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Atypical Friedreich Ataxia Caused by Compound Heterozygosity for a Novel Missense Mutation and the GAA Triplet–Repeat Expansion

To the Editor:

Friedreich ataxia (FRDA) is an autosomal recessive, progressive neurodegenerative disease with an age at onset within the 2d decade of life. It has an incidence of 1–2/50,000 and is the most common inherited ataxia (McKusick 1994). Clinical features include progressive gait and limb ataxia, areflexia, loss of position sense, dysarthria, and positive Babinski sign (Harding 1981). The majority of patients have evidence of hypertrophic cardiomyopathy, which is also the leading cause of death. Nearly a third of patients have either diabetes or abnormal glucose tolerance (Finocchiaro et al. 1988). The disease is incurable, and, after a decade of considerable morbidity, death usually ensues in the 4th decade. The FRDA phenotype can display a broad range of severity and, thus, confound diagnosis. Some patients are known to have an unusually delayed age at onset (De Michele et al. 1994), and others are known to have intact deep-tendon reflexes (Palau et al. 1995). FRDA in Acadian patients progresses unusually slowly (Barbeau et al. 1984).

More than 95% of FRDA patients are known to be homozygous for a GAA triplet–repeat expansion in the first intron of the *X25* gene (Campuzano et al. 1996). Although a strong negative correlation was detected between the age at onset and the size of the GAA triplet–repeat expansion (Dürr et al. 1996; Filla et al. 1996), no such molecular explanation was found for either the retention of deep-tendon reflexes or the gradual progression of FRDA in Acadians. Rare patients who are heterozygous for the GAA triplet–repeat expansion are expected to have other mutations within the *X25* gene. Some compound heterozygotes have been identified elsewhere (Campuzano et al. 1996; Filla et al. 1996), thus confirming the etiologic role of *X25*. The few point mutations reported to date have included mainly premature translation-terminating mutations. A single missense mutation, I154F, was reported in 3/43 apparently unrelated Italian FRDA families (Campuzano et al. 1996; Filla et al. 1996). The phenotype observed in these compound heterozygotes is essentially indistinguishable from that in individuals homozygous for the GAA triplet–repeat expansion.

We have investigated a Caucasian family with three FRDA patients showing atypically mild disease with gradual progression of symptoms. Here we report the identification of the second missense mutation (G130V), the first associated with a variant FRDA phenotype, within the *X25* gene. We show that affected individuals in this family are compound heterozygotes for the missense mutation and the GAA triplet–repeat expansion. We show that, although the GAA expansion results in a reduction in *X25* mRNA, the missense mutation is not associated with such a reduction. This indicates that most of the expressed *X25* mRNA in compound heterozygotes is mutant and is therefore likely to be the key determinant of the observed atypically mild FRDA phenotype, possibly arising from a subtle loss of function. This novel mutation and the only other reported missense mutation (I154F) map within the highly conserved sequence domain in the carboxy terminus of frataxin, the putative protein encoded by *X25* (Campuzano et al. 1996; Gibson et al. 1996), further emphasizing its functional significance and the direct role of frataxin in FRDA. Identification of *X25* mutations in this family broadens the clinical spectrum of FRDA and characterizes a potential molecular basis for the observed clinical variation.

The single nuclear family analyzed (fig. 1*A*) consisted of six offspring, three of whom, ages 42 (PAT75), 39 (PAT66 [proband]), and 35 (PAT78) years, were clinically affected. Onset of disease was in the early teens, starting with weakness in the lower limbs and followed by a gradual progression over the ensuing 20 years. The older two brothers are presently ambulatory, using either a walking stick or walker, and lead fully productive working lives. Physical examination of PAT75 and PAT66 revealed that their upper limbs were affected to a lesser extent and lacked several key signs. They have sensory loss over the distal lower limbs, mild to moderate motor weakness, impaired position and vibration sense, hypo- or areflexia, and bilateral Babinski sign (seen only in PAT66). There was no atrophy, and muscle tone was normal. Notably, there was no dysarthria, and coordination was either very mildly affected or normal. Nerve conduction studies revealed slowing of motor-conduction velocities and absent sensory-evoked re-

sponses. Magnetic-resonance imaging revealed cervical spinal cord atrophy. No cardiac abnormalities were detected by electrocardiography and/or echocardiography. Blood glucose levels were borderline elevated, and mild glucose intolerance was revealed in a 5-h glucose-tolerance test. Although PAT78 was not personally examined by us, examination of her clinical records revealed little intrafamilial phenotypic variation; however, she is slightly more physically incapacitated than her older two brothers.

Long-range PCR analysis of the GAA triplet–repeat expansion in *X25* intron 1 (Campuzano et al. 1996) revealed that the proband (PAT66) had one expanded allele, with an estimated 840 triplet repeats, and a normal unexpanded allele. Analysis of immediate family members revealed that all those clinically diagnosed with FRDA had a maternally derived expanded allele (estimated range 840–875 repeats) and a paternally derived unexpanded allele (fig. 1*A*). This indicated that the affected individuals may be compound heterozygotes, with one allele expanded and the other bearing a distinct mutation in another location of the *X25* gene. All six *X25* coding exons (exons 1–4, 5A and 5B) from the proband (PAT66) were amplified by PCR using genomic DNA as template; and these revealed no obvious alterations in size. Chemical mismatch–cleavage analysis of each of these exons revealed a single distinct cleavage product in exon 4 after modification of the heteroduplex by hydroxylamine (data not shown). A similar analysis was performed by use of exon 4 from all family members; and this revealed that the cleavage product was paternal in origin and was inherited by each of the six offspring (fig. 1*B*). Thus, it was apparent that only those offspring who inherited both the maternally derived expansion and the paternally derived exon 4 mutation had developed FRDA. The exon 4 PCR product from the proband was cloned into a plasmid vector (pMOS*Blue;* Amersham), and five independent clones were sequenced. Three of the five clones revealed a G→T transversion at position 389 (data not shown), which is predicted to result in a substitution of a valine for a glycine at amino acid position 130 (G130V).

Since all six offspring had inherited the missense mutation from the father, it was imperative to determine that the father, who was clinically unaffected, was not homozygous for the same. Since the single base substitution did not affect a naturally occurring restriction-enzyme site, we designed an allele-specific PCR assay that would engineer a new *Afl*III restriction site after amplification of the mutant allele. This analysis revealed that the father was indeed heterozygous and had passed his mutant *X25* allele to each of his six offspring (fig. 1*C*). The same assay was also used to screen 51 unrelated control individuals, none of whom were found to have this mutation. This mutation was also absent in a further 16 FRDA patients homozygous for the GAA triplet–repeat expansion.

We performed an RNase protection assay to assess the relative effects of the GAA triplet–repeat expansion and the G130V mutation on steady-state *X25* mRNA levels (fig. 2). Transformed lymphoblastoid cell lines were established and used as a source of RNA. The father and all three offspring who are only heterozygous for the missense mutation had normal mRNA levels, indicating that the missense mutation did not affect steady-state levels of *X25* mRNA. The mother (PAT73), who is only heterozygous for the GAA expansion, showed less than half the normal mRNA level. Furthermore, the three affected offspring who are compound heterozygotes showed approximately half the normal level of mRNA, similar to what we have found elsewhere in clinically normal heterozygous carriers of the GAA expansion (Cossèe et al. 1997). This indicates that, in the compound heterozygotes, a major proportion of *X25* transcript available for translation is likely to contain the point mutation.

The G130V mutation maps within an evolutionarily conserved sequence tract encoded partially by *X25* exons 3, 4, and 5A, and the glycine at position 130 is invariably conserved (fig. 3). In addition to the previously published sequences from *Caenorhabditis elegans* and *Saccharomyces cerevisiae* (Campuzano et al. 1996), we recently have identified the homologous frataxin sequence from *Mus musculus,* in which this glycine residue also is conserved. A recent phylogenetic analysis (Gibson et al. 1996) revealed that the carboxy terminus of frataxin is highly homologous to the products of the prokaryotic *CyaY* genes in *Escherichia coli, Erwinia chrysanthemi,* and *Yersinia pestis,* in which this glycine residue is also conserved (fig. 3). The *CyaY* genes have been identified only in the γ subdivision of purple bacteria, the closest living relatives of mitochondria, and not in either gram-positive bacteria (*Mycoplasma genitalium* and *Bacillus subtilis*) or the archaeon *Methanococcus jannaschii.* Gibson et al. (1996) have considered this to be indicative that *X25* may have evolved, after mitochondrial endosymbiosis, from a *CyaY* gene present in a mitochondrial ancestor and that frataxin may function as a nuclear-encoded mitochondrial protein. It is interesting to note that the only other reported missense mutation (I154F), which also maps within the same conserved domain, produces a typical FRDA phenotype in compound heterozygotes that is indistinguishable from those homozygous for the GAA expansion. This indicates that the G130V mutation may have resulted in a more subtle loss of function than was produced by I154F. We believe that the G130V mutation is pathogenic for the following reasons: (*a*) only compound heterozygotes are clinically affected; (*b*) there were no other mutations found in a screening of the entire coding se-

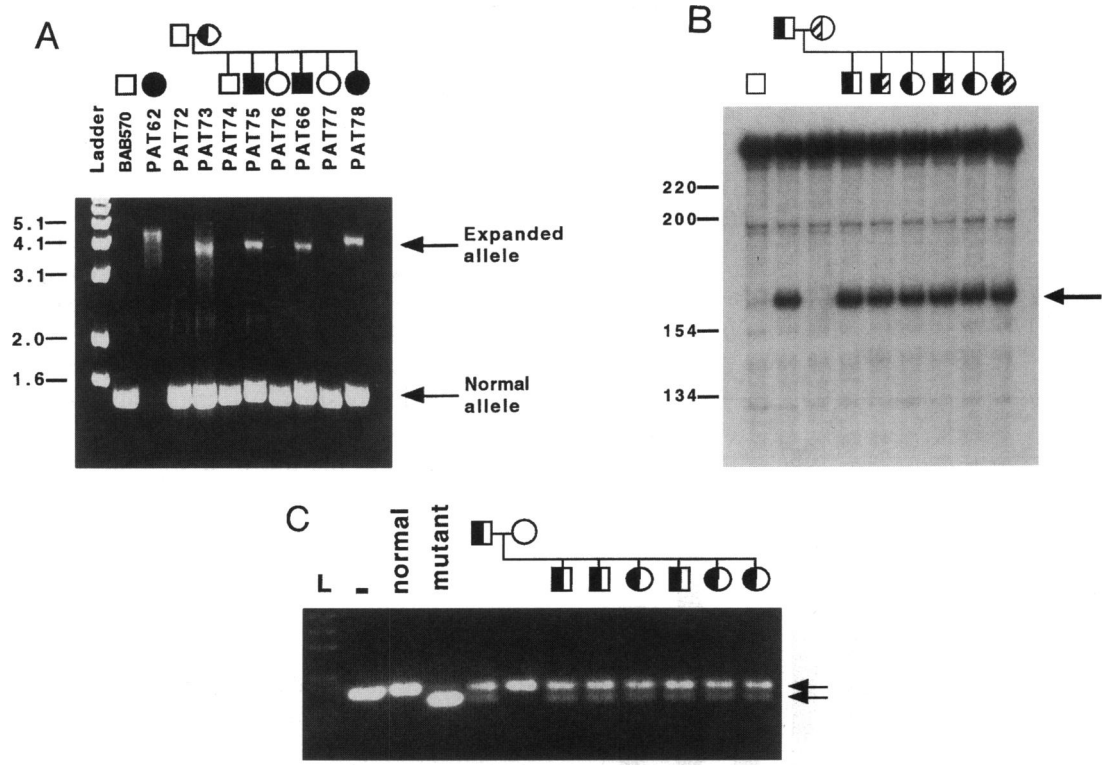

Figure 1 *A*, Long-range PCR analysis showing that clinically affected individuals, indicated by fully blackened symbols, are heterozygous for a maternally derived GAA triplet–repeat expansion in intron 1. PCR primers and conditions are exactly as described previously (Campuzano et al. 1996). There is slight variation in the size of the expanded alleles, indicative of some instability in germline transmission. The youngest affected offspring (PAT78) bears a slightly larger expansion (n = 875) than her two affected sibs (PAT66 and PAT75; n = 840). Known normal (BAB570) and homozygously expanded (PAT62) controls were also used in the same assay, which showed expected results. The first lane contains a size marker with band sizes indicated in Kb. *B*, Chemical mismatch cleavage analysis of exon 4 demonstrating inheritance of a paternally derived cleavage product in each of the six offspring. Products obtained after treatment of heteroduplexes with hydroxylamine (2.3 M for 2 h at 37°C) followed by cleavage of modified cytosines with piperidine (10% for 30 min at 90°C) are shown electrophoresed on a 8% denaturing polyacrylamide gel. Half-blackened boxes indicate carriers of the point mutation responsible for the cleavage product, and half-hatched boxes indicate carriers of the GAA triplet–repeat expansion. A single normal control and the mother who is a carrier of the GAA expansion, shown in lanes 1 and 3, respectively, do not have the same cleavage product. Sizes (in bp) are indicated, based on a coelectrophoresed radiolabeled size marker. *C*, Allele-specific PCR assay for mutant allele recognition following *Afl*III restriction digestion. The upstream primer (4mutF: 5′-TTT TTT CCA CCT AAT CCC CTA ACG TG-3′) was designed to terminate just short of the G→T transversion, and nucleotide substitutions (*underlined*) were introduced so as to generate an *Afl*III recognition sequence following PCR, in the event of the G→T substitution. PCR with the downstream primer for amplification of exon 4 (Campuzano et al. 1996), followed by *Afl*III restriction digestion, demonstrated that the father and each of his six offspring are heterozygous for the G130V missense mutation. The same assay was also performed on PCR products generated using the cloned "normal" and "mutant" sequences. The mutant sequence shows the presence of the mutation, which was also used as an indicator of the completeness of restriction digestion. "L" indicates the lane with a DNA ladder, and "-" indicates the lane containing undigested PCR product.

quence and splice junctions by use of chemical mismatch–cleavage analysis, a very sensitive mutation screening strategy in our hands (Bidichandani et al. 1995); (*c*) Gly-130 is highly conserved in evolution; and (*d*) this mutation was absent in a screening of >100 control chromosomes.

At least two studies have shown that the size of the GAA expansion in the smaller of the two expanded alleles is an important indicator of the phenotypic severity (Dürr et al. 1996; Filla et al. 1996). A strong negative correlation accounting for ~50% of the variability in age at onset was observed. There was also a significant positive correlation between the size of the smaller expansion and both the rate of disease progression and the presence of cardiomyopathy, skeletal complications, lower-limb muscle weakness and wasting, upper-limb areflexia, and extensor-plantar response (Dürr et al. 1996). The reason for the correlation with the smaller expansion is thought to be the proportional suppression of transcription, which is caused by the GAA expansion, in which the larger allele produces an almost complete blockade and the smaller allele continues to allow limited expression. However, there is considerable interindividual phenotypic variability seen in patients with sim-

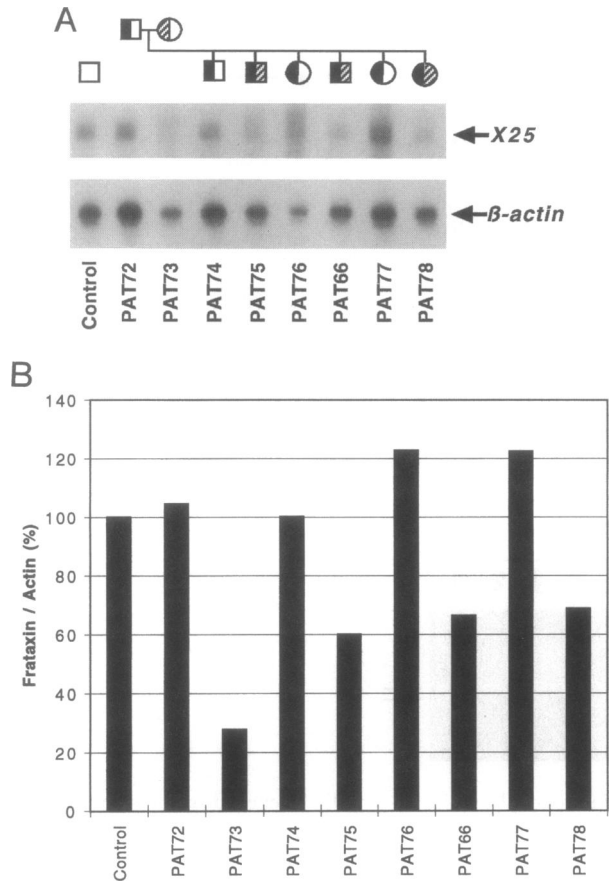

Figure 2 *A*, RNase protection assay demonstrating reduced steady-state *X25* mRNA levels in carriers of the GAA expansion (half-hatched symbols), and normal levels in carriers of the G130V mutation (half-blackened symbols). The unblackened square represents a sample from a normal male control. The riboprobe used for detection of the *X25* transcript spanned the spliced junctions of exons 1 and 2 (cDNA positions +58 to +252). Levels of β-*actin* transcript were simultaneously estimated in the same RNase protection reaction using a riboprobe of low specific activity. RNase protection assays were performed with 20 μg of total lymphoblast RNA using the RPAII kit (Ambion) essentially as recommended. *B*, Bar graph showing results of a densitometric analysis of relative band intensities from the RNase protection assay shown in *A*. Heterozygous carriers of the missense mutation (PAT72, PAT74, PAT76, and PAT78) show normal levels of *X25* mRNA. The mother (PAT73) who is heterozygous for the GAA expansion has less than half the normal level of *X25* mRNA. Compound heterozygotes for the G130V missense mutation and the GAA expansion (PAT75, PAT66, and PAT78) show transcript levels that are approximately half of normal. Densitometry was performed using the Collage™ image analysis software package (Fotodyne). Ratios of the *X25* and β-*actin* signals were calculated as a percentage of the normal control (considered as 100%).

ilar-sized repeats, with unknown factors estimated to account for ≤30% of the variance (Dürr et al. 1996). A likely explanation for this is the presence of somatic heterogeneity in the size of the GAA expansion, directly demonstrated in at least one FRDA patient (S. I. Bidichandani and P. I. Patel, unpublished data) and also seen in the other triplet-repeat diseases (Bidichandani and Patel 1996).

We have shown that the missense mutation per se does not affect *X25* mRNA levels and that compound heterozygotes have approximately half the normal level. Given that the levels of *X25* mRNA in cells from compound heterozygotes are comparable to those from otherwise asymptomatic heterozygous carriers of the expansion, we believe that the missense mutation in the residual transcript is the underlying cause of the atypical phenotype. Although the age at onset in the individuals studied was in the early teens, disease progression has been remarkably slow. Affected individuals continue to be ambulatory into their 4th and even 5th decade of life, albeit with the aid of a walking stick or walker. The mildness of the ataxia, the absence of dysarthria, and the absence of cardiac involvement are remarkable. Furthermore, the oldest affected sib has no extensor-plantar responses, and his knee jerks are still detectable. The abnormal glucose tolerance in compound heterozygotes is consistent with the results reported by Dürr et al. (1996), who found no significant correlation between the size of the GAA repeat and the presence of diabetes or abnormal glucose tolerance. The reason for the slightly more severe phenotype in the younger sister (PAT78) is not known. A slightly larger expanded allele in comparison with those of her affected brothers (fig. 1) is a possible although less likely explanation. However, the role of somatic mosaicism in expansion size, other environmental factors, or as yet unknown factors cannot be disregarded.

The identification of *X25* mutations in affected individuals in this family further broadens the clinical spectrum of FRDA. Dürr et al. (1996) have shown that 46% of their patients who did not meet all the diagnostic criteria of FRDA (Harding 1981) were homozygous for the expansion, indicating that the clinical spectrum is broader than previously had been thought. Here we have shown that an atypical FRDA phenotype associated with a remarkably slow rate of disease progression in a Caucasian family can be caused by compound heterozygosity for a missense mutation and the GAA expansion. Although it is now apparent that the wide variation in clinical phenotype seen in FRDA is a function of the large number of FRDA alleles with varying degrees of GAA expansion, our report characterizes yet another potential determinant of phenotypic variance. Characterization of the molecular basis of FRDA in this family has also finally put to rest differences in opinion regarding the clinical diagnosis of the disease phenotype in this family.

In a recent report, Carvajal et al. (1996) cloned rare transcripts after nested PCR amplifications, which, on sequencing, revealed splicing of exons of the *X25* gene to those of the neighboring *STM7* gene. Since the *STM7*

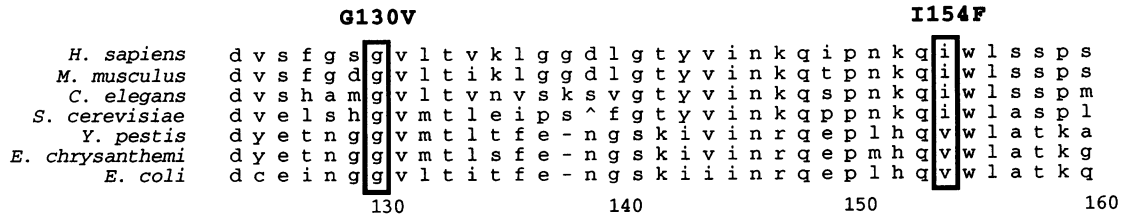

Figure 3 Sequence alignment demonstrating that the glycine (Gly) at position 130 in frataxin is highly conserved in evolution (highlighted by the box). The position of the previously identified missense mutation I154F is also depicted. This result was partially derived following a BLAST search and partially by modifying the data from Gibson et al. (1996). The source of the sequences are as follows: *Homo sapiens* frataxin (GenBank U43753), *Mus musculus* frataxin (WashU-HHMI mouse EST AA073270), *Caenorhabditis elegans* (GenBank U29537), *Saccharomyces cerevisiae* (GenBank T38910), *Yersinia pestis* CyaY (GenBank P46356), *Erwinia chrysanthemi* CyaY (GenBank P40128), and *Escherichia coli* CyaY (GenBank P27838). The amino acid positions are indicated for the human frataxin sequence. A single frameshift error in the *S. cerevisiae* sequence is indicated by a caret (^).

coding sequence (without any contribution from *X25*) encodes a phosphatidylinositol-4-phosphate 5-kinase, they concluded that FRDA was caused by a defect in this enzyme. Our group and several others have expressed the view that the significance of these rare transcripts (all of which exclude *X25* exon 1) is questionable and that, at least at present, there is insufficient evidence to arrive at their conclusion (Cossèe et al. 1997). Interestingly, there was no exon 2–only protected fragment detected in our RNase protection assay, which would have been predicted from the alternate splicing, between exon 2 and exons of the *STM7* gene, reported by Carvajal et al. (1996). Since those transcripts were detected after nested PCR amplifications, it is likely that they are much less abundant than the *X25* exon 1–exon 2 spliced product detected here. Given that the G130V mutation is unlikely to affect the ability of the first 16 exons of the neighboring *STM7* gene to encode a functional phosphatidylinositol phosphate kinase (Carvajal et al. 1996), we believe that this mutation questions the role of *STM7* and, moreover, further confirms the role of frataxin in FRDA.

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Anticipation in Pediatric Malignancies

To the Editor:

In the recent article in the *Journal* entitled "Anticipation in Familial Leukemia," Horwitz et al. (1996) note that, in addition to decreasing age at presentation in parent-child pairs, anticipation is suggested in families with affected cousins without disease in intervening relatives. We have found this latter pattern of inheritance in a family (fig. 1) recently evaluated in our clinic with increased occurrence of neuroblastoma. Review of the literature demonstrates at least two additional such families. In the family shown in figure 1, two siblings and a second cousin have been diagnosed with neuroblastoma at <1 year of age. The father of the sibling has a history of Wilms tumor in childhood, but neither the grandparents nor great-grandparents of the affected children were known to be affected. The affected family is of German descent, and there is no history of consanguinity. A recent article (Maris et al. 1996) details a similar family with two siblings and a second cousin affected with neuroblastoma. The sexes of the affected children are different from those in the family reported here, and therefore it is not the same family. They also report one additional family with affected half siblings and an unaffected mother. The genetics of neuroblastoma was previously investigated (Knudson and Strong 1972) in a review of 529 cases of neuroblastoma. Analysis of the age at onset of neuroblastoma in familial versus sporadic cases was consistent with a two-hit mechanism and would argue against autosomal recessive inheritance. In their series of patients, Knudson and Strong found 13 cases of multiple occurrences of neuroblastoma within a family and noted one case with two second cousins

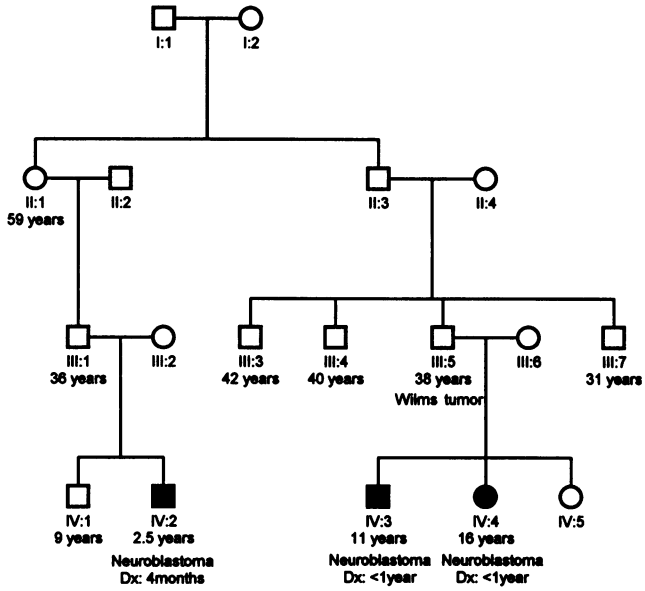

Figure 1 Pedigree from a family with three cases of neuroblastoma presenting at <1 year of age. All known cases of malignancy of any type are shown.

affected. The remainder of the 12 families have multiple sibs affected, including one half-sib pair. Taken together, these reports represent five families in which unaffected adults may have transmitted a predisposition to their offspring. Although in the case of the families with affected half-sibs this may be the result of germline mosaicism of the transmitting parent, this cannot be the explanation in the three families with affected second cousins. It is difficult to rule out a chance association of three cases of neuroblastoma in these families, although neuroblastoma has a prevalence of only 1/8,000 live births (Brodeur and Castleberry 1993). Also arguing against chance occurrence is the finding in two families with affected half-sibs of multiple tumors. The occurrence of multiple tumors is associated with inherited malignancy (Knudson and Strong 1972). These families provide evidence that neuroblastoma may represent another syndrome that has anticipation as a feature of its phenotypic expression.

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