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Genetic Alterations in Thyroid Tumor Progression: Association with p53 Gene Mutations

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To identify the genetic events that must be involved in thyroid tumor progression, we initially investigated p53 gene alterations in 10 papillary adenocarcinomas, 4 follicular adenocarcinomas, and 8 undifferentiated carcinomas. Base substitutional mutations in exons 5 to 8 and loss of heterozygosity (LOH) of the p53 gene were not detected in papillary or follicular adenocarcinomas. However, 7 of 8 undifferentiated carcinomas were carrying base substitutional mutations, and LOH was detected in 3 of 5 informative cases. Furthermore, to verify that the p53 gene alterations are truly involved in tumor progression, DNA from individual foci of the four undifferentiated carcinomas coexisting with a differentiated focus and from one follicular adenocarcinoma with an undifferentiated focus was analyzed by direct sequencing and polymerase-chain-reaction-restriction-fragment-length polymorphism (PCR-RFLP). Base substitutional mutations in the p53 gene from exons 5 to 8 were identified exclusively in the undifferentiated foci, but not in the differentiated foci. LOH was observed in 3 of 4 informative undifferentiated foci. In one of these positive cases, LOH was observed in both papillary adenocarcinoma and undifferentiated carcinoma. However, a p53 gene mutation at codon 248 was detected in the undifferentiated carcinoma but not in the papillary adenocarcinoma. The results imply that LOH occurs first in papillary adenocarcinoma followed by a p53 mutation during the transition from papillary adenocarcinoma to undifferentiated carcinoma. Maintenance of LOH during tumor progression excludes the possibility that these different histological foci are derived from different origins and represents molecular evidence that undifferentiated carcinoma is very likely derived from preexisting papillary adenocarcinoma. Furthermore, these results strongly suggest that the mutated p53 gene plays a crucial role in de-differentiation during the progression of thyroid tumors.

Key words: Tumor progression — p53 — Thyroid carcinoma — De-differentiation — Multistep carcinogenesis

According to the current concept of carcinogenesis, tumor development is accompanied by multistep accumulation of adverse genetic and epigenetic events.[1] It has become clear that an accumulation of genetic events is associated with biological changes during tumor progression.[2–6] In this regard thyroid carcinoma might serve as an interesting model, because undifferentiated carcinomas are suspected of arising from well differentiated tumors, mainly papillary adenocarcinoma or follicular adenocarcinoma, from pathological observations of their coexistence in the same tumor.[7–9]

To date, as candidate gene alterations involved in the early stages of thyroid tumor progression, point mutations in the dominantly acting activated *ras* oncogenes have been detected in benign, well-differentiated, and undifferentiated carcinomas.[10, 11] In addition, rearrangements of the *ret* and *trk* genes observed in papillary adenocarcinoma are suspected of being early events in tumor progression resulting in undifferentiated carcinoma.[12–14] Recently, we found that alterations of the p53 gene, which is one of the best understood tumor suppressor genes,[15, 16] are associated uniquely with undifferentiated carcinomas but not with well-differentiated carcinomas.[17] However, it could not be established with certainty that these changes were the actual genetic events involved in tumor transition from well-differentiated to undifferentiated carcinoma because our previous findings were obtained by the analysis of thyroid tumors from separate persons. To substantiate the involvement of p53 gene mutations in de-differentiation during tumor progression, it would be ideal to trace these genetic events in a single tumor in which the histological features of differentiated and undifferentiated carcinomas were observed together with normal tissue.

In the present study, we investigated p53 gene alterations in foci that show various histological features, namely, differentiated and undifferentiated components, coexisting simultaneously in the same tumor.

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MATERIALS AND METHODS

Tumor and cell line Ten cases of papillary adenocarcinoma, four cases of follicular adenocarcinoma, seven cases of undifferentiated carcinoma, and one cell line 8305C (JCRB 0824) established from an undifferentiated carcinoma of the thyroid were investigated in this study. Among these cases, one follicular adenocarcinoma contained an undifferentiated focus with a weak trabecular pattern, three cases of undifferentiated carcinomas each contained a residual papillary adenocarcinoma focus, and one case of undifferentiated carcinoma contained a residual follicular adenocarcinoma focus, according to the histological classification guidelines of the World Health Organization.[18]

DNA extraction Tissues derived either from surgical resections or autopsies were fixed with 10% formalin and embedded in paraffin blocks. Five 5-μm-thick sections (12–100 mm^2) of each tissue were prepared for histological and genetic analyses. Among the five serial sections, the first and fifth were stained with hematoxylin and eosin for histological assessment. After microscopic identification, apparently normal portions and tumor portions that each showed uniform histological features were collected from the remaining three sections with stainless steel disposable scalpels. Subsequently, these tissue samples were deparaffinized with 1 ml of xylene, washed with 100% ethanol, and treated in 100 μl of digestion buffer (50 m*M* Tris-Cl [pH 8.5], 1 m*M* EDTA, and 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 using the proteinase K-phenol-chloroform extraction method.

PCR direct sequencing Genomic DNA was subjected to polymerase chain reaction (PCR) amplification in 20 μl of solution containing 50 m*M* KCl, 10 m*M* Tris-Cl (pH 8.3), 5.5 m*M* $MgCl_2$, 500 μ*M* dNTP (each of the four), 2 pmol of PCR primers, and 0.5 unit of *Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT), using 40 cycles of the following thermal conditions: 30 s at 94°C, 1 min at 60°C, and 30 s at 72°C. The PCR products were purified using low-melting agarose gel (3%) electrophoresis. With 1% of the purified products as templates, 35 cycles of asymmetric PCR were performed in 20 μl of the same solution described above except that an unequal molar ratio of the two primers was used (1 pmol:20 pmol). After purification, the products were sequenced using a modification of the dideoxy-termination method of Sanger *et al.*,[19] using 1 pmol of sequencing primers with Sequenase Version 2.0 reagent kit (U.S. Biochemical, Cleveland, OH). The primers used for PCR amplification and direct sequencing of exons 5, 6, 7, and 8 have been described by Hsu *et al.*[20]

Polymerase-chain-reaction-restriction-fragment-length polymorphism (PCR-RFLP) Genomic DNA (200 ng) was subjected to PCR amplification in 20 μl of solution containing 50 m*M* KCl, 10 m*M* Tris-Cl (pH 8.3), 1.5–2.5 m*M* $MgCl_2$, 200–600 μ*M* dNTP (each of the four), 2–3 pmol of PCR primers, and 0.5 unit of *Taq* DNA polymerase, using 35 cycles of the following thermal conditions: 30 s at 94°C, 1 min at 60°C, 30 s at 72°C. The primers, synthesized according to the p53 gene sequence, were sense TTGCCGTCCCAAGCAATGGATGA and antisense TCTGGGAAGGGACAGAAGATGAC for the *Bst*UI site in exon 4;[21] sense TGCCCTATGAGCCGCCTGAG and antisense TAGGGAGGTCAAATAAGCAG for the *Msp*I site in intron 6,[22] and sense ATCACACTGGAAGACTCCAG and antisense AAATGTGATGAGAGGTGGATG for the *Apa*I site in intron 7.[23] Each of the PCR-amplified products was digested with *Bst*UI, *Msp*I, and *Apa*I, respectively, and detected using 8% acrylamide gel electrophoresis.

RESULTS

PCR-direct sequencing analyses of the p53 gene, exons 5 to 8, and PCR-RFLP at the *Bst*UI site in exon 4, the *Msp*I site in intron 6, and the *Apa*I site in intron 7, were

Table I. Mutations of the p53 Gene in Thyroid Carcinomas

Histological type[a]	Mutations in p53 gene[b] (positive/tested)	Allelic loss[c] (positive/informative)
Papillary adenocarcinoma	0/10	0/4
Follicular adenocarcinoma	0/4	0/1
Undifferentiated carcinoma	7/8	3/5

a) After microscopic identification, DNAs were extracted selectively from tumor tissues.

b) Mutations of the p53 gene, exons 5–8, determined by PCR direct sequencing. All sequences were confirmed from forward and backward strands more than two times to exclude errors. All the positive cases showed base substitution mutations. In all cases, there was no mutation in the normal tissue. In the case of coexisting tumors, results from the main focus having the features corresponding to the final diagnosis are shown.

c) Allelic loss in the p53 gene determined by PCR-RFLP at the *Bst*UI site in exon 4, at the *Msp*I site in intron 6, and at the *Apa*I site in intron 7 using tumor and normal tissues. Information was obtained from eight cases using the *Bst*UI site, from one case using the *Msp*I site, and from seven cases using the *Apa*I site. Using these three RFLP sites, 4 out of 10 cases of papillary adenocarcinoma, 1 out of 4 cases of follicular adenocarcinoma, and 5 out of 8 cases of undifferentiated carcinoma were informative.

Table II. Mutations of the p53 Gene in Individual Foci Coexisting in a Thyroid Carcinoma

Case	Histology[a]	Codon	Nucleotide[c]	Amino acid	Allelic loss[c]
1	Normal	248	CGG	Arg	NI[d]
	Follicular	248	CGG	Arg	NI
	Undifferentiated	248	CGG/CAG	Gln	NI
2	Normal	248	CGG	Arg	−
	Papillary	248	CGG	Arg	+
	Undifferentiated	248	CGG/GGG	Arg/Gly	+
3	Normal[b]	213	NT	NT	NT[e]
	Papillary	213	CGA	Arg	−
	Undifferentiated	213	CGA/TGA	Arg/stop	−
4	Normal	248	CGG	Arg	−
	Papillary	248	CGG	Arg	−
	Undifferentiated	248	CAG	Gln	+
5	Normal	178	CAC	His	−
	Follicular	178	CAC	His	−
	Undifferentiated	178	GAC	Asp	+

a) DNA was extracted from each histological part separately and analyzed for p53 mutations and allelic loss.
b) Normal tissue was not available for this case.
c) Nucleotide substitution and allelic loss were determined as described in Table I. The codon 72 *Bst*UI site and intron 7 *Apa*I site were informative in cases, 2, 3, and 4. The codon 72 *Bst*UI site was informative in case 5.
d) NI: not informative.
e) NT: not tested.

performed for 22 cases of thyroid carcinoma. No mutations and no allelic deletions were detected in either the papillary adenocarcinoma or follicular adenocarcinoma, whereas in undifferentiated carcinomas a base substitution mutation was found in seven out of eight cases, and an allelic deletion of the p53 gene was detected in three out of five informative cases, as shown in Table I. For coexisting tumors, results from the main focus showing the features corresponding to the final diagnosis are given.

Detailed results from four undifferentiated carcinomas and one follicular adenocarcinoma that simultaneously showed differentiated and undifferentiated histological features in the same tumor are summarized in Table II. Case 1 was diagnosed as follicular adenocarcinoma with a small undifferentiated focus. Mutations were not observed in the normal thyroid follicle or in follicular adenocarcinoma, whereas a G:C to A:T transition at the second base of codon 248 was observed in the undifferentiated focus. Allelic loss was not informative in this case.

Cases 2–5 were diagnosed as undifferentiated carcinoma and contained residual well-differentiated papillary or follicular foci. Mutations were not observed in normal tissues or in well-differentiated carcinoma foci. Mutations were found in all four of the undifferentiated carcinomas: a C:G to G:C transversion at the first base of codon 248 in Case 2, a C:G to T:A transition at the first base of codon 213 in Case 3, a G:C to A:T transition at the second base of codon 248 in Case 4, and a C:G to G:C transversion at the first base of codon 178 in Case 5. Allelic loss was detected in both the residual papillary component and in the undifferentiated carcinoma of Case 2. There was no allelic loss in Case 3. Allelic loss was detected in undifferentiated carcinomas but not in the coexisting well-differentiated foci of Cases 4 and 5. These results of PCR-RFLP could be obtained from the codon 72 *Bst*UI site, which was informative in cases 2, 3, 4, and 5, and from the intron 7 *Apa*I site, which was informative in cases 2, 3, and 4. The mutational spectrum of undifferentiated carcinoma, for which accompanying well differentiated adenocarcinomas were not available, was as follows. In one case two non-sense mutations and one missense mutation were found; all were C:G to T:A transitions at the third base of codon 135 and 141 and at the first base of codon 248. Loss of heterozygosity (LOH) could not be observed at the intron 7 *Apa*I site, which was informative. Mutations in the remaining two cases were C:G to T:A transitions at the first base of codon 273 and a G:C to A:T transition at the second base of codon 248. In these two cases, PCR-RFLP was not informative.

Examples of pathological findings and p53 gene analysis for Case 4 are shown in Fig. 1. This tumor simul-

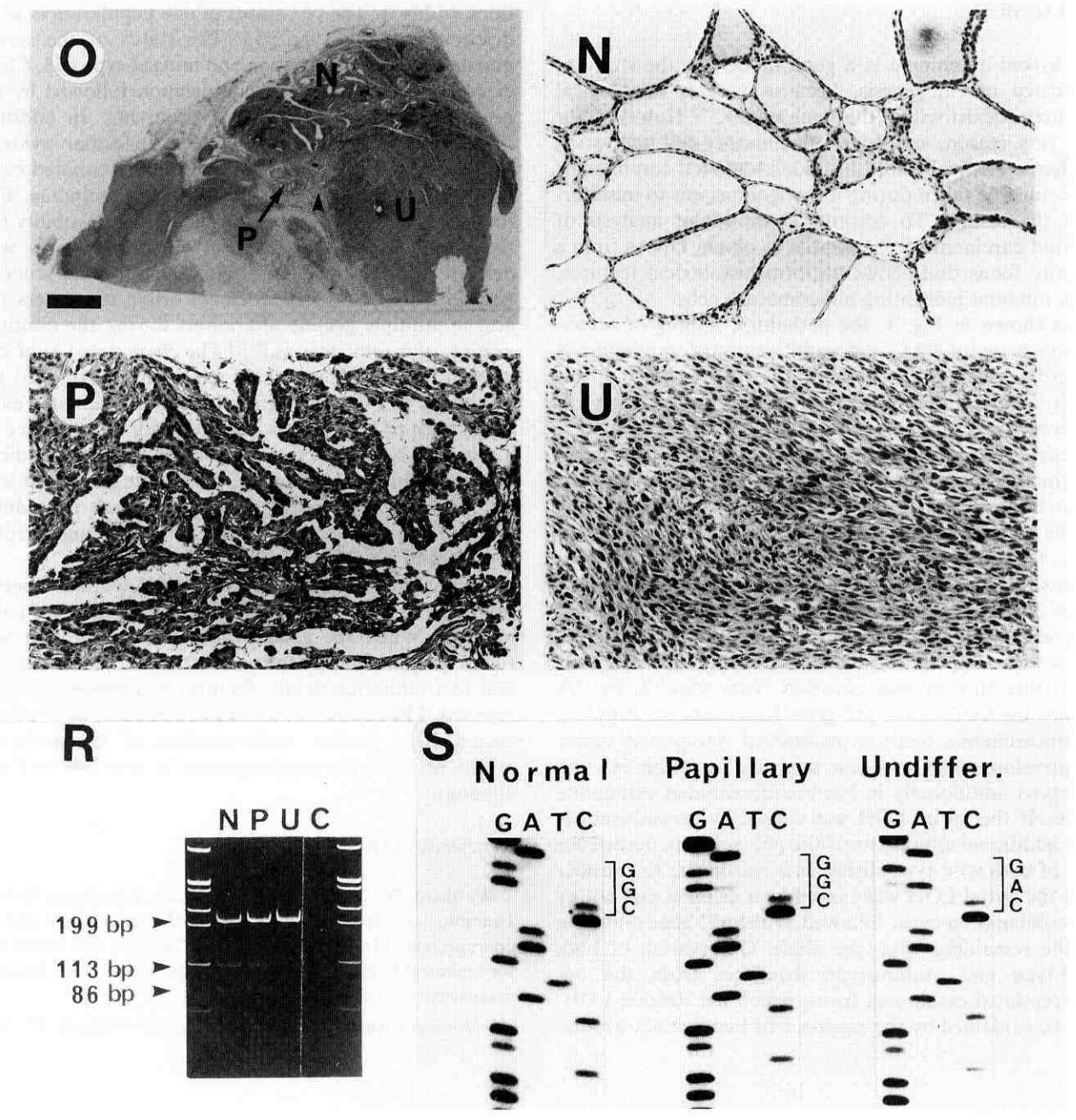

Fig. 1. Histological findings and p53 gene analysis of undifferentiated carcinoma coexisting with papillary adenocarcinoma. O; an overview of the section, which contains normal thyroid tissue (N), papillary adenocarcinoma (P), and undifferentiated carcinoma (U). The arrowhead shows the papillary adenocarcinoma-undifferentiated carcinoma continuity. Scale=0.5 cm. N, P, U; enlargement of each focus ×100 magnification.

taneously contained normal thyroid tissue (Fig. 1N), a papillary adenocarcinoma (Fig. 1P), and an undifferentiated carcinoma (Fig. 1U). The DNAs extracted from specific foci, the smallest of which was 4×3 mm, were subjected to PCR amplification. An allelic deletion and a base substitutional mutation were demonstrated exclusively in the undifferentiated focus but not in the differentiated (papillary) focus (Figs. 1R and 1S).

DISCUSSION

Thyroid carcinoma is a good model for the study of multistep carcinogenesis, because various histological features coexist within the same tumor.[7-9] However, for this very reason, and since inflammatory cell infiltration is characteristically seen in undifferentiated carcinomas, care must be taken during investigations not to misinterpret the results. To accomplish molecular analysis of thyroid carcinoma, it is essential to obtain DNAs from a definite focus that shows uniform histological features, with minimal infiltrating inflammatory cells.

As shown in Fig. 1, the pathologic finding of a continuity between PAC and undifferentiated carcinoma is consistent with the hypothesis that in the thyroid, undifferentiated carcinoma arises from a preexisting well-differentiated carcinoma. Furthermore, the observation of base substitutional mutations in the undifferentiated carcinoma but not in the differentiated carcinoma of the same tumor supports the involvement of p53 mutations in the histological de-differentiation of tumors. In addition, the observation of base substitutional mutations exclusively in the undifferentiated foci in the other four cases may indicate the involvement of a p53 gene alteration in the tumor transition (Table II).

Further support for the commonality in lineage of coexisting tumors was obtained from Case 2. In this tumor, the LOH of the p53 gene detected in the papillary adenocarcinoma focus is maintained throughout tumor progression, and a p53 gene mutation at codon 248 was observed additionally in the undifferentiated carcinoma focus. If the initial LOH was caused by recombination, two additional alterations of the p53 gene are needed for loss of both wild-type alleles. It is reasonable to consider that the initial LOH was caused by a deletion and not by a recombination event followed by the p53 gene mutation of the remaining wild-type allele. Observation of both wild-type and mutant-type sequences from the undifferentiated carcinoma focus, which has definite LOH, can be explained by the existence of two distinct populations within it. The p53 status of one population is allelic deletion and wild-type p53. The status of the second population is allelic deletion and mutant-type p53. Clonal expansion of cells with allelic deletion followed by p53 gene mutation is suspected of occurring. In addition, these findings are the first direct molecular evidence supporting the hypothesis that an undifferentiated carcinoma arises from a well-differentiated carcinoma. Furthermore, this result seems to exclude the possibility that these foci showing various histological features were derived from independent origins. This concept is consistent with that of the monoclonal origin of cancers that accrue multiple genetic alterations during the multistep process of carcinogenesis.[24, 25] The characteristics of slow growth of well-differentiated carcinoma having no p53 mutations and the rapid growth of undifferentiated carcinoma with p53 mutations together with the evidence for a common lineage of these two types of tumors indicate that p53 mutations occurring during the growth of well-differentiated adenocarcinoma might result in undifferentiated carcinomas due to an effect on uncontrolled growth and de-differentiation.

Tumor progression is a fundamental feature observed not only in thyroid carcinomas but also in other carcinomas.[6] When the genetic alterations associated with tumor progression are to be investigated, preferably several foci exhibiting unique features in a tumor should be selected. The experimental results presented in this paper suggest that further understanding of the molecular events of multistep carcinogenesis *in vivo* will be forthcoming.

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