Mutations of the Human Homolog of Drosophila *patched* in the Nevoid Basal Cell Carcinoma Syndrome

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Summary

The nevoid basal cell carcinoma syndrome (NBCCS) is an autosomal dominant disorder characterized by multiple basal cell carcinomas (BCCs), pits of the palms and soles, jaw keratocysts, a variety of other tumors, and developmental abnormalities. *NBCCS* maps to chromosome 9q22.3. Familial and sporadic BCCs display loss of heterozygosity in this region, consistent with the gene being a tumor suppressor. A human sequence *(PTC)* with strong homology to the Drosophila segment polarity gene, *patched*, was isolated from a YAC and cosmid contig of the *NBCCS* region. Mutation analysis revealed alterations of *PTC* in NBCCS patients and in related tumors. We propose that a reduction in expression of the *patched* gene can lead to the developmental abnormalities observed in the syndrome and that complete loss of *patched* function contributes to transformation of certain cell types.

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Introduction

Tumor suppressor genes are important regulators of cell growth and differentiation (Knudson, 1971; Weinberg, 1995a). The paradigm for their role in cancer is that they are *trans*-acting and recessive at the cellular level, i.e., loss of one homolog has no effect on cell function, and homozygous inactivation is required for carcinogenesis (Cavenee et al., 1983). However, there is increasing evidence that tumor suppressor genes are not completely recessive at the level of the cell. In the heterozygous state, mutations in the *APC* gene may promote excessive proliferation of the colon epithelium (Fearon and Vogelstein, 1990; Powell et al., 1992). A more dramatic effect is seen with heterozygous mutations in the Wilms' tumor (*WT1*) gene, which lead to congenital anomalies of the genitourinary system (Pelletier et al., 1991). Some of these mutations may be dominant negative, but hemizygous null mutations can have the same effect.

Murine knockout models support a role for tumor suppressors in development. Homozygous inactivation of *Rb1* leads to embryonic lethality with multiple developmental anomalies including a hunchback appearance, migration defects and massive cell death in portions of the brain, and dysregulation of hepatic hematopoiesis (Jacks et al., 1992; Lee et al., 1992). *Wt1* deficiency results in complete renal agenesis as well as cardiac hypoplasia and diaphragmatic defects (Kreidberg et al., 1993). *Nf1* knockout mice have ventricular septal defects of the heart, persistent truncus arteriosus, and hyperplasia of sympathetic ganglia (Jacks et al., 1994; Brannan et al., 1994). Exencephaly has been described in a small percentage of *p53*-deficient mouse embryos (Sah et al., 1995). Because these mice are homozygously mutant for *p53*, a mechanism involving environmental teratogens (Nicol et al., 1995) or somatic events in additional genes must be postulated to explain the appearance of exencephaly in some embryos but not in others.

The nevoid basal cell carcinoma syndrome (NBCCS), also known as Gorlin syndrome and the basal cell nevus syndrome, is an autosomal dominant disorder that predisposes to both cancer and developmental defects (Gorlin, 1995). Its prevalence has been estimated at 1 per 56,000, and 1%–2% of medulloblastomas and 0.5% of basal cell carcinomas (BCCs) are attributable to the syndrome (Springate, 1986; Evans et al., 1991). In addition to BCCs and medulloblastomas, NBCCS patients are also at an increased risk for ovarian fibromas, meningiomas, fibrosarcomas, rhabdomyosarcomas, cardiac fibromas, and ovarian dermoids (Evans et al., 1991, 1993; Gorlin, 1995). Nonneoplastic features including odontogenic keratocysts (which are most aggressive in

the second and third decades of life), pathognomonic dyskeratotic pitting of the hands and feet, and progressive intracranial calcification (usually evident from the second decade) are very common. There is a broad range of skeletal defects (Gorlin, 1995; Shanley et al., 1994) including rib, vertebral and shoulder anomalies, pectus excavatum, immobile thumbs, and polydactyly. Craniofacial and brain abnormalities include cleft palate, characteristic coarse facies, strabismus, dysgenesis of the corpus callosum, macrocephaly, and frontal bossing (Gorlin, 1995). Generalized overgrowth (Bale et al., 1991) and acromegalic appearance are common, but growth hormone and IGF1 levels are not elevated.

Implications for the affected individual can be severe, predominantly due to the prolific BCCs, which can number more than 500 in a lifetime (Shanley et al., 1994). Expression of many features of the syndrome are variable, both within and between families (Anderson et al., 1967; unpublished data). This variation between families may reflect specific phenotypic effects of different mutations, modifier genes, or environmental factors (sunlight exposure is likely to play a role in the age of onset and incidence of BCCs). One-third to one-half of patients have no affected relatives and are presumed to be the product of new germ cell mutations (Gorlin, 1995). Unilateral and segmental NBCCS are probably attributable to somatic mutation in one cell of an early embryo (Gutierrez and Mora, 1986).

Mapping of the gene for this syndrome (*NBCCS*) to chromosome 9q22–31 (Gailani et al., 1992; Reis et al., 1992; Farndon et al., 1992) and the demonstration that the same region is deleted in a high percentage of BCCs and other tumors related to the disorder (Gailani et al., 1992) provided strong evidence that the gene functions as a tumor suppressor. Inactivation of this gene may be a necessary if not sufficient event for the development of BCCs (Shanley et al., 1995; Gailani et al.; 1996, Holmberg et al., 1996).

Results

Fine Mapping by Linkage and Tumor Deletion Studies

Since the original mapping of the gene in 1992, linkage studies have narrowed the *NBCCS* region to a 4 cM interval between *D9S180* and *D9S196* (Goldstein et al., 1994; Wicking et al., 1994). Farndon et al. (1994) reported recombination involving an unaffected individual that tentatively placed the gene proximal to *D9S287*. In addition, we identified one recombination between *D9S287* and the Fanconi's anemia complementation group C (*FACC*) gene in a three-generation family that would place the gene proximal to *D9S287*. The recombinant individual was a one-and-a-half-year-old female presumed to be affected on the basis of macrocephaly, strabismus, and frontal bossing. Some of the key features of the syndrome, such as BCCs, jaw cysts, and palmar pits, were lacking, but these features have age-dependent expression, and their presence in a young child would not be expected.

Allelic loss in BCCs was concordant with linkage mapping in placing the gene between *D9S196* and *D9S180* (Figure 1). Most hereditary tumors with allelic loss deleted the entire region between the flanking markers. However, one hereditary cardiac fibroma showed loss at *D9S287* but not *D9S280* on the nondisease-carrying allele, suggesting that the gene is located distal to *D9S280*. In sporadic BCCs, four tumors were found that retained *D9S287* and lost more distal markers, suggesting that the gene is distal to *D9S287*, in conflict with findings from linkage analysis. In addition, we also saw two tumors that lost the proximal marker *D9S280* but not *D9S287*, consistent with the linkage data. Several hypotheses can be proposed to explain these discrepancies, including the existence of a second gene responsible for the allelic loss in some sporadic tumors. Taken together, our data suggested that the most likely location of the *NBCCS* gene was between markers *D9S280* and *D9S287*. Nevertheless, physical mapping and cDNA isolation was undertaken in the entire region between *D9S196* and *D9S180*.

Physical Mapping

We obtained 29 yeast artificial chromosomes (YACs) containing markers from this region from the CEPH megaYAC library, and 18 formed an overlapping contig between *D9S196* and *D9S180* with at least 2-fold redundancy. Based on this contig, the minimum distance between the flanking markers was 1.5 Mb, but virtually all large YACs had internal deletions as judged by sequence-tagged site content. Additional YACs were obtained from the ICI library to provide redundancy in areas apparently prone to deletion. Cosmid and bacterial artificial chromosome (BAC) contigs were constructed around known sequence-tagged sites and genes, and additional cosmids from the region were isolated by hybridizing YACs to the Lawrence Livermore gridded cosmid library. In total, over 800 cosmids specific to this region were gridded into 96-well plates, and contigs of BACs, P1s, and cosmids covering 1.5 Mb were constructed (Figure 1). Because of deletions in YACs and some gaps in the cosmid and BAC contig, pulsed-field gel electrophoresis (PFGE) and fluorescence in situ hybridization (FISH) were used to integrate the cloned regions. Based on the sizes of restriction fragments in this region and FISH estimates, the physical distance from *D9S180* to *D9S196* was estimated at not less than 2 Mb.

Isolation of cDNAs

Several methods were used to find genes that map to chromosome 9q22, including sample sequencing of cosmids, exon trapping, CpG island cloning, and direct selection of cDNAs from BACs and cosmids. In addition, genes known to lie in this general area were more finely mapped by use of somatic cell hybrids made from two NBCCS patients with visible 9q22 deletions (submitted to the National Institute of General Medical Sciences repository), YAC contigs, and FISH. Ten genes, ten expressed sequence tags (ESTs) with sequences in GenBank, and 31 anonymously selected cDNA fragments, CpG island clones, and trapped exons with no known homology were identified (Table 1).

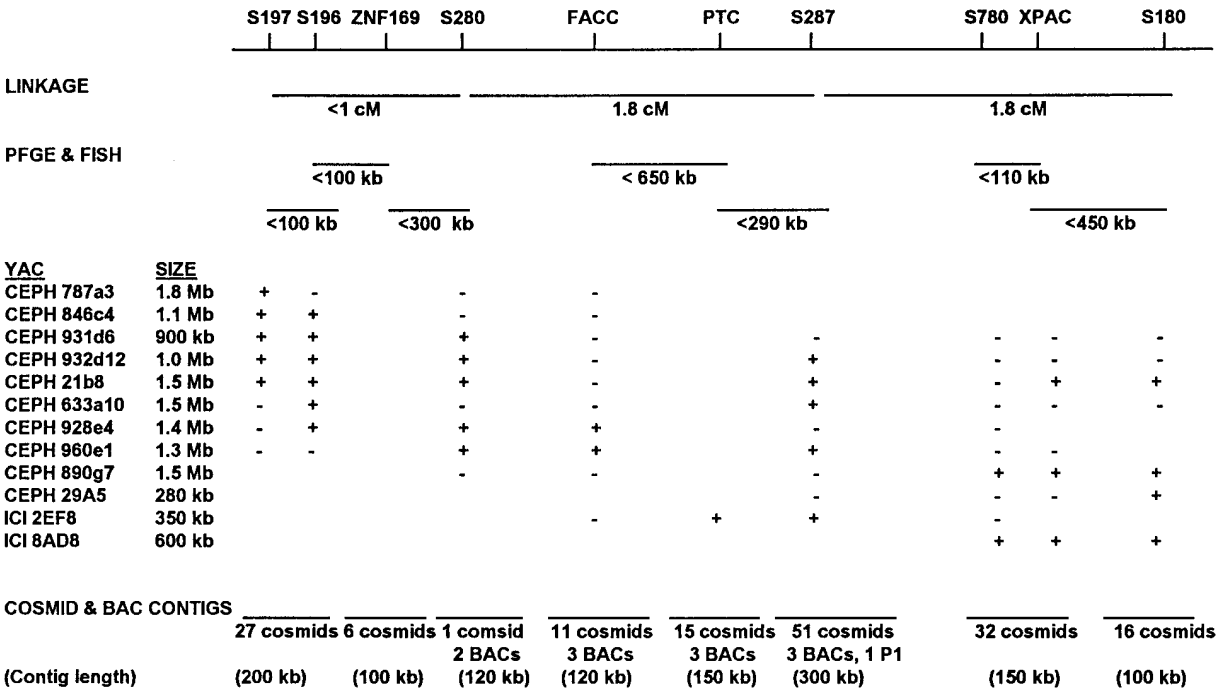

Figure 1. Integrated Framework Map of the *NBCCS* Region

Both linkage and tumor deletion studies place *NBCCS* between *D9S196* and *D9S180* but are conflicting with regard to whether the gene lies proximal or distal to *D9S287* (discussed in text). The order of six polymorphic markers, *D9S197, D9S196, D9S280, FACC, D9S287, D9S180*, is derived from genetic linkage data (Farndon et al., 1994; Pericak-Vance et al., 1995). *D9S196* and *D9S197* show no measurable recombination. PFGE and FISH give a minimum distance of 2 Mb between *D9S196* and *D9S180*. Key information about YAC, BAC, and cosmid contigs in the *NBCCS* region is shown. In total, 22 overlapping YACs and more than 800 cosmids were isolated from this region. BAC and cosmid contigs covering more than 1.2 Mb have been submitted to the Genome Data Base.

Screening Patients for Germline Deletions or Rearrangements

Because chromosome 9q22 appeared to be very gene rich, an attempt was made to localize the *NBCCS* gene more precisely by searching for submicroscopic rearrangements in patients. We hybridized 15 cosmids at approximately 100–200 kb intervals spanning the region between *D9S196* and *D9S180* to PFGE blots of 82 unrelated NBCCS patients. In addition, probes from genes known to map to the interval as well as those identified in the course of the study were included in this analysis. PFGE variants were identified in three patients with probes from within the *FACC* gene. All three were heterozygous for SacII bands approximately 30 kb shorter than normal (310 kb versus 280 kb). The limit of resolution of PFGE was about 10 kb, so that it was not possible to determine whether the apparently identical variant SacII bands were exactly the same size. Other restriction enzymes, including NotI, BssHII, MluI, SfiI, and NruI, did not show variant bands. The variations were not consistent with germline deletions in these patients but could conceivably be caused by point mutations creating new restriction sites or other small alterations, such as the recurrent inversions seen in the *F8C* gene of many hemophiliacs (Lakich et al., 1993).

The nature of the DNA alterations causing these changes on PFGE has not yet been elucidated, but the data relating them to the disease are compelling. The families of two of the patients with variations were not available for study, but the third patient was a sporadic case of NBCCS, and neither parent had the SacII alteration. The finding of this variant in a patient but not in her parents could be interpreted as the result of hypermutability of some CG-rich region near *FACC*, but no variation in this region was identified in PFGE blots of over 100 normal chromosomes.

Evaluation of *PTC* as a Candidate Gene

Because variant PFGE bands were identified near *FACC* and linkage and tumor data suggested this region as a likely location, we evaluated cDNAs that mapped to this area. *FACC,* itself, was not considered a candidate because heterozygous mutations in this gene do not cause NBCCS (Strathdee et al., 1992). *FACC* and *PTC* (a human gene with strong homology to Drosophila *patched* [*ptc*]) hybridized to the same 650 kb NotI fragment and 675 kb and 1000 kb (partial) MluI fragments. Mouse interspecies backcross analysis determined that there were no recombinants between the *PTC* and *FACC* genes out of 190 meioses (Hahn et al., 1996a). *PTC* and *D9S287* were both present on ICI YAC 2EF8, having a size of 350 kb, strongly suggesting that *PTC* lies between *D9S287* and *FACC*.

To screen for mutations in the *PTC* gene, the intron/exon boundaries of the gene were determined from genomic clones and long-range polymerase chain reaction (PCR) products. *PTC* consists of 23 exons and the gene

Table 1. cDNA Clones from the *NBCCS* Region on Chromosome 9q22.3

Clone Designation[a]	Clone Type[b]
cDNAs previously mapped to chromosome 9q and more finely mapped with somatic cell hybrids, PGFE, and YAC contigs	
FACC	Gene
NCBP	Gene
HSD17B3	Gene
TMOD	Gene
XPA	Gene
SYK	Gene
WI-11139	EST (R14225)
WI-11414	EST (T88697)
WI-8684	EST (R14413)
D9S1697	EST (R06574)
D9S1145	EST (contains R17127 and Z38405)
Novel clones or clones not previously mapped to chromosome 9q identified by sample sequencing, exon trapping, HTF island cloning or cDNA selection	
ZNF169	Gene
FBP1	Gene
PTC	Gene
Coronin homolog	Gene
2F1a	EST (R39928)
2F1b	EST (T11435)
11F21	EST (Z43835)
31F3	EST (R16281)
yo20g05.s1	EST (Merck EST)
Plus 31 anonymous selected cDNA fragments, HTF island clones, and trapped exons from YACs and cosmid pools[c]	

[a]For known genes and anonymous cDNAs that have been submitted to the Genome Data Base (http://gdbwww.gdb.org/gdb), standard locus nomenclature is used. For ESTs without a GDB number, WI indicates a Whitehead Institute clone (http://www-genome.wi.mit.edu).

[b]GenBank accession numbers, where available, are given in parentheses for anonymous ESTs. Additional information can be obtained at http://www.ncgr.org/gsdb.

[c]Additional sequence data will be required to determine whether some of the anonymous cDNAs represent different portions of the same genes.

spans approximately 34 kb (Figure 2). Panels of unrelated NBCCS patients and BCCs were screened by single strand conformation polymorphism (SSCP) analysis (primers used for amplification of *PTC* exons are shown in Table 2). Patients displaying variations were compared with unaffected individuals of the same race, and variants found only in affected individuals were further characterized by DNA sequencing. Of the mutations identified in unrelated patients, four were deletions or insertions resulting in frameshifts and two were point mutations leading to premature stops (Table 3; Figures 3 and 4). An additional finding confirming the relationship between mutations in *PTC* and the disease was identification of a frameshift mutation in a sporadic NBCCS patient that was not present in either of her unaffected parents (Figure 4).

To analyze the role of *PTC* in neoplasia, tumors related to the syndrome were screened for mutations. Two sporadic BCCs with allelic loss of the *NBCCS* region had inactivating mutations of the remaining allele (Table 3; Figures 5 and 6). A tumor removed from the cheek had a CC to TT alteration, typical of ultraviolet B mutagenesis. The second tumor from the nose had a 14 bp deletion, a mutation that cannot be related to any specific environmental agent. Mutations have not yet been identified in any sporadic BCC not showing allelic loss of chromosome 9q22, and alternative modes of pathogenesis may be operative in these tumor types.

Discussion

Tumorigenesis in humans is a multistep process involving activation of oncogenes and inactivation of tumor suppressor genes. Genetic analysis of hereditary cancer predisposition syndromes has been critical in elucidating the nature of tumor suppressors. The gene underlying hereditary predisposition to retinoblastoma (*RB1*) (Friend et al., 1986) was the first of this class to be isolated. *RB1* and several other tumor suppressors function in the nucleus to regulate transcription and the cell cycle (Weinberg, 1995b). Increasing evidence indicates that modulation of proliferation and differentiation may also take place at the cell membrane. Tumor suppressors with presumed functions in intercellular adhesion and signaling include *DCC* (Fearon et al., 1990), *APC*

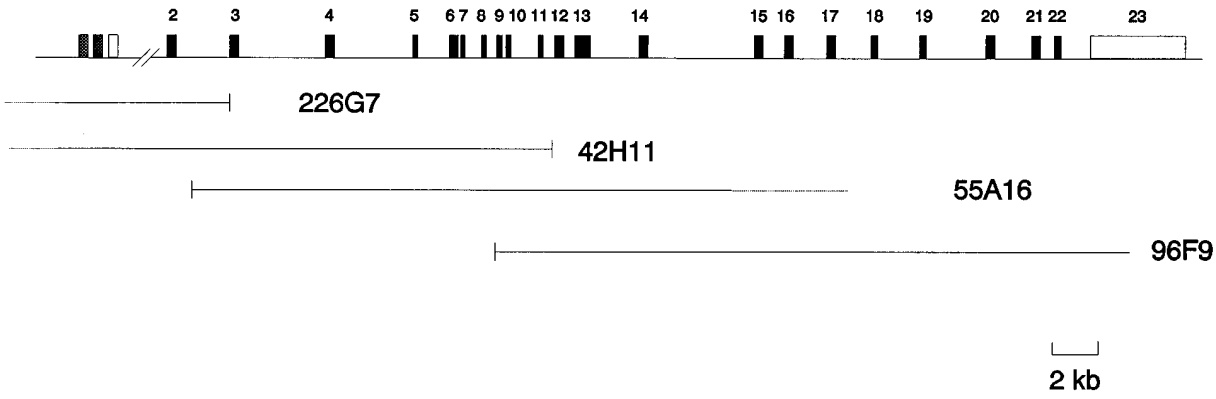

Figure 2. Map of the *PTC* Locus

The gene lies on four overlapping cosmids including 226G7, 42H11, 55A16, and 96F9 (LL09NC01, 96-well coordinates). The coding exons of the gene are shown as closed boxes and noncoding exons as open boxes. Splice variants of the 5′ noncoding region of the gene indicate at least two alternate first exons and possibly a third alternate exon (see Discussion).

Table 2. Primers to Amplify *PTC* Exons

Exon	Position[a]	Exon Size (bp)	Primers[b]
1	1-189	189	PTCF18 GAAGG CGAGC ACCCA GAC PTCR18 TCTTT CCCTC CTCTC CCTTC
1A	Alternate First exon	>239	PTCF22 GCTAT GGAAA TGCGT CGG PTCR22 CAGTC CTGCT CTGTC CATCA
2	190-382	193	PTCF19 GTGGC TGAGA GCGAA GTTTC PTCR19 TTCCA CCCAC AGCTC CTC
3	383-582	200	PTCF27 CTATT GTGTA TCCAA TGGCA GG PTCR27 ATTAG TAGGT GGACG CGGC
4	583-642	60	PTCF20 GAG AAATTTTTGTCTCTGCTTTTCA PTCR20 CCTGA TCCAT GTAAC CTGTT TC
5	643-734	92	PTCF21 GCAAA AATTT CTCAG GAACACC PTCR21 TGGAA CAAAC AATGA TAAGCAA
6	735-933	199	PTCF15 CCTAC AAGGT GGATG CAGTG 18R2 TTTGC TCTCC ACCCT TCTGA
7	934-1055	122	11e18F GTGAC CTGCC TACTA ATTCCC 18R3 GGCTA GCGAG GATAA CGGTTTA
8	1056-1203	148	PTCF2 GAGGC AGTGG AAACT GCTTC PTCR2 TTGCA TAACC AGCGA GTCTG
9	1204-1335	132	PTCF23 GTGCT GTCGA GGCTT GTG PTCR23 ACGGA CAGCA GATAA ATGGC
10	1336-1492	157	PTCF23 GTGCT GTCGA GGCTT GTG PTCR23 ACGGA CAGCA GATAA ATGGC
11	1493-1591	97	PTCF5 GTGTT AGGTG CTGGT GGCA PTCR5 CTTAG GAACA GAGGA AGCTG
12	1591-1835	245	PTCF24 TCTGC CACGT ATCTG CTCAC PTCR24 CATGC TGAGA ATTGC AGGAA
13	1836-2238	403	PTCF16 GGCCT ACACC GACAC ACAC PTCR16 TTTTT TTGAA GACAG GAAGA GCC PTC13R GTCAG CAGAC TGATT CAGGT PTC37R AAGAT GAGAG TGTCC ACTTCG
14	2239-2548	310	PTCF14 GACAG CTTCT CTTTG TCCAG PTCR14 ACGCA AAAGA CCGAA AGGAC GA
15	2549-2691	143	PTCF13 AGGGT CCTTC TGGCT GCGAG PTCR13 TCAGT GCCCA GCAGC TGGAG TA
16	2692-2875	185	PTCF7 AACCC CATTC TCAAA GGCCTCTGTTC PTCR7 CACCT CTGTA AGTTC CCAGA CCT
17	2876-3156	281	PTCF12 AACTG TGATG CTCTT CTACC CTGG PTCR12 AAACT TCCCG GCTGC AGAAA GA
18	3157-3294	138	PTCF8 TTTGA TCTGA ACCGA GGACACC PTCR8 CAAAC AGAGC CAGAG GAAATGG
19	3295-3437	143	PTCF11 TAGGA CAGAG CTGAG CATTT ACC PTC21R TACCT GACAA TGAAG TCG
20	3437-3537	101	PTCF11 TAGGA CAGAG CTGAG CATTT ACC PTC21R TACCT GACAA TGAAG TCG
21	3538-3792	255	PTCF25 AACAG AGGCC CCTGA AAAAT PTCR25 GATCA CTTGG TGGGC AGG
22	3793-4330[c]	537	PTCF10 TCTAA CCCAC CCTCA CCCTT PTC31R ATTGT TAGGG CCAGA ATGCC PTCF26 AGAAA AGGCT TGTGG CCAC PTCR26 TCACC CTCAG TTGGA GCTG

[a]Positions of exons are shown in bp according to the numbering of the human cDNA sequence. The size of the exon is known in all cases except for exon 1A, for which the 5′ end is not yet defined.

[b]The sequence of the primers is 5′-3′. There is an approximately 600 bp intron between exons 9 and 10 (primers F23 and R23), and a 45 bp intron between exons 18 and 19 (primers F11 and 21R). Updated primer sequences and PCR conditions will be available by anonymous FTP (contact dean@fcrfv1.ncifcrf.gov).

[c]Position of the last bp of the final codon.

Table 3. Mutations in the *PTC* Gene

Sample Type[a]	Inheritance[b]	Exon	Type of Mutation	Designation
NBCCS	F	8	Premature stop	C1081T
NBCCS	F	6	37 bp deletion	del 804-840
NBCCS	F	8	Premature stop	G1148A
NBCCS	F	13	2 bp insertion	2047insCT
NBCCS	S	13	1 bp insertion	2000insC
NBCCS	S	15	1 bp deletion	2583delC
BCC	S	8	Premature stop	CC1081TT
BCC	S	16	14 bp deletion	del 2704-2717

[a]NBCCS, germline mutations in a patient with the syndrome; BCC, somatic mutation in a basal cell carcinoma.
[b]F, familial; S, sporadic.

(Rubinfeld et al., 1993; Su et al., 1993), and *NF2* (Trofatter et al., 1993).

Our current study provides strong evidence that mutations of the human homolog of Drosophila *ptc* cause NBCCS. Alterations predicted to inactivate the PTC gene product were found in six unrelated NBCCS patients. Frameshift mutations were found in two sporadic patients but not in their parents, and somatic mutations were identified in two sporadic tumors of the types seen in the syndrome.

The Drosophila *ptc* Gene in Differentiation and Development

The *patched* gene is part of a signaling pathway that is conserved in eukaryotic organisms (Goodrich et al., 1996; Hahn et al., 1996a). The Drosophila gene (*ptc)* encodes a transmembrane glycoprotein that plays a role in segment polarity (Hooper and Scott, 1989; Nakano et al., 1989). Many alleles of *ptc* produce an embryonic lethal phenotype with mirror-image duplication of segment boundaries and deletion of the remainder of the segments (Nüsslein-Volhard and Wieschaus, 1980), but hypomorphic alleles produce viable adults with overgrowth of the anterior compartment of the wing, loss of costal structures, and wing vein defects (Phillips et al., 1990). Genetic and functional studies have shown that one of the wild-type functions of *ptc* is transcriptional repression of members of the *Wnt* and *TGF*β gene families (Ingham et al., 1991; Capdevila et al., 1994). The mechanism of this repression is not known, and several downstream targets of *ptc* activity may exist. The action of *ptc* is opposed by the *hedgehog* (*hh*) gene, which encodes a secreted glycoprotein that acts to transcriptionally activate both *ptc*-repressible genes and *ptc* itself (Tabata and Kornberg, 1994; Basler and Struhl, 1994). Thus, in a given cell type the activity of target genes results from a balance between *hh* signaling from adjacent cells and *ptc* activation.

Mammalian Homologs of *ptc*

We isolated a human homolog (*PTC*) of Drosophila *ptc* by sequence-sampling cosmids from the *NBCCS* region. The gene displays up to 67% identity at the nucleotide level and 60% similarity at the amino acid level to the Drosophila gene (Hahn et al., 1996a). The identification of a human homolog would have been difficult either using a hybridization approach or by screening an expression library with antibodies to the Drosophila protein. Goodrich et al. (1996) were able to isolate a murine

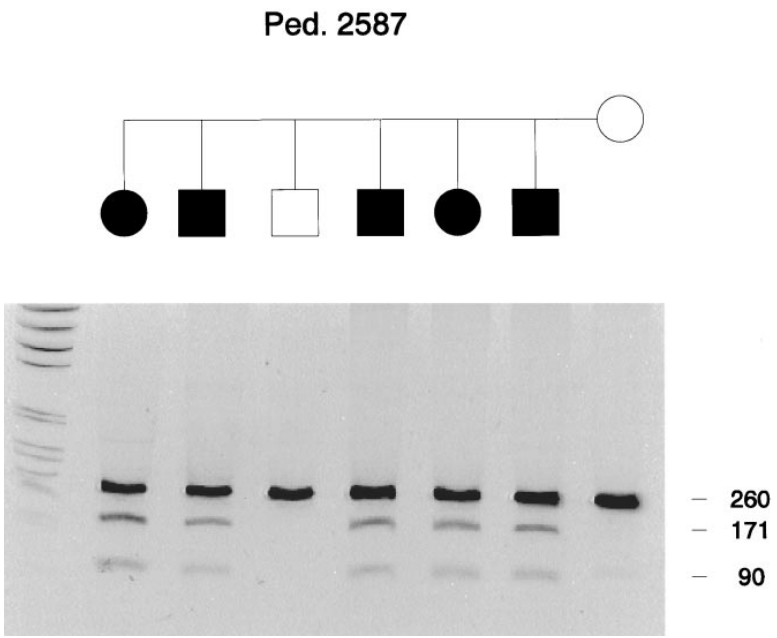

Figure 3. Segregation of a Premature Termination Mutation of *PTC* in an NBCCS Pedigree

The C1081T (Q210X) mutation segregating in this kindred creates a BfaI site. PCR with flanking primers produces a 260 bp product, which digests to 171 bp and 90 bp fragments in affected family members. The PCR product remains undigested in unaffected family members.

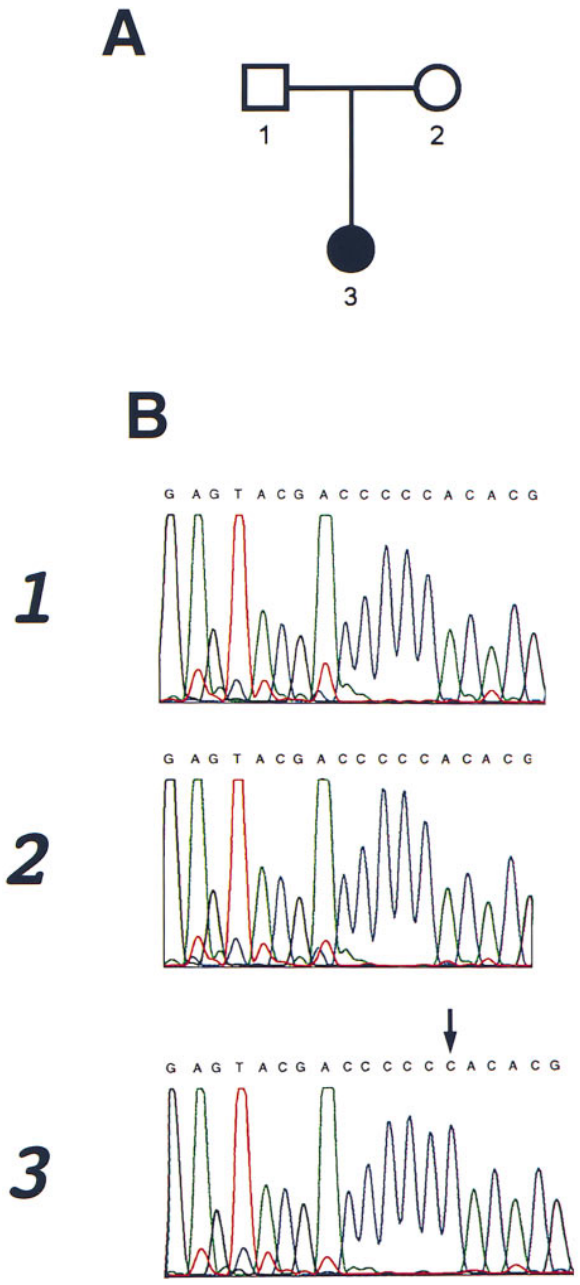

Figure 4. A Frameshift Mutation of *PTC* in a Sporadic NBCCS Patient

(A) Both parents (1 and 2) of an affected individual (3) were free of phenotypic features of NBCCS.
(B) The patient was heterozygous for an SSCP variant in exon 13 that was not present in her parents, and sequencing of a PCR product from genomic DNA showed two sequences out of frame following base 2000 (data not shown). The abnormal conformer was sequenced and contained a 1 bp insertion (2000insC), resulting in a premature stop 9 amino acids downstream. Sequences of PCR products from both parents were normal.

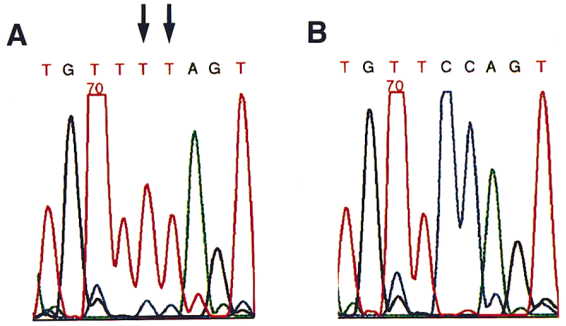

Figure 5. An Ultraviolet B-Induced Mutation of *PTC* in a Sporadic BCC

(A) CC to TT mutation in the remaining allele of a tumor with allelic loss of the *NBCCS* region. This DNA alteration, which results in a premature stop, is typical of ultraviolet B mutagenesis.
(B) Constitutional DNA from the patient has a normal sequence.

patched gene (*Ptc)* utilizing degenerate primers to conserved domains of the Drosophila sequence. Hydropathy plots of the human and Drosophila sequence show remarkable similarity and predict the presence of 8–12 transmembrane domains. Both human and mouse data (Hahn et al., 1996a; Goodrich et al., 1996) strongly suggest that *patched* is a single copy gene in mammals.

Analysis of fetal brain cDNA clones, RACE (rapid amplification of cDNA ends) experiments with epidermal RNA, and genomic sequencing revealed the presence of three different 5′ ends for the human *PTC* gene (Figure 2; data not shown). Two of the human alternative first exons diverge from the mouse *Ptc* cDNA at the same position, and the third is highly homologous to the mouse N-terminus. The mouse N-terminus most closely matches the N-terminus of the Drosophila gene and a related Caenorhabditis elegans sequence (GenBank accession number Z46812). These data suggest that there are at least three different forms of the PTC protein in mammalian cells: the ancestral form represented by the murine sequence, and the two human forms. The first in-frame methionine codon for one of the human forms is in the third exon, suggesting that this form of the mRNA either encodes an N-terminally truncated protein or uses an alternate initiation codon. The second human form contains an open reading frame that extends through to the 5′ end and may be initiated by upstream sequences that have not yet been isolated. The third form contains an in-frame methionine codon at a position conserved with the predicted initiation codon in the mouse (Goodrich et al., 1996). Additional characterization of the *PTC* 5′ end is required to resolve the location of the start site in the different forms of human PTC. The identification of several potential forms of the PTC protein provides a mechanism whereby a single *PTC* gene could play a role in different pathways. It will be important to determine the regulation of the different splice forms of *Ptc* mRNA, as this may shed light on the apparent role of the gene in both embryonic development and growth control of adult cells.

The Role of *PTC* in Neoplasia

The data presented in this study strongly support earlier observations (Gailani et al., 1992) suggesting that the *NBCCS* gene functions as a tumor suppressor. We previously demonstrated that hereditary tumors display loss of the wild-type homolog of the *NBCCS* region. Here, we show that the germline mutations detected to date are inactivating and that, therefore, hereditary

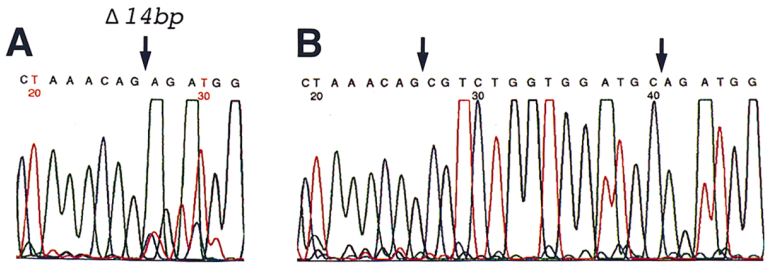

Figure 6. A *PTC* Deletion in a Sporadic BCC

(A) A 14 bp deletion in the remaining allele of a tumor with allelic loss of the *NBCCS* region. Despite the fact that this tumor was removed from the nose, a highly sunlight-exposed area, the mutation cannot be related specifically to ultraviolet radiation.

(B) Constitutional DNA had the normal sequence.

tumors have no functional copy of the gene. In addition, this report provides direct evidence that sporadic BCCs can arise with somatic loss of both copies of the gene. The role of *PTC* alterations in other tumor types associated with NBCCS, such as medulloblastoma, remains to be explored.

That two known targets of *ptc* repression in Drosophila represent gene families involved in cell–cell communication and cell signaling provides a possible mechanism by which *ptc* could function as a tumor suppressor. The *ptc* pathway has recently been implicated in tumorigenesis by the cloning of the pancreatic tumor suppressor gene, *DPC4* (Hahn et al., 1996b), which shows sequence similarity to Drosophila mothers against *dpp* (*mad*). The *mad* gene interacts with *dpp*, a Drosophila *TGF*β homolog specifically repressed by *ptc.*

The Role of *PTC* in Developmental Anomalies

During murine development, *Ptc* is initially detected within the somites and ventral neural tube and later in the posterior ectoderm of each limb bud. Other sites of expression include the pharyngeal arches, cells surrounding the placodes of the vibrissae, and the genital eminence (Hahn et al., 1996a). In the mouse, expression of *Ptc* is linked to expression of the vertebrate homologs of Drosophila *hh* such that the transcripts are found mostly in adjacent nonoverlapping tissues (Goodrich et al., 1996). *Sonic hedgehog* (*Shh*) is detected in the notochord and the overlying floorplate region of the neural tube (Echelard et al., 1993). Apart from being involved in midline signaling in vertebrates, *Shh* is also expressed in the developing limbs, where it appears to mediate the activity of the zone of polarizing activity (Riddle et al., 1993).

Many of the features of NBCCS can be correlated with the presumed sites of expression of *PTC* in the developing human embryo (Table 4). However, under the classical two-hit model for the action of tumor suppressors (Knudson, 1971), the finding of developmental defects in a syndrome caused by hemizygous inactivation of this type of gene constitutes a paradox, because loss of one copy is thought to have little or no effect on cell function.

It is possible that some of the discrete defects in NBCCS (e.g., spina bifida occulta, bifid ribs, and jaw cysts) can be explained by a two-hit mechanism. Like the neoplasms in cancer predisposition syndromes, many of these defects are multiple and appear in a random pattern. However, isolated defects of the same type are seen occasionally in the general population. These anomalies might result from homozygous inactivation of *PTC* in an early progenitor cell of the relevant tissue, leading to either abnormal migration, abnormal differentiation, or failure to undergo programmed cell death. Allelic loss studies have shown that keratocysts of the jaw are clonal abnormalities that arise with homozygous inactivation of the *NBCCS* gene (Levanant et al., 1996). However, generalized or symmetric features, such as overgrowth, macrocephaly, and facial dysmorphology, defy the two-hit paradigm and probably result from perturbation of a dosage-sensitive pathway during embryonic development.

NBCCS is unusual among autosomal dominant cancer predisposition syndromes for its prominent developmental defects. No known tumor suppressor gene has sequence similarity to *PTC*, and, functionally, *PTC* may represent a new type of neoplasia-related gene. Further characterization of the protein product and its interaction with HH should yield important information on the link between differentiation and growth control.

Table 4. Mouse *Ptc* Expression and Human NBCCS Phenotype

Site of Expression in Mouse	NBCCS Phenotype
Pharyngeal arches	Facial malformations Jaw cysts (dental lamima derivative) Cleft palate
Neural tube	Dysgenesis of the corpus callosum Eye anomalies
Somites	Spina bifida Vertebral fusion Rib anomalies
Limb buds	Short fourth metacarpals Polydactyly

Experimental Procedures

Subjects and Samples

DNA samples were collected from 363 individuals in 128 NBCCS kindreds. Patients were examined by a clinical geneticist, and diagnosis of Gorlin syndrome was based on at least two major features of the syndrome, e.g., jaw cysts, palmar pits, multiple BCCs, or a family history of typical Gorlin syndrome. Lymphoblastoid cell lines were made from at least one affected member of 82 kindreds. A total of 252 BCCs were collected as either fresh or paraffin-embedded specimens.

Short Tandem Repeat Polymorphisms

PCR reactions were performed in 50 μl volumes containing 100 ng of template DNA, 200 μM dNTPs, 1.5 mM $MgCl_2$, 0.25 mM spermidine, 10 pM of each primer, 1 μCi [α-^{32}P]dCTP (Amersham), and 1.25 U Taq polymerase (Promega) in Promega buffer (10 mM Tris HCl [pH 9], 50 mM KCl, 0.1% Triton X-100). An Ericomp Dual Block

thermocycler was set with the following parameters for 25 cycles: 94°C for 1 min; 55°C for 30 s; 72°C for 2 min. PCR products were analyzed on 5% polyacrylamide gels, and exposed at −70°C with Kodak XAR film. Loci are shown in Figure 1, and primer sequences are available from the Genome Data Base (http://gdbwww.gdb.org).

PFGE

Cultured lymphoblastoid cells were embedded in LMP agarose (Bio-Rad) at a concentration of approximately 2×10^6/220 μl block, and DNA was extracted according to standard methods (Sambrook et al., 1989). Quarter blocks were digested with SacII, MluI, NotI, BssHII, NruI, and SfiI under conditions recommended by the manufacturer (New England BioLabs). Electrophoresis was carried out with the Bio-Rad CHEF DR II apparatus, using 1% agarose gels run for 20 hr at 200 V with a pulse time of 75 s. For higher resolution of fragments under 500 kb, a 25 s pulse time was used. Transfer to nylon membranes (Du Pont Gene Screen Plus) was performed according to the instructions of the manufacturer after exposure of the gel to UV (6–7 mW/cm^2) for 2 min. Probes were labeled to a specific activity of approximately 10^9 DPM/μg with [α-^{32}P]dCTP (Amersham) by the random primed synthesis method (Boehringer Mannheim Kit). Hybridization was carried out for 18 hr at 65°C in 0.5 M sodium phosphate (pH 7.2), 7% SDS, 1% BSA, 1 mM EDTA, and 200 μg/ml herring sperm DNA. For probes containing repetitive sequences, sheared sonicated human placental DNA (Sigma) was added to the hybridization solution (500 μg/ml) and preassociated with the probe at 65°C for 45 min prior to hybridization to the filter. Filters were washed in 0.1× SSC with 1% SDS at 65°C and exposed to autoradiographic film with an intensifying screen at −70°C from 12 hr to 3 days. Probes that detected similar sized fragments on different blots were directly compared for comigrating fragments by hybridization to the same blot. Blots were stripped in 0.4 N NaOH for 30 min at room temperature between uses.

FISH

Cosmid clones were labeled by nick translation with biotin-11-dUTP, digoxigenin-11-dUTP, or both, and hybridized to metaphase and interphase chromosomes under suppression conditions. Biotinylated probes were detected with 5 μg/ml FITC-conjugated avidin DCS. Digoxigenin-labeled probes were detected with 2 μg/ml anti-digoxigenin Fab, conjugated to rhodamine. The chromosomes were counterstained with 200 ng/ml of DAPI. Images were obtained using a microscope coupled to a cooled CCD camera. The digitalized images were processed, pseudocolored, and merged, and the distances between signals were measured.

Cosmid and BAC Screening

A gridded chromosome 9 cosmid library (LL09NC01) was replicated onto nylon filters (Du Pont Gene Screen Plus) and screened according to the recommendations of the Human Genome Center, Lawrence Livermore National Laboratory (J. Alleman, J. Garnes, J. McNinch, B. S. Wong, P. J. deJong, personal communication). Positive clones were streaked out to single colonies and confirmed to contain the appropriate markers by PCR or hybridization. Gridded BAC filters were screened by hybridization according to the recommendations of the manufacturer (Research Genetics). Because of the small chance of chimerism in cosmids and BACs, fragments from the ends of contigs were mapped with a panel of human–hamster somatic cell hybrids to confirm their localization on chromosome 9q22.

Isolation of cDNAs

We used four methods to isolate candidate cDNAs. Direct cDNA selection (Parimoo et al., 1991) was applied to pools of cosmids and BACs. Following two rounds of selection, the PCR products were size-fractionated and cloned into PCRII (Invitrogen). Transformants were gridded into 96-well plates, and replica filters were probed with the genomic template DNA to identify cDNAs that hybridized the correct genomic region.

Exon trapping was performed using the method developed by Buckler et al. (1991) and later modified by Church et al. (1994). BamHI–BglII digests of pools of five or six cosmids were cloned into the BamHI site of the splicing vector pSPL3b (Burn et al., 1995). Trapped DNAs were sequenced and mapped back to the *NBCCS* candidate region by hybridization to the cosmids from which they were derived.

For CpG island cloning, YACs were size-fractionated by PFGE, excised from the gel, and digested with BssHII. Subsequently, vectorette linkers were added and PCR amplification was performed using a vectorette primer and a 5′ Alu primer (Valdes et al., 1994). After an initial denaturation at 100°C for 5 min, 30 amplification cycles were performed with denaturation for 1 min at 98°C, annealing for 1 min at 60°C, and extension for 3 min at 72°C. Taq polymerase (10 U) was used in a total volume of 100 μl consisting of 50 mM KCl, 10 mM Tris (pH 9), 2 mM $MgCl_2$, 0.1% Triton X-100, and 200 μM dNTP. The PCR products were electrophoresed on a 1% agarose gel, in order to determine their size, and subsequently cloned into the pGEM T vector (Promega) by a shotgun procedure.

For sequence-sampling the ends of chromosome 9, specific cosmids or cosmid subclones were directly sequenced (Smith et al., 1994). Sequencing was performed on an ABI 373 DNA sequencer. The resulting end sequences were manually trimmed, examined for simple sequence repeats, and used to search the DNA sequence databases. Both nucleotide and amino acid searches were performed. In addition, sequences were examined for potential coding regions by GRAIL (Uberbacher and Mural, 1991).

Short cDNA fragments obtained by the methods outlined above were extended by screening brain or epidermal cDNA libraries and by rapid amplification of cDNA ends (Marathon Kit, Clontech).

Intron/Exon Structure of the Human *PTC* Gene

Oligonucleotides were chosen at approximately 150 bp intervals spanning the cDNA of the human *PTC* gene. PCR products were generated from cosmids 226G7, 42H11, 55A16 or 96F9. Reactions were performed in a 50 μl volume containing 25 pmol of various oligonucleotide combinations, 200 μmol dNTPs, 1.5 mM or 1.85 mM or 2.2 mM $MgCl_2$, 5 U Taq polymerase, and amplified for 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 2.5 min. Some samples were amplified by long range PCR using the Expand Long Template PCR system (Boehringer Mannheim) according to the instructions of the manufacturer. PCR products were resolved on a 1% agarose gel and isolated by a DNA purification kit (Jetsorb, Genomed). Sequencing of PCR fragments was performed with the Taq Dyedeoxy Terminator Cycle Sequencing Kit (Applied Biosystems). Sequencing reactions were resolved on an ABI 373A automated sequencer. Positions of introns were determined by predicted splice donor or splice acceptor sites.

Mutation Detection

A combined SSCP (Orita et al., 1989) and heteroduplex analysis (White et al., 1992) approach was used under optimized conditions (Glavač and Dean, 1993). DNA samples (100 ng) were amplified in PCR buffer containing 1.5 mM $MgCl_2$ and [α-^{32}P]dCTP for 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s. Products were diluted 1:3 in stop solution, denatured at 95°C for 2 min, and 3 μl loaded directly on gels. Gel formulations used were the following: first, 6% acrylamide:Bis (2.6% cross-linking), 10% glycerol at room temperature, 45 W; second, 6% acrylamide:Bis (2.6% cross-linking) at 4°C, 60 W; third, 10% acrylamide:Bis (1.3% cross-linking), 10% glycerol at 4°C, 60 W; fourth, 0.5× MDE (ATGC Corporation, Malvern, PA), 10% glycerol at 4°C, 50 W. Gels were run for 3–16 hr (3000 Vh/100 bp), dried, and exposed to X-ray film for 2–24 hr. Heteroduplexes were identified from the double-stranded DNA at the bottom of the gels, and SSCPs were identified from the single-stranded region.

Samples showing variation were compared with other family members to assess segregation of the alleles, or with normal DNA from the same patient in the case of tumors. PCR products with SSCP or heteroduplex variants were treated with shrimp alkaline phosphatase and exonuclease I (United States Biochemical) and cycle-sequenced with AmplitaqFS (Perkin Elmer). The products were analyzed on an Applied Biosystems model 373A DNA sequencer.

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