UV Fingerprints Predominate in the *PTCH* Mutation Spectra of Basal Cell Carcinomas Independent of Clinical Phenotype

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Basal cell carcinoma (BCC) shows a wide interpatient variation in lesion accrual. To determine whether certain tumorigenic fingerprints and potentially predisposing patched (*PTCH*) tumor suppressor single-nucleotide polymorphisms (SNPs) are distributed differently among sporadic BCC patients, we compared the *PTCH* mutation spectra in early-onset BCC (first lesion at age <35 years), regular BCC (first lesion at age $\geqslant 35$ years and <10 lesions), and multiple BCC ($\geqslant 10$ lesions). The *PTCH* gene was mutated in 29 of 60 cases (48%). Most of the *PTCH* mutations bore the UV fingerprint (i.e., C→T or tandem CC→TT transitions at dipyrimidine sites). However, neither the proportion nor the spectra of exonic *PTCH* mutations differed significantly among the three groups. A large number of SNPs (IVS10 + 99C/T, IVS11-51G/C, 1665T/C, 1686C/T, IVS15 + 9G/C, IVS16-80G/C, IVS17 + 21G/A, and 3944C/T or its combinations) were also detected, but again their incidence did not differ significantly among the groups. Interestingly, expression of the IVS16-80G/C and the IVS17 + 21G/A genotype did not achieve the Hardy–Weinberg equilibrium in patients with regular and/or early-onset BCC. These data suggest that a (UV-) mutated *PTCH* gene is important for sporadic BCC formation independent of clinical phenotype and that the IVS16-80G/C and/or IVS17 + 21G/A SNP site might be important for tumorigenesis in certain BCC patients.

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INTRODUCTION

Basal cell carcinoma (BCC) is the most common cancer in humans. The American Cancer Society estimates that, in the United States alone, more than 800,000 new cases are diagnosed each year (http://www.skincancer.org/). The pathogenic role of UV radiation in BCC tumorigenesis is clear. Most BCCs occur on sun-exposed body sites, and BCC incidence correlates unambiguously with sun exposure (Scotto *et al.*, 1996; Gailani *et al.*, 1996a; Kerb *et al.*, 1997, and citations therein). Xeroderma pigmentosum (XP), a hereditary disease involving defective DNA repair and the generation of UV-induced DNA photoproducts, is associated with an increased incidence of skin cancers including BCC. Non-melanoma skin cancers including BCC characteristically exhibit UV fingerprint mutations (i.e., C→T or tandem CC→TT transitions at dipyrimidine sites) in tumor suppressor genes such as *p53*, *patched* (*PTCH*), and *INK-4-ARF* and in proto-oncogenes such as *Ras* (Seidl *et al.*, 2001; Kreimer-Erlacher *et al.*, 2001, 2003; Wolf *et al.*, 2004). Moreover, according to evidence from transgenic animal studies with *PTCH*+/− mice, UV exposure leads to the formation of BCC-like lesions (Aszterbaum *et al.*, 1999).

However, even though UV exposure is considered the major risk factor for BCC, recent research suggests that some individuals may also be genetically predisposed to the disease (Heagerty *et al.*, 1994; Hoban *et al.*, 2002; Strange *et al.*, 2002). Indeed, a striking characteristic of BCC is the extent to which tumor accrual varies among patients (Ramachandran *et al.*, 2000). In the case of sporadic BCC, some patients may have only a solitary lesion at presentation and during follow-up, whereas others may exhibit multiple lesions at presentation and clustering of lesions during follow-up (Ramachandran *et al.*, 1999; and references cited therein). At our institution, we have identified over the last decade more than 16,000 patients with more than 32,000 BCC lesions (Ferra, 2002; P Wolf *et al.*, unpublished data). Of interest is that more than 200 patients in this patient population had early-onset BCC (i.e., their first BCC lesion was diagnosed before the age of 35 years), whereas another 245 patients had multiple BCCs (i.e., $\geqslant 10$ lesions). As others have previously suggested (Ramachandran *et al.*, 1999), it seems unlikely that differences in UV exposure alone would account for these

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Abbreviations: BCC, basal cell carcinoma; ECL, extracellular loop; LOH, loss of heterozygosity; NBCCS, nevoid basal cell carcinoma syndrome; PTCH, *patched gene; ROS, reactive oxygen species; TMD, transmembrane domain; XP, xeroderma pigmentosum*

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different BCC phenotypes. Indeed, a multivariate statistical analysis of phenotype, genotype, and environmental factors in our patient population revealed that the most significant independent risk factor in having single *versus* multiple BCC lesions, besides a history of painful or blistering sunburns (odds ratio of 2.4), was a family history of skin cancer (odds ratio of 6.0) (P Wolf *et al.*, unpublished data).

The most important recent advance in understanding the molecular biology of BCC has been the discovery that germline mutations in the human homologue of the *Drosophila PTCH* gene cause nevoid basal cell carcinoma syndrome (NBCCS) (Hahn *et al.*, 1996a; Johnson *et al.*, 1996). This rare autosomal-dominant disorder, which is also known as Gorlin syndrome (Gorlin, 1987), is characterized by frequent BCC lesions and is associated with an increased risk of developing other tumors (e.g., medulloblastomas, ovarian fibromas, meningiomas, fibrosarcomas, rhabdomyosarcomas, and cardiac fibromas). Phenotypically, patients with NBCCS exhibit odontogenic jaw cysts, palmar and plantar pits, and developmental abnormalities such as rib and craniofacial skeletal alterations. These patients inherit in all of their normal cells a mutation on one allele of the *PTCH* gene; moreover, BCCs and other tumors in these patients exhibit a second somatic mutation and/or loss of heterozygosity (LOH), thereby fulfilling the criteria of the two-hit model of molecular carcinogenesis and identifying *PTCH* as a tumor suppressor gene (Hahn *et al.*, 1996b; Unden *et al.*, 1996).

Shortly after this identification of *PTCH* as a tumor suppressor gene, Hahn *et al.* (1996a) isolated the complete human *PTCH* cDNA and mapped the gene to chromosome 9q22.3. Others subsequently determined that the *PTCH* gene encodes a transmembrane protein that functions as a receptor for the hedgehog family of proteins (Stone *et al.*, 1996). Binding of hedgehog to PTCH induces the release and activation of another cellular-membrane protein called smoothened. Release of the smoothened protein activates the downstream signalling pathway, which leads to the induction of a number of proteins via the Gli1 transcription factor, including transforming growth factor-β (Heberlein *et al.*, 1993), platelet-derived growth factor receptor-α (Xie *et al.*, 2001), Gli (Dahmane *et al.*, 1997), and even PTCH itself (Unden *et al.*, 1997). From studies in *Drosophila*, it is known that in the absence of hedgehog, *PTCH* and smoothened form a complex that keeps smoothened in an inactive state (Bale and Yu, 2001). A recent report by Bijlsma *et al.* (2006) showed that smoothened can be repressed by *PTCH*-dependent (pro-)vitamin D3 secretion. In skin, hedgehog signalling has been implicated in hair follicle growth and morphogenesis; mutational loss of normal PTCH function generates a constitutive hedgehog signal that promotes proliferation rather than differentiation. Although these data provide indirect support for the role of *PTCH* in BCC tumorigenesis, the exact mechanisms by which dysregulated hedgehog signalling leads to BCC tumorigenesis remain to be determined.

In any case, *PTCH* mutations occur frequently in sporadic BCC (Gailani *et al.*, 1996b; Hahn *et al.*, 1996b; Unden *et al.*, 1996) and XP-associated BCC (Bodak *et al.*, 1999; D'Errico *et al.*, 2000). They are also just as likely to occur in minute BCC lesions as in larger ones; and all histological subtypes of BCC, whether primary or recurrent, frequently exhibit LOH or *PTCH* mutation (Gailani *et al.*, 1996b; Unden *et al.*, 1996; Bodak *et al.*, 1999; D'Errico *et al.*, 2000). In this study, we analyzed the *PTCH* gene sequences in tumor specimens from three phenotypically distinct groups of patients in order to identify potential differences in their *PTCH* mutation spectra and potential tumor-causing agents, risk factors, and tumor-predisposing *PTCH* variants.

RESULTS

Patient demographics and clinical characteristics

Selected demographic and clinical characteristics of the patients whose archived tumors were analyzed in this study are summarized in Materials and Methods.

High overall frequency of exonic UV fingerprints on the *PTCH* gene in BCC

As shown by PCR and subsequent direct automated sequencing of the *PTCH* gene (exons 2–23), exonic missense/stop base and/or intronic splice site *PTCH* mutations were found in 29 of 60 BCC (48%). Exonic *PTCH* mutations ($n=28$) occurred frequently in all three patient groups. These mutations originated most frequently from group A (43% (12/28)) and group C (39% (11/28)) and less from group B (18% (5/28)) (Table 1 and Figure 1). Of note, one tumor from group A (A14) and two tumors from group C (C2 and C4) carried two *PTCH* mutations. Statistical analysis revealed no significant differences in the number and type of exonic mutations among the different groups. Most of the exonic mutations (68% (19/28)) bore the UV fingerprint of C→T transition (63% (12/19)) or tandem CC→TT transitions (37% (7/19)) at dipyrimidine sites. The remaining mutations were classified as being of either the reactive oxygen species (ROS) fingerprint (i.e., G:C→T:A transversion) (11% (3/28)) or other type (21% (6/28)). Only two tumors (A12 and C4) had the same mutation (i.e., a C→T transition at codon 1051). Interestingly, most of the exonic mutations that were detected (54% (15/28)) were nonsense mutations, whose predicted amino-acid sequence encoded a stop signal (Table 2). Mutations in the PTCH protein occurred predominantly in the extracellular loop (ECL) (54% (15/28)) and the transmembrane domain (TMD) (25% (7/28)). The remainder occurred in the cytoplasmic loop (11% (3/11)), N-terminus (7% (2/28)), and C-terminus (4% (1/28)). The ECL mutations were further distributed among the ECL1 (8 (29%)), ECL3 (1 (4%)]), and ECL4 (6 (21%)). Those in the TMD were further distributed among the TMD1 (1 (4%)), TMD2 (1 (4%)), TMD3 (1 (4%)), TMD4 (1 (4%)), TMD5 (2 (7%)), and TMD12 (1 (4%)). Those in the cytoplasmic loop were further distributed among the cytoplasmic loop 2 (1 (4%)) and cytoplasmic loop 4 (2 (7%)). However, when analyzed for homogeneity of mutation distribution with regard to the size of the different domains, the predominance of mutations at the ECL1, ECL4, and TMD sites did not reach statistical significance, as the observed mutation frequencies in those regions were close to the expected mutation frequencies and fell within the 95% confidence intervals (data not shown). In addition, eight silent mutations were detected in group A and four in group B.

Table 1. *PTCH* mutations in BCCs from different patient groups

Group[1]	Tumor	Exon/intronic position	Codon	Codon surrounding sequence[2]	Base change	Strand[3]	Amino-acid change	Location in protein[4]	Type of mutation[5]
A	A2	12	568	t**CC**g	CC→TT	T	Pro→Leu	TMD 5	UV
	A4	12	539	g**G**AGt	G→T	T	Glu→Stop	CPL 2	ROS
	A7	12	563	cG**C**Gt	C→T	T	Ala→Val	TMD 5	Other
	A9	6	278	c**C**CAg	C→T	NT	Trp→Stop	ECL 1	UV
	A12	18	1051	t**C**CAg	C→T	NT	Trp→Stop	CPL 4	UV
	A13	5	237	cTT**C**c	C→T	NT	Glu→Lys	ECL 1	UV
	A14	11	504	aT**G**Gc	G→T	NT	Pro→Gln	TMD 4	ROS
		22	1237	gCT**Cc**	CC→TT	NT	Glu→Lys	N-term	UV
	A15	9	417	t**C**AAa	C→T	T	Gln→Stop	ECL 1	UV
	A18	23	1396	gTC**C**c	C→T	NT	Gly→Arg	N-term	UV
	A19	17	926	c**CC**Aa	CC→TT	NT	Trp→Stop	ECL 4	UV
	A20	3	184	**c**CAGg	CC→TT	T	Gln→Stop	ECL 1	UV
B	B1	15	816	c**C**AGc	C→T	T	Gln→Stop	ECL 4	UV
	B2	10	494	tT**C**Ct	C→T	T	Ser→Phe	TMD 2	UV
		IVS10-19	—	cc**c**cc	C→T	T	—	—	UV
		IVS10+68	—	gc**c**ag	C→T	T	—	—	UV
	B5	IVS19+16	—	tt**c**aa	C→T	NT	—	—	UV
	B6	3	159	g**A**TAc	A→T	T	Ile→Leu	ECL 1	Other
	B7	2	115	aT**CC**c	CC→TT	NT	Gly→Lys	TMD 1	UV
	B9	IVS13+34	—	ac**c**ca	C→T	NT	—	—	UV
	B12	IVS20+1	—	ta**c**CT	C→T	NT	—	—	UV; splice
	B14	IVS13-1	—	GC**c**tg	C→T	NT	—	—	UV; splice
	B18	IVS9-6	—	ga**c**ca	C→T	T	—	—	UV
	B20	12	576	c**C**AGg	C→T	T	Gln→Stop	ECL 3	UV
C	C2	3	149	ga**G**AAga	G→T	T	Glu→Stop	ECL 1	ROS
		18	1016	tc**C**TCtt	C→T	T	Leu→Phe	ECL 4	UV
	C3	10	480	tg**T**CAgt	C→G	T	Ser→Stop	TMD 3	Other
		IVS13+3	—	gt**a**ca	A→T	T	—	—	Other splice
	C4	2	96	aa**A**AAaa	A→T	T	Lys→Stop	C-term	Other
		18	1051	gt**C**CAgg	C→T	NT	Trp→Stop	CPL 4	UV
	C5	8	390	a**gG**ACaa	GG→AT	T	Asp→Tyr	ECL 1	Other
	C6	5	236	t**cC**CAga	CC→TT	NT	Trp→Stop	ECL 1	UV
	C8	18	1018	t**cC**CAga	CC→TT	NT	Trp→Stop	ECL 4	UV
		IVS19+34	—	ga**a**aa	A→C	T	—	—	ROS
	C9	15	791	ttA**T**Tgc	T→C	T	Ile→Thr	ECL 4	Other
	C14	21	1167	agCCCat	C→T	NT	Gly→Arg	TMD 12	UV
	C18	IVS+5	—	ta**c**tg	C→T	NT	—	—	UV; splice
	C19	15	816	c**C**AGc	C→T	T	Gln→Stop	ECL 4	UV
	C20	IVS19+1	—	ta**c**CA	C→T	NT	—	—	UV; splice

BCC, basal cell carcinoma; *PTCH*, patched gene.
[1]Groups are defined in Materials and Methods: group A, early-onset BCC; group B, multiple BCC; group C, regular BCC.
[2]The sequence for the strand (transcribed or non-transcribed) containing a pyrimidine at the mutation site is shown in the 5′→3′ direction. Nucleotides of the affected codon are written in capital letters. Mutated bases are shown in bold letters.
[3]Strand with affected pyrimidine: T, transcribed strand; NT, non-transcribed strand.
[4]Location in the PTCH protein: TMD, transmembrane domain; ECL, extracellular loop; CPL, cytoplasmatic loop; C-term, carboxyl-terminus; N-Term, amine-terminus.
[5]Types of mutation are defined in Materials and Methods. UV, UV fingerprint; ROS, reactive oxygen species fingerprint. Splice, mutation that alters the splice-site consensus sequence.

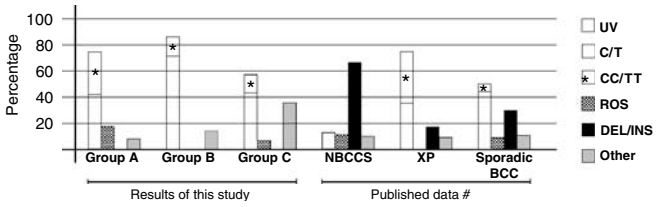

Figure 1. *PTCH* mutation spectra of BCC lesions in this study and in the literature. Tumor samples from three groups of patients seen at our institution were analyzed for *PTCH* mutation spectra ($n=20$ patients, each group): group A, early-onset BCC; group B, multiple BCC; group C, regular BCC. *Percentages for groups A–C are based on the numbers of patients in our study population ($n=60$); percentages for NBCCS-associated BCC, XP-associated BCC, and sporadic BCC are based on the numbers of patients in the studies reported in the literature (NBCCS, $n=132$; XP, $n=31$; sporadic BCC, $n=86$) (Lindström *et al.*, 2006). Tumor samples were analyzed for the presence of UV fingerprint (UV), deletion/insertion (DEL/INS), ROS fingerprint, and other mutations. Overall, the mutation spectra in our study population differed significantly from those reported for NBCCS-associated BCC ($P<0.001$; χ^2-test) and sporadic BCC ($P=0.04$) in the literature.

Similar to exonic mutations most of these silent mutations (75% (9/12)) had the UV fingerprint. Subsequent comparison of DNA from tumor and adjacent normal tissue samples revealed that every specific mutation, whether silent or not, occurred only in tumor tissue, thus indicating that each mutation was somatic.

Presence of intronic *PTCH* mutations in BCC

In addition to mutations in the coding region of the *PTCH* gene, a total of seven intronic mutations were detected in 30% (6/20) of group B lesions and a total of four intronic mutations in 20% (4/20) of group C lesions; none were detected in group A lesions (Table 1). The difference between the number of intronic mutations in groups B and C *versus* group A was also significant ($P=0.02$). As in the case of the exonic mutations, most of the observed intronic mutations (82% (9/11)) bore the UV fingerprint; the remainder were classified as being of either the ROS fingerprint (9% (1/11)) or other type (9% (1/11)). On the basis of predicted amino-acid sequences, most of the intronic mutations (55% (6/11)) would have had no affect on the splice site of the *PTCH* gene; approximately one-third (36% (4/11)) would have affected the 5′ splice site, and only one would have affected the 3′ splice site. Interestingly, one tumor (B2) bore two intronic C→T mutations as well as an exonic C→T mutation at codon 10 (Table 1). In addition, two other tumors (C3 and C8) each carried one intronic and one exonic mutation. Important to note is that intronic mutations in tumors B12, B14, C3, C18, and C20 apparently led to aberrant or less efficient splicing. This was indicated by the lowering of the consensus splice site score in those tumors (Shapiro and Senapathy, 1987; www.genet.sickkids.on.ca/~ali/splicesitescore.html) (data not shown).

Comparison of *PTCH* mutation spectra of BCC in this study *versus* in the literature

In our study population, mutation of the *PTCH* gene, whether by exonic (missense/stop base) mutation or intronic (splice site) mutation, was observed in 11 (55%), 7 (35%), and 11 (55%) of BCC in groups A, B, and C, respectively (Table 1 and Figure 1). The mutation spectra in these three different groups were compared with mutation spectra for NBCCS-associated BCC and sporadic BCC recently reported by Lindström *et al.*, (2006). Lindström *et al.* (2006) derived their mutation spectra by pooling data from previous sequencing studies of the *PTCH* locus in sporadic, NBCCS-associated, and XP-associated BCC. Comparison of our mutation spectra against those reported by Lindström *et al.* (2006) of 249 mutations that had been included in the *PTCH* mutation database (http://www.cybergene.se/cgi-bin/w3-msql/ptchbase/index.html) as of 30 June 2005 (Figure 1) revealed statistically significant differences between our overall mutation spectra and those for NBCCS-associated BCC ($P<0,001$; χ^2-test) and sporadic BCC ($P=0.04$). These significant differences were mainly due to the predominance of deletion/insertion mutations in the mutation spectra of NBCCS-associated BCC lesions (66% (87/132)) and the relatively frequent occurrence of these mutations in sporadic BCC lesions (30% (26/86)), as reported by Lindström *et al.* (2006). In contrast, we were unable to detect any deletion or insertion mutations in the BCC lesions obtained from our archives. Instead, except for seven tandem CC→TT transitions and one two-nucleotide (GG→AT) substitution in tumor C5, most of the mutations we detected were single-nucleotide substitutions (Table 1). There was no statistically significant difference between our mutation spectra data and those of XP-associated BCC (Lindström *et al.*, 2006).

Differences in frequency of *PTCH* single-nucleotide polymorphism variants and LOH in BCC among patient groups

Eight previously described single-nucleotide polymorphism (SNP) variants (IVS10+99C/T, IVS11–51G/C, 1665T/C, 1686C/T, IVS15+9G/C, IVS16-80G/C, IVS17+21G/A, and 3944C/T) were detected in a large number of BCC in our study population, and their genotype frequencies for the SNPs and ancestral alleles are presented in Table 2. Statistical analysis using a global test (Goeman *et al.*, 2004) revealed a significant difference in SNP genotype distribution among the different groups ($P=0.033$). Statistical analysis after Bonferroni correction revealed no significant difference in the frequencies of SNPs among the different BCC groups. Moreover, with two exceptions, all *PTCH* SNPs achieved the Hardy–Weinberg equilibrium in all three BCC groups (Table 2). The exceptions were the IVS16-80G/C and the IVS17+21G/A locus. Indeed, samples heterozygous for IVS16-80G/C were significantly less frequent than expected from the allele frequencies generated for groups A ($P=0.0232$) and C ($P=0.0004$) individually and for all three groups as a whole ($P=0.0003$). Those samples heterozygous for IVS17+21G/A were significantly more frequent than expected from the allele frequencies generated for group C ($P=0.0198$). Importantly, similar results were obtained for the SNP analysis when data were subjected to statistical analysis separately for tumor tissue samples with mutations or normal tissue samples of tumor adjacent skin of tumors with mutations or a combination of both (data not shown). Moreover, when the SNP sequences of interest were

Table 2. Genotype and allelic frequencies of *PTCH* variants in BCCs from different patient groups

SNP	Group[1]	p11[2]	p12[3]	p22[4]	p gt[5]	p1[6]	p2[7]	p allele[8]	D-HWE[9]	P HWE[10]
IVS10+99C/T	A	0.85	0.1500	—		0.93	0.07		0.011	
	B	1.00	—	—		1.00	0.00		NA	
	C	0.80	0.20	—		0.90	0.10		0.020	
	All	0.88	0.12	—	NS	0.94	0.06	NS	0.007	NS
IVS11-51 C/T	A	0.55	0.35	0.10		0.73	0.27		−0.049	
	B	0.65	0.20	0.15		0.75	0.25		−0.175	
	C	0.50	0.25	0.25		0.63	0.37		−0.219	
	All	0.57	0.26	0.17	NS	0.70	0.30	NS	−0.153	NS
1665 T/C	A	0.80	0.10	0.10		0.85	0.15		−0.155	
	B	0.90	0.10	—		0.95	0.05		0.005	
	C	0.60	0.35	0.05		0.78	0.22		0.001	
	All	0.77	0.18	0.05	NS	0.86	0.14	NS	−0.060	NS
1686 C/T	A	0.75	0.25	—		0.88	0.12		0.031	.
	B	0.80	0.20	—		0.90	0.10		0.020	
	C	0.90	0.10	—		0.95	0.05		0.005	.
	All	0.82	0.18	—	NS	0.91	0.09	NS	0.017	NS
IVS15+9G/C	A	0.30	0.40	0.30		0.50	0.50		−0.100	
	B	0.40	0.40	0.20		0.60	0.40		−0.080	
	C	0.25	0.50	0.25		0.50	0.50		0.000	
	All	0.32	0.43	0.25	NS	0.53	0.47	NS	−0.064	NS
IVS16-80 G/C	A	0.70	0.15	0.15		0.78	0.22		−0.199	**0.0232**
	B	0.25	0.40	0.35		0.45	0.55		−0.095	NS
	C	0.55	0.10	0.35		0.60	0.40		−0.380	**0.0004**
	All	0.50	0.22	0.28	0.0278	0.61	0.39	0.0121	−0.260	**0.0003**
IVS17+21 G/A	A	0.20	0.60	0.20		0.50	0.50		0.100	
	B	0.10	0.45	0.45		0.32	0.68		0.011	
	C	0.15	0.80	0.05		0.55	0.45		0.305	**0.0198**
	All	0.15	0.62	0.23	0.0460	0.46	0.54	NS	0.120	NS
3944 C/T	A	0.35	0.55	0.10		0.63	0.37		0.081	
	B	0.15	0.50	0.35		0.40	0.60		0.020	
	C	0.30	0.60	0.10		0.60	0.40		0.120	
	All	0.27	0.55	0.18	NS	0.54	0.46	NS	0.053	NS

BCC, basal cell carcinoma; NA, not applicable; NS, not significant; PTCH, patched gene; SNP, single-nucleotide polymorphism.
[1]Groups are defined in Materials and Methods: group A, early-onset BCC; group B, multiple BCC; group C, regular BCC,
[2]Genotype frequency for homozygous ancestral allele.
[3]Genotype frequency heterozygous ancestral allele.
[4]Genotype frequency for homozygous SNP.
[5]χ^2 test comparing genotype frequencies among the groups. The global test over all SNPs was significant (P=0.033).
[6]Allelic frequency for ancestral allele.
[7]Allelic frequency for SNP.
[8]χ^2 test comparing allelic frequencies among groups. The global test over all SNPs was not significant (P=0.054).
[9]D-HWE (difference from Hardy–Weinberg equilibrium) defined as p12/2 – p1* p2. Negative values indicate less heterozygocity than expected.
[10]χ^2 test for Hardy–Weinberg equilibrium (HWE) within each group and for the pooled data. The HWE within groups was only tested when the overall test revealed a significant difference.
Significant P-values after Bonferroni correction are shown in boldface type.

compared in *PTCH*-mutated tumor tissue and adjacent normal tissue, a change from heterozygosity to homozygosity suggestive of LOH was noted in many cases (i.e., 5 (45%) in group A, 3 (60%) in group B, and 5 (55%) in group C) (Table 3).

DISCUSSION

In one of the largest studies (if not the largest study) of *PTCH* gene mutation spectra in sporadic BCC, we have found that almost half of the tumors we examined bore *PTCH* gene mutations (48% (29/60)). Our data, which we obtained by analyzing archived tumors from three phenotypically distinct patient groups (i.e., early-onset, multiple, and regular BCC), also indicate that most of these mutations occurred as exonic missense/stop base mutations or intronic changes. Moreover, most of these exonic and intronic mutations (68 and 82%, respectively) bore the UV fingerprint (i.e., C→T or tandem CC→TT transitions at dipyrimidine sites), whereas the remainder bore ROS fingerprint (Ruggeri *et al.*, 1993) or other types of mutations.

That the percentage and type of exonic *PTCH* mutations did not differ significantly among our three patient groups indicates that a (UV-) mutated *PTCH* gene is important for sporadic BCC formation independent of clinical phenotype (i.e., regular, multiple, or early-onset BCC). It could be argued that the high overall proportion of UV fingerprint mutations observed in our study might be due to the overall predominance of UV fingerprint-bearing lesions originating in the head and neck region (59% (34/58)). It is presumed that the head and neck receive cumulatively more sunlight than do other body sites. Surprisingly, however, taking together exonic and intronic mutations the proportion of UV fingerprint-bearing BCC lesions originating on the trunk and extremities was even greater than the proportion of those originating in the head and neck region (93% (13/14) *vs* 58% (14/24)) (data not shown).

Some investigators have proposed cigarette smoking as a potential factor or cofactor in BCC formation (Cohen and Rogers, 1995; Strange *et al.*, 2002), and this hypothesis certainly has merit. Indeed, one specific mutation identified in our analyses (i.e., G:C→T:A transversion) has been attributed to oxidative damage of the kind inflicted by the polycyclic aromatic hydrocarbon, benzo[*a*]pyrene, found in cigarette smoke and crude coal tar products. However, the relative scarcity of such mutations in our study population argues against a causative role for this mutation. Only three such mutations were found in our study population: two in group A and one in group C. Moreover, it must be remembered that G:C→T:A transversions may also arise from oxidative UV(A) damage inflicted via singlet oxygen-induced production of 8-hydroxyguanosine (Ichihashi *et al.*, 2003 and references cited therein; Agar *et al.*, 2004).

The overall percentage of *PTCH*-mutated sporadic BCC in our study population (48% (29/60)) is consistent with the percentages (i.e., 40–50%) reported in the literature (Chidambaram *et al.*, 1996; Gailani *et al.*, 1996b; Hahn *et al.*, 1996b; Unden *et al.*, 1996; Wolter *et al.*, 1997; Aszterbaum *et al.*, 1998). However, the main difference between the mutation spectra of sporadic BCC reported here and in the literature is the lack of deletions and insertions (Figure 1). Consequently, the proportion of UV fingerprint mutations in our study is relatively higher. Indeed, the frequency of C→T and tandem CC→TT transitions in our population of patients with sporadic BCC is similar to that reported for a population of patients with XP-associated BCC (Figure 1). Although we can offer no definite explanation for this difference, one possibility is that the *PTCH* mutation data used in the previously published studies came from a ''contaminated'' open data bank. For example, mutations in sporadic BCC that have been recorded in the *PTCH* data bank (http://www.cybergene.se/cgi-bin/w3-msql/ptchbase/index.html) might have been contaminated by the entry of data obtained from *PTCH*-mutated non-sporadic BCC lesions associated with NBCCS or other syndromes. Such contamination can be ruled out in this study, however, as all of the BCC specimens that we analyzed came from a very well-defined and thoroughly screened population of patients seen at our institution (Ferra, 2002).

A substantial proportion of exonic mutations (36% (10/28)) in our study population occurred at *PTCH* mutation sites already entered in the *PTCH* data bank. Indeed, five tumors (i.e., A13, A15, A20, B20, and C14) exhibited base changes identical to those previously entered for the applicable mutation sites. Conversely, another five tumors (i.e., A4, A14 (codon 22 mutation), A19, B1, and C8) exhibited base changes different from those previously entered for the applicable mutation sites. Consistent with previous work regarding sporadic BCC (Lindström *et al.*, 2006), most of the exonic *PTCH* mutations in our study population were clustered in the ECL (54% (15/28)), particularly ECL1 (29% (8/28)) and ECL4 (21% (6/28)), a region shown to be functionally important in several studies (Marigo *et al.*, 1996; Johnson *et al.*, 2000; references cited in Lindström *et al.*, 2006). It has been suggested that hedgehog binding occurs at ECL1 and ECL4 (Marigo *et al.*, 1996) and that smoothened is inhibited at the large intracellular loop and the N-terminus (Ming *et al.*, 2002). In our study, a quarter of the exonic *PTCH* mutations (25% (7/28)) were located in different parts of the TMD, including TMD2 through TMD6, a region that is associated with the sterol-sensing domain. Some investigators have suggested that the sterol-sensing domain is an important region in the PTCH protein, as its proper function is thought to be required for the lipid binding of Niemann-Pick type C protein, a peptide that is closely related to PTCH and involved in lipid trafficking (Ohgami *et al.*, 2004). In addition to the exonic mutations, a substantial proportion of intronic mutations were detected in patients with the multiple BCC and regular BCC phenotypes. Importantly, almost half of the intronic mutations (45% (5/11)) affected splice sites, which in turn lowered the splice site consensus scores of the affected lesions considerably (Shapiro and Senapathy, 1987; www.genet.sickkids.on.ca/~ali/splicesitescore.html) and apparently led to aberrant or less efficient splicing.

LOH at chromosome 9q22 has been observed in 60–70% of BCC tumors, although such estimates of the extent of loss remain imprecise (Danaee *et al.*, 2006). Previous investigators

Table 3. Differences in SNP variant sequences in *PTCH*-mutated tumors and adjacent normal tissue

Sample	Sequence difference[1]	Tissue	SNP[2]							
			IVS10+99C/T	IVS11 −51C/G	1665 T/C	1686 C/T	IVS15 +9G/C	IVS16-80G/C	IVS17+21G/A	3944 C/T
A2	No									
A4	Yes	N	C[3]	G/C	T	C	G/C	G/C	G/A	C
		T	C	G/C	T	C	G*	G*	G/A	C
A7	Yes	N	C/T	C	T/C	C	G/C	G/C	G/A	C
		T	C/T	C	C*	C	C*	C*	G/A	C
A9	No									
A12	No									
A13	Yes	N	C/T	G/C	T	C/T	G/C	G	G/A	C/T
		T	C/T	G/C	T	C*	G/C	G	G/A	C/T
A14	Yes	N	C	G/C	T/C	C	G/C	G/C	G/A	C/T
		T	C	C*	T*	C	G*	G*	A*	C/T
A15	No									
A18	Yes	N	C	C	T	C/T	G/C	G/C	G/A	C/T
		T	C	C	T	C*	C*	G*	G*	C/T
A19	No									
A20	No									
B1	Yes	N	C	G	T/C	C	G/C	G/C	G/A	C/T
		T	C	G	T*	C	G/C	G/C	G/A	C/T
B2	No									
B6	Yes	N	C	C	T	C	G	G/C	G/A	C/T
		T	C	C	T	C	G	C*	A*	T*
B7	No									
B20	Yes	N	C	G	T/C	C/T	G/C	G/C	G/A	C/T
		T	C	G	T*	C*	G*	G*	A*	C/T
C2	Yes	N	C	G	T	C	C	G	G/A	C
		T	C	G	T	C	C	G	G*	C
C3	No									
C4	Yes	N	C	C	T/C	C/T	G/C	G/C	G/A	C/T
		T	C	C	T/C	C/T	C*	C*	G*	C/T
C5	Yes	N	C	C	T/C	C/T	G/C	G	G/A	C/T
		T	C	C	T*	C*	C*	G	A*	C/T
C6	Yes	N	C	G/C	T/C	C	G	G	G/A	C/T
		T	C	G/C	T*	C	G	G	G/A	C/T
C8	Yes	N	C	C	T/C	C	G/C	G	G/A	C/T
		T	C	C	T*	C	G/C	G	G/A	C/T
C9	No									
C14	No									
C19	No									

N, normal tissue; *PTCH*, patched gene; SNP, single-nucleotide polymorphism; T, tumor tissue.
[1]Sequence difference at any SNP site.
[2]Database of single-nucleotide polymorphisms (dbSNP), National Center for Biotechnology Information, National Library of Medicine (Bethesda, MD, (dbSNP accession). Build ID: rs574688 IVS11-51C/G, rs1805155 1665T/C, rs2066836 1686C/T, rs2066829 IVS15+9G/C, rs2274692 IVS16-8G/C0, rs2236406 IVS17+21G/A, rs357564 3944C/T. Available at: http://www.ncbi.nlm.nih.gov/SNP/.
[3]Single letter indicates homozygosity for that base at a specific SNP locus.
*In 13 of 25 samples with *PTCH* mutations (52%), comparison of normal (heterozygous) and tumor tissue (homozygous) revealed a sequence difference indicating loss of heterozygosity (LOH).

have inferred the loss of *PTCH* alleles from the loss of microsatellite markers adjacent to the *PTCH* gene (Hahn *et al.*, 1996b; Holmberg *et al.*, 1996; Johnson *et al.*, 1996; Shen *et al.*, 1999; Ling *et al.*, 2001; Kim *et al.*, 2002; Asplund *et al.*, 2005; Reifenberger *et al.*, 2005). We did look for LOH by taking advantage of the presence of a large number

of previously described SNPs (i.e., IVS10 + 99C/T, IVS11–51G/C, 1665T/C, 1686C/T, IVS15 + 9G/C, IVS16–80G/C, IVS17 + 21G/A, and 3944C/T or its combinations). In brief, we analyzed the sequences of various SNP-containing *PTCH* exons in *PTCH*-mutated tumor tissue and corresponding adjacent normal tissue. By our estimate, over half of the tissue pairs we examined (52% (13/25) showed LOH, as manifested by SNP homozygosity in the tumor specimen but heterozygosity in the normal tissue specimen (Table 3). However, despite this apparent prevalence of LOH in tumors-bearing *PTCH* mutations, it was notable that the tumors did not always lose the entire *PTCH* allele. Indeed, some of these tumors became homozygous for one or more SNPs but remained heterozygous for others. This is consistent with a recent report by Soufir *et al.* (2006), in which microsatellite analysis showed LOH for large parts (exons 4–23) but not the entire *PTCH* gene in a NBCCS case. Large deletions (Soufir *et al.*, 2006) or, in the case of *PTCH*-mutated tumors, other mechanisms, such as nondisjunction and duplication, mitotic recombination, or gene conversion (Alberts *et al.*, 2002), may have been responsible for LOH observed in our study.

Although exposure to UV radiation is thought to be the main risk factor for BCC development, recent studies suggest that susceptibility to BCC may also be enhanced by allelic SNP variations in the *PTCH* gene (Strange *et al.*, 2004a, b; Asplund *et al.*, 2005; Liboutet *et al.*, 2006). For instance, Liboutet *et al.* (2006) reported that the 3944C/C polymorphism (as opposed to the 3944C/T polymorphism) is significantly overrepresented in patients with multiple BCC. Strange *et al.* (2004a, b) have suggested that *PTCH* haplotypes including the 3944C/T polymorphism in combination with IVS15 + 9G/C or 1686C/T have tumorigenic potential. Thus, we looked for an association between SNP distribution and the risk of early-onset and multiple BCC; we found none. However, we did identify an apparent, although not significant difference in IVS16-80G/C and IVS17 + 21G/A genotype frequency among groups (Table 2). We also noted that the IVS16-80G/C and IVS17 + 21G/A genotype frequency did not achieve the Hardy–Weinberg equilibrium in the regular and/or early-onset patient group (Table 2), indicating that, in particular, this SNP sites might be critical for BCC formation. Finally, we observed that the overall frequency of the 3944C/C genotype in our study was notably lower than that in the study by Strange *et al.* However, because we had not gathered control data from a normal population of BCC-free individuals, we could not draw any general conclusions about the significance of the 3944C/C genotype in BCC.

Finally, the fact that we were unable to detect *PTCH* mutations in over half of the BCC specimens we examined (52% (31/60)) raises the possibility that other mechanisms besides *PTCH* mutations may be involved in inactivating the gene in many cases of sporadic BCC. For instance, somatic mosaicisms (Soufir *et al.*, 2006), promoter methylation, haploinsufficiency, or other genes involved in the hedgehog signalling pathway (Dahmane *et al.*, 1997; Xie *et al.*, 1998; Reifenberger *et al.*, 2005) may play a role. Consequently, we have begun such studies.

MATERIALS AND METHODS

Patients and tumor samples

The BCC samples analyzed in this study were obtained from patients who had been previously enrolled in an epidemiologic study of BCC occurrence in the State of Styria, Austria (Ferra, 2002). That study had been approved by the ethical committee of the Medical University of Graz, Austria. All patients in that study had provided tumor samples that were embedded in paraffin and subsequently archived in the Histopathology Unit of the Department of Dermatology at the Medical University of Graz. Those patients who were still alive at the time of this study gave their informed consent to DNA analysis of those tumor samples. The study was conducted according to the Declaration of Helsinki Principles.

For this study, archived tumor samples were taken for DNA analysis from three phenotypically distinct groups of 20 randomly selected patients each. The three groups were defined as early-onset BCC (i.e., a first BCC occurring before the age of 35 years) (group A) (3 men and 17 women; median age, 32.5 years; range 21–34 years; median number of BCC, 1; range, 1–18), multiple BCC (i.e., $\geqslant$10 lesions) (group B) (11 men and 9 women; median age, 67 years; range 26–90 years; median number of BCC, 15; range, 10–36), and regular BCC (i.e., a first BCC at the age of 35 years or older and $<$10 lesions) (group C) (11 men and 9 women; median age, 69 years; range 45–77 years; median number of BCC, 1; range, 1–6). The body site location of the BCC in the different patient groups was as follows: head and neck, 10; trunk, 8; extremities, 0; and body site location not available, 2 for group A; head and neck, 11; trunk, 5; extremities, 4 for group B; and head and neck, 13; trunk, 5; extremities, 2 for group C.

DNA extraction

DNA was extracted from the paraffin-embedded tumor samples as follows. Formalin-fixed, paraffin-embedded tissue samples were sliced into 5-μm-thick sections, placed on slides, deparaffinized with xylene for 10 minutes, incubated in ethanol (100%) for 10 minutes, and air dried. Samples were manually dissected to reduce the proportion of non-tumor cells in the samples. Tissues were then scraped off the slides and processed for DNA extraction using the QIAquick gel extraction kit (Qiagen, Vienna, Austria).

PCR amplification of *PTCH* gene

The *PTCH* gene in tumor samples was amplified by PCR as follows. The template for PCR consisted of 50–100 ng of tumor DNA in 50 μl of a solution containing PCR-10 $\times$ buffer (100 mM Tris-HCl, pH 8.3; 500 mM KCl), 1.5 mM $MgCl_2$, 200 μM of each deoxyribonucleoside triphosphate, 15 pmol (200 nM) of the upstream and downstream primers for the respective exons 3–23, and 2.5 U of AmpliTaq Gold Polymerase (Applied Biosystems, Vienna, Austria). PCR of exon 2 was performed in 50 μl of a solution containing PCR-10 $\times$ buffer (100 mM Tris-HCl, pH 8.3; 50 mM KCl, 15 mM $MgCl_2$), 200 μM of each deoxyribonucleoside triphosphate, 15 pmol (200 nM) of the appropriate upstream and downstream primers, and 1.25 U of HotStar polymerase (Applied Biosystems). The primer sequences of the *PTCH* gene had been previously published (Hahn *et al.* 1996b; Xie *et al.* 1997; Fujii *et al.*, 2003). Reaction mixtures were subjected to 40 cycles of amplification in a thermocycler (MyCycler™; www.bio-rad.com). Before the first cycle, tubes containing PCR mixtures were incubated for 12 minutes at 94°C (15 minute at 94°C in the case of *PTCH* exon 2). Each cycle consisted of denaturation at 94°C for

30 seconds, annealing at 55 or 60°C for 30 seconds, and polymerization at 72°C for 1 minute. After the last cycle, PCR mixtures were incubated at 72°C for 10 minutes. PCR reaction tubes containing no template DNA were included in each PCR run as negative controls for potential contamination of PCR reactions. The amplified products were then purified by gel electrophoresis on a 2.5% MetaPhor gel (Cambrex; Rockland, ME, USA) and extracted from the gels using a commercially available system, according to the manufacturer's recommended protocol (Wizard® SV Gel and PCR clean-up system; Promega; Mannheim, Germany).

Direct DNA sequencing

All purified DNA samples were sequenced using 3.2 pM of primer, 30 ng of DNA, and sequencing reagents (Big Dye® Terminator v1.1 Cycle Sequencing Kit; Applied Biosystems); purified on spin post-reaction clean-up plates (Sigma-Aldrich, Vienna, Austria), and separated in an ABI 3130 system (Applied Biosystems). Both DNA strands were sequenced, and mutations on one strand were always confirmed on the opposite strand. DNA samples from normal tissue and placenta were analyzed simultaneously with the purified DNA samples from tumor tissue in order to rule out PCR-generated mutations. DNA sequence analysis was performed on a personal computer equipped with SeqScape® software (Applied Biosystems). Sequences were aligned according to GenBank entry U59464.

PTCH mutation classification

PTCH mutations were defined and classified as described previously (Ichihashi *et al.*, 2003 and references cited therein; Agar *et al.*, 2004). UV fingerprints were defined as C→T or tandem CC→TT transitions at dipyrimidine sites. ROS fingerprints were defined as G:C→T:A transversions. All other mutations (except deletions and insertions) were defined as ''other''.

Statistical analysis

All statistical tests were two-sided at the 5% error level. In cases of multiple testing, the family-wise error level was maintained by Bonferroni correction (i.e., by dividing the significance level by the number of tests performed). Fisher's exact test was used for two-group comparisons of relative mutation frequencies. A global test (Goeman *et al.*, 2004) was performed in order to test the difference of SNP genotypes between groups. The homogeneity of allele frequencies and the Hardy–Weinberg equilibrium were analyzed using the exact χ^2-test and the R-packages ''coin'' and ''genetics'' (www.r-project.org). The uniformity of distribution of mutations on the PTCH protein was tested using the exact tests for homogeneity of Poisson distributions and the χ^2-test for independence of r by c tables using StatXact 7 statistical software (www.cytel.com).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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