p53 Gene Mutations Associated with Anaplastic Transformation of Human Thyroid Carcinomas

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Anaplastic carcinoma of the thyroid gland, which is one of the most aggressive, malignant tumors in humans, is considered to originate from preexisting differentiated thyroid cancer. To define the genetic alterations associated with such progression, we examined nine cases of anaplastic thyroid carcinoma for mutation in exons 4–9 of the p53 tumor suppressor gene. Preliminary screening for mutation by RNase protection analysis demonstrated that two out of nine anaplastic carcinomas contained sequence alterations in the p53 gene. Subsequent DNA sequencing identified the mutated nucleotides in these two cases; one was a nonsense mutation at codon 165, and the other was a single-base deletion at codon 176 resulting in the creation of a stop codon downstream due to frameshift. The fact that no mutations were detected in coexisting foci of papillary carcinomas from the same patients shows that these mutations of the p53 gene occurred after development of papillary carcinomas. These results suggest that p53 gene mutation triggers the progression from differentiated into anaplastic carcinoma in the human thyroid gland.

Key words: p53 gene — Anaplastic thyroid carcinoma — Progression — RNase protection analysis

Anaplastic carcinoma of the human thyroid gland is a relatively uncommon malignancy comprising 5–14% of thyroid cancers.[1] It is also the most malignant of the thyroid cancers, with aggressive characteristics such as rapid proliferation, invasive growth and metastasis. In contrast with patients with other types of thyroid cancers, those with anaplastic carcinoma have an extremely poor prognosis in spite of radiotherapy and chemotherapy. Most patients die within a year after diagnosis.[2,3] Anaplastic carcinoma is often observed to coexist with well-differentiated carcinomas such as papillary or follicular carcinoma.[4,5] On the basis of such histopathological evidence, it has been hypothesized that anaplastic carcinoma arises through anaplastic transformation of preexisting differentiated carcinoma.[6,7] So far, however, there is little information regarding the molecular mechanism underlying such progression, including activation of oncogenes and inactivation of tumor suppressor genes. In particular, the role of tumor suppressor genes in the development of thyroid cancers has not been studied in detail.

The p53 tumor suppressor gene is well-known as a frequent target for mutations and allele losses in a wide variety of human malignancies.[8,9] There is also some evidence that the p53 gene alteration is related to tumor progression. Fearon and Vogelstein[10] proposed that 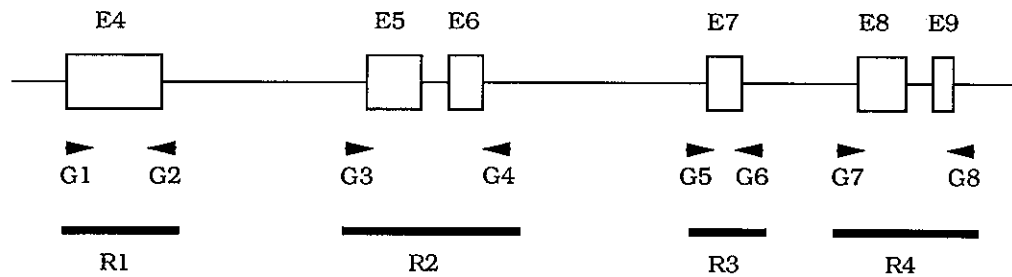mutational inactivation of the p53 gene is a critical event in the later stages of colorectal tumorigenesis. In chronic myelocytic leukemia, mutation in the p53 gene has been observed in conjunction with its progression and its evolution to blast crisis.[11] A recent report[12] has demonstrated that progression of brain tumors is associated with clonal expansion of cells containing the mutated p53 gene. These data taken together suggest a relationship between p53 gene mutation and clinical progression in at least several types of tumors. We report here that p53 gene mutations were found in two anaplastic carcinomas and not in coexisting papillary carcinomas of the same patients, indicating that p53 gene mutation is one of the crucial genetic changes triggering the transformation of differentiated into undifferentiated carcinomas in the human thyroid gland.

MATERIALS AND METHODS

Clinical materials The cancer tissues used in this study were removed surgically from patients with anaplastic thyroid carcinoma between 1987 and 1992. The specimens consisted of five frozen and four paraffin-embedded cancer tissues.

Preparation of genomic DNA High-molecular-weight DNAs were extracted from frozen cancer tissues as described.[13] DNAs were also purified from cancer tissues embedded in paraffin blocks. In brief, 50–100 paraffin sections were incubated overnight at 50°C in lysis buffer containing 600 μg/ml Proteinase K without deparaffini-

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Fig. 1. Schematic representation of the amplified region in the p53 gene and primers used. Open boxes E4–E9 indicate the coding exons of the p53 gene. Regions R1–R4 (bold lines) were amplified by PCR using primers G1–G8 (arrowheads). The sequences for the primers were taken from the published exon and intron sequences of the genomic p53 gene.[20] Underlined sequences are extraneous EcoRI or HindIII recognition sites to facilitate cloning of PCR products.

zation, and purified by phenol/chloroform extraction and ethanol precipitation.

The concentrations of these DNA stocks were estimated by both spectrophotometric and electrophoretic methods. We prepared 100 ng/μl aliquots as templates for the following polymerase chain reaction (PCR).

PCR PCR[14, 15] was performed in 25 μl volumes with 200 ng of genomic DNA and 1.2 μM primers in 67 mM Tris-HCl (pH 8.8), 6.7 mM MgCl$_2$, 16.6 mM (NH$_4$)$_2$SO$_4$, 10 mM β-mercaptoethanol, 6.7 μM EDTA, 10% DMSO, 1.5 mM dNTPs containing 2.5 units of *Thermus aquaticus* (*Taq*) DNA polymerase (Boehringer Mannheim). 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). The sequences of oligonucleotide primers used are shown in Fig. 1. All primers had extraneous nucleotides comprising either EcoRI or HindIII sites at their 5′ ends to facilitate subsequent cloning and sequencing of PCR products.

To control for DNA contamination of PCR, all experiments included one reaction tube in which diluted water was added instead of template DNA. Pipetting devices, tubes, and reagents used in PCR were kept strictly separated from any PCR products.

RNase protection analysis PCR products amplified from genomic DNA without any mutations in the p53 gene were cloned to be used as templates for RNA probes. Prior to preparation of RNA probes, we confirmed by DNA sequencing that the cloned PCR products had no mutations in the p53 gene. The ^{32}P-labeled RNA probes for each region representing the normal genomic sequences were synthesized by using an RNA transcription kit (Stratagene) and [α-^{32}P]CTP (Amersham). Amplified PCR products were 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) 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 (500 μg/ml) treatment and phenol/chloroform extraction. The RNA fragments were visualized by

8% polyacrylamide/8 M urea denaturing gel electrophoresis followed by autoradiography.[16-18] Two separate analyses were performed for each region, one with the sense and one with the antisense strand.

Cloning and sequencing of PCR products The PCR products were double-digested with *Eco*RI and *Hind*III, and fractionated by electrophoresis through 2% agarose gel. The DNA fragments were eluted from excised gel slices with the aid of SUPREC™-01 (Takara Shuzo), and ligated to Bluescript SK vector (Stratagene). After transformation of the *E. coli* DH5αF' strain, double-strand plasmid DNA was isolated from the pools of 50 clones as described by Del Sal *et al.*,[19] and used as a template for the sequencing reaction. Both sense and antisense strands were sequenced with the dideoxy chain-termination method using a T7 sequencing kit (Pharmacia) and [α-^{35}S]dATP (Amersham). The primers used for sequencing were T3 primer, 5'-ATTAACCCTCAC-TAAAG-3' (sense) and T7 primer, 5'-AATACGACTC-ACTATAG-3' (antisense). Reaction products were electrophoresed on 6% polyacrylamide/8 M urea gels for 2–4 h. Gels were fixed in 5% acetic acid/5% methanol, dried, and exposed to Kodak X-AR5 films at room temperature for 1–3 days.

RESULTS

Screening for the p53 gene mutation 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 (Fig. 1). Each region was amplified by PCR using genomic DNAs purified from five frozen and four paraffin-embedded anaplastic cancer specimens as templates. The PCR products were screened for mutation by RNase protection analysis. Two of nine cases of anaplastic thyroid carcinoma exhibited abnormal RNase cleavage patterns in region R2. Moreover, we could extract DNAs from coexisting foci of papillary carcinomas of these two cases; when they were analyzed in the same way, no variations in RNase cleavage patterns were observed. Fig. 2 shows the variation in RNase cleavage patterns detected in the anaplastic carcinoma of case 1.

Identification of the mutated nucleotide To confirm the sequence alterations detected by RNase protection analysis, we determined the nucleotide sequences of cloned PCR products, and identified the sites and characteristics of the alterations. Pools of 50 clones generated from PCR products were sequenced together in order to detect the mutation on one allele, and to minimize false-positive signals resulting from misincorporation of dNTPs during PCR reactions. In case 1, a single-base substitution (C to T transition) at codon 165 in exon 5, changing glutamine

Fig. 2. RNase protection analysis of region R2 of the p53 gene. Abnormal cleavage products were detected in a focus of anaplastic carcinoma of case 1 (lane 5), but not in a normal thyroid tissue (lane 3) or a coexisting focus of papillary carcinoma (lane 4) of case 1. Case 3 (lane 1) and case 4 (lane 2) showed normal cleavage patterns. Subsequent sequence analysis revealed that 411-base RNA probe was cleaved by RNase A into 276- and 135-base subfragments. Nucleotide lengths of uncleaved and cleaved RNA fragments are indicated on the right.

(CAG) into a stop codon (TAG), was identified (Fig. 3a). Case 7 contained a deletion of a guanine nucleotide at codon 176 (TGC to T-C) in exon 5 (Fig. 3b). This single-base deletion resulted in a frameshift, which created a stop codon downstream in exon 7. Both mutations were confirmed by sequencing of the opposite DNA strand complementary to that sequenced in the initial experiment. PCR products of region R2 from corresponding papillary carcinomas were also sequenced. As the RNase cleavage patterns indicated, no mutations were detected.

DISCUSSION

We explored nine cases of anaplastic thyroid carcinomas, and found that two of them contained the p53 gene with point mutations. Both of the mutations detected in
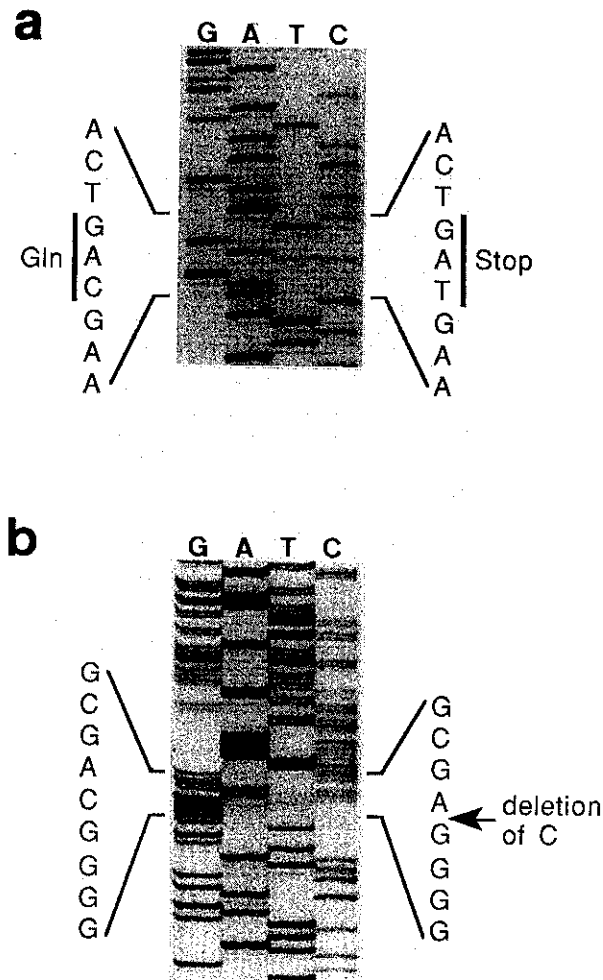
a

b

Fig. 3. Sequence analysis of PCR products that showed variations in RNase protection analysis. A part of the sense strand sequence of R2 in case 1 (a) and a part of the antisense strand sequence of R2 in case 7 (b) are shown. The sequences on the left and the right sides of each panel denote the wild-type sequence and the mutant sequence, respectively. In case 1, a C-to-T transition in codon 165 that resulted in a change from glutamine to a stop codon was observed. Case 7 showed a single-base deletion in codon 176, which caused the sequencing ladders to show double (normal and mutant) bands beyond the position of the mutation.

these two cases led to creation of stop codons; one was a nonsense mutation, and the other was a single-base deletion which gave rise to a stop codon in the downstream exon. Although we could not examine allelic deletion of the p53 gene in all cases, each of these two cases was thought to have the mutant p53 gene on one allele because both mutant and wild-type nucleotide bands were detected on the sequencing gels (Fig. 3). Newly

generated stop codons interrupt the normal synthesis of full-length p53 protein, and a truncated protein which lacks its putative functional domains is produced. Therefore, we inferred that at least one allele of the p53 gene was functionally inactivated in these two cases.

The frequency of mutation detected in our study was not as high as that for carcinomas of the colon[20, 21] or lung.[22-24] We regard our value as a conservative estimate for the following reasons. We designed all primers so that they included several of the first or last nucleotides of exons, as the nucleotide sequences of introns remain unknown except for the exon-intron junction.[25] Therefore, mutations occurring in the regions included in the primers, such as splice site mutation, could not be detected. It is also possible that mutations were present in a region of the gene not targeted in our study. If p53 gene inactivation in anaplastic carcinoma is frequently caused by generation of a stop codon, mutations which occur in upstream exons outside the five highly conserved domains[26] are also expected to affect the biological activities of p53 protein. It is known that some types of mismatches cannot be cleaved sufficiently by RNase A. To compensate for the mismatches undetectable by the analysis of one strand, we examined both sense and antisense strands. Under these conditions, more than 50% of all possible mismatches are detectable.[27] Moreover, mismatches can be detected as abnormal cleavage patterns if only they can be partially cleaved by RNase A. Therefore, the sensitivity of the RNase protection analysis is considered to be a minor reason for infrequent detection of the mutations.

Several types of genetic lesions have been observed in anaplastic thyroid carcinomas. Activation of cellular oncogenes such as K-*ras*, N-*ras*, and c-*myc* has already been reported.[28-30] A novel gene with transforming ability, termed *cot*, has been isolated from a human anaplastic carcinoma by means of the focus-formation assay.[31] On the other hand, there have been few studies concerning the mutational inactivation of tumor suppressor genes in this malignancy. Wright *et al.*[32] reported that point mutation of the p53 gene was found only in a differentiated follicular thyroid carcinoma cell line, but not in 20 other thyroid cancers including four anaplastic carcinomas. More recently, it was reported that p53 gene mutation is associated with anaplastic, but not with differentiated thyroid carcinoma.[33] The authors detected the mutation in six of seven (86%) anaplastic carcinomas including primary carcinomas and cultured cells, but not in ten papillary carcinomas from other patients.

In contrast, we detected the p53 gene mutation at relatively low frequency. However, both mutations detected in our study created stop codons which are expected to alter greatly the biological activities of the p53 protein. We have also shown that these mutations

were present in anaplastic carcinoma cells, but not in coexisting differentiated papillary carcinoma cells in the same patients. These observations, coupled with previous histopathological evidence, suggest that mutation resulting in the functional inactivation of the p53 gene plays a critical role in the progression (anaplastic transformation) of the differentiated thyroid carcinoma into anaplastic carcinoma. The relationship between the p53 gene mutation and tumor progression has also been supported by recent studies on brain tumors[12] and chronic myelocytic leukemias.[11] Acquisition of the p53 gene mutation may be a key step on the molecular pathway to the more malignant stage in human thyroid cancer.

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