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Heterogeneity of *p53* Mutational Status in Esophageal Squamous Cell Carcinoma

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In esophageal squamous cell carcinoma, *p53* gene mutations have been analyzed for inter- or intra-patient heterogeneity but only a few studies have investigated intratumoral heterogeneity. We investigated this question within individual esophageal cancers, and also in their lymph-node metastases in 8 cases. Analyzing the *p53* gene sequence by direct sequencing of polymerase chain reaction products, we found heterogeneity for *p53* mutations in the pre-invasive area in 3 esophageal cancers. In all areas sampled in the invasive portion of each cancer, the *p53* mutational status was identical in a given tumor. In heterogeneous tumors, the invasive area showed one of the *p53* mutations found in the pre-invasive area. In nodal metastases, the *p53* mutation was identical to that in the invasive area of each primary tumor. These data suggest that the timing of *p53* alteration is not as early as might have been expected, indicating that, in regard to *p53* gene alteration, some esophageal cancers are composed of various subclones in the pre-invasive stage with invasiveness developing in one of them, which becomes predominant through clonal selection.

Key words: Esophageal cancer — *p53* — Heterogeneity

With the development of molecular biologic techniques, it has become widely accepted that cancers are clonal neoplasms arising through multistep accumulation of somatic gene mutations in the progeny of a single cell of origin.[1)] Through these steps, intratumoral genetic heterogeneity arises. A homogeneous distribution of a certain gene mutation in a tumor implies that the mutation occurred before its clonal expansion, whereas a heterogeneous distribution implies that the mutation occurred relatively late during the tumor progression.

Mutations of the *p53* gene are the most frequent genetic changes observed in human cancers,[2, 3)] occurring within highly conserved regions of the gene[4, 5)] and resulting in loss of regulatory control of cell proliferation,[6)] as the normal *p53* gene becomes a dominant oncogene. Most *p53* gene mutations in tumors occur as point mutations, deletions or insertions within exons 5 through 9. The timing of *p53* mutational events has been shown to differ by organ. In colorectal cancer, *p53* mutations appear to occur during the transition from adenoma to cancer.[7–9)] In cancers of the thyroid,[10)] ovary,[11)] and liver,[12)] the mutations of *p53* are reported to take place at a later stage of tumor progression, while in cancers of the brain,[13)] adrenal gland,[14)] and breast,[15)] the mutations appear to be among the first detectable genetic alterations.

In esophageal cancer, many investigations concerning *p53* gene mutation have been performed[16–18)] and the *p53* mutational events occur at an early stage of carcinogenesis.[19–21)] However, only a few reports have considered in detail the intratumoral heterogeneity of *p53* gene mutations to establish whether the mutational status is truly homogeneous within a given tumor.

In this study, we set out to clarify whether intratumoral heterogeneity of *p53* exists in esophageal cancer by investigating the topographic distribution of *p53* mutational status within individual tumors, using a microdissection technique. We also searched for differences in *p53* mutational status between primary esophageal cancers and their matched lymph-node metastases.

MATERIALS AND METHODS

Samples Eight esophageal squamous cell carcinomas were used for the study. The patients all underwent esophagectomy and lymph-node dissection at Niigata University Hospital (6 males, 2 females; mean age 65 years). All patients had the habits of smoking and drinking, but had no history of cancer in the upper aerodigestive tract. No preoperative chemotherapy or radiotherapy was given. Resected tumors and corresponding lymph-nodes from dissected specimens were fixed in 10% formalin. Primary tumor extent initially was examined macroscopically using iodine staining. Tumor tissues were cut into serial blocks and embedded in paraffin. Sections (3 μm thick)

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were cut and stained with hematoxylin and eosin (HE). Tumor extent, depth of invasion, and lymph-node metastasis were confirmed microscopically in HE sections. Histopathologic classification followed the recommendations of the Japanese Society for Esophageal Diseases.[22]

Immunohistochemical staining of p53 protein Three serial 3 μm-thick sections were cut from paraffin blocks of each primary tumor and matched with sections from their lymph-node metastasis. The first section was stained with HE, and the second was immunostained for p53 protein using monoclonal antibody PAb1801 (Oncogene Science, Manhasset, NY) and the streptavidin-peroxidase complex method.[23] Expression of p53 protein was evaluated under a light microscope. Cells positive for p53 protein were defined as those with brownish nuclear staining, regardless of its intensity. Expression of p53 protein was classified as: (–), negative; (+), scattered positive cells; (++), focal aggregates of positive cells; (+++), diffuse areas of positive cells. Staining patterns of (++) and (+++) were considered to represent overexpression of p53 protein, as in our previous reports.[23–25]

DNA extraction Representative sections were selected after study of HE and *p53* immunostaining sections. After identification of invasive areas, pre-invasive areas (carcinoma *in situ*) adjoining invasive areas, and lymph-node metastases in HE sections, multiple areas were selected by a microdissection technique from each part for DNA extraction. The areas were selected to contain at least 90% malignant cells. DNA was extracted from 10 serial 10 μm-thick paraffin sections cut between the first set of HE sections and *p53* immunostain and the final set of these sections cut from the block. Microdissection was carried out manually with a microscope at a magnification of 40×, using commercially available sterilized disposable 25G×1″ needles (ϕ 0.5 mm) (Terumo, Tokyo) with a syringe. Each microdissected area contained about 1500 to 4500 cells. DNA was isolated using the DNA Isolater PS Kit (Wako, Osaka) following the manufacturer's protocol, and dissolved in 20 μl of sterile water.

Polymerase chain reaction (PCR) The DNA was amplified by nested PCR.[26] The target sequences of the *p53* gene, exon 5 (codons 126–186), exon 6 (codons 187–224), exon 7 (codons 225–261), and exon 8 (codons 262–306), were amplified independently. Oligonuculeotide primers were synthesized by Takara Shuzo, Kyoto. The sequences of the first and second PCR primers and the amplifying reactions were described previously.[25] One of the second primers was labeled by biotin at the 5′ terminal. Agarose gel electrophoresis (3%) was performed to confirm the products of the second PCR. To obtain a pure target sequence, the amplified band was cut from the agarose gel with a sterile scalpel and purified with SUPREC-01 (Takara Shuzo). Biotinylated purified second PCR products were dissolved in 50 μl of sterile water and subjected to direct sequencing without cloning.

Direct DNA sequencing Direct DNA sequencing was performed by the dideoxy procedure[27] using an "Auto Load" Solid-Phase Sequencing Kit (Pharmacia Biotec, Uppsala, Sweden), and an automated DNA sequencer (ALF-II, Pharmacia) equipped with ALF Manager (Version 2.5). Each PCR product was sequenced in both direc-

Table I. Mutational Analysis of *p53* in Pre-invasive Area, Invasive Area, and Metastases to Lymph-nodes

Tumor	Pre-invasive area			Invasive area			Lymph-node		
	No. of samples	IHC	Mutated sequence: codon (sequence$^{\text{amino acid}}$)	No. of samples	IHC	Mutated sequence: codon (sequence$^{\text{amino acid}}$)	No. of samples	IHC	Mutated sequence: codon (sequence$^{\text{amino acid}}$)
1	4	–	ND	2	–	ND	2	–	ND
2[a]	4	+++	220 ($TAT^{Tyr}\rightarrow GAT^{Asp}$)	No invasive area			No metastasis		
3	10	–	ND	No invasive area			No metastasis		
4	3	+++	283 ($CGC^{Arg}\rightarrow CCC^{Pro}$)	2	+++	283 ($CGC^{Arg}\rightarrow CCC^{Pro}$)	No metastasis		
5	5	+++	282 ($CGG^{Arg}\rightarrow TGG^{Trp}$)	3	+++	282 ($CGG^{Arg}\rightarrow TGG^{Trp}$)	2	+++	282 ($CGG^{Arg}\rightarrow TGG^{Trp}$)
6	7	+++	173 ($GTG^{Val}\rightarrow TTG^{Leu}$)	3	+++	173 ($GTG^{Val}\rightarrow TTG^{Leu}$)	4	+++	173 ($GTG^{Val}\rightarrow TTG^{Leu}$)
7[b]	3	–	286 ($GAA^{Glu}\rightarrow TAA^{Stop}$)	2	–	286 ($GAA^{Glu}\rightarrow TAA^{Stop}$)	2	–	286 ($GAA^{Glu}\rightarrow TAA^{Stop}$)
8[a]	5	3 +++	255 ($ATC^{Ile}\rightarrow TTC^{Phe}$)	5	+++	255 ($ATC^{Ile}\rightarrow TTC^{Phe}$)	2	+++	255 ($ATC^{Ile}\rightarrow TTC^{Phe}$)
		2 +++	ND						
9	3	2 –	ND	4	–	ND	2	–	ND
		1 –	306 ($CGA^{Arg}\rightarrow TGA^{Stop}$)						
10[b]	10	8 +++	205 ($TAT^{Tyr}\rightarrow TGT^{Cys}$)	6	+++	205 ($TAT^{Tyr}\rightarrow TGT^{Cys}$)	3	+++	205 ($TAT^{Tyr}\rightarrow TGT^{Cys}$)
		1 +++	205 ($TAT^{Tyr}\rightarrow TGT^{Cys}$)						
			269 ($AGC^{Ser}\rightarrow AAC^{Asn}$)						
		1 +++	290 ($CGC^{Arg}\rightarrow CAC^{His}$)						

a, *b*) Same patient; synchronous double primary cancer.
IHC, immunohistochemistry; ND, mutation in exons 5–8 not detected.

tions (forward and reverse) at least twice to confirm the reproducibility of results.

RESULTS

Table I shows the results of *p53* immunohistochemistry (IHC) and *p53* mutational status in different samples from 10 primary esophageal squamous cell carcinomas in 8 patients and in 7 lymph-node metastases. In each tumor, 2 to 10 invasive, pre-invasive, and metastatic samples were subjected to DNA analysis. Total numbers of areas investigated were 54 pre-invasive areas, 27 invasive areas and 17 nodal metastases.

Patterns of *p53* immunostaining were homogeneous within tumors where both pre-invasive and invasive areas either stained diffusely (6 tumors) or remained unstained (4 tumors). The staining pattern of the lymph-node metastases was identical to that seen in the corresponding primary tumors. Staining was localized strictly to the nuclei.

Ten different mutational codons were detected. All mutations were single base-pair substitutions that involved exon 8 in 5 tumors, exon 6 in 2 tumors, and exon 5 or 7 in 1 tumor each. Missense mutation resulting in an amino acid substitution represented 8 of 10 mutations, while 2 were nonsense mutations in codon 286 or 308 in exon 8, resulting in a stop codon.

Seven tumors showed identical *p53* mutational status throughout pre-invasive and invasive areas. In contrast, three tumors showed heterogeneous *p53* mutational status, with *p53* heterogeneity being observed only in pre-invasive areas, while mutational status in invasive areas was homogeneous. Within two tumors (Nos. 8 and 9), heterogeneity in the pre-invasive area represented a *p53* mutation occurring together with intact *p53* in other samples. In another tumor (No.10), three different mutational patterns (mutation in codon 205, codons 205 plus 269, and codon 290) were detected (Figs. 1 and 2). The *p53* mutations found in the invasive areas of these three tumors were all identical with a mutation in the pre-invasive area of the same tumor. Mutational status of the lymph-node metastases was identical to that in the invasive area of the corresponding primary tumor.

Most areas positive for *p53* immunostaining (62 of 64, 96.9%) revealed a *p53* mutation by DNA sequence analysis. Most areas negative for immunostaining (26 out of 34, 76.5%) failed to show any mutation in exons 5 through 8, though 8 negative areas showed a nonsense mutation in codon 306 (No. 9) or codon 286 (No. 7).

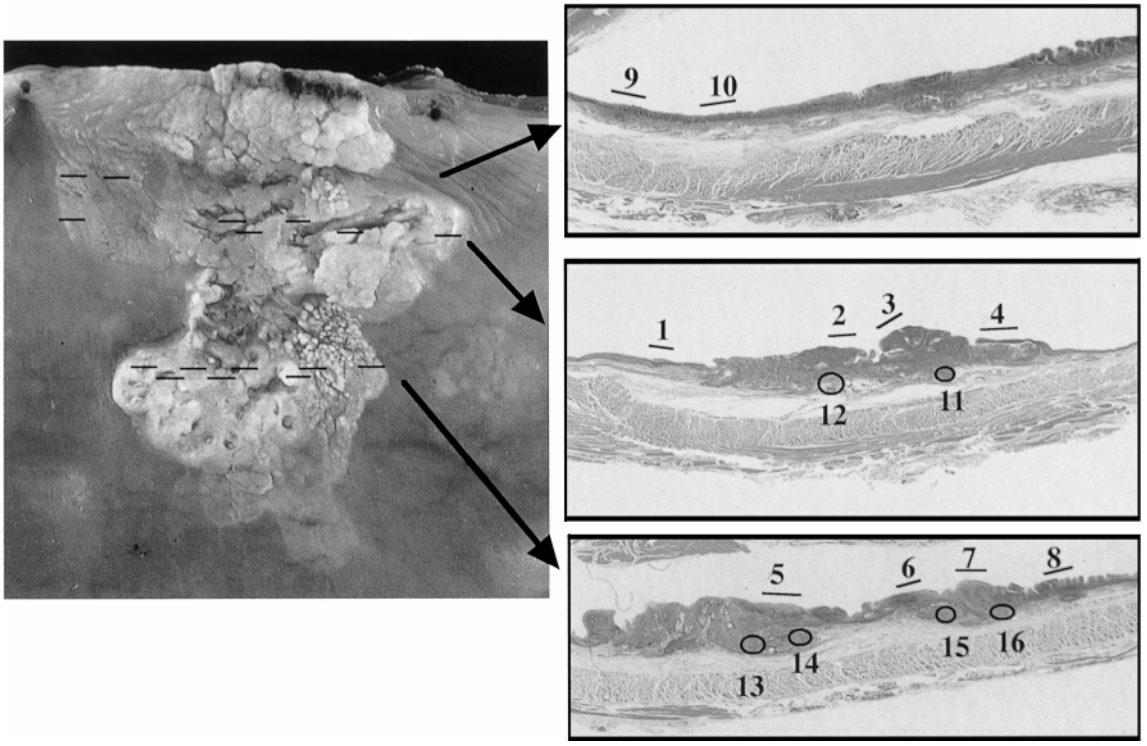

Fig. 1. A tumor (No. 10) showing heterogeneity for *p53* gene mutation. Topographic distributions in pre-invasive areas (1–10) and invasive areas (11–16) are shown. DNA was extracted from all areas for *p53* mutational analysis (see Fig. 2).

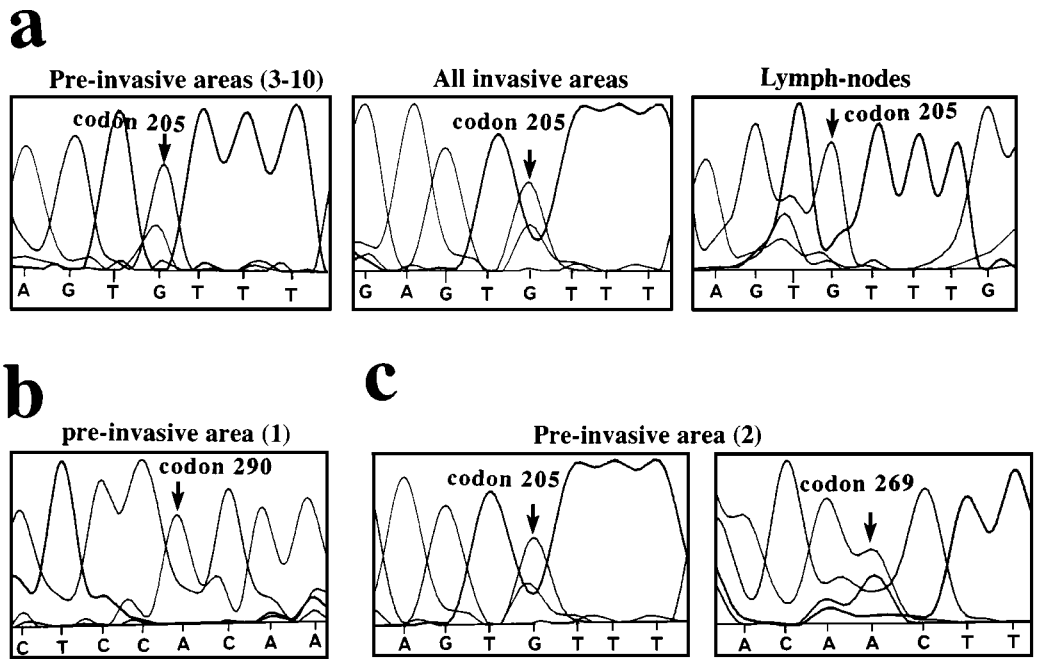

Fig. 2. Mutation of *p53* detected by direct sequencing of DNAs derived from a primary tumor and corresponding metastases to lymph-nodes (No. 10). a, Pre-invasive areas (3–10), all invasive areas, and involved lymph-nodes showed the same mutation at codon 205 ($TAT^{Tyr} \rightarrow TGT^{Cys}$). b, Pre-invasive area (1) showing a different mutation involving codon 290 ($CGC^{Arg} \rightarrow CAC^{His}$). c, Pre-invasive area (2) showing double mutation, at codons 205 ($TAT^{Tyr} \rightarrow TGT^{Cys}$) and 269 ($AGC^{Ser} \rightarrow AAC^{Asn}$). One of the two mutations is identical to that seen in pre-invasive areas (3–10), in all invasive areas, and metastases to lymph-nodes.

DISCUSSION

In the present study, 7 of 10 (70%) esophageal squamous cell carcinomas showed a homogeneous distribution of *p53* mutations while 3 of 10 (30%) revealed intratumoral heterogeneity in *p53* mutational status. Heterogeneity was observed only within the pre-invasive area. Nagel *et al*.[28] demonstrated a homogeneous distribution of *p53* mutation in esophageal cancers, and Hori *et al*.[29] and Mayama *et al*.[30] showed identical *p53* mutational status between primary esophageal cancers and metastatic lymph-nodes. With respect to the invasive area of the tumors, our data agree with the previous studies. Any discrepancy with the previous reports for pre-invasive areas may be explained by differences in tissue sampling system. The other group sampled DNA only from the invasive area or small numbers of pre-invasive areas, while our sampling involved 5 to 16 areas per tumor, including the pre-invasive area.

Three explanations are possible for *p53* heterogeneity in the pre-invasive area of esophageal carcinoma. The first follows the "field cancerization" theory[31] that extensive areas of tissues or organs become genomically unstable and predisposed to aberrant growth as a result of prolonged exposure to carcinogens. According to this theory, *p53* heterogeneity in the present study could be explained in terms of multiple clones with different *p53* mutations arising independently in a small area of esophageal mucosa and colliding with each other to form an apparently single tumor. Recent studies have strengthened support for this theory in terms of *p53* mutation,[32, 33] by means of microsatellite analysis including 17p13, where the *p53* gene is located.[34] However, none of our patients with *p53* heterogeneity had any history of upper aerodigestive tract cancers, and no association was found between the heterogeneity and the etiological background. The second possible explanation would be that *p53* mutations occurred within a single tumor after clonal expansion. Such mutational events would occur multiply and independently in several foci to impart clonal heterogeneity for *p53* to the tumor. The third possible explanation would be a combination of the former two possibilities, that multiple clones bearing no *p53* mutation acquire *p53* mutations independently after colliding with each other. In accordance with the latter two hypotheses, we found that the timing of *p53* mutation in esophageal carcinoma may

be later during carcinogenesis than has generally been believed. Similar data have been obtained for lung cancer, although *p53* mutation is thought to occur in early stages of carcinogenesis in the lung.[35] Li *et al*.[36] reported that 1 of 10 lung cancers showed intratumoral heterogeneity for *p53* mutational status. Discordance of *p53* mutation was also observed between primary lung cancers and their metastases,[37] indicating that in some lung cancers *p53* mutation may occur in later stages.

The topographic distribution of *p53* mutational heterogeneity is noteworthy. In tumors with heterogeneous status (Nos. 8, 9, 10), the invasive area was homogeneous for one of the *p53* mutations detected in the pre-invasive portion. These data suggest that only one of various subclones in the pre-invasive area becomes invasive. That is, only a certain subclone gained a sufficient growth advantage to become established as an invasive carcinoma through clonal selection in pre-invasive areas. Similar phenomena have been reported in colorectal cancer. Shibata *et al*.[38] demonstrated a heterogeneous distribution of c-K-*ras* gene in adenoma, but a homogeneous distribution in adenocarcinoma. The *p53* mutational status of nodal metastases was homogeneous and was identical to that of invasive areas in the primary tumor. This indicates that in esophageal cancer, *p53* mutation occurs before the nodal metastases.

Our present data also suggest a need for careful genetic evaluation before pre-operative chemotherapy and radiotherapy for esophageal cancer. Recently, genetic diagnosis has been attempted before radical cancer therapy by using endoscopically obtained biopsy samples. Furthermore, gene therapy based on augmenting normal *p53* levels within cells may ultimately contribute to treatment and possibly prevention of cancer.[39, 40] However, in such evaluations one should consider intratumoral *p53* heterogeneity and analyze multiple foci in the pre-invasive area.

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