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An Inherited Mutation Outside the Highly Conserved DNA-Binding Domain of the p53 Tumor Suppressor Protein in Children and Adults with Sporadic Adrenocortical Tumors

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Mutations of the p53 tumor suppressor gene are the single most common genetic alterations in human cancers. Recently, a distinct nucleotide substitution was identified in exon 10 of the p53 gene, leading to an Arg337His mutation in 97% of children with adrenocortical tumors from Southern Brazil. In the present study, we investigated the presence of this mutation in a larger series of 55 patients (37 adults and 18 children) with benign and malignant sporadic adrenocortical tumors. None of the patients had family cancer histories that conformed to the criteria for Li-Fraumeni syndrome. Twenty-one asymptomatic close relatives of patients with p53 mutations and 60 normal unrelated individuals were also studied. The missense Arg337His mutation was identified in 19 patients (14 children and 5 adults), and 8 of 11 cases studied had LOH. Among the 19 patients with the Arg337His mutation, only one boy and three adults showed fatal evolution or recurrent metastases. This mutation was also identified in heterozygous state in asymptomatic first-degree relatives of the patients, indicating that Arg337His mutation was inherited in most cases. In contrast, this mutation was not found in 120 alleles of normal unrelated controls. In conclusion, the germ line Arg337His mutation of p53 protein is present at a high frequency (77.7%) in children with benign or malignant sporadic adrenocortical tumors, but it is not restricted to the pediatric group, since 13.5% of adults with adrenocortical tumors also had this mutation. The presence of this mutation was related to unfavorable prognosis in most of the adults, but not in the children with adrenocortical tumors. (*J Clin Endocrinol Metab* 86: 4970–4973, 2001)

TUMORIGENESIS IS CHARACTERIZED by a series of genetic alterations in both oncogenes and tumor suppressor genes. A hallmark of tumor suppressor genes is that both alleles are generally altered, which usually represents a loss of function phenotype (1). The p53 tumor suppressor is a phosphoprotein that acts as a transcription factor in the cell cycle regulation, inducing cell cycle arrest or cell death in response to DNA-damaging agents, such as virus, radiation, and chemotherapeutics (1–4).

The p53 tumor suppressor gene is located on the short arm of human chromosome 17. LOH at chromosomal locus 17p has been consistently observed in adrenocortical carcinomas (5). Reincke *et al.* (6) investigated p53 mutations in exons 5–8, a highly conserved region, in 16 human adrenocortical tumors and two adrenocortical tumor cell lines. Single point mutations were detected in 3 of 11 patients with adrenal carcinomas (27%) and in both carcinoma cell lines (6). Point mutations were found at codons 151/152, 193, and 248, which resulted in nonconservative amino acid substitutions. In addition, large insertions or rearrangements of exons 7 and 8 were identified in two other patients (6). Interestingly, Lin *et al.* (7) reported a new mutational "hot spot" within exon 4 in benign adrenal tumors (60% of adrenocortical adenomas and 50% of pheochromocytomas) in Taiwanese patients. In this study, point mutations were identified at codons 100, 102, and 104 in the majority of these adrenal tumors (7). However, these findings were not confirmed in a larger series of Caucasian patients from the United States and Europe, suggesting that ethnic and environmental factors may be responsible for p53 mutational spectrum (8).

For unknown reasons, the incidence of childhood adrenocortical tumor in Southern Brazil is approximately 10 times greater than the worldwide incidence that ranges from only 0.3–0.38 million per year (9). Recently, a high incidence of a p53 germ line mutation was demonstrated in 36 children with adrenocortical tumors originated from Southern Brazil (10). This mutation, located at exon 10 of the p53 gene, resulted in the substitution of arginine for histidine at position 337 of the protein. To date, there are no reports of germ line p53 mutations in adults with sporadic adrenocortical tumors. In the present study, we investigated the frequency of this mutation in another Brazilian series including children and adults with benign and malignant sporadic adrenocortical tumors without significant family history of cancer. We, therefore, explored the significance of the Arg337His mutation located outside the highly conserved domain of the p53 tumor suppressor protein in the clinical evolution of adrenocortical tumors in children and adults.

Patients and Methods

The study was approved by the Ethics Committee of Hospital das Clinicas (São Paulo, Brazil), and informed written consent was obtained from all patients. We studied 55 patients (18 children and 37 adults) with sporadic adrenocortical tumors who belong to one medical center, Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo, in São Paulo city, which is located in Southwestern Brazil. Clinical data of all patients are shown in Tables 1 and 2. Fifty patients had functioning tumors. None of the patients had family cancer histories that conformed to the criteria for Li-Fraumeni syndrome.

Twenty-one asymptomatic close relatives (age ranging from 4–45 yr) of 11 children and 1 adult with adrenocortical tumors who harbored a p53 mutation and 60 normal unrelated individuals, including 30 children, were also studied.

DNA analysis

Genomic DNA was extracted from frozen tumor specimens using a QIAmp DNA mini Kit (QIAGEN, Hilden, Germany) of all patients. Genomic DNA was also extracted from peripheral blood from 11 patients, 21 asymptomatic close relatives, and 60 normal unrelated individuals according to standard protocols. A tissue sample was derived from the central portion of the tumor to minimize the possibility of contamination with normal tissue. The entire exon 10 was amplified by PCR using the following intronic primers: 5′-GCTGTATAGGTACT-TGAAGTGCAG-3′ and 5′-GATGAGAATGGAATCCTATG-3′ (11). A 100-μl PCR mixture containing deoxynucleotide triphosphates (200 μmol/liter each), upstream and downstream oligonucleotides primers (30 pmol each), 500 mM KCl, 15 mM MgCl2, 100 mM Tris HCl (pH 9.0), and 2.5 U *Taq polymerase* was used for amplification. The amplification protocol consisted of denaturing at 94 C for 5 min, followed by 35 cycles consisting of annealing at 50 C for 30 sec, primer extension at 72 C for 30 sec, and denaturing at 94 C for 30 sec. All amplified fragments were examined on 2% agarose gel electrophoresis. The PCR products were pretreated with an enzymatic combination of exonuclease I and shrimp alkaline phosphatase (United Stated Biochemical Corp., Cleveland, OH) and directly sequenced using the BigDye terminator cycle sequencing ready reaction kit (PE Applied Biosystems, Foster City, CA) in an ABI PRISM 310 automatic sequencer (Perkin-Elmer Corp.).

LOH

Patients with adrenocortical tumors who had evidence of the Arg337His mutation in the DNA extracted from adrenal tumor were further investigated for the presence of this same mutation in the DNA extracted from peripheral blood. LOH was defined by the presence of heterozygosity for the Arg337His mutation in blood DNA (germ line mutation) associated with homozygosity in tumor DNA in the same location (somatic mutation). The latter finding indicates that the remaining allele was lost by some deletion mechanism or suffered a second mutational hit (12). Therefore, LOH was investigated in 11 patients who harbored the somatic Arg337His mutation.

Results

The automatic sequencing of exon 10 revealed a nucleotide substitution of guanine for adenine at position 1010, which resulted in the substitution of the amino acid arginine for histidine at position 337 of the p53 tumor suppressor protein in 19 patients (14 children and 5 adults). Only patient 17 was homozygous for this mutation in blood DNA. LOH for Arg337His mutation was found in 8 of 11 cases (Tables 1 and 2).

Analysis of leukocyte DNA was also performed in 21 asymptomatic individuals who were close relatives (parents and/or siblings) of 11 children (patients 1, 2, 3, 4, 5, 8, 12, 14, 15, 17, and 18) and 1 adult (patient 51) with the Arg337His mutation of the p53. The mothers of patients 1, 3, 4, 14, 17, and 51, as well as the fathers of patients 5, 12, 17, and 18 carried the Arg337His mutation in heterozygous state. The mothers of patients 2, 8, and 15 showed normal p53 sequence. The youngest sister of patient 5 also carried the Arg337His mutation. Interestingly, both parents of patient 17, who had the mutation in homozygous state in blood DNA, carried the mutation in heterozygous state. Our findings indicate the 75% of the patients inherited the Arg337His mutation. At this moment, none of the family members with the Arg337His mutation in heterozygous state developed adrenocortical or any other tumor.

Among the patients who harbored the Arg337His mutation of the p53 tumor suppressor protein, two developed distant metastases (patients 5 and 51, Tables 1 and 2, respectively) and two patients (patients 33 and 49, Table 2) died due to progression of the adrenocortical carcinoma. The DNA analysis of 120 normal alleles from 30 children and 30 adults

TABLE 1. Clinical and molecular data of children with adrenocortical tumors

Case	Age (yr)	Sex	Clinical features	Tumor weight/size		Follow-up (yr)	Molecular analysis	
				g	cm		R337H	LOH
1	1.3	M	V	30		6	+	+
2	1.2	M	V	90	6.5	6	+	+
3	0.6	F	V	5	2	3	+	+
4	1.4	M	C/V	40	5	2	+	+
5	2.7	M	V		8.5	Alive with metastasis	+	+
6	2.2	F	V	30	5	6	+	NA
7	9	F	C		9	Dead after 2 yr	−	
8	2.2	M	C/V	135	6	2	+	NA
9	1	F	C/V	45	5	3	−	
10	2	F	V		3	7	−	
11	1.9	F	V/C	38	7.5	3	−	
12	1.6	F	V		5	0.2	+	+
13	3.2	F	C/V	40	5	13	+	NA
14	2	F	V		4.5	10	+	NA
15	0.66	F	C/V	40		12	+	NA
16	2.8	F	C/V	20	4.5	14	+	NA
17	1	F	C/V	60	6	0.3	+	[a]
18	2	F	V	55	6.5	0.2	+	+

V, Virilization; C, Cushing; NA, not available.
[a] Homozygous in tumoral and blood DNAs.

TABLE 2. Clinical and molecular data of adult patients with adrenocortical tumors

Case	Age (yr)	Sex	Clinical features	Tumor weight/size		Follow-up (yr)	Molecular analysis	
				g	cm		R337H	LOH
19	30	M	C	665		Dead after 1 yr	−	
20	36	F	C	15		1	+	−
21	18	F	C	20	3.2	1.4	−	
22	52	F	C	15	3.5	1	−	
23	37	F	H		3.5	6	−	
24	27	M	C		7.5	3	+	NA
25	52	F	NF	160	12	Dead after 1 yr	−	
26	37	F	C	15		4	−	
27	29	F	C	20		5	−	
28	38	F	C		2.5	7	−	
29	19	F	C		11	Dead after 2 yr	−	
30	47	F	C	25	3.5	5	−	
31	43	M	C		10	8	−	
32	40	F	NF		1.4	5	−	
33	19	F	V/C		13	Dead after 1 yr	+	+
34	55	M	NF	65	4.5	7	−	
35	32	F	V		13	9	−	
36	53	F	C		4	−	−	
37	27	F	C	25	4.5	5	−	
38	45	F	NF		7/7[a]		−	
39	17	F	V	825	15	Alive with metastasis	−	
40	18	F	V/C		16.5	Alive with metastasis	−	
41	45	M	NF	31	7	Dead after 5 yr	−	
42	44	F	V/C	2600	30	Dead after 1 yr	−	
43	45	F	V/C	320	10	Dead after 2 yr	−	
44	23	F	C	15	3	4	−	
45	39	F	C		8.5	9	−	
46	42	F	C		5	9	−	
47	38	F	V		2	8	−	
48	32	F	C		4.5	5	−	
49	26	F	C	960		Dead after 1 yr	+	NA
50	46	F	V	450	10	1.7	−	
51	23	F	V/C	550		Dead after 1.5 yr	+	−
52	50	F	V	35	4.5	0.5	−	
53	18	F	C		3	8	−	
54	30	M	F	330	11	Alive with metastasis	−	
55	39	F	C	17	3.5	9	−	

C, Cushing; V, virilization; H, hyperaldosteronism; NF, nonfunctioning; F, feminilization; NA, not available.
[a] Bilateral tumor.

did not reveal mutations in exon 10 of the p53 tumor suppressor gene.

Discussion

The p53 is a transcription factor whose function is to maintain genome integrity. In response to stress insults, the p53 activates a network of genes whose products mediate vital biological functions in cell cycle progression. Inactivation of p53 by mutation is a key molecular event, which is detected in more than half of all human cancer. The vast majority of all acquired mutations discovered in carcinomas have been detected in four hot spot areas that lie between exons 5 and 8 (2, 3, 6).

Here, we demonstrate that a missense Arg337His mutation located outside the highly conserved DNA-binding domain of the p53 tumor suppressor protein was present in 77.7% (14 of 18) of children with benign and malignant sporadic adrenocortical tumors. Our finding confirmed the high incidence of the Arg337His mutation in Brazilian children with adrenocortical tumors previously reported (10). In our series, this mutation was not exclusive of the pediatric group, and it was also found in 13.5% (5 of 37) of adult patients with adrenocortical tumors. The DNA analysis of 120 alleles of unrelated controls did not reveal any mutation in exon 10 of the p53 gene, indicating that Arg337His mutation is not widespread in Brazilian population.

The high incidence of loss of constitutional heterozygosity for a distinct position of the p53 gene in our patients with adrenocortical tumors was consistent with the classical action model of a tumor suppressor gene, where one mutation is inherited and the remaining allele is either lost by some deletion mechanism or suffers a second mutational hit (12).

p53 mutations are rarely observed in benign tumors and are generally associated with poor prognosis in several types of cancer (13, 14). In our series, only 1 (patient 5, Table 1) of 14 children with the Arg337His mutation developed metastases, suggesting that this mutation was not related to unfavorable prognosis in most children with adrenocortical tumors. It is noteworthy that a 2-yr-old girl who harbored the mutation on both maternal and paternal alleles (patient 17, Table 1) developed a large hormone-secreting adrenocortical tumor in the first year of life without signs of tumor invasion or metastases. On the other hand, three of five adults (patients 33, 49, and 51, Table 2) with this mutation developed metastasis and died due to progression of the tumoral disease.

We demonstrated that the mutation was transmitted at least by one parent in 9 of the 11 children and in one adult with adrenocortical tumors. These findings clearly indicate that the germ line Arg337His mutation was inherited in the vast majority of cases, but adrenocortical tumor had developed in only one member of each family during this study. The inherited forms of p53 mutations are commonly described in Li-Fraumeni syndrome (15–17). This rare familial cancer syndrome is characterized by a high incidence of sarcoma diagnosed early in life and at least two first-degree relatives with cancer occurring before the age of 45 yr, such as breast cancers and other diverse neoplasms, particularly brain tumors, leukemia, and adrenocortical carcinomas (15). In contrast with these features, the germ line Arg337His mutation was found in patients with sporadic adrenocortical tumors and in their normal relatives who showed no evidence of bearing a tumor into their 40s. Therefore, it is important to stress that inherited Arg337His mutation apparently confers relatively low penetrance for predisposition to the development of adrenocortical tumor in these families.

Interestingly, the G:A transition in exon 10 of the 53 gene described here is at CpG dinucleotide mutational hot spot (18). The unusual mutability of CpG dinucleotides is well documented and is attributed to the presence of 5-methylcytosine residues found at these dinucleotides in mammalian genome (18). Nearly one third of all human p53 tumor mutations are transitions at hot spot codons with CpG sites. The transitions at CpG sites are considered an important source of spontaneous generation of point mutations in human cells (18). Therefore, it is possible that the Arg337His mutation is not restricted to Brazilian cohorts. In fact, Varley *et al.* (19) recently identified p53 germ line mutations in 85% of 14 children with adrenocortical tumors who were ascertained from Manchester Children's Tumor Registry. The spectrum of mutations was remarkably limited, occurring predominantly in codons 152 and 158 within the core DNA-binding domain of the p53 (19). However, one patient with adrenocortical carcinoma in this series had the Arg337His mutation in the tumor DNA associated with two other mutations in introns 6 and 10. In this study, it was not possible to determine whether Arg337His mutation was inherited or acquired (19).

Mutation analysis of p53 gene have been confined principally to exons 4 through 8, and screening for the new hot spot within exon 10 in patients with adrenocortical tumors, especially at young ages from different parts of the world will clarify the frequency and importance of this mutation outside Brazil (20). Although many questions remain about the role of this distinct germ line p53 mutation in the origin of the adrenocortical tumors, there is no doubt about its extremely high incidence in Brazilian children with adrenocortical tumors as well as in their asymptomatic close relatives. There is also a clear evidence of LOH in most of the adrenocortical tumors. In addition, we demonstrated that the presence of the Arg337His mutation was not associated with an unfavorable prognosis in the great majority of children with adrenocortical tumors in our series.

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