[CANCER RESEARCH 52, 1369–1371, March 1, 1992]

Advances in Brief

Unique Association of *p53* Mutations with Undifferentiated but not with Differentiated Carcinomas of the Thyroid Gland

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Abstract

Thyroid neoplasms show a wide variety of lesions varying from slowly growing differentiated adenocarcinomas to rapidly proliferating undifferentiated carcinomas. There has been some histopathological evidence that the undifferentiated thyroid carcinomas are derived from differentiated carcinomas. Moreover, it is suspected that some genetic events might be associated with such changes. In the present study, mutations in the *p53* gene were investigated by direct sequencing analysis after polymerase chain reaction amplification of exons 5 to 8, using paraffin-embedded primary tumors and cultured cells.

No mutations in exons 5 to 8 were detected in 10 differentiated papillary adenocarcinomas, whereas 6 of 7 undifferentiated carcinomas were found to carry base substitution mutations. Sequencing analysis confirmed mutations at codons 135 (TGC → TGT), 141 (CCC → CCT), 178 (CAC → GAC), 213 (CGA → TGA), 248 (CGG → CAG, CGG → TGG), and 273 (CGT → TGT). The spectrum of mutations (G:C to A:T transitions in 7 of 8) might be a specific feature of the spontaneous cancers. The results strongly suggest that, in human thyroid glands, *p53* mutations play a crucial role in the progression of differentiated carcinomas to undifferentiated ones.

Introduction

According to the current concept of carcinogenesis, tumor development consists of multistep accumulations of adverse genetic and epigenetic events (1). These genetic events include activation of dominantly acting oncogenes by point mutations, rearrangements, or amplification and inactivation of tumor suppressor genes via point mutations or deletions. Among the tumor suppressor genes identified, the *p53* gene is the best understood and its mutation has been shown to be associated with many types of common cancers, such as colon, lung, liver, esophagus, and breast cancer (2). Interestingly, the majority of such *p53* gene mutations are clustered in four hot spot regions (codons 117–142, 171–181, 234–258, and 270–286) that are highly conserved among different species (3, 4).

In colorectal carcinomas, where the multistep nature of carcinogenesis has been studied in detail, the *p53* mutation is suspected to be a late event and relates to the adenoma-carcinoma transition (5). In this regard, thyroid carcinoma also might serve as an interesting model. It has been recognized that most of the thyroid neoplasms are differentiated tumors with high curability, whereas occasional undifferentiated carcinomas kill the host shortly after their diagnosis. Furthermore, it is believed that undifferentiated carcinomas of the thyroid gland mostly arise from well-differentiated tumors (6–9). In thyroid carcinomas, dominantly acting activated oncogenes of *ras* point mutations and *ret* translocations have been detected (10, 11), whereas mutations of the *p53* gene in thyroid neoplasms have not been investigated. In the present study, we describe a new finding that *p53* mutations are uniquely associated with undifferentiated thyroid carcinomas but not with differentiated papillary adenocarcinomas.

Materials and Methods

Ten cases of differentiated papillary adenocarcinoma, six cases of undifferentiated carcinoma, and one cell line 8305C (JCRB 0824) established in our laboratory from an undifferentiated carcinoma of the thyroid were investigated in this study. Tissues derived either from surgical resections or autopsies were fixed with 10% formalin and embedded in paraffin blocks. Five 5-μm-thick sections (9–100 mm^2) of each tissue were sufficient for the PCR[2] study. Among the five serial sections, the first and fifth ones were stained with hematoxylin and eosin for histological assessment. After microscopic identification, apparent normal portions and tumor portions were collected from the remaining three sections with stainless steel disposable scalpels. Subsequently, they were deparaffinized with 1 ml of xylene, washed with 100% ethanol, and treated in 100 μl of digestion buffer (50 mM Tris-Cl, pH 8.5–1 mM EDTA-0.5% Tween 20) with 100 μg of proteinase K at 37°C for 48 h. After phenol-chloroform extraction, genomic DNA was precipitated with ethanol. Genomic DNA from cell line 8305C was also prepared by the proteinase K-phenol chloroform extraction method.

Genomic DNA (200 ng) was subjected to PCR amplification in 20 μl of solution containing 50 mM KCl, 10 mM Tris-Cl (pH 8.3), 5.5 mM $MgCl_2$,500 μM concentrations each of four deoxynucleotide triphosphates, 2 pmol of PCR primers, and 0.5 unit of *Taq* DNA polymerase (Perkin-Elmer Cetus) under 40 cycles of thermal conditions as follows: 30 s at 94°C (denaturation); 1 min at 60°C (annealing); and 30 s at 72°C (polymerization). The products of PCR were purified by low melting agarose gel (3%) electrophoresis. Using 1% of the purified product as templates, 35 cycles of asymmetrical PCR were performed in 20 μl of the same solution described above except with an uneven molar ratio of the two primers (1 pmol:20 pmol). The products of asymmetrical PCR were purified by precipitation with ethanol and isopropyl alcohol in the presence of ammonium acetate. Sequencing primers were labeled on their 5′ ends with [r-^{32}P]ATP and T4 polynucleotide kinase. Purified asymmetrical PCR products were sequenced directly by the dideoxy chain termination method of Sanger *et al.* (12). Template DNA was denatured at 95°C for 5 min, annealed with 1 pmol of labeled sequencing primer at 65°C for 10 min, and sequenced with Sequenase version 2.0 kit reagents (U. S. Biochemical). Mutations were confirmed in the sequence of sense and antisense strands. The primers used for PCR amplification and direct sequencing of exons 5, 6, 7, and 8 have been described previously (13).

Received 12/4/91; accepted 1/17/92.

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[2] The abbreviation used is: PCR, polymerase chain reaction.

Results

Direct sequence analysis of *p53* gene exons 5 to 8 was performed after PCR amplification in 17 cases of thyroid carcinoma. No mutations could be detected in 10 differentiated papillary adenocarcinomas, whereas base change mutations were detected in 6 of 7 (86%) of the undifferentiated carcinomas, as described in Table 1. Details of the mutations are summarized in Table 2. The base substitution found in the anaplastic cell line 8305C was a C:G to T:A transition at the first base of codon 273 with loss of the wild-type nucleotide at the site of the substitution (Fig. 1).

Mutations in cases 2 and 3 were G:C to A:T transitions at the second base of codon 248, with a wild-type nucleotide band of equal intensity in case 2 and with a weak wild-type nucleotide in case 3 (Fig. 1) at the site of the base substitution. The first base of codon 213 in case 4 was mutated from C:G to T:A with a wild-type nucleotide band of equal intensity (Fig. 1).

Two nonsense mutations and one missense mutation were found in case 5; they were all C:G to T:A transitions at the third base of codons 135 and 141 and at the first base of codon 248. All three mutations in case 5 were accompanied with wild-type nucleotide bands of equal intensity. The mutation in case 6 was a C:G to G:C transversion at the first base of codon 178 with a faint band of the wild-type nucleotide. To investigate the possibility of germline mutations, the sequence of the corresponding exon was examined in adjacent tissue in cases 3, 4, and 6. There were no detectable germline abnormalities in these cases. In other cases we could not obtain normal tissues in order to investigate possible germline mutations.

Table 1 *p53 gene mutations and histological type*

Histological type	*p53* gene mutation (positive/tested)
Papillary adenocarcinoma	0/10 (0)[a]
Undifferentiated carcinoma	6/7 (86)

[a] Numbers in parentheses, percentages of mutations.

Table 2 *p53 mutations in undifferentiated carcinomas*

Case[a]	Codon	Nucleotide substitution	Amino acid change	Loss of wild type[b]	Germline mutation[c]
1	273	CGT → TGT	Arg → Cys	+	NT
2	248	CGG → CAG	Arg → Gln	−	NT
3	248	CGG → CAG	Arg → Gln	+	−
4	213	CGA → TGA	Arg → stop	−	−
5	135	TGC → TGT	Nonsense	NS	NT
	141	CCC → CCT	Nonsense	NS	NT
	248	CGG → TGG	Arg → Trp	NS	NT
6	178	CAC → GAC	His → Asp	+	−

[a] Case 1 was an undifferentiated carcinoma cell line (8305C) and cases 2–6 were primary undifferentiated carcinomas.
[b] Loss of wild-type allele at *p53* was determined by direct sequencing. NS, not sufficient to be judged by direct sequencing.
[c] Germline mutations were investigated using adjacent tissues. NT, not tested.

Discussion

Mutations of the *p53* gene are the most commonly observed genetic alterations in human cancers. However, such mutations have not been investigated previously in thyroid cancers. In the present study, we have found a very strong association of *p53* mutations in undifferentiated thyroid carcinomas, but no association in differentiated papillary adenocarcinomas. These mutations, except in one case, were clustered in four regions, which are highly conserved among different species (14). Interestingly one exceptional mutation that occurred out of the well-conserved regions resulted in a stop codon. In many other human tumors, most of the *p53* mutations are localized in these regions and among them there are at least three mutational hot spots affecting codons 175, 248, and 273 (3). The frequency of mutations occurring in each of the hot spot regions and their type of base substitution differs depending on the cancer type. In lung and esophageal cancers related to cigarette smoking, G:C to T:A transversions are the most frequent (2, 15, 16). In hepatocellular carcinoma, related to hepatitis virus infection and ingestion of contaminated food with aflatoxin B_1 in China and southern Africa, G:C to T:A transversions at codon 249 are the most frequent gene alteration (13, 17). These findings suggest a close correlation between etiological agents in the environment and specific types of base substitutions.

Unfortunately, there is nothing known about the exogenous risk factors of undifferentiated carcinomas of the thyroid gland. According to the sequential analysis in this study, seven of eight mutations were G:C to A:T transitions, among which three were G:C to A:T transitions at the CpG dinucleotide of codon 248. This base substitution pattern is similar to that of most other cancers such as colon, breast, lung (small cell), and leukemia and might be characteristic of spontaneous mutations in mammalian cells (2).

In the thyroid carcinomas, one-half of the undifferentiated carcinomas showing only a single mutant band on the sequencing gel represent a loss of the normal allele. On the other hand, two cases showed both normal and mutant bands, which might be due to the remaining normal allele of the *p53* gene. Considering the retention of the normal allele from these findings, it cannot be excluded that the wild-type allele might be derived from contamination by infiltrating inflammatory cells and stromal cells and that the residual allele might be mutated elsewhere besides exons 5 to 8. However, these data can also suggest the possibility that the single *p53* mutation of one allele with a remaining normal allele can be involved in tumor pro-

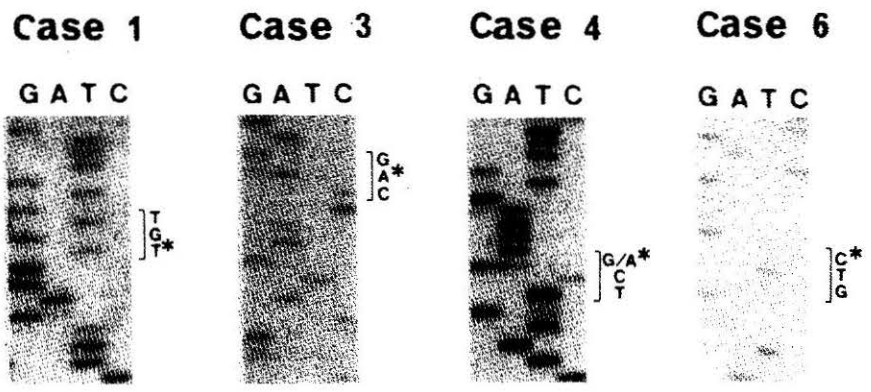

Fig. 1. Direct sequencing of the *p53* gene in primary thyroid cancers. In Case 1, a C:G to T:A transition (T*) in codon 273 is indicated in the sequence of the sense strand. In Case 3, a G:C to A:T transition (A*) in codon 248 is indicated in the sequence of the sense strand. In Case 4, a C:G to T:A transition (A*) in codon 213 is indicated in the sequence of the antisense strand. The mutated nucleotide band was accompanied by the wild-type nucleotide band of equal intensity. In Case 6, C:G to G:C (C*) transversion in codon 178 is indicated in the sequence of the antisense strand.

gression through a dominant negative effect, which might be mediated by the binding of the mutant *p53* product to the wild-type product (18, 19). Subsequently, during further tumor progression, another loss of growth control can be exerted when the wild-type allele is lost (4).

In the thyroid carcinoma it is generally agreed that undifferentiated thyroid carcinomas mainly arise from preexisting differentiated carcinomas; this hypothesis is supported by much clinical and pathological evidence (6–9). Further, *p53* mutation was detected exclusively at the undifferentiated foci in a thyroid carcinoma where various histological features of normal, differentiated, and undifferentiated carcinomas were observed simultaneously.[3] The characteristics of slow growth of tumor cells with no *p53* mutations in differentiated carcinomas and rapid growth of cells with an extremely high frequency of *p53* mutations in undifferentiated carcinomas suggest that *p53* mutations, which emerged during the growth of differentiated carcinomas, might result in undifferentiated carcinomas due to their uncontrolled growth (18, 19) and dedifferentiation (20).

Acknowledgments

We thank Drs. James E. Trosko, Yukio Takeshima, and Rumi Haruta for fruitful discussions and encouragement. We are very grateful to Masumi Enno, Hiromi Tagawa, and Norie Ishii for technical support. We also thank Michiko Takagi for manuscript preparation and Michael Edington for his review of the manuscript.

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[3] Manuscript in preparation.