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Are There Low-Penetrance *TP53* Alleles? Evidence from Childhood Adrenocortical Tumors

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Summary

We have analyzed a panel of 14 cases of childhood adrenocortical tumors unselected for family history and have identified germline *TP53* mutations in >80%, making this the highest known incidence of a germline mutation in a tumor-suppressor gene in any cancer. The spectrum of germline *TP53* mutations detected is remarkably limited. Analysis of tumor tissue for loss of constitutional heterozygosity, with respect to the germline mutant allele and the occurrence of other somatic *TP53* mutations, indicates complex sequences of genetic events in a number of tumors. None of the families had cancer histories that conformed to the criteria for Li-Fraumeni syndrome, but, in some families, we were able to demonstrate that the mutation had been inherited. In these families there were gene carriers unaffected in their 40s and 50s, and there were others with relatively late-onset cancers. These data provide evidence that certain *TP53* alleles confer relatively low penetrance for predisposition to the development of cancer, and they imply that deleterious *TP53* mutations may be more frequent in the population than has been estimated previously. Our findings have considerable implications for the clinical management of children with andrenocortical tumors and their parents, in terms of both genetic testing and the early detection and treatment of tumors.

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Introduction

Childhood adrenocortical tumors are rare, with an incidence of 0.3/million children/year. Adrenocortical carcinoma (ACC [MIM 202300]) shows an apparent bimodal age distribution, with a median age at onset of childhood disease of 3 years, and a median age at onset of adult disease of 55–59 years of age (data based on information from the Manchester Children's Tumor Registry, England, and the Office of National Statistics, Wales). Childhood ACC is known to occur at a considerably increased frequency in patients with Beckwith-Wiedemann syndrome (BWS [Wiedemann 1983]), and in families with Li-Fraumeni syndrome (LFS [Li et al. 1988]). In a high proportion of the latter, the cancer-prone condition is associated with inheritance of a germline mutation in the *TP53* gene (Malkin et al. 1990; Birch et al. 1994*a*; Varley et al. 1997*a*). In families affected by LFS and including childhood ACC, the disease appears to be highly penetrant, with other carriers of the germline *TP53* mutation presenting with typical LFS tumors (e.g., bone and soft-tissue sarcomas and breast and brain tumors) at exceptionally young ages (Birch et al. 1994*a*; Varley et al. 1997*a*). In a series of families studied by our own group, all of those in which there was an adrenocortical tumor carried a germline *TP53* mutation (Varley et al. 1997*a*). In spite of strong associations between LFS, ACC, and germline *TP53* mutations, there has so far been only one study which has attempted to determine the frequency of germline *TP53* mutations in a consecutive series of children with ACC, and, in that study, three of six patients analyzed were shown to carry *TP53* mutations (Wagner et al. 1994). There are no reports of germline mutations in sporadic adult ACCs.

We have obtained material from 14 cases of adrenocortical tumors in children aged <15 years, who have been selected as having no significant family history of cancer or a family history which did not conform to LFS or Li-Fraumeni–like syndrome (LFL [Birch et al. 1994*a*]). We examined the material for the presence of

germline and somatic *TP53* mutations, loss of heterozygosity (LOH), p53 and mismatch-repair protein expression, and microsatellite instability. The majority of patients with childhood adrenocortical tumors have germline *TP53* mutations, which can be associated with a dramatic accumulation of further mutations in the tumors. Although these patients were selected for the absence of family histories of cancer that are consistent with LFS/LFL, detailed examination of their family histories identified individuals with tumors and suggested that the *TP53* mutations we have found predispose to cancer with low penetrance. Thus, germline *TP53* mutations may be more frequent than has been estimated previously.

Patients and Methods

Clinical Samples

Eleven patients were ascertained as a population-based series of children with adrenocortical tumors (Manchester Children's Tumor Registry). From this same registry, two additional families conforming to the definition of LFS had been ascertained and studied as part of a separate cohort (Birch et al. 1994*a*; Varley et al. 1997*a*). Three further patients who had been referred to our hospital, but resided outside the boundaries of the Registry's catchment area, were also included. The series therefore comprised, in total, 14 cases of adrenocortical tumor. Three of the patients had a personal history of multiple independent primary tumors, all within the radiation field of the original tumor, and all of types highly suggestive of a germline *TP53* mutation (i.e., soft-tissue sarcomas and osteosarcomas). The clinical details of the patients included in this study are given in table 1. The patients' ages at diagnosis of the adrenocortical tumors had a range of <1–14 years. Twelve of the tumors were confirmed as ACC; the remaining two were classified as adrenocortical adenomas. In both of the latter cases, the patients are alive as of this writing (December 1998) and well into their 20s. One of these patients (patient 10) was diagnosed with mild hemihypertrophy, and his mother was recorded to have a bifid left renal pelvis and duplication of the ureter in the upper quarter. The research project had the approval of the local district's medical-research ethics committee.

Sections (10 μm) were cut from fixed, paraffin-embedded tumors, as indicated in table 2. Normal tissue was obtained from nine patients, either as fixed and embedded normal tissue, as lymphocytes, or as a lymphoblastoid cell line. In addition, normal material was obtained from the mothers of two affected children who were first cousins (see below). DNA was extracted according to standard protocols (Varley et al. 1997*a*, 1997*b*). All tumor sections from which DNA was prepared contained $\geq$80% tumor cells, with most comprising ~100%.

Mutation Detection

Exons 2–11 of *TP53* were amplified from each sample (normal and tumor), and the products were screened initially by SSCP. Included in the analysis were all the intron-exon boundaries. The primers that were used are listed in table 3. In each case, a forward (F) and a reverse (R) primer were used to amplify each entire exon; however, for some exons, different combinations of primers also were used (exons 4, 5, and 7). Initially, DNA was amplified in a first-round standard hot-start PCR of 35 cycles (at 94°C, 55°C, and 72°C, for 1 min each), except for exon 7, which was amplified using a touchdown protocol (Don et al. 1991). The products were then diluted 1:10 into a second-round SSCP-PCR mix containing the same reagents as in the first-round PCR, except with a reduced concentration (10 μM) of unlabeled dCTP, plus 1 μCi α^{32}P-dCTP per 10-μl reaction (Munn et al. 1996). The cycling parameters were 10 cycles of 94°C, 50°C, and 72°C, for 1 min each. The products were separated onto 0.5 × MDE gels (FMC Biochemicals) at 4–6 W for 12–16 h, dried, and subjected to autoradiography as described elsewhere (Munn et al. 1996). Controls (no DNA and normal DNA) were included on every gel. Any samples showing abnormal band shifts were reanalyzed by repeating both first- and second-round SSCP-PCR reactions. In a number of cases, the results were verified in an independent laboratory using fresh material.

DNA Sequencing and LOH

The sequence variation that caused the abnormal band shift was determined by sequencing the product of the first-round PCR, either with tailed primers or by carrying out direct sequencing using genomic DNA as a template and nested tailed PCR primers (Varley et al. 1997*a*). In all cases in which both these methods were used identical mutations were seen, and DNA sequencing was performed in both directions in multiple independent reactions to confirm the results. In five cases, a duplicate blood/DNA sample was analyzed independently in a second laboratory, and our results were confirmed. LOH was determined either from the SSCP gel (i.e., loss of bands representing the normal allele were compared to the control on the same gel) or from the sequencing electropherogram (Varley et al. 1997*b*).

Where an SSCP band shift or sequencing alteration was detected (in either normal or tumor material), the entire experiment was repeated with fresh material. The results were consistent in every case. Wherever a mutation was detected in normal DNA, the mutant band/nucleotide appeared to be in the heterozygous state, by

Table 1

Clinical Details of the Children Included

Patient Number (Sex)	Family History (Age at Diagnosis, in Years)[a]	Clinical History (Age at Diagnosis, in Years)	XRT[b]
1 (F)	3d-degree relative, stomach/pancreatic cancer (61); 4th-degree relative, breast cancer (42)	ACC (<2); chondrosarcoma (11); sclerosing osteosarcoma (12); died (12)	Yes
2 (F)	Uncle, NHL rectum (15)	ACC (13); died (13)	nk
3 (F)	Grandmother, cerebral tumor (35)	ACC (2); died (4)	Yes
4 (F)	NFH	ACC (<1); spindle-cell sarcoma, breast (14); died (15)	Yes
5 (F)	Mother, lung cancer (58 [smoker])	ACC (<1); osteochondroma (13); leiomyosarcoma (26); died (27)	Yes
6 (F)	NFH	ACC (3); died (5)	Yes
7 (F)	Cousin (patient 8) ACC (4); grandmother, uterine cancer (57)	ACC (14); died (15)	No
8 (F)	Cousin (patient 7) ACC diagnosed at 14 years; grandmother, uterine cancer (57)	ACC (4); died (6);	Yes
9 (F)	Grandmother, cervical cancer (56); breast cancer (65)	Adrenocortical adenoma (4); alive (23)	No
10 (M)	NFH (mild hemihypertrophy)	Adrenocortical adenoma (7); alive (25)	No
11 (F)	NFH	ACC (5); alive (17)	No
12 (M)	Grandfather, astrocytoma (44); 4th-degree relative, chondosarcoma (35)	ACC (<3); alive (3)	nk
13 (F)	Grandmother, brain tumor (42)	ACC (<2); alive (2)	nk
14 (F)	Father, glioma (35); grandfather, prostate cancer (70)	ACC (9); died (10)	Yes

[a] "NFH" denotes no significant family history of cancer and "NHL" denotes non-Hodgkins lymphoma.
[b] Treatment of the patient with radiotherapy for the primary adrenocortical tumor; "nk" denotes not known.

comparison with known examples of germline *TP53* mutations analyzed in parallel. Somatic mutations in tumors were also confirmed by repeating first- and second-round PCRs and sequencing. We are therefore confident that PCR artifacts have been excluded.

Immunohistochemistry

Immunohistochemical detection of p53 protein was performed with the CM1 antibody (Bartek et al. 1991; Midgley et al. 1992) on sections from fixed and embedded material, as described elsewhere (Birch et al. 1994*b*). The extent of the staining was assessed as the percentage of cells staining and was expressed with respect to the intensity of staining within those cells (see table 2). Negative and positive controls were processed at the same time, and they are the same as those described elsewhere (Birch et al. 1994*b*). Expression of the mismatch-repair proteins MLH1 and MSH2 was performed with commercial antibodies, according to the manufacturer's instructions (PharMingen). Staining was assessed as being "moderate" or "strong," as "nuclear or cytoplasmic," and, when appropriate, as "negative."

Mismatch Repair and Microsatellite Analysis

Four mononucleotide tracts (hMSH3, hMSH6, BAT26, and BAT40 [Parsons et al. 1995; Malkhosyan et al. 1996]) and four dinucleotide-repeat sequences (D2S123, D3S1076, D8S255, and D13S175 [Dib et al. 1996]) were analyzed. DNA was amplified in a 10-μl reaction mix that contained reduced unlabeled dCTP and 1 μCi ^{32}P-dCTP per reaction. All sequences were amplified for 35 cycles of 94°C, 56°C, and 72°C each for 1 min, following an initial 4-min denaturation at 94°C. Microsatellites were analyzed by electrophoresis on a 6% denaturing acrylamide gel and were visualized by autoradiography.

Results

Germline Mutations in TP53

Germline *TP53* mutations were identified in nine cases (table 2). In addition, although we were unable to obtain normal DNA from two other patients, analysis of constitutional DNA from cancer-affected relatives identified germline *TP53* defects (shown as inferred mutations in table 2). Of the remaining three cases, a germline *TP53* mutation was excluded by SSCP analysis in two patients, including one with mild hemihypertrophy. In this latter case, it is possible that the occurrence of an adrenocortical tumor is associated with BWS and thus is more likely to be linked to a gene or genes on chromosome 11p15 rather than to *TP53*. In the final case, we were unable to obtain normal DNA for analysis, although we detected *TP53* mutations in the tumor DNA. However, it is not possible to determine whether these mutations represent germline or somatic events. One of the patients with an adrenocortical adenoma (patient 9) had an in-

Table 2

***TP53* Gene Analysis in Childhood Adrenocortical Tumors**

Patient Number	Germline Mutation[a]	Tumor Type (age at diagnosis, in years)	Somatic Mutation[b]	LOH[c]	p53 Expression (% of Cells) in	
					Tumor[d]	Normal Tissues
1	152, Pro→Leu, CCG→CTG inferred	ACC (<2)	nt*	nt*	++ (>95)	++ (>95)
		Chondrosarcoma (11)	(152, Pro→Leu, CCG→CTG) Multiple, including 144, Gln→Stop, CAC→TAG 158, Arg→Tyr, CGC→TAC Intron 6 T→C SSCP shift exon 7 SSCP shift exon 10	Loss of this allele LOH	+++ (>95)	
		Osteosarcoma (12)	(152, Pro→Leu, CCG→CTG) 152, Pro→Arg, CCG→CGG	Loss of this allele LOH	nd	
2	No	ACC (13)	Intron 9 T→A SSCP shift in exon 5 SSCP shift in exon 7	LOH	++ (>95)	++/+++ (>95)
3	158, Arg→His, CGC→CAC	ACC (2)	158, Arg→His, CGC→CAC No other SSCP shift in exons 4–9	LOH	nd	+ (<5)
4	158, Arg→His, CGC→CAC	ACC (<1)	158, Arg→His, CGC→CAC No other SSCP shift in exons 2–11	No LOH	++ (>95)	++ (>95)
		Sarcoma breast (14)	158, Arg→His, CGC→CAC No other SSCP shift in exons 2–11	LOH	+++ (>95)	
5	152, Pro→Leu, CCG→CTG	ACC (>1)	(152, Pro→Leu, CCG→CTG) 158, Arg→His, CGC→CAC 230, Thr→Leu, ACC→ATC 248, Arg→Gln, CGG→CAG (only exons 4–9 analyzed)	Loss of this allele LOH	++ (>95)	++ (>95)
		Osteochondroma (13)	(152, Pro→Leu, CCG→CTG) 130, Leu→Phe, CTC→TTC (only exon 5 analyzed)	Loss of this allele LOH	+++ (>95)	

6	na	ACC (3)	337, Arg→His, CGC→CAC C→T intron 6 C→T intron 10	LOH	+++ (>95)	+++ (>95)
7	152, Pro→Leu, CCG→CTG	ACC (14)	152, Pro→Leu, CCG→CTG; SSCP shift in exon 7	LOH	++/+++ (50)	nd
8	152, Pro→Leu, CCG→CTG	ACC (4)	152, Pro→Leu, CCG→CTG 240, Ser→Asn, AGT→AAT 250, Pro→Pro, CCC→CCT	LOH No LOH at 240/250	++/+++ (>95)	nd
9	152, Pro→Leu, CCG→CTG inferred	AC adenoma (4)	(152, Pro→Leu, CCG→CTG) 250, Pro→Pro, CCC→CCT	Loss of this allele No LOH	+/++ (>95)	nd
10	No	AC adenoma (7)	No SSCP shift exons 2–11	No LOH	++/+++ (60)	+/++ (>95)
11	152, Pro→Leu, CCG→CTG	ACC (5)	(152, Pro→Leu, CCG→CTG) 176, Cys→Tyr, TGC→TAC (only exons 4–9 and 11 analyzed)	Loss of this allele LOH	+ (>95)	+ (>95)
12	158, Arg→His, CGC→CAC	ACC (<3)	158, Arg→His, CGC→CAC No other SSCP shift in exons 2–11	No LOH	+/++ (>95)	+++ (>95)
13	213, Arg→Stop, CGA→TGA	ACC (<2)	213, Arg→Stop, CGA→TGA No other SSCP shift in exons 2–11	LOH	+/++ (50)	nd
14	251, Ile→Ile, ATC→ATT Homo/Hemizygous	ACC (9)	nt*	nt*	++ (>95)	++ (>95)

[a] Germline mutations were identified by screening normal DNA from the patient except in the case of patients 7 and 8, where an identical mutation was found in their respective tumors, and the mutation was confirmed to be germline by analysis of their mothers' DNA. In patients 1 and 9, normal material was unavailable from the patients themselves, but a germline mutation was identified in relatives in the same lineage. The abbreviation "na" denotes normal tissue unavailable for analysis.

[b] Somatic mutations were identified by screening DNA isolated from the tumor indicated. The notation "*nt" denotes no tumor. In the two cases indicated, no tumor material was available. In some cases, SSCP shifts were seen, but sequencing to characterize the precise mutation was not performed. In cases where there is a germline mutation, the details given in this column are for additional mutations in the tumor.

[c] LOH was assessed from either the SSCP gel or from the sequencing electropherogram as loss of one allele.

[d] p53 expression was assessed as weak (+), moderate (++), or strong (+++), and the percentage of cells showing such staining are given. Normal tissues evaluated include normal adrenal gland, adjacent tissues, or normal material from distant sites.

Table 3

PCR Primers Used for SSCP Analysis of *TP53*

Exon	Primers		PCR Conditions[a]
	Forward	Reverse	
2	2F1 (5′-AAGCGTCTCATGCTGGATCCC-3′)	2R (5′-GCCCTTCCAATGGATCCACT-3′)	Standard + 10% DMSO
3	3F (5′-GAAGCGAAAATTCATGGGACTGA-3′)	3R1 (5′-CCCCAGCCCAACCCTTGTC-3′)	Standard + 10% DMSO
4A	4F (5′-GACCTGGTCCTCTGACTGCTC-3′)	4R2 (5′-TGCAGGGGCCGCCGGTGTAG-3′)	Standard + 10% DMSO
4B	4F2 (5′-CTACACCGGCGGCCCCTGCA-3′)	4R (5′-GCATTGAAGTCTCATGGAAG-3′)	Standard
5	5F (5′-ATCTGTTCACTTGTGCCCTG-3′)	5R (5′-ATCAGTGAGGAATCAGAGGC-3′)	Standard
5A	5F (5′-ATCTGTTCACTTGTGCCCTG-3′)	5R2 (5′-CTCACAACCTCCGTCATGTG-3′)	Standard (1.5μM $MgCl_2$)
5B	5F2 (5′-TTGCCAACTGGCCAAGACCT-3′)	5R (5′-ATCAGTGAGGAATCAGAGGC-3′)	Standard
6	6F (5′-GCCTCTGATTCCTCACTGAT-3′)	6R (5′-GGAGGGCCACTGACAACCA-3′)	Standard (1.5μM $MgCl_2$)
7	7F (5′-CTTGCCACAGGTCTCCCCAA-3′)	7R (5′-AGGGGTCAGCGGCAAGCAGA-3′)	Touchdown (Don et al. 1991)
7A	7F (5′-CTTGCCACAGGTCTCCCCAA-3′)	7R2 (5′-TGATGATGGTGAGGATGGGCC-3′)	Standard + 10% DMSO
7B	7F2 (5′-ATCTCCTAGGTTGGCTCTGAC-3′)	7R (5′-AGGGGTCAGCGGCAAGCAGA-3′)	Standard + 10% DMSO
8	8F (5′-TTCCTTACTGCCTCTTGCTT-3′)	8R (5′-TGAATCTGAGGCATAACTGC-3′)	Standard
9	9F (5′-GCAGTTATGCCTCAGATTCA-3′)	9R (5′-ACTTTCCACTTGATAAGAGG-3′)	Standard
10	10F (5′-CCATCTTTTAACTCAGGTAC-3′)	10R (5′-TATGGCTTTCCAACCTAGGAA-3′)	Standard
11	11F (5′-CTCACTCATGTGATGTCATC-3′)	11R (5′-CAAAATGGCAGGGGAGG-3′)	Standard

[a] Standard PCR conditions were as follows (final concentrations): 1 × *Taq* buffer (Promega Corporation), 200 μM each dNTP, 1.0 mM $MgCl_2$, 0.25 μM each primer, 0.2 units *Taq* polymerase (Promega). Some reactions as indicated were performed using an increased concentration of MgCl, or with the addition of DMSO. Amplification of exon 7 was performed using a touchdown protocol. Further details of cycling parameters are given in the text.

ferred mutation (see table 2). We have previously reported that benign lesions occur in germline *TP53* mutation carriers and that, indeed, there can be loss of the wild-type allele in such lesions (Varley et al. 1996).

The spectrum of mutations detected is remarkable. Among patients with a confirmed or inferred germline *TP53* mutation, six had identical mutations at codon 152 (Pro→Leu, CCG→CTG), and three had identical mutations at codon 158 (Arg→His, CGC→CAC). Apart from two affected first cousins (see table 1 andtable 2), as far as we have been able to determine from a very detailed pedigree analysis, none of the patients are related. One other patient (patient 13) was found to have a nonsense mutation at position 213. Identical germline mutations have been reported previously at this position in patients with LFS (Frebourg et al. 1995) and in patients with a strong family history of cancer (Horio et al. 1994). In the remaining case with a germline mutation, we identified a silent mutation at codon 251, which is homo- or hemizygous. There are no reports of this nucleotide change in the *TP53* database (Beroud and Soussi 1998), and the substitution is not predicted to alter splicing or mRNA stability. One explanation for this finding is that one allele of *TP53* has the codon 251 substitution, whereas the other has a deletion of all or part of the gene. We have not been able to study this finding further, because of the limited availability of material from this patient.

Family History

Although all the cases in this report were selected because they were not members of families that conformed to the definitions of LFS/LFL, nonetheless, a number did have suspicious family histories (table 1). Two of the patients were first cousins, affected with ACC at ages 4 years and 14 years (patients 7 and 8). The mothers of these children are unaffected at ages 47 years and 42 years, respectively, although both have been shown to carry the germline *TP53* mutation. The maternal grandmother developed carcinoma of the uterus at age 57 years and also carried the identical mutation (with no LOH in the tumor). In three other cases in which we have detected a germline mutation, although all parents are unaffected (at ages $\leqslant$51 years), one grandparent in each family developed a brain tumor in their 30s or 40s. We have not been able to obtain material from these brain tumors to analyze the mutation status. Furthermore, in one of these families, a second cousin developed a chondrosarcoma in his 30s. In one other family in which a germline mutation was detected, the father of a 9-year-old child with ACC developed a brain tumor in his mid-30s. The mother of patient 5 developed a lung adenocarcinoma at age 58 years. Although this is not an unusually young age for a smoker to develop such a tumor, she was shown to carry the identical germline mutation to that of her daughter.

In families 1 and 9, although we were unable to analyze normal DNA from the proband, we were able to obtain archival normal material from affected relatives. In neither family was the history of cancer particularly striking, with respect either to the ages at onset or to the tumor types. However, in both families, we were able to detect a germline mutation in the normal DNA from affected relatives.

Somatic Mutations and LOH

In all cases except two, we were able to examine the adrenocortical tumor DNA for somatic *TP53* mutations and LOH. The simplest model of action for a tumor-suppressor gene is that one mutation (usually missense or nonsense) is inherited and the remaining allele either is lost by some deletion mechanism or suffers a second mutational hit (Ponder 1988). Thus, one might expect that in an adrenocortical tumor of a patient with an inherited germline *TP53* gene, there will be either loss of the wild-type allele or a second mutational event. The situation in some of the tumors examined in the present study is far from being so simple.

Among the four tumors examined from three patients with a codon 158 mutation (patients 3, 4, and 12), there is LOH in two of the tumors and no LOH in the other two, with no additional SSCP band shifts in any tumor. In earlier studies, we (Varley et al. 1997*b*) and others (Sedlacek et al. 1998) have shown that there is LOH in about half of all tumors from patients with germline *TP53* mutations, so this finding is not unusual. However, it is somewhat surprising that we have not detected a second mutation in the two tumors in which there is no LOH. In the tumor from the patient with a germline codon 213 nonsense mutation, there is LOH.

In all the tumors of patients with a germline codon 152 mutation, there are multiple additional somatic mutations. In three tumors there is loss of the confirmed germline mutant allele (patient 5, ACC and osteochondroma; patient 11, ACC), and in three other tumors there is loss of the inferred germline mutant allele (patient 1, chondrosarcoma and osteosarcoma; patient 9, adrenocortical adenoma). In our previous study of LOH in tumors, we found two cases in which there was loss of the germline mutant allele in tumors, one of which was an ACC (Varley et al. 1997*b*). In all tumors with codon 152 mutations, there are additional missense mutations within both the exons and the flanking intronic sequences (table 2). In some of the tumors, the situation is extremely complex, with both loss of the inherited mutant allele and multiple additional somatic mutations. There are some intriguing findings in both tumors from patient 5, most strikingly in the ACC. Analysis of exons 4–9 from this tumor show that there are three somatic mutations, affecting codons 158, 230, and 248 (table 2). None of these mutations are present in the constitutional DNA isolated from a lymphoblastoid cell line from this patient, and all of them are present in the homozygous/hemizygous state in the tumor. One explanation for this finding is that there have been a number of mutational events affecting the normal germline allele of *TP53*, including the classic hot-spot codon 248 mutation. There has subsequently been loss of the inherited mutant copy of *TP53*, possibly as a result of selective pressure imposed by the presence of one of the other candidate tumor-suppressor genes on chromosome 17 (see fig. 1*a*). Perhaps the most interesting aspects of this finding of multiple mutational hits in the adrenocortical tumor are the young age at onset (<1 year) and the number of missense mutations within a single gene. It is tempting to speculate that either there has been some exposure to DNA-damaging agents in utero, or that there has been a somatic mutation in a second gene whose product is involved in the maintenance of genomic stability. In two patients with an inferred germline codon 152 mutation (patients 1 and 9), we have observed similar findings to the above, namely loss of the inherited mutant allele and further somatic mutations, all showing LOH.

In the ACC from patient 8, there is LOH at position 152 (the germline mutation), with loss of the wild-type sequence at this position, but there are two somatic mutations within exon 7, at positions 240 and 250, which are present in the heterozygous state. The latter mutation is silent (CCC→CCT, Pro→Pro). The simplest explanation for this finding is that the wild-type chromosome is lost and the mutant chromosome reduplicated, with subsequent mutational hits occurring in one allele (fig. 1*b*). Although this model suggests that both mutations occur in the same allele, we cannot rule out the possibility that they occur on different alleles. Interestingly, we also detected a codon 250 CCC→CCT change in DNA from another adrenocortical tumor (patient 9). This change has not been previously reported as a polymorphism, and we have never seen this change in any control or cancer-affected individual ($n > 100$, data not

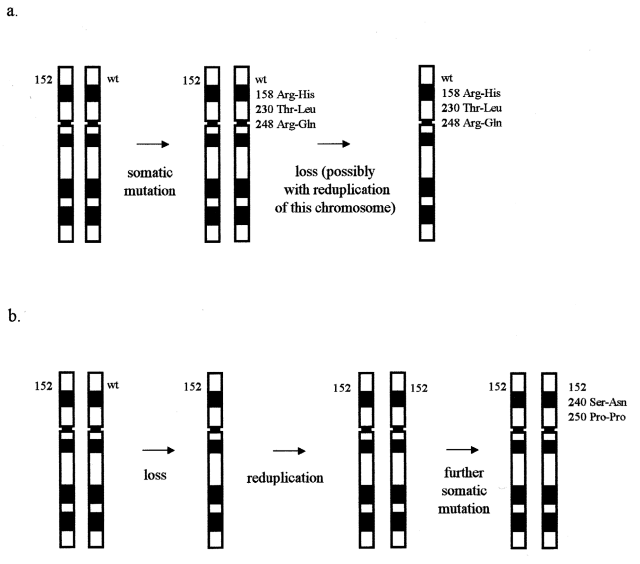

Figure 1 Diagrammatic interpretation of the somatic changes in tumors from patients 5 (a) and 8 (b).

shown), although there is one report of an identical change in a squamous carcinoma of skin (Campbell et al. 1993). The significance of this silent alteration is unclear, but it might again be a consequence of increased genomic instability.

In one further patient (patient 6), mutations were observed in the tumor, with complete loss of the wild-type allele. We were unable to determine whether these mutations were inherited or acquired. Finally, in one of the two cases (patient 2) in which we have been unable to find a germline *TP53* mutation, we have detected multiple somatic *TP53* mutations.

Detection of p53 by Immunohistochemistry

We were able to stain all the adrenocortical tumors except one (see table 2) for the expression of p53 protein. In addition, normal tissues were stained from the majority of patients and, wherever possible, from other primary tumors (see table 2). The extent of staining was assessed using two criteria: the percentage of cells staining, and the intensity of staining in the positive cells. In all cases examined, there was detectable p53 staining. In most tumors, there was staining in the majority of tumor cells, with a moderate-to-strong intensity indicating increased stabilization of the protein, relative to the normal wild-type state. Interestingly, in all cases in which we were able to examine normal tissue, the staining was at least as strong as in the tumors, and, in some cases, even more intense (see table 2). In two cases in which we were unable to analyze normal DNA for germline *TP53* mutations, there was detectable staining for p53 protein in normal cells.

Expression of MSH2 and MLH1

Twelve adrenocortical tumors (patients 1–12) were studied for the expression of MLH1 and MSH2 by immunostaining. Adrenocortical tumors from two patients (patients 5 and 7) showed loss of expression of both proteins, and two others (patients 2 and 4) showed complete loss of expression of MLH1 but not of MSH2. Cytoplasmic staining of MLH1 was apparent in two tumors (from patients 1 and 11). The remaining six tumors showed nuclear expression of both proteins, although in no case were all the tumor cells stained with both antibodies. It would appear, therefore, that a significant proportion of the childhood adrenocortical tumors studied have compromised mismatch repair. There is no correlation between the position of the *TP53* mutation and loss of expression of the mismatch repair genes.

Microsatellite Instability

We looked for evidence of microsatellite instability (MI) in the tumors by means of both mononucleotide and dinucleotide repeats. Only one tumor from a patient with a codon 152 mutation (patient 1, chondrosarcoma) showed evidence of MI, but because of the limited availability of normal material for comparison and difficulties amplifying some repeats from several of the other samples, we cannot draw any firm conclusions about the occurrence of MI in the adrenocortical tumors.

Discussion

Of 14 patients analyzed in this study, we have been able to obtain normal DNA from 11, 9 (82%) of whom have confirmed *TP53* germline mutations. Two of the remaining three patients have inferred germline mutations. This is a remarkably high frequency of germline mutation and represents the highest incidence of a germline mutation reported in any cancer. Since we have used SSCP as a primary screen for *TP53* mutations, the figure may be an underestimate. The spectrum of mutations found in childhood adrenocortical tumors in the present study is extremely interesting. All the patients with germline mutations except two have alterations to codons 152 or 158 (table 2). There is one other report in the literature of a germline mutation in codon 152, intriguingly in the only other published study of children with ACC (Wagner et al. 1994). There are no reports of a germline codon 158 mutation. These two residues lie within the core DNA-binding domain of the *TP53* gene, but are outside the highly conserved domains. Both are, however, conserved in all species examined to date (Soussi et al. 1990), strongly indicating that they have an essential role in the function of the normal p53 protein. We have analyzed fibroblasts from an unaffected individual with a germline codon 152 mutation, and have demonstrated that the cells show reduced transient G_1 arrest and an increased cell survival following irradiation and that the mutant p53 fails to transactivate in a yeast functional assay (Boyle et al. 1999). These data confirm that the mutant protein is functionally abnormal.

This is only the second study to examine the frequency of germline *TP53* mutations in childhood adrenocortical tumors. The patients were unselected for any family history and cases consistent with LFS/LFL were excluded, which may explain the limited spectrum of mutations detected. There are numerous other reports of germline *TP53* mutations in families in which there is a child with an adrenocortical tumor, many of which conform to the definitions of classic LFS or LFL (Sameshima et al. 1992; Bardeesy et al. 1994; Birch et al. 1994*a*; Hamelin et al. 1994; Stolzenberg et al. 1994; Strauss et al. 1995; Varley et al. 1997*a*; Sedlacek et al. 1998; Vital et al. 1998). In addition, there are a number of reports of children with adrenocortical tumors who either have some family history of cancer (Grayson et al. 1994; Pivnick et al. 1998)

or no family history of cancer at all, some of which represent de novo mutations (Gutierrez et al. 1994; Wagner et al. 1994; Giunta et al. 1996; Moutou et al. 1996). Finally, there are some reports of individuals with germline mutations and adult-onset adrenocortical tumors (Warneford et al. 1992; Felix et al. 1993; Lubbe et al. 1995; Varley et al. 1998). The spectrum of germline mutations seen in the above studies is broad, in contrast to the narrow range in this present report. However, in the majority of the above reports, there was a strong family history of cancer associated with the ACC cases, and three other cases carried de novo mutations. The present series was population-based but did not include those cases meeting the LFS/LFL criteria, and this could explain the limited spectrum of mutations observed. It is noteworthy that in the only other series of ACC that was unselected for family history one of the three germline *TP53* mutations detected was at codon 152 (Wagner et al. 1994). It seems unlikely that there is a population difference, because, in LFS/LFL studied in our laboratory in which there is a case of childhood ACC, we see a variety of mutations, including missense mutations at codons 220, 248, 180, and 175, a complex deletion-insertion event, and a splicing mutation (Birch et al. 1994*a*; Varley et al. 1995; Varley et al. 1997*a*).

A number of the patients with germline *TP53* mutations have relatives with tumors characteristic of LFS, although the clustering of cancers in those families does not fulfill the clinical criteria for classic LFS or for LFL. In one family, first cousins were affected with ACC at ages 4 years and 14 years, and, although their mothers are unaffected at ages 42 years and 47 years, they both carry the mutant allele. In addition, in this same family, a second-degree relative of the probands is an germline carrier unaffected at age 45 years. In the report by Wagner et al. (1994), the mother of a child with an ACC and an identical germline codon 152 mutation, although unaffected at the time of the study, developed breast cancer at age 46 years. Four other children in the present study have a first- or second-degree relative affected with a brain tumor at age <45 years, with obligate carriers unaffected at ages up to the mid-60s. These data, therefore, suggest that there exists a subset of germline *TP53* mutations that are of lower penetrance than those detected in families that conform to the definitions of LFS or LFL. This hypothesis is strengthened by the finding that in two families, although normal material from the probands with the adrenocortical tumors was unavailable, analysis of an affected relative revealed the existence of a germline mutation, and in both families there were unaffected relatives who are inferred obligate carriers.

The current estimates for lifetime penetrance in individuals with a germline *TP53* mutation approach 90% (LeBihan et al. 1995), and analysis of first primary cancers in 20 families affected by LFS or LFL that contain germline *TP53* mutations showed that mean age at diagnosis was 28 years (Birch and Varley, unpublished data). These data apparently would place the unaffected carriers in the families reported here at very high risk. However, these risk estimates of *TP53* germline mutation carriers are based on the analysis of a relatively small number of families ascertained according to the clinical definitions of LFS or LFL. There could, therefore, be a considerable bias in these risk estimates. In the series of families in the present study, the ascertainment has been made to exclude families with clinical LFS or LFL, but otherwise without regard to family history, and it was based solely on the occurrence of a case of childhood adrenocortical cancer. Apart from the index case, there are no additional cases of childhood cancer, and the ages at onset of the brain and breast tumors in these families appears to be later than in those with classic LFS who carry a germline mutation affecting a known mutational hotspot (Birch et al. 1998). Finally, a number of relatives of children with adrenocortical tumors who have been shown to carry germline mutations have developed tumors that are not normally associated with LFS, including lung, uterine, and cervical tumors. These data all support the proposal that the mutations we have detected, predominantly in codons 152 and 158 of *TP53,* are of relatively low penetrance, both in terms of the incidence of cancer and in terms of the tumor types.

Low-penetrance alleles have been identified in other cancer-predisposition syndromes, most notably retinoblastoma (Onadim et al. 1992; Dryja et al. 1993; Lohmann et al. 1994; Bremner et al. 1997; Schubert et al. 1997; Cowell and Bia 1998). The spectrum of mutations seen in such families is distinctly different from that seen in patients with severe phenotypes or in families showing high penetrance (Cowell and Bia 1998; Harbour 1998). The mutations that we have detected at high frequency in patients with childhood adrenocortical tumors and with no or mild family histories of cancer do not, at first sight, appear to be of a different class than those seen in higher penetrance LFS/LFL families. Further biological studies are in progress to address this important point and to determine the consequences of the codon 152 and 158 mutations.

The findings reported here have considerable implications for patients with childhood adrenocortical tumors and their families. Genetic screening should be considered, and, if a mutation is found, it might be appropriate to offer carriers screening for breast and brain tumors using protocols which minimize exposure to ionizing radiation (e.g., magnetic resonance imaging or ultrasound). Three of the children who were treated with radiotherapy survived >2 years, and all three developed additional primary tumors within the radiation field (table 1). None had received chemotherapy. These

secondary tumors were typical of those seen in carriers of germline *TP53* mutations: bone and soft-tissue sarcomas. There are other reports in the literature of therapy-induced secondary tumors in germline *TP53* mutation carriers (Felix et al. 1996, 1998; Hisada et al. 1998), but those reported in the present study are particularly striking.

We performed very detailed analysis of *TP53* for LOH in the tumors and for the occurrence of second mutations. These data proved extremely interesting. In the tumors from patients with a codon 158 or 213 mutation, there are few other alterations, limited to loss of the wild-type allele. In contrast, tumors from patients with codon 152 mutations showed an unusually high number of somatic mutations. The selective drive for the accumulation of such mutations is not apparent and may reflect an increased genomic instability as a result of the specific mutation. This is a testable hypothesis and, if verified, would be the first demonstration of a *TP53* mutation–specific function in the recognition or accumulation of DNA damage. We tested a number of the tumors for expression of the mismatch-repair genes *MLH1* and *MSH2*, to determine whether the high incidence of somatic missense mutations in tumors from patients with codon 152 mutations reflected a generalized defect in mismatch repair, possibly as a direct consequence of the germline mutation. Although mismatch repair appears to be compromised in a number of tumors, there is no correlation with the type of *TP53* mutation. If specific mutations do lead to an increased genomic instability as proposed above, it does not manifest as an increase in chromosomal gain and loss as judged by CGH (James et al. 1999).

Of the 17 somatic mutations that we have characterized, 14 involve either C→T or G→A transitions. Of these 14, 6 occur at CpG dinucleotides (see table 2). Spontaneous deamination of cytosine residues at either CpG or ^{me}CpG is considered to be the most significant source of transition mutations of the type seen in the tumors reported in the present study. Since there are specific DNA glycosylases which recognize the U:G and T:G mismatches generated by deamination of CpG or ^{me}CpG, respectively, the increase in mutations may be associated with defects in these pathways, or, perhaps, a significantly increased spontaneous deamination rate could account for the observations reported here. Studies of components of excision repair pathways are underway, in order to shed light on these intriguing observations.

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Electronic-Database Information

The accession number and URL for data in this article are as follows:

Online Mendelian Inheritance in Man (OMIM): http://www.ncbi.nlm.nih.gov/Omim (for ACC [MIM 201300])

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