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Inactivation of the p53 Gene Is not Required for Tumorigenesis of Medullary Thyroid Carcinoma or Pheochromocytoma

Ikuo Yana,[1,2] Tsutomu Nakamura,[1] Eisei Shin,[2] Katsu Karakawa,[2] Hiroki Kurahashi,[1] Yoshihiro Kurita,[2] Tetsuro Kobayashi,[2] Takesada Mori,[2] Isamu Nishisho[1,3] and Shin-ichiro Takai[1]

[1]*Division of Clinical Genetics, Department of Medical Genetics, Biomedical Research Center, Osaka University Medical School, 2-2 Yamadaoka, Suita 565 and* [2]*Second Department of Surgery, Osaka University Medical School, Fukushima 1-1-50, Fukushima-ku, Osaka 553*

A polymerase chain reaction (PCR)-mediated RNase protection analysis was performed to detect subtle genetic alterations of p53 in medullary thyroid carcinoma (MTC) and pheochromocytoma. None of the 30 pheochromocytomas showed abnormal RNase protection patterns. Only one of 32 MTCs showed an abnormal pattern, and subsequent DNA sequencing of the PCR product revealed that it had a G to C transversion in codon 49 that resulted in a change from aspartic acid to histidine. However, this was a sporadic MTC with no specific clinicopathological characteristics. On the basis of a previous report that genes on chromosome 17p were not deleted in MTCs and were relatively infrequently deleted in pheochromocytomas, our results suggest that the p53 gene is not involved in tumorigenesis of MTC or pheochromocytoma.

Key words: MTC — Pheochromocytoma — p53 — RNase protection analysis — Multiple endocrine neoplasia type 2

Medullary thyroid carcinoma (MTC) and pheochromocytoma, classified as neuroendocrine tumors, occur in both sporadic and hereditary forms. In their hereditary form, they are transmitted as an autosomal dominant trait either independently or as part of a multiple endocrine neoplasia type 2 (MEN 2). The gene predisposing to MEN 2 syndrome has been mapped at the pericentromeric region of chromosome 10 by linkage analysis.[1,2] In MEN 2 tumors, frequent loss of heterozygosity (LOH) on chromosome arms 1p, 3p, and 22q has been demonstrated,[3–8] while loss of genes at the pericentromeric region of chromosome 10 is infrequent.[9–11] These data suggest that inactivation of multiple tumor suppressor genes is required for tumorigenesis in MEN 2, as is the case in colorectal cancers.[12–14]

Inactivation of the p53 tumor suppressor gene plays an important role in carcionogenesis of a wide variety of human tumors.[15–18] In these tumors, the p53 gene is often eliminated somatically through loss of the chromosomal region 17p13.1 that has been identified as a form of LOH through molecular genetic analysis.[19,20] The LOH on 17p in MTCs and pheochromocytomas has been quantitated as 0% and 24%, respectively.[6] To detect more subtle genetic alterations that inactivate the p 53 gene, we performed a polymerase chain reaction(PCR)-mediated RNase protection analysis of these two types of tumors.

[3] To whom correspondence should be addressed.

Thirty-two MTCs (20 sporadic and 12 hereditary) and 30 pheochromocytomas (20 sporadic and 10 hereditary) were obtained between June 1984 and December 1991. The average sizes of MTCs and pheochromocytomas were 2.8 cm and 4.5 cm in diameter, respectively. Fresh tumor tissues were frozen immediately at the time of surgical operation and kept at −80°C until DNA extraction. High-molecular-weight DNAs were prepared from frozen tumor tissues by proteinase K digestion and phenol/chloroform extraction. A portion of the genomic p53 gene including exon 4 to exon 9 was divided into four regions, R1 to R4, and oligonucleotide primers for PCR were designed so that all the coding exons in each region were covered (Table I). PCR was performed with a thermal programmer (Nippon Genetics, Tokyo) in 25 μl volumes with 200 ng of genomic DNA and 1.2 μM primers in 10% DMSO, 67 mM Tris-HCl (pH 8.8), 6.7 mM $MgCl_2$, 16.6 mM $(NH_4)_2SO_4$, 10 mM β-mercaptoethanol, 6.7 mM EDTA, and 1.5 mM dNTPs containing 2.5 units of *Thermus aquaticus* (Taq.) DNA polymerase (Boehringer Mannheim, Penzberg, Germany). PCR reactions consisted of the initiation cycle (2 min at 95°C; 3 min at 55°C; 2 min at 70°C), repeated cycles (37 cycles of 30 s at 95°C; 2 min at 55°C; 2 min at 70°C), and the final cycle (1 min at 95°C; 2 min at 55°C; 4 min at 70°C). All primers had extraneous nucleotides comprising either the *Eco*RI or *Hin*dIII site at their 5′ends to facilitate subsequent cloning and sequencing of PCR products. The

Table I. Oligonucleotide Primers Used in PCR Amplification

Primer		Amplified product		
Name	Sequence	Region	Length (bp)	Codon included
G1	5′-GTAGGAATTCGTCCCAAGCAATGGATGAT-3′	R1	276	43–121
G2	5′-CTCAAGCTTAACTGACCGTGCAAGTCACA-3′			
G3	5′-GTAGGAATTCCTCTTCCTGCAGTACTC-3′	R2	408	127–223
G4	5′-CTCAAGCTTAGTTGCAAACCAGACCTCAG-3′			
G5	5′-GTAGGAATTCTCCTAGGTTGGCTCTGA-3′	R3	123	228–256
G6	5′-CTCAAGCTTCTGACCTGGAGTCTTCCAG-3′			
G7	5′-GTAGGAATTCCTATCCTGAGTAGTGGTAA-3′	R4	339	263–329
G8	5′-CTCAAGCTTAAGACTTAGTACCTGAAGG-3′			

The sequences for the primers were taken from the published exon and intron sequences of the genomic p53 gene. Underlined sequences are extraneous *Eco*RI or *Hin*dIII recognition sites used to facilitate cloning of PCR products.

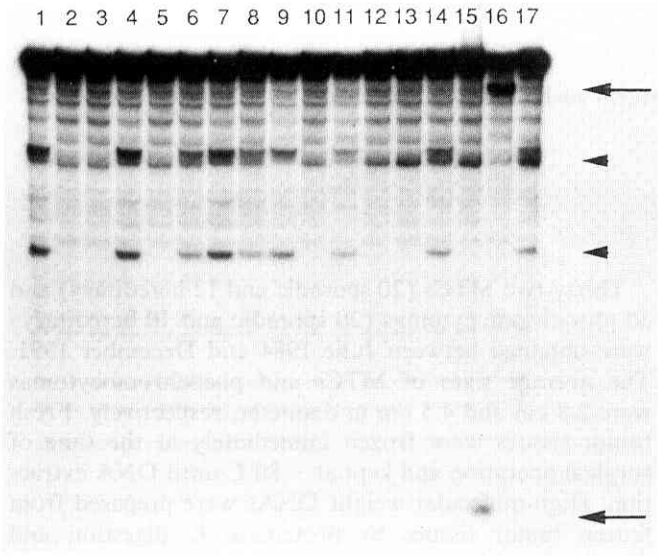

Fig. 1. RNase protection analysis of region R1 of the p53 gene in MTCs. The amplified genomic fragments were 276 bp in length and contained codons 43 to 121. Lane 16 (MTC 16) shows abnormal RNase cleavage products (arrows). Arrowheads indicate polymorphism in codon 72.

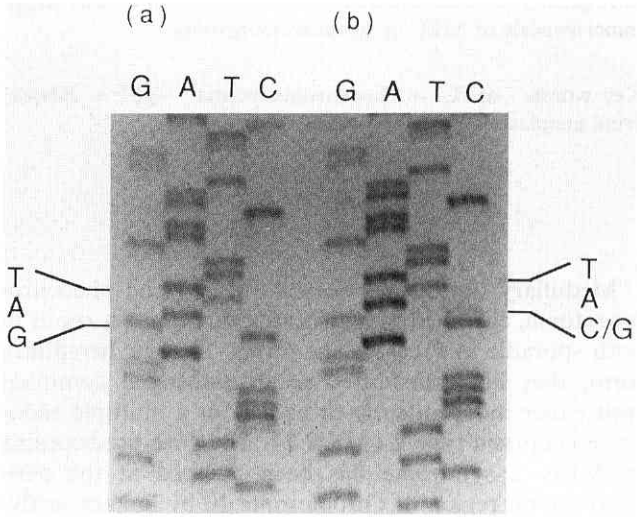

Fig. 2. Sequence analysis of the variant PCR product. This shows the C [in germ line, (a)] to G [in MTC, (b)] transversion on one allele in codon 49 that resulted in a transformation from aspartic acid to histidine in MTC 16.

PCR products were screened for mutation by RNase protection analysis. The ^{32}P-labeled RNA probes for each region representing the normal genomic sequences were prepared by using an RNA transcription kit (Stratagene, La Jolla, CA) and [α-^{32}P]CTP (Amersham, Little Chalfont, England). One μl of amplified PCR products was hybridized with labeled RNA probes (1.5×10^5 cpm) at 50°C for 2 h, and the hybrids were digested with RNase A (22.5 μg/ml) (Sigma, St. Louis, MO) at 37°C for 1 h. RNase A can cleave at single base pair mismatches within DNA-RNA hybrids. RNase A was inactivated by proteinase K (50 μg/ml) treatment and phenol/chloroform extraction. The RNA fragments were visualized by 8% polyacrylamide/8 *M* urea denaturing gel electrophoresis followed by autoradiography. Two separate analyses were performed for each region, one with the sense and one with the antisense strand. The variant PCR products were double-digested with *Eco*RI and *Hin*dIII, and fractionated by electrophoresis through 2% agarose gel. The eluted DNA fragments were cloned in the *Eco*RI and *Hin*dIII sites of Bluescript SK and pools of at least 50 clones were sequenced together by means of T7 polymerase. The primers used for sequencing were T3 primer, 5′-

ATTAACCCTCACTAAAG-3′ (sense) and T7 primer, 5′-AATACGACTCACTATAG-3′ (antisense).

As shown in Figure 1, only one MTC (MTC 16) showed an abnormal RNase protection pattern in region R1 (encoding amino acids 43 to 121). Cloning and sequencing of the MTC 16 PCR product indicated that it had a G-to-C transversion in codon 49 that resulted in a change from aspartic acid to histidine (Fig. 2). However, this was a sporadic MTC having no specific characteristics with regard to tumor size (2.5 cm in diameter), histopathological feature, and clinical course. According to recent reports, the amino acid substitution was found germinally and is considered a rare polymorphism.[21, 22] As we could not obtain the constitutional DNA of the patient, we are unable to conclude whether the relatively unconservative substitution is related to MTC tumorigenesis or not. No other tumors showed any abnormal RNase protection patterns in the four regions examined in this study. It is known that RNase A cannot effectively cleave some types of mismatches. To improve the sensitivity of RNase protection analysis, we examined both sense and antisense strands. Under these conditions, more than 50% of all possible mismatches can be detected.[23] Moreover, we can recognize mismatches as abnormal cleavage patterns only if they can be partially cleaved by RNase A. Therefore, we consider that the sensitivity of the RNase protection analysis is a minor reason for infrequent detection of the mutations. Polymorphism in codon 72 (CCC or CGC), reported by Buchman *et al.*,[24] was also detected as a result of RNase protection analysis of region R1 (Fig. 1). The gene frequency calculated in 60 tumors was 0.58 for CCC and 0.42 for CGC.

In summary, only one of the 32 MTCs (3.1%) was found to have a point mutation in the p53 gene, while none of the 30 pheochromocytomas showed even a subtle alteration in the gene. The MTC 16 with a mutated p53 gene was histologically a typical medullary thyroid carcinoma with no specific clinical characteristics. Although LOH on 17p was not examined in this study, our results, taken together with those of a previous report that LOH on 17p was not found in MTCs and was not frequent in pheochromocytomas, strongly suggest that inactivation of the p53 tumor suppressor gene is not essential for tumorigenesis of these two types of tumors, which are components of the MEN 2 syndrome.

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