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Mutations of the *KIT* (Mast/Stem Cell Growth Factor Receptor) Proto-Oncogene Account for a Continuous Range of Phenotypes in Human Piebaldism

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Summary

Piebaldism is a rare autosomal dominant disorder of pigmentation, characterized by congenital patches of white skin and hair from which melanocytes are absent. We have previously shown that piebaldism can result from missense and frameshift mutations of the *KIT* proto-oncogene, which encodes the cellular receptor tyrosine kinase for the mast/stem cell growth factor. Here, we report two novel *KIT* mutations associated with human piebaldism. A proximal frameshift is associated with a mild piebald phenotype, and a splice-junction mutation is associated with a highly variable piebald phenotype. We discuss the apparent relationship between the predicted impact of specific *KIT* mutations on total *KIT*-dependent signal transduction and the severity of the resultant piebald phenotypes.

Introduction

Piebaldism is an autosomal dominant genetic disorder of pigmentation, characterized by patches of white skin and overlying hair (Keeler 1934; Froggat 1951; Cooke 1952). Melanocytes are absent from the regions of hypopigmented skin, apparently resulting from a defect of early melanoblast proliferation or migration from the neural crest during embryonic development (Breathnach et al. 1965; Jimbow et al. 1975). In contrast to vitiligo, with which it is often confused, piebaldism is both congenital and nonprogressive. Piebaldism is also distinguished from Waardenburg syndrome, which also includes abnormalities of the bony facies and deafness. Because of its distinctive phenotype, piebaldism has been recognized since at least ancient Greek times (Fowler 1905), and it was one of the first genetic disorders for which a pedigree was presented (Morgan 1786).

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The human piebaldism locus was provisionally mapped to chromosome segment 4q12 on the basis of patients with interstitial deletions of this region (Funderburk and Crandall 1974; Lacassie et al. 1977; Hoo et al. 1986; Yamamoto et al. 1989). Recently we demonstrated that human piebaldism can result from both missense and frameshift mutations of the *KIT* (mast/stem cell growth factor receptor) proto-oncogene located in this chromosomal region (Giebel and Spritz 1991; Spritz et al. 1992).

Here we describe two novel *KIT* gene mutations associated with piebaldism. A frameshift at codon 85 is associated with a very mild piebald phenotype in an American Caucasian family. A mutation at the 5′ splice site of the 12th intervening sequence is associated with an extremely variable piebald phenotype in a South African black family. The codon 85 frameshift constitutes a pure loss of function mutation, demonstrating that human piebaldism can result from simple haplo-insufficiency for KIT-dependent signal transduction. The IVS12 splice-junction mutation likely results in a combination of loss of function and also dominant-negative inhibition of KIT-dependent signal transduction. In general, the severity of the piebald phenotypes in different families correlates well with

the predicted decrease of total KIT-dependent signal transduction resulting from the associated *KIT* gene mutations.

Subjects and Methods

Description of the Probands

Proband 1, individual III-2 of family 1 (fig. 1, *top*), was an adult Caucasian male with mild piebaldism, including a small, somewhat indistinct white forelock, a small leukodermal patch on the lower central abdomen, and indistinct, mottled leukoderma of the anterior left leg. There was no dysmorphia or heterochromia irides, and hearing was apparently normal. Numerous family members were also affected with a consistently very mild piebald phenotype characterized by a small patch of central abdominal leukoderma and, in only some cases, small white forelocks or occasional white frontal hairs and limited mottled leukoderma of one leg. There was no history of dysmorphia, heterochromia irides, deafness, constipation, anemia, or infertility in any of the affected individuals.

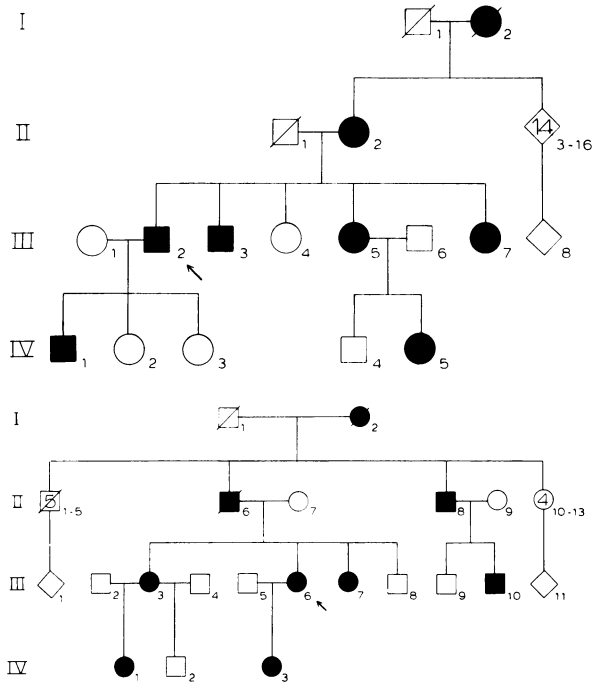

Figure 1 Pedigrees of piebaldism. *Top*, Family 1. The arrow indicates the proband. Blackened symbols denote individuals with piebaldism, and unblackened symbols denote normal individuals. Individuals denoted by the diamonds were not examined, and no phenotypic information was available. *Bottom*, Family 2. The symbols are as in the top panel.

Proband 2, individual III-6 of family 2 (fig. 1, *bottom*), was an adult black female with quite severe piebaldism, including white forelock and extensive striking leukoderma of the forehead, chest, abdomen, arms, and legs. There was no dysmorphia or heterochromia irides, and hearing was normal. A complete clinical description of this family, descended from the Xhosa tribal group of the Transkei region in South Africa, has recently been published elsewhere (Winship et al. 1991). The piebald phenotypes of seven affected family members in three generations vary considerably, ranging from extremely severe (individual III-6) to very mild (individual IV-3).

Southern Blot Hybridization Analysis

DNA was isolated from peripheral blood leukocytes and was analyzed by blot hybridization (Southern 1975; Vandenplas et al. 1984). For family 2 only, 7 mg from each individual was digested with *Hin*dIII or *Sac*I, and filters were hybridized to [^{32}P] radiolabeled *KIT* probe pv-kit, obtained from the American Type Culture Collection. After hybridization for 48 h at 65°C, filters were washed twice in 3 × SSC, 0.1% SDS and twice in 0.1 × SSC, 0.1% SDS, all at 65°C for 30 min each. Genetic linkage analysis was performed using the MLINK program, version 5.03 (Lathrop and Lalouel 1984); it was assumed that piebaldism exhibits complete penetrance and occurs with a frequency of 1/100,000.

PCR Amplification of the Human KIT Gene

The 21 exons of the human *KIT* gene and their flanking sequences (Giebel et al., in press) were amplified from genomic DNA of probands 1 and 2 (see fig. 1) and an unrelated, normally pigmented individual, by 30 cycles of the PCR (Saiki et al. 1988) exactly as described elsewhere (Spritz et al. 1992), except that annealing temperatures were modified slightly to maximize specificity for each exon. For exons 2, 3, 7, 12, 14–16, and 18–21, annealings were performed at 59°C; for exons 1, 4, 5, 6, 8–11, 13, and 17, annealings were at 53°C.

Single-Strand Conformation Polymorphism (SSCP)/ Heteroduplex and DNA Sequence Analyses

Three milliliters of the 100-ml PCR for each of the 21 *KIT* exons were denatured by adding an equal volume of 95% formamide, 10 mM NaOH, 20 mM EDTA, 0.025% bromphenol blue, 0.025% xylene cyanol FF and heating for 2.5 min at 95°C. The samples were immediately applied to 20 × 40 × 0.04-cm non-

denaturing 25% Hydrolink 5000 (AT Biochemical) gels and electrophoresed for 4–5 h at 30 W constant power, at room temperature and with a cooling plate and fan. After electrophoresis, the DNA bands were visualized by silver staining. Aberrant SSCP bands were excised from the gel, and the DNA was eluted and used as templates for PCR amplification as described above. The amplified fragments were cloned in M13mp18, and three independent clones of each were subjected to DNA sequence analyses (Sanger et al. 1977).

For proband 1 exons 2 and 6 and for proband 2 exons 8–16 and 18 were also amplified directly from genomic DNA as described above, and the products of two independent PCR reactions for each exon were pooled and cloned in M13mp18. At least six independent clones were subjected to DNA sequence analyses (Sanger et al. 1977).

Segregation Analyses

A 281-bp fragment containing *KIT* exon 12 plus portions of the adjacent intervening sequences was PCR-amplified from DNA of proband 2, her unaffected brother (individual III-8; see fig. 1, *bottom*), and her mildly affected daughter (individual IV-3). The amplification products were analyzed by SSCP/heteroduplex analysis as described above and were scored for the aberrant exon 12 heteroduplex. Similarly, a 330-bp fragment containing *KIT* exon 18 plus portions of the adjacent intervening sequences was PCR-amplified from DNAs of the same individuals and was cleaved with *Sac*I, electrophoresed through a 6% polyacrylamide gel, and scored for a *Sac*I RFLP at codon 862Leu.

Results

The Proband of Family 1 Is Heterozygous for a Frameshift at Codon 85

Combined SSCP/heteroduplex analysis of the 21 *KIT* exons amplified from DNAs of proband 1 and an unrelated normally pigmented individual demonstrated an aberrant pattern for exon 2 of the proband. As shown in figure 2*a*, exon 2 of proband 1 exhibited abnormalities of both the SSCP and heteroduplex band patterns. Analyses of the remainder of the *KIT* exons of the patient appeared normal (data not shown).

The aberrant SSCP bands of *KIT* exon 2 of the proband were eluted from the gel and used as templates

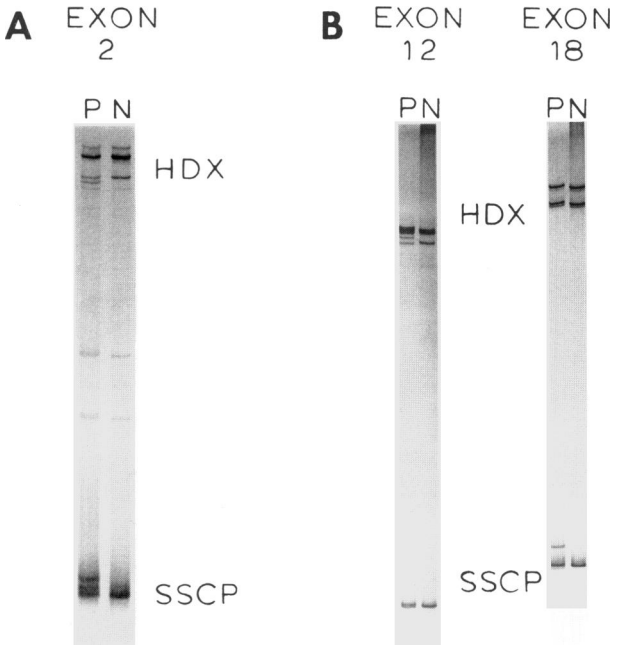

Figure 2 SSCP/heteroduplex analyses of *KIT* exons. The SSCP and heteroduplex (HDX) patterns are indicated. Lane P, Proband. Lane N, Unrelated normal individual. *a*, Analyses of exon 2 in proband of family 1. *b*, Analyses of exons 12 and 18 in proband of family 2.

for a second round of PCR amplification, and the resulting PCR products were cloned in M13mp18. DNA sequence analyses demonstrated that proband 1 is heterozygous for a single-base deletion (GAA→-AA), within codon 85 (fig. 3), near the beginning of the extracellular ligand-binding domain. This results in a frameshift distal to codon 85, with an 18-amino-acid nonsense peptide terminating at a novel in-frame TAA at codons 103–104. The codon 85 frameshift was confirmed by independent PCR amplification and sequencing of exon 2 from the proband's genomic DNA.

The Proband of Family 2 Is Heterozygous for a Mutation at the 5′ Splice Site of IVS12

Preliminary genetic linkage analysis of family 2 was carried out using two different human *KIT* RFLPs. A *Hin*dIII polymorphism (Berdahl et al. 1988) was not informative in this family. A *Sac*I RFLP (Curtis 1991) was informative, although at a low level (data not shown). Two-point linkage analysis, performed using the MLINK program (Lathrop and Lalouel 1984), yielded a maximal Z (Z_{max}) of 0.419 at $r = .05$, with

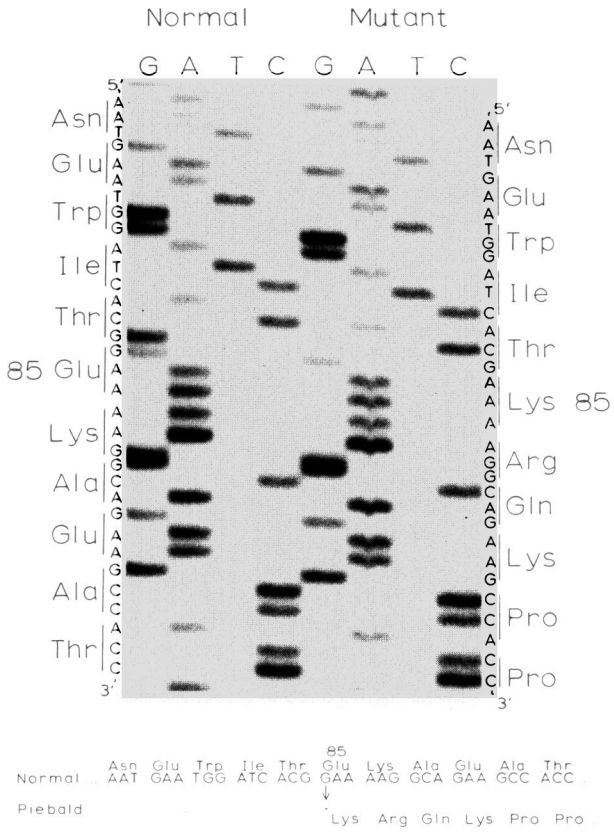

Figure 3 Sequences in region of codon 85 *KIT* gene frameshift from proband of family 1. Both the mutant and normal alleles are shown. The sequence indicated is that of the coding strand.

no apparent recombinants. This was consistent with, although it did not prove, genetic linkage of the *KIT* locus and the piebald phenotype in this family.

Combined SSCP/heteroduplex analysis of the 21 *KIT* exons amplified from DNAs of proband 2 and an unrelated normally pigmented individual demonstrated aberrant patterns for both exon 12 and exon 18 of the proband. As shown in figure 2*b,* exon 12 exhibited an extra heteroduplex band; an extra SSCP band was also apparent, although it resolved poorly from the normal band in this gel system. Exon 18 exhibited an abnormal SSCP pattern but showed no apparent abnormality of the heteroduplexes. Analyses of the remainder of the *KIT* exons of proband 2 appeared normal (data not shown).

The aberrant SSCP bands of *KIT* exons 12 and 18 of the proband were eluted from the gel, PCR amplified, and cloned as above. DNA sequence analyses demonstrated that the atypical exon 18 pattern results from a silent DNA sequence polymorphism within codon 862Leu, CTG versus CTC, for which proband 2 is heterozygous. This polymorphism, which we have observed among only 1 of 30 additional unrelated individuals we have studied to date (Giebel and Spritz 1991; Spritz et al. 1992; Giebel et al., in press; S. A. Holmes and R. A. Spritz, unpublished data), alters a *Sac*I cleavage site (GAGCTC, *Sac*I+; GAGCTG, *Sac*I−) and thus presumably corresponds to the *Sac*I RFLP discussed above (Curtis 1991). Proband 2 is also heterozygous for a pathologic *KIT* gene mutation, a guanine-to-adenine substitution at the first base of IVS12 (fig. 4). This mutation abolishes the 5′ splice site of IVS12 and would thus prevent normal splicing of the corresponding *KIT* mRNA precursor. This mutation was confirmed by independent PCR amplification and sequencing of exon 12 from the proband's genomic DNA. Exons 8–16 were also amplified and sequenced completely and were found to be normal.

As shown in figure 5*a,* the proband's mildly affected daughter (individual IV-3) also exhibits the aberrant exon 12 heteroduplex band pattern, but her unaffected brother (individual III-8) and two unrelated, normally pigmented individuals showed only the normal pattern. Thus, the IVS12 mutation appears to cosegregate with the piebald phenotype in this family. However, the codon 862 polymorphism does not. DNA fragments containing *KIT* exon 18 were PCR-amplified from proband 2 and individuals III-8 and IV-3 and were cleaved with *Sac*I. As shown in figure 5*b,* proband 2 and her unaffected brother (III-8) were both heterozygous for the codon 862Leu CTC *Sac*I polymorphism, whereas her affected daughter (IV-3) was homozygous for the absence of a *Sac*I site within exon 18, corresponding to codon 862Leu CTG. Thus, the codon 862Leu CTC polymorphism does not cosegregate with the piebald trait in this family and is *trans* to the IVS12 splice-junction mutation in proband 2.

Discussion

Piebaldism is a rare autosomal dominant disorder of melanocyte development characterized by congenital patches of white skin and hair. These white patches completely lack melanocytes (Breathnach et al. 1965; Jimbow et al. 1975), and the piebald phenotype is thought to result from defective proliferation or migration of melanoblasts from the neural crest to the epidermis during embryogenesis. We previously demonstrated that piebaldism can result from missense or frameshift mutations in the tyrosine kinase domain

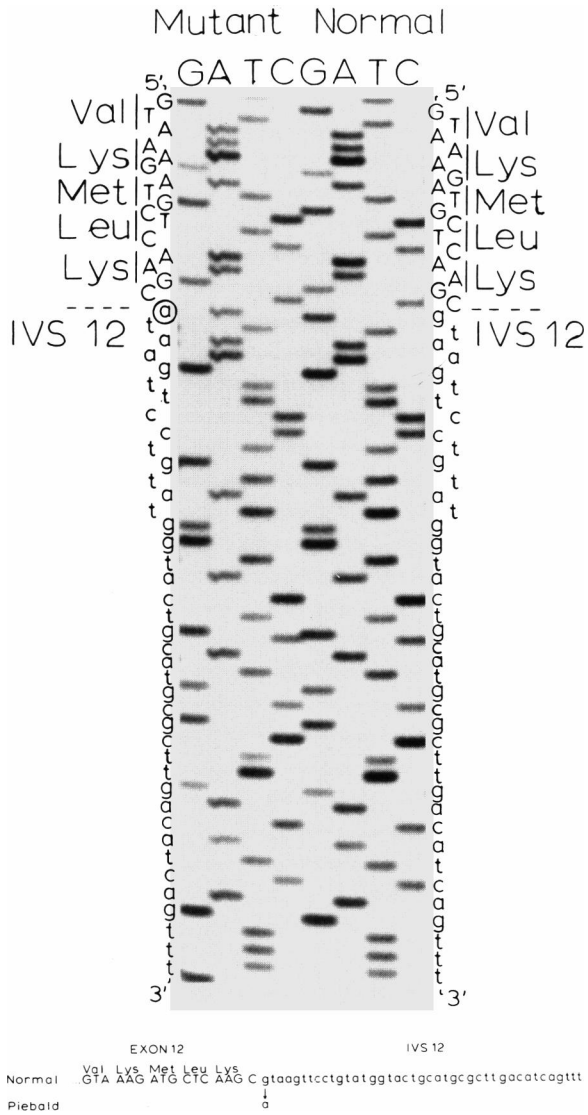

Figure 4 Sequences in region of *KIT* gene IVS12 splice-junction mutation from proband of family 2. Both the mutant and normal alleles are shown. The sequence indicated is that of the coding strand. The abnormal nucleotide at the first base of IVS12 is circled.

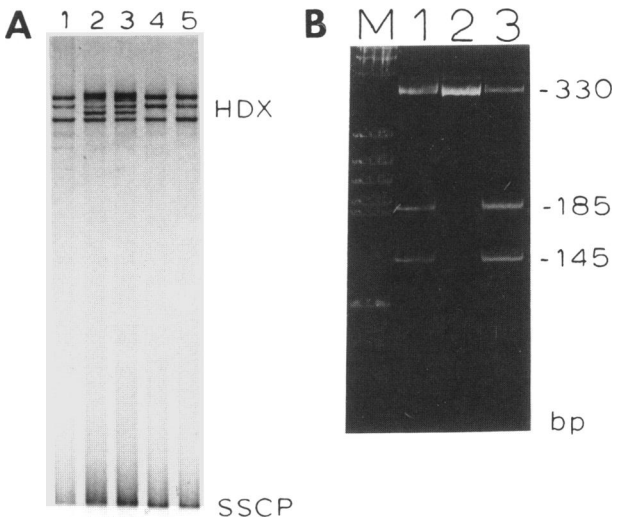

Figure 5 Segregation analysis of the *KIT* IVS12 splice-junction mutation and the codon 862Leu CTG/CTC polymorphism in family 2. *a*, SSCP/heteroduplex analysis of PCR-amplified exon 12. Lanes 1 and 5, Unrelated normally pigmented controls. Lane 2, Proband (individual III-6). Lane 3, Proband's mildly affected daughter (individual IV-3). Lane 4, Proband's unaffected brother (individual III-8). *b*, *Sac*I cleavage analysis of exon 18. Lanes 1, Proband (individual III-6). Lane 2, Proband's mildly affected daughter (individual IV-3). Lane 3, Proband's unaffected brother (individual III-8). Lane M, Molecular size standard (pBR322 digested with *Bst*NI + *Hae*III).

of the *KIT* proto-oncogene (Giebel and Spritz 1991; Spritz et al. 1992). The *KIT* gene encodes the type III receptor tyrosine kinase that functions as the transmembrane receptor for the mast/stem cell growth factor. The 976-amino-acid KIT polypeptide consists of a pentarepetitive extracellular ligand-binding domain, a transmembrane domain, and a bipartite intracellular tyrosine kinase domain (Yarden et al. 1987). The KIT receptor monomer apparently dimerizes in response to ligand binding, thought to be a crucial step in the signal transduction process (Yarden and Ullrich 1988; Ullrich and Schlessinger 1990).

Here we have described two novel *KIT* gene mutations in patients with piebaldism: a frameshift at codon 85 and a 5′ splice-junction mutation of IVS12. The codon 85 frameshift would result in the biosynthesis of a truncated KIT polypeptide consisting only of the signal peptide and the very beginning of the ligand-binding domain. This very short KIT fragment would obviously not be functional, and this allele would thus result in a 50% reduction in the amount of functional KIT receptor. It has not previously been clear whether haploinsufficiency for KIT function could result in piebaldism. Two patients have been reported in whom piebaldism has been associated with hemizygous *KIT* gene deletions (Fleischman et al. 1991; Spritz et al., in press). However, both of these large deletions have also included at least part of the adjacent platelet-derived growth factor a receptor (*PDGFRA*) locus. As deletion of the *PDGFRA* gene alone is associated with piebald-like pigmentary ab-

normalities in the patch (*Ph*) mouse (Smith et al. 1991; Stephenson et al. 1991), the piebald phenotype in these two patients might thus have resulted from the combined effects of deletion of the *KIT, PDGFRA,* and perhaps additional genes in this chromosomal region. The *KIT* codon 85 frameshift described here is associated with a consistently mild phenotype among affected family members, demonstrating that haploinsufficiency for KIT-dependent signal transduction can result in mild piebaldism in humans.

We previously described two missense mutations of the *KIT* gene, at codons 584 and 664, both within the intracellular tyrosine kinase domain. Both of these substitutions, as well as a third at codon 583 (Fleischman, in press), are associated with a consistently severe piebald phenotype among affected members of the respective families (Giebel and Spritz 1991; Spritz et al. 1992), and we therefore suggested that the nonfunctional KIT polypeptides might be incorporated into nonfunctional KIT receptor heterodimers, inhibiting KIT-dependent signal transduction in a dominant-negative manner. The associated consistently severe piebald phenotypes might thus result from as much as 75% reduction in the amount of functional KIT receptor dimer.

Between these two extremes, we have observed several families in which the piebald phenotypes are quite variable, ranging from very mild to very severe even within each family. In two of these we found distal *KIT* frameshifts, at codons 561 and 642 (Spritz et al. 1992), both of which would result in termination of translation near the beginning of the intracellular tyrosine kinase domain. The resultant truncated KIT polypeptides would consist of the extracellular ligand-binding domain, the transmembrane domain, and only the most proximal portion of the intracellular tyrosine kinase domain, and we hypothesized that these distal frameshifts constitute pure loss of function mutations (Spritz et al. 1992). Recently, however, analogous experimentally truncated forms of the platelet-derived growth factor receptor (Ueno et al. 1991), the epidermal growth factor receptor (Kashles et al. 1991), and the fibroblast growth factor receptor 1 (Ueno et al. 1992) have been shown to associate with normal receptor polypeptide to form nonfunctional receptor heterodimers, dominant-negatively inhibiting receptor function in cultured cells and even disrupting early embryonic development in vivo (Amaya et al. 1991). If the truncated KIT polypeptides resulting from the distal frameshifts exert similar dominant-negative effects, these alleles might reduce the total amount of functional KIT receptor dimer by 50%–75%, depending on the stability of the truncated KIT polypeptides. The resultant intermediate level of KIT-dependent signal transduction may in some manner account for the variability of the piebald phenotypes associated with these mutant alleles.

Similarly, the IVS12 splice-junction mutation described here is also associated with a highly variable piebald phenotype and likewise is likely associated with a combination of loss of function and dominant-negative effects. The guanine-to-adenine substitution at the first base of IVS12 abolishes the 5′ splice site of this intervening sequence, preventing normal splicing of the corresponding *KIT* mRNA precursor. IVS12 is only 83 bases in length, the shortest of the 20 intervening sequences of the human *KIT* gene (Giebel et al., in press), and there are no potential in-frame cryptic 5′ splice sites either within the intron or in the upstream exon. Retention of the 83-bp IVS12 segment in the spliced *KIT* mRNA would result in a frameshift at codon 626, near the beginning of the tyrosine kinase domain, with termination of translation following a short nonsense peptide. This truncated KIT polypeptide would thus be very similar to those resulting from the codon 561 and 642 frameshifts, described above. However, a *KIT* IVS10 splice-junction mutation in the classic mouse *W* allele results in exon skipping in vivo (Hayashi et al. 1991). The human *KIT* IVS12 5′ splice-junction mutation might likewise result in aberrant splicing of the corresponding *KIT* mRNA precursor between the 5′ splice site of IVS11 and the 3′ splice site of IVS12. Although the resultant *KIT* mRNA would be translatable in-frame, it would lack exon 12 (codons 592–626) at the beginning of the tyrosine kinase domain, including the ATP-binding site. The corresponding deleted polypeptide would thus be nonfunctional but might still be incorporated into nonfunctional receptor heterodimers, dominant-negatively inhibiting KIT-dependent signal transduction. Depending on the efficiency of the potential aberrant splice and on the stabilities of the truncated and deleted KIT polypeptides, the IVS12 splice-junction mutation might thus result in a very complex combination of loss of function and dominant-negative effects on KIT-dependent receptor function, with a net 50%–75% reduction in the amount of functional KIT receptor dimer, and a corresponding variable piebald phenotype.

Thus, the severity of the piebald phenotypes segregating in each family appears to be related to the nature of the associated *KIT* gene mutations. A mutation

resulting from haploinsufficiency is associated with a mild phenotype. Mutations associated with dominant-negative inhibition of KIT receptor function are associated with severe phenotypes. Mutations that result in combined loss of function and dominant-negative effects are associated with highly variable phenotypes, ranging from mild to severe even among members of individual families. Furthermore, although the total number of known *KIT* gene mutations is still very small, it may be significant that no missense substitutions have been observed within the extracellular ligand-binding domain. This portion of the KIT polypeptide is pentarepetitive, and many amino acid substitutions in this region may thus not reduce KIT-dependent signal transduction sufficiently to result in the piebald phenotype.

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