ONLINE MUTATION REPORT

De novo *HRAS* and *KRAS* mutations in two siblings with short stature and neuro-cardio-facio-cutaneous features

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Mutations in genes involved in Ras signalling cause Noonan syndrome and other disorders characterised by growth disturbances and variable neuro-cardio-facio-cutaneous features. We describe two sisters, 46 and 31 years old, who presented with dysmorphic features, hypotonia, feeding difficulties, retarded growth and psychomotor retardation early in life. The patients were initially diagnosed with Costello syndrome, and autosomal recessive inheritance was assumed. Remarkably, however, we identified a germline *HRAS* mutation (G12A) in one sister and a germline *KRAS* mutation (F156L) in her sibling. Both mutations had arisen de novo. The F156L mutant K-Ras protein accumulated in the active, guanosine triphosphate-bound conformation and affected downstream signalling. The patient harbouring this mutation was followed for three decades, and her cardiac hypertrophy gradually normalised. However, she developed severe epilepsy with hippocampal sclerosis and atrophy. The occurrence of distinct de novo mutations adds to variable expressivity and gonadal mosaicism as possible explanations of how an autosomal dominant disease may manifest as an apparently recessive condition.

Mutations in interacting components of cellular signalling cascades may lead to inherited syndromes that display significant phenotypic overlap. A particularly instructive example is that of the genes associated with the Ras/mitogen-activated protein kinase (MAPK) pathway. In these genes mainly activating mutations cause a group of clinically related disorders with reduced growth and neuro-cardio-facio-cutaneous anomalies. These genetic diseases include Noonan syndrome (NS),¹⁻⁵ LEOPARD syndrome,^{6,7} neurofibromatosis type I (NF1),⁸⁻¹⁰ Costello syndrome (CS)¹¹ and cardio-facio-cutaneous (CFC) syndrome.^{12,13}

The clinical diagnosis of a Ras-related syndrome is often difficult and uncertain, especially in the neonatal period and during infancy. In particular, there are many overlapping features between NS, CS and CFC syndrome. Therefore, in such patients the identification of a mutation in the MAPK pathway not only provides a molecular explanation for the disease, but may also serve to classify it correctly with regard to diagnosis, prognosis and possible complications.

We describe two sisters initially considered to have CS due to autosomal recessive inheritance. Intriguingly, *RAS* mutational analysis revealed different de novo mutations in these individuals, leading to a re-evaluation of the diagnosis of the younger sister. This finding allowed us to compare the clinical manifestations of two closely related disorders of the MAPK signalling cascade on the same genetic background and over a time scale of 3–4 decades. Moreover, our data underline the importance of establishing the causative genetic lesion in syndromes with short stature and neuro-cardio-facio-cutaneous

features, and raise questions regarding inherited factors in this family that might predispose to the occurrence of offspring with germline *RAS* gene mutations.

METHODS

The study procedures were performed according to the Declaration of Helsinki. Written informed consent was obtained from the patients' mother, as was permission to present facial pictures of them. A preliminary clinical description of the two patients has been presented in abstract form by Søvik.¹⁴

Molecular genetic studies

DNA samples were available for the five living family members and for the deceased father. A panel of seven highly polymorphic microsatellite markers from four chromosomes was used to check that the genotypes were fully consistent with the samples belonging to two parents and their four children. The coding exons and flanking introns of the *HRAS* and *KRAS* genes were amplified by PCR and screened by direct DNA sequencing in all six family members. We found only gene variants listed in the NCBI single nucleotide polymorphism (SNP) database (at www.ncbi.nlm.nih.gov) except for the two mutations described below. DNA from patient 2 was also sequenced for mutations in the *PTPN11* gene, and gave normal results. For patient 1, nested allele-specific amplification of *HRAS* exon 2 was obtained using the primer pairs 5'-TGGGGCCTGGGG/5'-TATTCGTCCACAAA and 5'-TGGGGCC TGGGC/5'-TATTCGTCCACAAAG, which were specific for SNPs of the maternal and paternal alleles, respectively.¹⁵

Biochemical analysis of the F156L K-Ras mutant protein

The Ras-guanosine triphosphate (GTP) assay and immunoblotting have been described previously.² Briefly, we transiently transfected COS-7 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, California, USA) with pDEST12.2 vectors (Invitrogen) encoding normal, F156L or G12D K-Ras mutant proteins. The cell medium was Dulbecco's modified Eagle's medium-H21 containing 10% fetal bovine serum; 24 hours after transfection, the serum concentration was reduced to 0.1% by changing the medium for starvation conditions or left unchanged for basal conditions. The cells were lysed and Ras-GTP levels measured using Raf-1 RBD agarose (Upstate, Charlottesville, Virginia, USA). The antibodies used for immunoblotting were anti-Pan-Ras (Ab-3) (Calbiochem, San Diego, California, USA), anti-phospho-MEK1/2 (Ser217/221) and anti-MEK1/2 (both from Cell Signaling Technology, Danvers, Massachusetts, USA).

Abbreviations: CFC, cardio-facio-cutaneous; CS, Costello's syndrome; GTP, guanosine triphosphate; MAPK, mitogen-activated protein kinase; NF1, neurofibromatosis type 1; NS, Noonan's syndrome; SNP, single nucleotide polymorphism

RESULTS

Family history

Figure 1A shows the family pedigree. The parents were healthy and non-consanguineous with no known inherited diseases on either side. The father died at 68 years of age from multiple myeloma. One girl (II:1) was born prematurely after 29 weeks of gestation and died a few hours later. The mother had two spontaneous abortions, one in week 16 (II:2, male fetus) and another in week 10 (II:6, sex unknown). The affected sisters have two healthy brothers, born at term with birth weights of around 4.2 and 4.4 kg. The final heights of the father and mother were 186 cm and 176 cm, respectively.

Figure 1 *HRAS* and *KRAS* mutations in two sisters with Costello syndrome (CS) and cardio-facio-cutaneous (CFC) syndrome. (A) Family pedigree; diamonds indicate miscarriages, slashes indicate deceased family members. The current age and the age at death are given for living and deceased members, respectively. II:3 (patient 1) with CS is represented by a grey shaded circle, and below she is depicted at the ages of 11 and 30 years. II:7 (patient 2) with CFC syndrome is represented by a black circle, and the photographs show her at the ages of 7 and 30 years. (B) A heterozygous 35G→C mutation (arrow) in *HRAS* exon 2, changing glycine to alanine at codon 12 (G12A), was identified in patient 1. A heterozygous 466T→C mutation (arrow) in *KRAS* exon 4B, changing phenylalanine to leucine at codon 156 (F156L), was identified in patient 2. The normal DNA and protein sequences (with codons numbered) of the mutated regions are given above the panel. Neither G12A nor F156L were present in DNA from the parents, and they therefore represent de novo mutations. Allele-specific amplification of the *HRAS* exon 2 region in patient 1 showed that the mutation (asterisk) had occurred on the paternal chromosome. Parental informed consent was obtained for publication of this figure.

Table 1 Clinical features in sisters with Costello syndrome and cardio-facio-cutaneous syndrome due to activating *HRAS* and *KRAS* germline mutations

Clinical features	Patient 1	Patient 2
	G12A in <i>HRAS</i>	F156L in <i>KRAS</i>
Growth and development		
Birth weight >90 centile	+	+
Feeding problems in infancy	+	+
Relative macrocephaly	+	+
Short stature	+	+
Psychomotor retardation	+	+
Dysmorphic features		
Curly/sparse hair	+	–
Coarse facial features	+	+
Warts/papillomas (facial)	+	–
Hyperkeratosis	+	+
Acanthosis nigricans	+	–
Hypertelorism	+	+
Low-set ears	+	+
Redundant/loose skin	+	+
Low posterior/trident hairline	+	+
Dystrophic nails	+	+
Organ dysfunction		
Hoarse voice	+	–
Cardiomyopathy	–	+
Tonic-clonic seizures	–	+

+, present; –, not present.

Clinical findings in patient 1

This patient (II:3 in fig1A) was born in 1961, 6 weeks preterm after a normal pregnancy. Her birth weight was 3950 g (>97.5th centile), length 50 cm (>97.5th centile), and head circumference 35.5 cm (90–97.5th centile). In the first year of life, pronounced hypotonia and severe feeding difficulties with persistent vomiting caused recurrent hospitalisations and necessitated an orogastric tube. A cardiac murmur was detected at the age of 5 months. Increased skin pigmentation was also noted. By the age of 1 year, psychomotor retardation was obvious, and nystagmus and a hoarse cry were noted. Coarse facial features and loose skin suggested Hurler syndrome, which was excluded by biochemical analysis. At the age of 2 years, the child could stand with support and express a few words. The cardiac murmur persisted, but there was no evidence of cardiac enlargement or hypertrophy. High-grade myopia was detected at 5 years of age. The patient fractured her femoral neck after a fall at the age of 16 years. She had her first menstrual bleed when she was 19 years old. Four asymptomatic, low-grade papillary bladder carcinomas were removed at 44 years of age.

The patient has always had curly hair and pronounced skin changes resembling acanthosis nigricans. Her hair is now sparse and she has no body hair. She has numerous papillomas/warts on the face. Histological examination of one of these showed hyperplastic squamous epithelium with hyperkeratosis, but no malignancy. The skin changes were progressive with increased pigmentation and formation of papillomas, which have regularly been removed by cryotherapy. These and other clinical features summarised in table 1 were highly suggestive of CS.¹⁶ At 39 years of age, the patient's height was 153 cm (<2.5th centile), weight 58 kg (>97.5th centile related to height) and head circumference 56 cm (50–75th centile). She is considered moderately mentally disabled with a warm and social personality.

Clinical findings in patient 2

This patient (II:7 in fig 1A) was born at term in 1976 after a normal pregnancy. Her birth weight was 4800 g (>97.5th

Figure 2 Normalisation of the cardiac hypertrophy in patient 2. The thickness (broken line) of intraventricular septum (IVS) was measured by echocardiography in patient 2 at different ages. For comparison, normal values for different ages are shown as a solid line (mean (2 SD)). At the age of 15 years, the IVS thickness of patient 2 was within the normal range.

centile), length 55 cm (>97.5th centile), and head circumference 36 cm (75th centile). She also had hypotonia and feeding problems as a newborn, but not as severely as her older sister. At the age of 4 months, an enlarged liver and a cardiac murmur were detected. Cardiac catheterisation revealed obstructive cardiomyopathy. Echocardiography showed asymmetrical left ventricular hypertrophy with more pronounced involvement of the intraventricular septum than of the posterior wall. The patient subsequently developed marked mitral valve abnormalities with long, thickened leaflets and mitral prolapse, but no mitral regurgitation. A liver biopsy showed increased fat content, but was otherwise normal. At 4 years of age, the patient developed epileptic seizures, and was treated with anticonvulsant medication. At 9 years of age, a CT scan showed slight cerebral atrophy. At 13 years of age, an electroencephalogram revealed generalised slowing indicative of a generalised encephalopathy. A skin biopsy showed hyperkeratosis and acanthosis at the extensor surfaces of the arms. Her first menstrual bleed occurred when she was 16 years old. At 24 years of age, her height was 157 cm (2.5–10th centile),

weight 41 kg (10–25th centile related to height), and head circumference 56 cm (50–75th centile).

This patient is considered as mentally disabled as her sister, with the same warm and social personality. Intriguingly, over the years, her cardiac disorder has regressed considerably and the intraventricular septum hypertrophy has resolved (fig 2). Her main clinical problem is epilepsy, with 12–14 complex partial seizures per month, partly as clusters over a few days, and secondary generalised tonic-clonic seizures once a year. She had a convulsive status epilepticus at 26 years of age. Brain MRI showed gliosis and atrophy of the right hippocampus (fig 3). Overall, we consider the clinical features (table 1) most compatible with CFC syndrome.¹⁷

Molecular and biochemical studies

As stated, the clinical features of patient 1 were highly suggestive of CS. Based on a report demonstrating germline *HRAS* mutations in most patients with CS,¹¹ we examined DNA from patient 1. In *HRAS* exon 2 we found a heterozygous nucleotide substitution, 35G→C, which changes glycine to alanine at amino acid position 12 (G12A) (fig 1B). The mutation occurred on the paternal *HRAS* allele, and we verified that it was not present in either parent or in the brothers, thus it was a de novo mutation. The *HRAS* mutation identified in patient 1 has been reported in other patients with CS.^{11–20} It is also a rare somatic alteration in cancer,^{21–22} but has to our knowledge not been reported for bladder carcinomas. The G12A H-Ras substitution alters the phosphate-binding loop of the protein, transforms cultured Rat1 cells, and is predicted to decrease intrinsic Ras GTPase activity.^{23–24}

Surprisingly, patient 2 did not have the same *HRAS* mutation as her sister. We instead detected a de novo, heterozygous 466T→C nucleotide substitution in exon 4B of the *KRAS* gene (fig 1B), leading to a change of phenylalanine with leucine at amino acid position 156 (F156L). In this case, we were unable to determine the germline origin (maternal or paternal) of the mutation as more than 10 kb of sequenced genomic DNA around exon 4B did not contain any informative polymorphisms.

A previous study of the F156L substitution in H-Ras revealed a rapid rate of guanine nucleotide dissociation and a modest increase in Ras-GTP levels in NIH 3T3 cells.²⁵ To assess the

Figure 3 Structural abnormalities of the brain in patient 2 revealed by MRI. Coronal T2-weighted images through the hippocampus in patients 1 and 2 at the ages of 29 and 45 years, respectively, are compared. For patient 2, a fluid-attenuated inversion recovery (FLAIR) image is also displayed. In patient 1, the brain appears normal, whereas in patient 2 hippocampal gliosis and atrophy is present on the right side (arrows). Patient 2 has had a convulsive status epilepticus as well as secondary generalised tonic-clonic seizures and complex partial seizures consisting of emesis, anxiety, oral and motor automatisms (hands) with impaired consciousness. The clinical semiology and the radiological findings concur with a mesial temporal or hippocampal focus for her epilepsy.

functional impact of this amino acid substitution in K-Ras, we expressed wild-type K-Ras, F156L K-Ras, or oncogenic G12D K-Ras protein in COS-7 monkey kidney cells. F156L K-Ras accumulated in the active, GTP-bound conformation in COS-7 cells that were cultured in serum and after 7 hours of serum deprivation (fig 4). In addition, F156L K-Ras expression resulted in elevated levels of phosphorylated MEK in these cells.

DISCUSSION

Two de novo mutations: connection or coincidence?

We describe the unexpected and challenging finding of distinct de novo *RAS* mutations in two growth-retarded sisters with overlapping but non-identical neuro-cardio-facio-cutaneous features. The similar clinical pictures of the patients initially suggested that they had inherited the same condition, with the phenotypic differences being due to variable expressivity. We assumed recessive inheritance because both parents and their respective families were unaffected and because chromosome analyses, including high-resolution comparative genomic hybridisation, were normal. Alternatively, one of the parents could have been a gonadal mosaic for a dominant condition.^{26, 27}

It was therefore extraordinary to discover different, de novo *RAS* mutations in the siblings, a finding that, together with two spontaneous abortions and the birth of a premature child, might suggest an unusual mutational predisposition in the kindred. Biological material from the unsuccessful pregnancies, which could have elucidated this hypothesis by further mutation analyses, was not available. Thorough questioning of the family did not reveal any predisposition to cancer or other diseases in the parents and their relatives. Furthermore,

the parents had no history of exposure to environmental risk factors such as chemicals or radiation.

The father was 21–39 years old at the seven conceptions, indicating that his age did not strongly influence the mutation probability.^{28–29} In sporadic cases of NS²⁹ and NF1,³⁰ and in other dominant diseases where de novo mutations are seen,²⁸ the genetic alterations preferentially occur on the paternal allele. This appears also to be the case for CS^{15–31} and was confirmed for the *HRAS* mutation of patient 1. However, we were unable to determine the parental origin of the *KRAS* mutation.

In summary, we could not establish an underlying risk factor in our pedigree. It is nevertheless conceivable that *RAS* mutations are relatively common in spermatogenesis but normally not transmitted due to cell-cycle control mechanisms. Disruption of a critical “checkpoint” protein that is activated in response to aberrant Ras signalling in developing male gametes might then explain the existence of affected siblings with independent de novo *RAS* mutations. If male germ cell progenitors that acquire a *RAS* mutation obtain a proliferative or survival advantage similar to that inferred by oncogenic Ras proteins, an increased incidence of such mutations might also be expected. Notably, a selective advantage of the mutated germ cells has been suggested for mutations in the *FGFR2* gene.^{32–33}

Clinical considerations

The two patients presented variations of the same features, but only the phenotype of patient 1 was fully consistent with CS. Distinguishing features are accelerated intrauterine growth followed by postnatal growth retardation, pronounced neonatal feeding difficulties, an appearance suggestive of a mucopolysaccharidosis, psychomotor retardation, coarse facial features, loose and hyperpigmented skin, and at a later stage the development of numerous facial papillomas and bladder cancer.^{16–18–20}

As germline *KRAS* mutations have been reported almost exclusively in individuals with NS or CFC syndrome,^{2–3–12–34} the molecular analysis of patient 2 required a revision of her initial clinical diagnosis. High birth weight, coarse facial features, skin changes and mental retardation are uncommon in NS, whereas feeding problems in infancy are often noted. For CFC syndrome, feeding problems are not a characteristic feature, but such cases have been described.³⁵ A general finding in CFC syndrome is sparse and curly hair, a feature not observed in patient 2. However, she did have other signs of ectodermal dysplasia (brittle nails, hyperkeratosis on the extensor surfaces of the extremities). Both her epilepsy and moderate degree of psychomotor retardation are more compatible with CFC syndrome than NS. Hypertrophic cardiomyopathy is commonly associated with NS, but is also often noted in CFC patients.¹⁷ Taken together, a CFC syndrome diagnosis seems most warranted for patient 2.

The *KRAS* mutation found in patient 2 was recently observed in a single patient (male, aged 14 months) of the large cohort studied by Zenker *et al.*³⁴ This case, too, had hypertrophic cardiomyopathy and a structural anomaly of the brain (Dandy–Walker malformation), but not increased birth weight. In a second patient (male, aged 8.5 years) from the same cohort, a different amino acid substitution of the same K-Ras position was found (F156I). This case did show increased birth weight, but neither heart defects nor brain abnormalities were reported.

Patient 2 has been followed up for 31 years. By monitoring her cardiac function, we were able to show that the intraventricular septum hypertrophy gradually resolved as she grew older (fig 2). She currently has no obvious cardiac symptoms. To our knowledge, complete normalisation of

Figure 4 Biochemical analysis of the F156L *KRAS* mutation of patient 2. COS-7 cells were transiently transfected with plasmids encoding normal K-Ras protein (WT), F156L K-Ras protein, a known oncogenic K-Ras protein (G12D), or empty plasmid vector. The activation of the K-Ras proteins and of a downstream effector was assayed in cells growing under basal and starved conditions as described in Methods. The top panel of the blot shows the Ras-guanine triphosphate (GTP) levels. A lane was intentionally left blank between the F156L and G12D mutants to clearly separate F156L from G12D, which gives a strong signal because a high percentage of the protein is GTP-bound. Levels of Ras-GTP were low in cells transfected with empty vector or normal K-Ras under basal and starved conditions, whereas oncogenic G12D K-Ras and F156L K-Ras accumulated in the GTP-bound conformation. The second panel shows that total Ras protein levels were raised to a similar extent for each transfection. The third panel presents immunoblotting data using an antibody that is specific for the phosphorylated (active) form of MEK, a major downstream effector of Ras-GTP in the mitogen-activated protein kinase signalling cascade. The levels of phosphorylated MEK (pMEK) were markedly elevated in COS-7 cells expressing F156L or G12D K-Ras and correlated with the amount of Ras-GTP. The bottom panel shows that the endogenous levels of total MEK protein are similar in all assays.

cardiac hypertrophy associated with CFC syndrome or NS has not previously been reported.

In contrast to the disappearance of the cardiac symptoms, the neurological problems of patient 2 have gradually worsened. The severity of epilepsy has increased, and MRI provides a structural correlate for this development (fig 3). The brain of patient 1, however, appears structurally normal, and she has not had epileptic seizures. Dysfunction of the Ras/MAPK pathway is probably relevant in explaining both mental retardation and epilepsy in patients with neuro-cardio-facio-cutaneous features. All key elements of the Ras signalling cascade, which has a central role in regulating cell growth and differentiation, are present in the brain, and the MAPK pathway is also linked to neurological functions such as synaptic and behavioral plasticity.³⁶ In the hippocampus, for example, both long-term potentiation of synaptic transmission and protein kinase modulation of dendritic K⁺ channels involve ERK activation.^{37,38} The fact that epilepsy and hippocampal atrophy with sclerosis was associated with a *KRAS* and not with an *HRAS* mutation in our family is interesting, as there appears to be a striking difference in K-Ras and H-Ras dynamics in hippocampal neurons, mediated by their distinct membrane-anchoring motifs.³⁹

Functional studies of the F156L K-Ras mutation

The mutation of patient 2 was tested in a cellular model system (fig 4). K-Ras F156L accumulates in the active, GTP-bound conformation and induces elevated levels of phosphorylated MEK in COS-7 cells. The identical amino acid substitution in H-Ras causes a markedly increased rate of guanine nucleotide exchange.²⁵ Because the concentration of GTP far exceeds that of GDP in living cells, mutations that increase the rate of guanine nucleotide exchange are predicted to increase Ras-GTP levels. The amino terminal end (amino acids 1–165) of H-Ras, K-Ras, and N-Ras comprise a highly conserved G domain that has a common structure and switch mechanism, and it is therefore likely that the biochemical properties of F156L K-Ras are similar to those of the H-Ras mutant protein. Interestingly, germline *SOS1* mutations that encode proteins with elevated guanine nucleotide exchange activity were recently found in about 10% of patients with NS.^{4,5} Direct analysis of intrinsic GTP hydrolysis rates, responsiveness to GTPase-activating proteins, and guanine nucleotide binding and dissociation by recombinant F156L K-Ras is required to fully characterise the biochemical consequences of this mutant protein.

Genotype–phenotype correlations

The discovery that neurofibromin-1 (NF1) is a GTPase-activating protein provided the first direct evidence implicating aberrant Ras signalling in a human developmental disorder.⁴⁰ Subsequently, Tartaglia and coworkers reported that point mutations in the *PTPN11* gene cause about half the cases of NS.^{1,41} The recent identification of mutations in *HRAS*, *KRAS*, *BRAF*, *MEK* and *SOS1* in patients with CS, NS and CFC syndrome^{2,4,5,11–13} then provided a unifying mechanism for the similarities of syndromes with short stature and neuro-cardio-facio-cutaneous anomalies. However, CS and CFC syndrome are generally associated with mental retardation, whereas patients with NS or NF1 tend to be intellectually normal, although they often have learning difficulties. These data suggest that the clinical phenotypes of individual patients will be influenced by the precise molecular lesion and how each perturbs the degree and duration of Ras signalling in various tissues.

Our data highlight the importance of molecular testing to reveal the underlying genetic mechanism in congenital disorders of the MAPK pathway. Such information can be helpful

KEY POINTS

- Mutations in genes of the Ras/MAPK signalling cascade cause syndromes characterised by short stature and neuro-cardio-facio-cutaneous anomalies.
- We report the molecular analysis and long-term clinical follow-up of two sisters, both initially diagnosed with CS.
- Surprisingly, the siblings had independent, de novo mutations in the *HRAS* and *KRAS* genes. The mutant K-Ras protein accumulated in the active, GTP-bound conformation and affected downstream signalling.
- The clinical diagnosis of the sister with *KRAS* mutation was changed to CFC syndrome. This patient had hypertrophic cardiomyopathy, which over the years has completely resolved. She experienced increasing problems with epilepsy, and a structural brain abnormality was noted.
- Distinct de novo mutations in siblings with phenotypic overlap is a possible, although extraordinary, explanation of how an autosomal dominant disease may masquerade as an apparently recessive condition.

in prognostic evaluation and reproductive decisions, and may guide future medical management and educational placement of the patients. Moreover, risk for the development of malignancies can be more adequately evaluated (higher for *PTNP11*, *NF1* and, in particular, *HRAS* mutations; probably lower for the others). Intriguingly, cancer-associated *HRAS* mutations that impair intrinsic Ras GTPase activity and confer resistance to GTPase-activating proteins are tolerated in the embryo, as they are common in CS, whereas the germline *KRAS* mutations detected to date are generally not found in cancer. Molecular and clinical dissection of syndromes of the NS spectrum may therefore add to our understanding of how the components of Ras signalling act as oncoproteins in tumorigenesis. Finally, the surprising molecular findings in our family demonstrate that distinct de novo mutations in siblings with phenotypic overlap is a possible explanation of how a dominant disease may manifest as an apparently recessive condition.

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