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ORIGINAL ARTICLE

Genotype-phenotype correlation in Costello syndrome: *HRAS* mutation analysis in 43 cases

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Background: Costello syndrome (CS) is a rare multiple congenital abnormality syndrome, associated with failure to thrive and developmental delay. One of the more distinctive features in childhood is the development of facial warts, often nasolabial and in other moist body surfaces. Individuals with CS have an increased risk of malignancy, suggested to be about 17%. Recently, mutations in the *HRAS* gene on chromosome 11p13.3 have been found to cause CS.
Methods: We report here the results of *HRAS* analysis in 43 individuals with a clinical diagnosis of CS.
Results: Mutations were found in 37 (86%) of patients. Analysis of parental DNA samples was possible in 16 cases for both parents and in three cases for one parent, and confirmed the mutations as de novo in all of these cases. Three novel mutations (G12C, G12E, and K117R) were found in five cases.
Conclusions: These results confirm that CS is caused, in most cases, by heterozygous missense mutations in the proto-oncogene *HRAS*. Analysis of the major phenotypic features by mutation suggests a potential correlation between malignancy risk and genotype, which is highest for patients with an uncommon (G12A) substitution. These results confirm that mutation testing for *HRAS* is a reliable diagnostic test for CS.

Costello syndrome (CS) is a rare multiple congenital abnormality syndrome, first described by Costello in 1971 and 1977.[1 2] The condition is associated in all cases with a characteristic facies, a distinctive hand posture and appearance, severe feeding difficulty, and failure to thrive.[3] Affected pregnancies are often associated with polyhydramnios, and birth weight is often high.

A number of congenital anomalies have a relatively high frequency. Cardiac abnormalities (congenital heart disease, atrial arrhythmia, cardiomyopathy) occur in two thirds of cases.[4] Cerebral imaging may be normal, but ventricular dilatation occurs in 40% of cases,[5] and Chiari malformation, sometime presenting in adult life,[6] occurs. Syringomyelia and cerebral atrophy have also been described.[5] Developmental disability is invariable, but may be mild or severe, and can include autistic spectrum behaviours. Short stature can be associated with growth hormone deficiency, while precocious puberty has also been reported.

The phenotype of CS evolves over time.[7] One of the more distinctive features in childhood is the development of facial warts, often nasolabial and in other moist body surfaces.

Since the first reports of rhabdomyosarcoma in CS[8 9] it has been recognised that children and adults with CS have an increased risk of malignancy. Gripp *et al*[10] reported five further cases of rhabdomyosarcoma in patients with CS and reviewed the literature. A total of 17 solid tumours had been reported, the majority being rhabdomyosarcoma. Most tumours showed embryonal histology, with one alveolar and one pleomorphic subtype. The age at presentation ranged from 6 months to 6 years. Eight of these tumours originated from the abdomen, pelvis, or urogenital area. Neuroblastoma occurred in three patients, ranging in age from 2 months to 4 years. From these data, Gripp *et al*[10] suggested a tumour frequency of up to 17%, based on the 17 solid tumours reported in about 100 known cases.

The adult tumour phenotype includes an isolated description of acoustic neuroma,[11] three cases of bladder carcinoma,[12–14] and one case of assumed benign choroid plexus papilloma.[6] Delayed puberty, hypogonadism, osteoporosis, benign breast disease, and the development of gastro-oesophageal reflux were the other health problems in a survey of affected adults.[6]

While the diagnosis of CS is relatively easy in the older child or adult with classic features, particularly in the presence of facial warts, in early life the facial and other phenotypic resemblance to both Noonan syndrome (NS) and cardiofaciocutaneous (CFC) syndrome can make a certain diagnosis difficult. No firm diagnostic criteria are available, although the frequency of clinical signs has been tabulated as an aid to diagnosis.[15]

In the absence of a definitive test, the diagnosis has remained a clinical one, with prognosis in affected individuals impossible to predict. Segregation analysis suggested the cause of CS to be a new dominant mutation.[16] Cloning of the breakpoints in the only published translocation[17] did not identify the causative gene,[18] while mutations in the *PTPN11* gene (which are found in around half of cases of NS) were not found in a series of CS patients.[19]

Recently heterozygous mutations in the *HRAS* gene on chromosome 11p13.3 were found in 12 of 13 patients with CS.[20] Four different mutations were seen: G12S in seven patients, G12A in two, G13D in two, and G12V in one. A rhabdomyosarcoma was only present in one patient, who had

Abbreviations: CFC, cardiofaciocutaneous; CS, Costello syndrome; NS, Noonan syndrome

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a G12S mutation. One of the two patients with a G12A mutation had a ganglioneuroblastoma and severe cardiomyopathy, and died suddenly at 3 years of age.

In a recently published analysis of a series of 40 patients with CS,[21] missense mutations in HRAS were found in 33 (82.5%). The commonest was again G12S, occurring in 30; two patients had the G12A substitution, and one patient had a novel G13C change. No genotype-phenotype correlation was possible, owing to the small numbers of patients with the less common mutations.

We report here the results of mutation analysis in 43 patients with a clinical diagnosis of CS. Mutational analysis was also undertaken in four cases with a differential diagnosis of CFC and CS, and five cases of NS without *PTPN11* mutations.

MATERIALS AND METHODS

Study population

Cases were ascertained through the long interest of our groups in CS. Many have been reviewed clinically by several of us (BK, M-AD, DL, SS, NP). Clinical data were collected and tabulated by questionnaire, summarising the most pertinent clinical features, with particular emphasis on the presence or absence of malignancy, and unusual phenotypic features.

Ethics approval was granted by the North Manchester Ethics Research Committee (BK). Analysis in France (BA, SS) was performed under conditions established by the French law. Informed consent was obtained from all participants or their parents or carers.

Mutation analysis of *HRAS* using genomic DNA

The structure of the *HRAS* gene had previously been defined from which primers for the amplification of genomic DNA were designed (available on request). Genomic DNA (40 ng) was suspended in a 80 ml reaction containing 20 pmols of each forward and reverse primer, 0.75 mmol/l each dNTP, 67 mmol/l Tris-HCl (pH 8.0), 3.7 mmol/l $MgCl_2$, 6.7 mmol/l EDTA, 16 mmol/l $(NH4)_2SO_4$, 0.085 mg/ml bovine serum albumin, and 0.1 U *Taq* DNA polymerase. Samples were processed through 30 cycles of amplification consisting of 45 seconds at 94℃ (denaturation), 45 seconds at 55℃ (annealing) and 1 minute at 72℃ (extension). The final step was lengthened to 10 minutes. PCR products were purified using montage PCR columns (Millipore) in accordance with the manufacturer's instructions. Direct sequencing of PCR products were performed using a commercial kit and fluorescent sequencer (BigDye Terminator cycle sequencing kit, version 2.0 and ABI 377 sequencer; both Applied Biosystems) following the manufacturer's instructions.

RESULTS

The mutations (including nucleotide substitutions) and clinical features of the mutation positive cases are summarised in table 1. Mutations were found in 37 of 43 (86%) patients with a clinical diagnosis of CS. No mutations were found in four patients with a possible diagnosis of either CS or CFC or five NS cases without a *PTPN11* mutation.

Analysis of parental DNA samples was possible in 16 cases for both parents and in three cases for one parent, and confirmed the mutations as de novo in all of these cases.

As in the Japanese and North American series, the commonest mutation in our series was the G12S missense substitution, which we found in 30 of 37 mutation positive cases. Two of these 30 patients had a rhabdomyosarcoma (patients 1 and 8). Patient 1 was included in the report of Gripp *et al*,[21] and is included here for completeness of phenotype analysis but was only counted once in the analyses of malignancy frequency (table 2). Three patients, two of whom had a rhabdomyosarcoma, had a G12A mutation. Three novel mutations were observed: G12C in two patients (one with a rhabdomyosarcoma), G12E in one patient, and K117R in one patient.

In our series, all the mutation positive cases had failure to thrive, and the facial appearance and hands characteristic of CS. Macrocephaly, relative or absolute, was found in 32 of 37 mutation positive cases; current head size is unknown in two cases, and another, patient 11, had cerebral atrophy secondary to ischaemia.

Warts in unusual sites are one of the defining features of CS. They were present in 12 patients with a G12S mutation, but absent in 18 analysed or seen at 9 years of age or younger. They were also absent in the 9 year old patient with the K117R mutation, and one 5 year old patient with a G12A mutation. The youngest patient with warts was aged 6 years, with a G12C mutation.

Cardiomyopathy was common, occurring in 19 patients, and was found in patients with all five mutations. Atrial arrhythmia occurred in 11 patients, all of whom had the common G12S mutation, as did the patients with arrhythmia in the series of Gripp *et al*.[21] Congenital heart disease occurred in only eight patients, and in the presence of mutations G12S, G12A, and K117R.

Hypoglycaemia occurred in three patients, growth hormone deficiency in four, and precocious puberty in one. Chiari malformation occurred in two patients, and ventricular dilatation in three. Four patients had epilepsy. None of these findings was sufficiently common for correlation with mutation type to be possible.

Several clinical features are of interest. Patient 7 (G12A) died aged 25 years of a short and severe respiratory illness, just prior to testing being undertaken. At postmortem, extensive lung fibrosis was described. This has not previously been reported in CS.

In two cases, the phenotype was so severe that the clinical diagnosis has been doubted. Patient 10 (G12S) remains hospitalised in the second year of life because of severe airway obstruction secondary to subglottic stenosis and persisting respiratory compromise despite surgery and tracheostomy. Patient 11 (G12E) presented as a newborn with severe hypoglycaemia secondary to hyperinsulinism, requiring treatment throughout her life. She had hepatomegaly and cardiomegaly noted on the first day of life, as well as persistent and severe feeding difficulty. At 5 weeks, she developed aponea during a respiratory infection, associated with a cardiac arrest. Despite initial improvement, she required re-ventilation. She had severe biventricular cardiomyopathy, and on bronchography and bronchoscopy, bronchomalacia of the left main bronchus with narrowing of the right main bronchus was found. Her head growth and development ceased, with evidence on magnetic resonance imaging scan of cerebral atrophy, assumed due to hypoxia. She developed a chylous ascites, and remained ventilator dependent despite maximum therapy, including tracheostomy. She died of respiratory failure. At postmortem, the clinical findings diagnosed in life were confirmed. She had a nesidioblastosis-like lesion, with hypertrophy and hyperplasia of the Langherhans' islets of the pancreas, evidence of cortical ischaemia, and hypertrophic cardiomyopathy. Unexpected findings were a fibromuscular dysplasia of the coronary arteries, an inflammatory subglottic tracheal polyp, multiple cysts of both ovaries, and a severe canicular cholestasis.

The K117R mutation described in patient 16 lies in exon 3 and was not described in the original report. This patient's physical phenotype was unusual in that there was micro-retrognatism and both plantar and palmar creases were slightly less pronounced than usually seen in CS patients. The

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Table 1 Genotype and major phenotypic features in mutation positive cases of CS

No.	Age (years)	Muta–tion	FTT	CF	CH	CHD	HCM	Arr	Tumour	Warts	M	CM	Other CNS	EF	Other
1*	11	G12S	+	+	+	+	+	+	Rhabdo	+	+	+		GH	Severe scoliosis
2	5	G12S	+	+	+	–	–	+	–	–	+	–	–	–	
3	20	G12S	+	+	+	–	+	+	–	+	+	–	–	–	
4	4	G12S	+	+	+	+	–	–	–	–	+	–	Epi	Hypo, GH	Partial response to clonidine stimulation
5	–	G12C	+	+	+	–	+	–	Rhabdo	–	+–	–	–	–	Died aged 7 months
6	14	G12S	+	+	+	–	–	–	–	+	+	–	–	–	Severe scoliosis
7	–	G12A	+	+	+	–	+	–	–	+	+	–	–	–	Died aged 25 years, respiratory failure
8	–	G12S	+	+	+	–	?	?	Rhabdo	–	+	–	–	–	Died aged 7 years,
9	14	G12S	+	+	+	–	–	–	–	– 8 years	+	–	–	–	
10	1	G12S	+	+	+	–	+	–	–	–	+		VD	Hypo	Subglottic stenosis
11	–	G12E	+	+	+	–	+	–	–	–	–	–	CA	Hypo	Died aged 6 months
12 *	–	G12A	+	+	+	+	–	–	Rhabdo		+	–	–	–	Died aged 5 years,
13	3	G12S	+	+	+	–	–	–	–	–	+	–	–	–	
14	10	G12S	+	+	+	–	+	–	–	–	+	–	VD	PP, GH	
15	3	G12S	+	+	+	–	–	–	–	–	+	–	–	–	
16	9	K117R	+	+	+	–	–	–	–	–	+	–	–	–	
17	4	G12S	+	+	+	–	–	+	–	–	+	–	–	–	
18	6	G12C	+	+	+	–	+	–	–	+	+	–	VD, Epi	_	
19	8	G12S	+	+	+	–	–	–	–	?	+	–	–	–	
20	1	G12S	+	+	+	–	+	+	–	–	+	–	–	–	
21	23	G12S	+	+	+	+	–	–	–	+	+	–	–	–	Recurrent breast lumps
22	10	G12S	+	+	+	–	+	+	–	+	+	+	–	GH	
23	2	G12S	+	+	+	–	–	+	–	–	+	–	–	–	
24	16	G12S	+	+	+	–	+	–	–	+	+	–	–	–	
25	5	G12S	+	+	+	–	–	–	–	–	+	–	–	–	
26	2	G12S	+	+	+	+	+	+	–	–	+	–	–	–	
27	9	G12S	+	+	+	–	+	+	–	–	+	–	–	–	
28	7	G12S	+	+	+	+	–	+	–	+	+	–	–	–	
29	10	G12S	+	+	+	–	–	–	–	+	+	–	–	–	
30	11	G12S	+	+	+	–	–	–	–	+	+	–	–	IGF-1 defic	
31	4	G12S	+	+	+	–	–	–	–	–	?	–	Epi	–	
32	10	G12A	+	+	+	–	+	–	Rhabdo	+	+	–	–	–	
33	27	G12S	+	+	+	–	+	–	–	+	–	–	–	–	
34	12	G12S	+	+	+	–	+	+	–	–	+	–	Epi	–	Laryngeal diastema
35	20	G12S	+	+	+	–	+	–	–	+	+	–	–	–	Gynaecomastia at 7 years,
36	4	G12S	+	+	+	–	+	–	–	–	–	–	–	–	Tracheomalacia
37	?	G12S	+	+	+	?	?	?	–	?	?	?	?	?	

FTT, Failure to thrive; CF, Classic facies; CS, Classic hands; CHD, Congenital heart disease; Arr, Arrythmia; HCM, Cardiomyopathy; M, Macrocephaly, relative or absolute; CM, Chiari malformation; EF, endocrine features; Rhabdo, rhabdomyosarcoma; GH, Growth hormone deficiency or treatment; Hypo, hypoglycaemia; PP, precocious puberty; Epi, epilepsy; defi, deficiency; CA, cerebral atrophy; VD, Ventricular dilatation. G12S, glycine to serine (nt. 34G→A); G12C, glycine to cysteine (nt. 34G→T); G12A, glycine to alanine (nt. 35 G→C); G12E glycine to glutamic acid (nt. 35G→GA);K117R lysine to arginine (nt. 350A→G). *First reported by Kerr *et al.*[8]

behavioural phenotype included autistic traits, with verbal stereotypies and hand biting. Otherwise she had classical CS features, with cardiac involvement (cardiomyopathy and ventricular septal defect), but no neurological malformation.

Residue 117 lies in a region of HRAS where no other mutation associated with CS has been found found. The mutation changes a lysine into an arginine. The de novo status of the mutation was demonstrated by its absence in

Table 2 Mutation spectrum and malignancy frequency in CS

	Aoki *et al*[21]		Gripp *et al*[22]		This series		
Mutation	Patient no.	Tumour	Patient no.	Tumour	Patient no.	Tumour	Frequency
G12S	7	1 rhabdo	30	2 rhabdo, 1 benign bladder	29 *	1 rhabdo*	4/65 (7%)
G12A	2	1 ganglion.	2	1 bladder carcinoma	3	2 rhabdo	4/7 (57%)
G13D	2	–	–	–	–	–	–
G12V	1	–	–	–	–	–	–
G12C	–	–	–	–	2	1 rhabdo	50%
G12E	–	–	–	–	1	–	–
K117R	–	–	–	–	1	–	–
G13C	–	–	1	–	–	–	–
Totals	12	2	33	3	36*	4*	9/81 (11%)

Rhabdo, rhabdomyosarcoma; *Patient 1 in this report was excluded from totals because first reported by Gripp *et al.*[21]

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both parents of this child. The lysine at position 117 is highly conserved through evolution. Interestingly, mutations at codons 116 and 119 were found in cancer.[20] This variant was not found in 100 independent control chromosomes tested. Altogether, these elements indicate that K117R is highly likely to be a mutation and not a polymorphism, although it does not change the amino acid class (both lysine and arginine are positively charged).

DISCUSSION

These results confirm that CS is caused, in most cases, by heterozygous missense mutations in the proto-oncogene *HRAS*, and that mutations of this type do not cause either CFC syndrome or *PTPN11* mutation negative NS. Together with the previously published series,[20 21] mutations in *HRAS* have now been found in 82 of 96 (85%) patients with a clinical diagnosis of CS. The mutation negative cases could be explained either by locus heterogeneity or by the difficulties of diagnosis of CS, given the number of overlapping phenotypes. The clinical diagnosis in the mutation negative cases is being reviewed.

For families, the most worrying aspect of the CS phenotype is the malignancy risk. Table II summarises this series and the published data on mutation type and tumour frequency in CS. Overall, the frequency of malignancy in the published mutation positive cases is 11%.

With the common G12S mutation, rhabomyosarcoma was observed in four cases (7%), with a benign bladder tumour in a single case. Of seven cases with a G12A mutation, one developed a ganglioneuroblastoma, two a rhabdomyosarcoma, and one a bladder carcinoma, a tumour rate of 57%. Another malignancy, a rhabdomyosarcoma, occurred in one of the two patients with a G12C mutation in our series. In the other patient with a G12C mutation, the phenotype at 6 years of age is unremarkable.

These data suggest a potential correlation between mutation type and malignancy in CS, with the malignancy risk for the common mutation lower than previously estimated,[10] but the G12A mutation associated with a very high risk. Owing to the small numbers, these data must be regarded as preliminary and will require testing on larger numbers of patients.

In our two cases with the most severe phenotype, excluding malignancy (patients 10 and 11), one had the common mutation, G12S. In the other, a novel mutation, G12E, was found. Whether or not this is significant in terms of clinical effects will also require study of more patients. The patient with the novel K117R mutation presented autistic features and microretrognatism.

The mechanisms by which mutations in *HRAS* cause this complex phenotype are unknown. Different mutations in *HRAS* are known to result in different transforming potentials, with G12V, not observed in our series, being of greater potency than G12S.[20] Further functional studies will be required to elucidate the effects of the different mutations.

These results confirm that CS is caused, in most cases, by heterozygous missense mutations in the proto-oncogene *HRAS*These results confirm that mutation testing for *HRAS* is a reliable diagnostic test for CS, with mutations found in at least 85% of those with a clinical diagnosis of CS. Failure to thrive, macrocephaly, and the characteristic appearance of the face and hands are confirmed as the most reliable diagnostic signs. Genotype-phenotype analysis suggests that the highest malignancy risk exists for those with a G12A mutation, with the malignancy risk for other more common mutations being less than previously thought. The existence of a broader genotype-phenotype correlation will require the analysis of more cases.

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