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Germline *PHOX2B* Mutation in Hereditary Neuroblastoma

To the Editor:

We read with interest the study by Trochet and colleagues (2004), published in the April 2004 issue of *The American Journal of Human Genetics,* that described germline mutations of the paired-like homeobox 2B gene (*PHOX2B* [MIM 603851]) in neuroblastoma (MIM 256700). We have also considered *PHOX2B* as a candidate gene for predisposition to neuroblastoma, and we now report on a germline *PHOX2B* mutation in a pedigree with neuroblastoma. However, we also show that there is no evidence for mutation of this gene in eight other pedigrees with neuroblastoma screened to date. We think these data establish *PHOX2B* as the first bona fide gene that can predispose to neuroblastoma when mutated in the germline, and the findings further emphasize the complex genetics of this important pediatric malignancy.

We previously demonstrated linkage of hereditary neuroblastoma to 16p12-13 by use of a genomewide screening strategy (Maris et al. 2002). Positional cloning of a putative 16p12-13 hereditary neuroblastoma-predisposition gene (*HNB1*) is ongoing, but the critical genomic region for this gene remains large. We had previously considered and excluded other genes known to be mutated in Hirschsprung disease (MIM 142623) and/or in congenital central hypoventilation syndrome (CCHS [MIM 209880]) as candidates for *HNB1,* because these disorders can occur coincident with both sporadic and hereditary neuroblastoma (Maris et al. 2002). Because of the recent reports that the vast majority of patients with CCHS harbor *PHOX2B* mutations, including two patients also affected with neuroblastoma (Amiel et al. 2003; Weese-Mayer et al. 2003), we initiated a screen for germline mutations in this gene in our series of pedigrees with neuroblastoma.

Oligonucleotide primer pairs flanking the coding regions of exons 1, 2, and 3 of *PHOX2B* were designed by use of the program Primer 3.0; these primer pairs were used for PCR amplification and bidirectional sequencing of purified PCR products (primer sequences available on request). We screened germline DNA from the proband and an unaffected family member for each of the seven families that showed cosegregation of a 16p haplotype with disease, as well as for two pedigrees that consisted of cousins with neuroblastoma with no cosegregation of 16p marker haplotypes (see Maris et al. [2002] for details of pedigrees). We also sequenced 109 control DNA samples from the Coriell SNP500 Cancer Panel (Coriell Cell Repositories). All sequence aberrations were confirmed by repeat sequencing after cloning of purified PCR products (TOPO TA Cloning Kit [Invitrogen]), and DNA samples from the remaining available members of the pedigree were also screened for the variant. The Children's Hospital of Philadelphia institutional review board approved this work.

A heterozygous single-base deletion (676delG) was discovered in a complex pedigree with neuroblastoma (fig. 1) (see dbSNP Home Page). This family has seven members in three generations affected with neuroblastoma, and two of these individuals were also shown to have Hirschsprung disease. The proband was affected with neuroblastoma, Hirschsprung disease, and neurofibromatosis type 1 (MIM 162200). The putative nonsense mutation 676delG segregated with neuroblastoma through all three generations, and the frameshift was predicted to produce a slightly truncated protein that would no longer code for the second polyalanine tract. This family had previously been shown to cosegregate a 16p12-13 haplotype with neuroblastoma, and the proband was also shown to have an inactivating mutation in *NF1* (3775delT) that was not present in either of her parents (Maris et al. 2002). Tumor material was available only for patient 1-001, and the tumor exon 3 sequence remained heterozygous for the 676delG mutation. In addition, loss-of-heterozygosity studies using microsatellite markers (D4S2912, D4S1587, D4S405, D4S2971, and D4S428) that are closely linked to the *PHOX2B* locus showed no evidence for allelic deletion. The only other sequence variant discovered in the remaining eight pedigrees was a putative SNP (C552T) in pedigree 12 that is not predicted to affect the resultant protein sequence (S184S) (see dbSNP Home Page). This

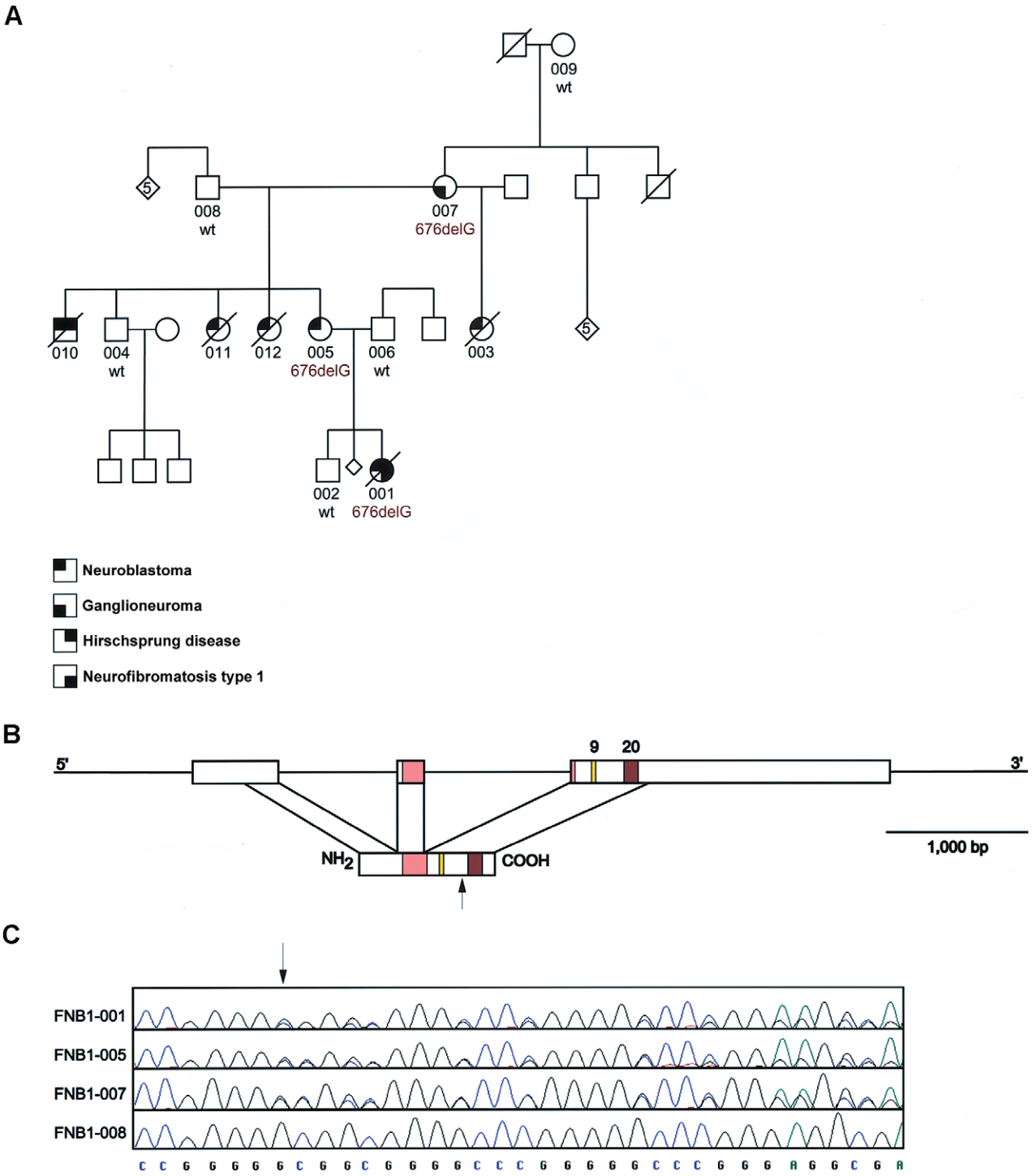

Figure 1 Germline *PHOX2B* mutation in a pedigree segregating neuroblastoma and Hirschsprung disease. *A*, Family 1 pedigree structure. DNA samples from this family with neuroblastoma were available only for patients with a *PHOX2B* result shown (wt = wild type; 676delG = heterozygous mutation segregating through three generations). Patients 1-001 and 1-010 also had Hirschsprung disease, and patient 1-001 was affected with neurofibromatosis type 1. *B*, Genomic organization of *PHOX2B* and schematic representation of the PHOX2B protein. The homeobox domain and the 9– and 20–amino-acid polyalanine repeats are shown for both schemas (*pink*, *yellow*, and *dark red boxes*, respectively). The location of the 676delG mutation is shown (*arrow*). *C*, DNA sequence electropherograms from exon 3 of the *PHOX2B* gene, showing a heterozygous single-base deletion at G676 (*arrow*) that segregates through all three generations but does not appear in a representative unaffected family member.

sequence variant was present in the proband but was not detected in the patient's affected father (no maternal DNA sample was available). It is important to note that neither sequence variant was identified in the bidirectional sequencing of 218 alleles from the control sample set. This strongly suggests that the 676delG sequence variant that segregates with the disease phenotype is a true mutation. The C552T sequence variant, which does not segregate with the disease, is more likely a very rare polymorphism, but we cannot formally exclude the possibility that there might be a functional effect of this presumably neutral polymorphism.

Accumulated data strongly implicate *PHOX2B* as an essential regulator of normal autonomic nervous system development (Pattyn et al. 1999; Brunet and Pattyn 2002). The discovery of polyalanine-expansion mutations in the majority of patients with CCHS clearly defines a role for this gene in human disease (Amiel et al. 2003; Weese-Mayer et al. 2003), and there appears to be a correlation between the severity of the respiratory symptoms and the length of polyalanine expansion (Weese-Mayer et al. 2003; Matera et al. 2004). Neuroblastoma represents perhaps the most aberrant phenotype that results from abnormal adrenergic tissue development. The rare but well-described synchronous appearance of neuroblastoma with other disorders of the autonomic nervous system has suggested a common genetic etiology often referred to as a "neurocristopathy" (Gaisie et al. 1979; Nemecek et al. 2003). Although other genes implicated in Hirschsprung disease and/or CCHS have not been excluded as hereditary neuroblastoma-predisposition genes (Maris et al. 2002; Perri et al. 2002), our data further establish *PHOX2B* as an important gene involved in the initiation of neuroblastoma tumorigenesis. However, the fact that the majority of pedigrees studied here do not show *PHOX2B* mutations clearly implicates locus heterogeneity for hereditary predisposition to neuroblastoma. Assuming that our inferences of linkage to 16p are correct, and in light of the observation of two germline mutations in the proband of the family presented here, we suggest that an oligogenic mechanism for neuroblastoma initiation should be considered, as has been shown for other diseases of neural crest–derived tissues (Gabriel et al. 2002).

It is not yet clear if the *PHOX2B* mutations discovered in patients with hereditary or sporadic neuroblastoma result in gain or loss of protein function. The hypothesis that *PHOX2B* functions as a tumor suppressor is supported by the potential predicted consequence of the five mutations described, to date, in patients with neuroblastoma. Weese-Mayer and colleagues discovered a nonsense mutation that predicts a significantly truncated protein that would miss most of exon 3, including all of the 20-alanine repeat motif (Weese-Mayer et al. 2003). The frameshift mutation described here is similar to that reported by Amiel and colleagues (2003) in a patient who also had CCHS, Hirschsprung disease, and neuroblastoma, and, in both cases, the changes in reading frame are predicted to abolish the polyalanine tract. Trochet and colleagues (2004) discovered two missense mutations, both of which map to a conserved portion of the homeodomain and thus may interfere with DNA binding. On the other hand, 4p12 is not a known site of frequent allelic deletion in neuroblastoma (Maris and Matthay 1999), and, to date, biallelic inactivation of *PHOX2B* has not been demonstrated, although far too few cases have been examined to assert this with confidence.

Taken together, these data suggest that *PHOX2B* mutations are involved in the initiation of neuroblastoma tumorigenesis, especially in patients with associated disorders of the autonomic nervous system. Our data also indicate that germline mutational events in this gene are not involved in the majority of hereditary neuroblastoma cases and that alternative genetic events may predispose to tumorigenesis. Examination of additional patients will facilitate the definition of *PHOX2B* mutation frequency in the genetic and (apparently) sporadic forms of neuroblastoma and will help to clarify the role of *PHOX2B* mutations in tumor initiation and progression.

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Electronic-Database Information

The URLs for data presented herein are as follows:

dbSNP Home Page, http://www.ncbi.nlm.nih.gov/SNP/
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for *PHOX2B,* neuroblastoma, Hirschsprung disease, CCHS, and neurofibromatosis type 1)

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Comparative Frequency of Fragile-X (*FMR1*) and Creatine Transporter (*SLC6A8*) Mutations in X-Linked Mental Retardation

To the Editor:

The study by Rosenberg et al. (2004), in the July 2004 issue of *The American Journal of Human Genetics,* and the previous work by Dr. Salomons's laboratory on the implications of the creatine transporter gene, *SLC6A8,* for X-linked mental retardation (XLMR) are very important contributions to the field (Salomons et al. 2001; Rosenberg et al. 2004). I wish, however, to qualify the concluding sentences in the abstract and the discussion section of the study by Rosenberg et al. (2004), which may lead readers to overestimate the incidence of mutations in the creatine transporter gene in mental retardation (MR). The authors write in the abstract that the "frequency of *SLC6A8* mutations in XLMR is close to that of CGG expansions in *FMR1*" (Rosenberg et al. 2004, p. 97). This is certainly incorrect. Rosenberg et al. (2004) found a 2.2% prevalence of *SLC6A8* mutations in families with proven or possible XLMR (the latter are families with at least two males affected by MR and compatible with X-linked inheritance). On the other hand, the *FMR1* expansion mutation associated with fragile-X syndrome is found in ~2%–3% of males with MR who were not selected for family history (these figures are based on cohorts with little clinical preselection apart from the exclusion of clearly chromosomal or syndromic forms of MR) (see de Vries et al. 1997; Hecimovic et al. 2002; Pandey et al. 2002; Grønskov et al. 2004; Biancalana et al., in press). In fact, when selection is based on possible X-linked inheritance, the proportion of individuals with fragile-X syndrome is much higher. For instance, in the study by Fishburn et al. (1983), fragile-X syndrome accounted for MR in 12 of 45 male sib pairs with "nonspecific" MR, a proportion (27%) that is thus >10 times higher than the reported incidence of *SLC6A8* mutations in a cohort containing sib pairs such as these as well as families with even more obvious XLMR. In fact, we have proposed recently that, unless there are clear hotspots of mutations and/or a very large mutation target size (such as for Duchenne muscular dystrophy, Rett syndrome, and hemophilia A), the population incidence of X-linked diseases implicating genes of average size that lead to highly decreased reproductive fitness is 10–20 times lower than the incidence of fragile-X syndrome (1/50,000–1/100,000 for most X-linked diseases, compared with 1/~5,000 males for the fragile-X syndrome) (Chelly and Mandel 2001). Thus, one expects that the contribution to XLMR of an average gene that does not present mutation hotspots would be 10–20 times lower than that of *FMR1*.