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業績報告書シリーズ

甲状腺の未分化癌とp53遺伝子の変異との関係[§]

Unique Association of p53 Mutations with Undifferentiated but Not with Differentiated Thyroid Gland Carcinomas

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要　約

甲状腺腫瘍は，ゆるやかに増殖する分化腺癌から急速に増殖する未分化癌まで多様な像を呈する。病理組織学的には，未分化癌は分化癌から発生するという証拠がある。さらに，遺伝子に起こった何らかの異常がこれらの変化に関連するのではないかと疑われる。この研究において，パラフィン包埋された原発腫瘍および培養細胞株を用い，エクソン5−8のPCRを増幅した後，直接，塩基配列決定法によりp53遺伝子の突然変異が検出された。

分化型乳頭腺癌10症例において，エクソン5−8の突然変異は認められなかったが，未分化癌では，7症例中6症例が塩基置換による突然変異を有することが分かった。塩基配列決定法により，コドン135（TGC→TGT），141（CCC→CCT），178（CAC→GAC），213（CGA→TGA），248（CGG→CAG，CGG→TGG），273（CGT→TGT）において突然変異を確認した。この突然変異のスペクトラム（7／8においてG：CからA：Tへの転位）は，自然発生した腫瘍に認められる p53 突然変異の特徴かも知れない。

これらの結果は，ヒトの甲状腺における分化癌から未分化癌への転化に，p53 の突然変異が重要な役割をはたしていることを強く示唆する。

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Technical Report Series

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Summary

Thyroid neoplasms show a wide variety of lesions varying from slowly growing differentiated adenocarcinomas to rapidly proliferating undifferentiated carcinomas. There is 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 detected by direct sequencing analysis after polymerase chain reaction amplification of exons 5–8, using paraffin-embedded primary tumors and cultured cells.

No mutations in exons 5 to 8 were detected in 10 differentiated papillary adenocarcinomas, whereas 6 out 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 muta-

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tions, 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 since it has been recognized that most thyroid neoplasms are differentiated tumors with high curability, whereas occasional undifferentiated carcinomas kill the host shortly after their diagnosis. Furthermore, it is felt 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 the discovery 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, 6 cases of undifferentiated carcinoma, and 1 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 polymerase chain reaction (PCR) study. Among the five serial sections, the first and fifth 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 disposable stainless steel 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, and 0.5% Tween 20) with 100 μg of proteinase K at 37°C for 48 hr. After phenol-chloroform extraction, genomic DNA was precipitated with ethanol. Genomic DNA from the 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 each of four dNTPs, 2 pmol of PCR primers, and 0.5 units 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 PCR products were purified by low melting agarose gel (3%) electrophoresis. Using 1% of the purified product as templates, 35 cycles of asymmetric PCR were performed in 20 μL of the same solution described previously, except with an uneven molar ratio of the two primers (1 pmol:20 pmol). The products of

asymmetric PCR were purified by precipitation with ethanol and isopropanol in the presence of ammonium acetate. Sequencing primers were labeled on their 5′ ends with [r-^{32}P]ATP and T4 polynucleotide kinase. Purified asymmetric 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 (US Biochemical, Cleveland, Ohio). Mutations were confirmed in the sequence of sense and antisense strands. The primers used for PCR amplification and direct sequencing of exons 5–8 have been described previously.[13]

Results

Direct sequence analysis of p53 gene exons 5–8 was performed after PCR amplification in 17 cases of thyroid carcinoma. No mutations were detected in 10 papillary adenocarcinomas, whereas base change mutations were detected in 6 of 7 (86%) of the undifferentiated carcinomas, as described in Table 1. The 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 (Figure 1, Case 1).

The 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 (Figure 1, Case 2) 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 (Figure 1, Case 3).

Two non-sense mutations and one mis-sense 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 by 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 the other cases we could not obtain normal tissues in order to investigate possible germline mutations.

Discussion

Mutations of the p53 gene are the most commonly observed genetic alterations in human cancers. However, such mutations have not been previously investi-

Table 1. The frequency of p53 gene mutations by histological tumor type

Histological type	No. of p53 gene mutations (positive/tested)
Papillary adenocarcinoma	0/10
Undifferentiated carcinoma	6/7

Table 2. Characteristics of p53 mutations in undifferentiated carcinomas

Patient[a]	Codon	Nucleotide substitution	Amino acid change	Loss of wild-type[b]	Germline mutation[c]
Case 1	273	CGT → TGT	Arg → Cys	+	NT
Case 2	248	CGG → CAG	Arg → Gln	–	NT
Case 3	248	CGG → CAG	Arg → Gln	+	–
Case 4	213	CGA → TGA	Arg → Stop	–	–
Case 5	135	TGC → TGT	Non-sense	NS	NT
	141	CCC → CCT	Non-sense	NS	NT
	248	CGG → TGG	Arg → Trp	NS	NT
Case 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. NT = not tested.

[c]Germline mutations were investigated using adjacent tissues.

gated in thyroid cancers. In the present study, we 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

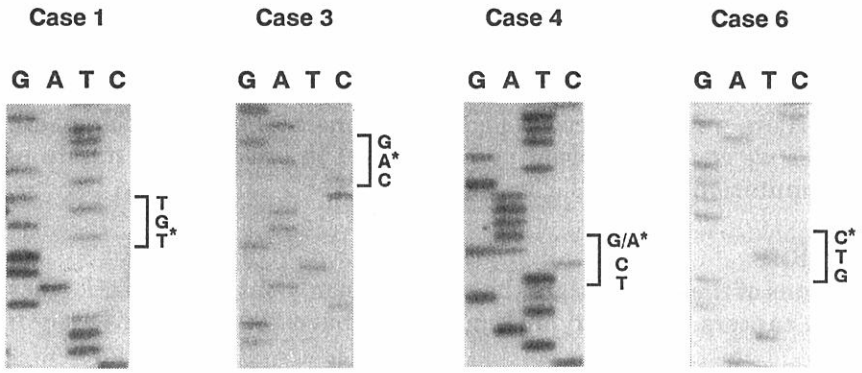

Figure 1. Direct sequencing of the p53 gene in primary thyroid cancers. Case 1 is a C:G to T:A transition (T*) in codon 273 as indicated in the sequence of the sense strand. Case 3 is a G:C to A:T transition (A*) in codon 248 as indicated in the sequence of the sense strand. Case 4 is a C:G to T:A transition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

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 differ 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 B1 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, nothing is 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, the single mutant band seen on the sequencing gel in half of the undifferentiated carcinomas represents a loss of the normal allele. However, two cases showed both normal and mutant bands, which might be due to the remaining normal allele of the p53 gene. Keeping in mind the retention of the normal allele, one should not exclude the possibility 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 5–8. However, these data also suggest the possibility that the single p53 mutation of one allele with a remaining normal allele can be involved in tumor progression 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] As to one negative case of p53 mutation found in the undifferentiated carcinoma, it is possible that other gene alterations of p53 besides those in exons 5–8 might exist.

In the thyroid carcinoma it is generally agreed that undifferentiated thyroid carcinomas mainly arise from preexisting differentiated carcinomas, and this hypothesis is supported by much clinical and pathological evidence.[6–9] Further, p53 mutation was detected exclusively at the differentiated foci in a thyroid carcinoma in which various histological features of normal, differentiated, and undifferentiated carcinomas were observed simultaneously (manuscript in preparation). The slow tumor-cell growth and lack of p53 mutations observed in differentiated carcinomas and the rapid tumor-cell growth and profuse p53 mutations observed in undifferentiated carcinomas, together, imply that p53 mutations that occur during the growth of differentiated carcinomas can give rise to undifferentiated carcinomas through the process of de-differentiation.[18–20]

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