NP_789765) amino acid sequences. See Catalogue of Somatic Mutations in Cancer (COSMIC) at http://www.sanger.ac.uk/genetics/CGP/cosmic/ for somatic mutations observed in *KRAS*.

Correspondence to: Dr Kerstin Kutsche, Institut für Humangenetik, Universitätsklinikum Hamburg-Eppendorf, Butenfeld 42, 22529 Hamburg, Germany; kkutsche@uke.uni-hamburg.de

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