Jpn. J. Cancer Res. **89**, 40–46, January 1998

Alteration of *p53* Clonality Accompanying Colorectal Cancer Progression

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The aim of this study was to clarify whether or not the status of gene alteration is heterogeneous in intramucosal carcinoma and homogeneous within invasive carcinoma. We selected 10 colorectal carcinoma cases (1 mucosal, 5 submucosal and 4 advanced carcinomas including 2 cases with lymph node metastasis) and analyzed the *p53* gene sequence. Six colorectal cancers in this study showed heterogeneity in *p53* mutations in cells from the intramucosal part. In the invasive part of a carcinoma, *p53* mutation status was homogeneous intratumorally in all cases. These data indicate that, in regard to *p53* gene alterations, colorectal cancers can be composed of various subclones when limited to the mucosa, but clonal selection occurs when one of these subclones commences invasion to the submucosa, generating a monoclonal invasive carcinoma.

Key words: Colorectal cancer — *p53* gene — Clonality — Heterogeneity — Intramucosal carcinoma

The development of neoplasia is thought to involve multiple mutations that culminate in malignancy.[1)] It is the result of clonal expansion of a single cell, which may progress through stages of hyperplasia, precancerous or benign neoplasia and finally invasive carcinoma[2)] through the generation of successive subclones. This clonal expansion involves activation or inactivation of various stages of neoplastic progression, which results in heterogeneity of the genetic alterations within histologically identical types of tumors from different individuals and/or within a single tumor at different clinical stages.

At least three tumor suppressor genes, *p53*,[3)] *APC*[4)] and *DCC*,[5)] and the activation of the dominant oncogene, K-*ras*[6)] are thought to play roles in the development of colorectal carcinoma. It is stressed in Fearon and Vogelstein's model that the progressive accumulation of gene alterations is more important than the order of occurrence in colorectal tumor progression.[2)] Among the genes responsible for development of colorectal carcinoma, inactivation of the *p53* gene is thought to play a role in transition from benign (adenoma) to malignant growth,[7)] because alteration of the *p53* gene occurs in 49–70% of colorectal carcinomas but is rare in adenomas.[7, 8)] However, this idea was derived from a comparison of *p53* gene inactivation frequencies between the pre-invasive (intramucosal) and invasive portions of the same carcinoma.

Recently, genetic diagnosis has been used to confirm histological diagnosis of malignancy.[9)] p53 immunohistochemistry (IHC) is an especially powerful method for distinguishing adenoma from carcinoma. Besides the diagnostic use, an advantage of IHC is that it permits correlation of genetic mutations with histological alterations. It has been noted that the IHC staining pattern of p53 in the invasive part of colorectal carcinomas is uniform (totally negative, scattered or diffusely positive), though staining in the mucosal part occasionally shows intermingling of some nested regions of p53-positive cells within a negative area (our unpublished observation). This observation led us to speculate that colorectal carcinomas are not monoclonal for *p53* gene alterations when they are limited to the mucosa, but they become monoclonal through clonal selection as they invade the submucosa.

The aim of this study was to clarify whether gene alterations in identical tumors are heterogeneous in intramucosal carcinomas and homogeneous within invasive carcinomas. The study revealed that *p53* mutations in some colorectal carcinomas can be strikingly heterogeneous when limited to the mucosa.

MATERIALS AND METHODS

Samples The samples were 10 endoscopically or surgically resected human colorectal carcinomas (1 mucosal, 5 submucosal and 4 advanced carcinomas[10)] including 2 cases with lymph node metastasis) from the archives of the First Department of Pathology, Niigata University School of Medicine. All samples were fixed in 10% formalin and embedded in paraffin. Because our aim was to investigate intratumoral heterogeneity of *p53* gene mutations within mucosal and invasive portions separately, invasive colorectal carcinomas with intact intramucosal carcinomatous portions and p53 protein over-expression were selected.

Histological diagnosis was done according to the criteria of Watanabe *et al*., and the carcinomas were divided into those with high-grade atypia (CAH) and those with low-grade atypia (CAL).[11-13)]

Immunohistochemistry Two serial, 3 μm-thick sections

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were made from all blocks of each sample. The first section was stained with hematoxylin and eosin (HE) and the second was immunostained for p53 protein using monoclonal antibody PAb-1801 (Oncogene Science Inc., Manhasset, NY). Immunohistochemical staining was performed using the streptavidin-peroxidase complex.[14] Cells positive for p53 were defined as those with brown-stained nuclei, regardless of their staining intensity. p53 staining patterns were classified into the following four types: (1) diffuse: many positive cells scattered throughout most of the lesion; (2) nested: positive cells aggregated in nested area(s); (3) scattered: a few isolated, scattered positive cells; and (4) negative: no positive cells. The staining patterns of types 1 and 2 were regarded as corresponding to over-expression of p53 protein according to our published studies.[14, 15]

DNA extraction Representative sections were selected by observation of HE and p53 IHC specimens. After identification of intramucosal and invasive portions using HE-stained sections, plural areas were selected by means of a microdissection technique from each part for DNA extraction (Fig. 1A). For advanced carcinomas, six invasive areas diagrammed in Fig. 2 were selected. The areas were selected so as to contain at least 80% malignant cells. Adenomatous areas that were completely free of malignant

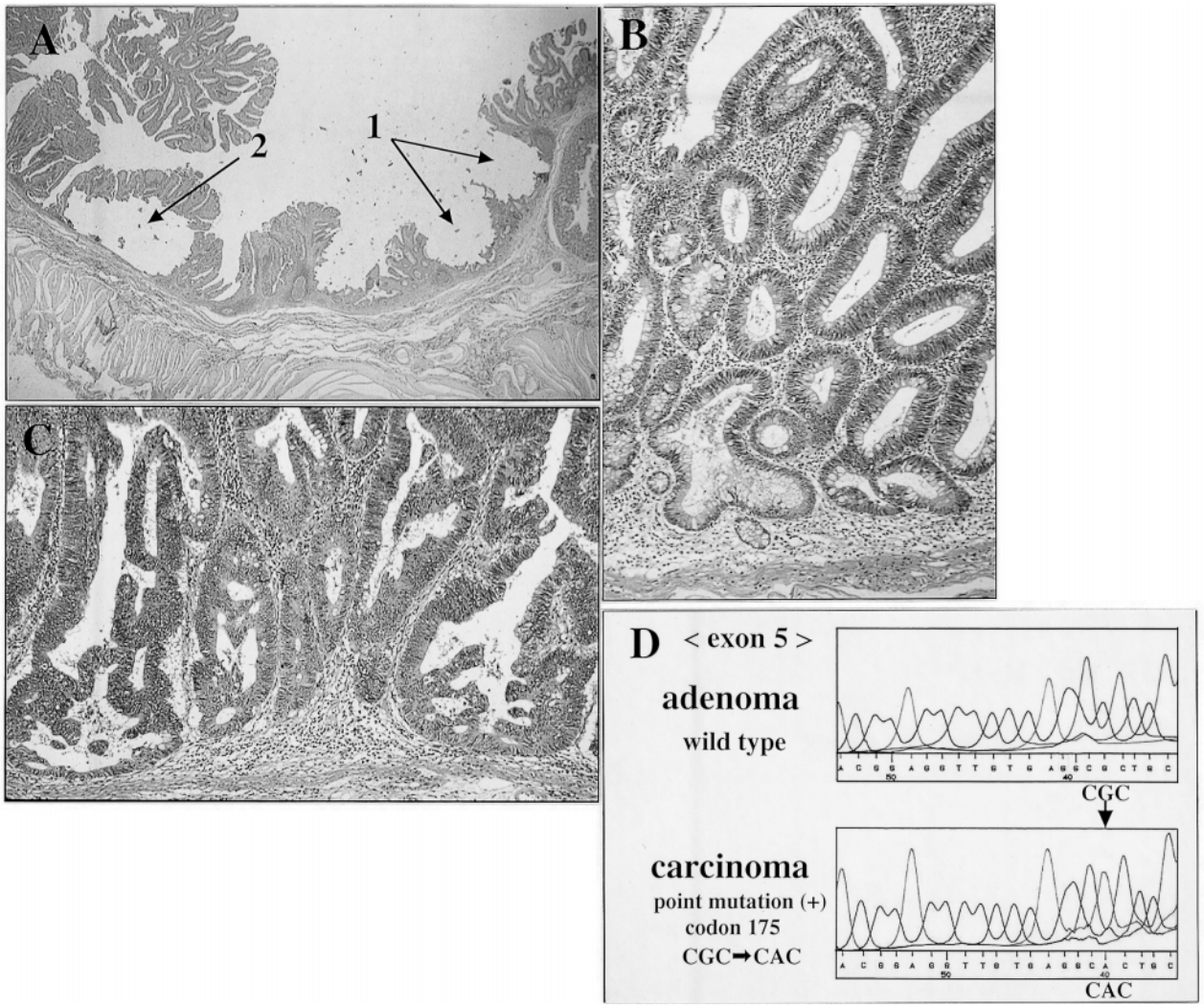

Fig. 1. Microdissection of a complex lesion from a single tumor (case 10 in Table I). A, Tumor after selective removal of tissue and staining with hematoxylin. Microdomain 1 is tubular adenoma and 2 is intramucosal carcinoma with low-grade atypia. B, High-power view of microdomain 1 (×50). C, High-power view of microdomain 2 (×50). D, Sequencing analysis of DNA from adenoma (B) and carcinoma (C). The lane of carcinoma shows a G-to-A base change in codon 175, which results in an amino acid change from Arg to His. The adenoma lane shows the wild-type.

cells were selected adjoining the carcinoma, and normal areas were chosen as well.

To extract genomic DNA from each of the areas discussed above, ten 10-μm thick serial sections were prepared. They were located between the first set of HE section and p53 immunostaining and the last set. The microdissection was carried out manually with a microscope at a magnification of 40×, using commercially available sterilized disposable 25G × 1″ needles (ϕ0.5 mm) (Terumo, Tokyo) with a syringe. The removed tissue was transferred to a 0.5 ml microfuge tube. DNA was isolated using the DNA Isolator PS Kit (Wako Pure Chemical Industries Ltd., Osaka) and dissolved in 20 μl of distilled water.

Mutational analysis by direct sequencing Four fragments of DNA, including exons 5, 6, 7 and 8 of the *p53* gene were amplified by nested polymerase chain reaction (PCR)[16] using two sets of primers for each exon as described previously.[15] The product of the first PCR was used as the template for the second PCR. The second set of PCR reactions, which had one of the primers labeled with biotin, was performed under the same conditions as the first. Products of the second PCR were electrophoresed for 40 min at 100 V in 4% agarose gels (NuSieve 3:1 Agarose: FMC BioProducts, Rockland, ME). The DNA was purified using a Mermaid Kit (Bio101, La Jolla, CA) or SUPREC-

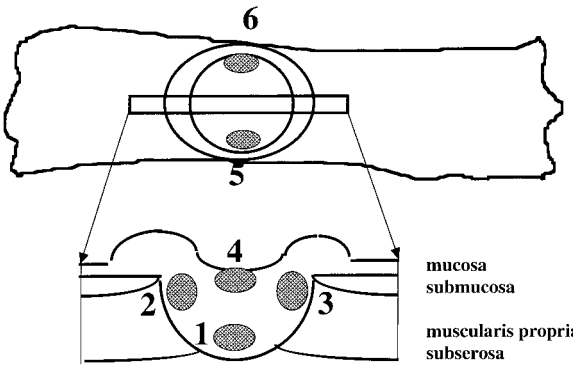

Fig. 2. For advanced cancers, DNA was extracted separately from six invasive areas; superficial and deep areas at the center (4, 1), and four peripheral areas (2, 3, 5, 6).

Table I. Difference of *p53* Gene Clonality between Mucosal and Invasive Part

Case	Depth	Mucosal part						Invasive part					
		Hist.	p53 IHC	No. of focus	Codon	Base change	Amino acid change	Hist.	p53 IHC	No. of focus	Codon	Base change	Amino acid change
1	m	CAL	+	7	216	GTG→TTG	Val→Leu						
2	sm	CAL	+	4	175	CGC→CAC	Arg→His	CAH	+	6	175	CGC→CAC	Arg→His
		CAH	+	6	175	CGC→CAC	Arg→His						
3	mp	CAH	+	2	232–238 (21 bp)		Deletion	CAH	+	6	232–238 (21 bp)		Deletion
4	ss	CAH	+	3	242	TGC→TGG	Cys→Trp	CAH	+	6	242	TGC→TGG	Cys→Trp
								CAH*	+	1	242	TGC→TGG	Cys→Trp
5	sm	CAH	–	3	Wild-type			CAH	+	2	175	CGC→CAC	Arg→His
		CAH	+	1	175	CGC→CAC	Arg→His						
		CAH	+	1	175	CGC→CAC	Arg→His						
					154	GGC→GGT	Gly→Gly						
6	sm	CAL	–	2	Wild-type			CAH	–	2	Wild-type		
		CAH	–	1	Wild-type								
		CAH	+	2	273	CGT→CAT	Arg→His						
7	sm	CAL	–	3	Wild-type			CAH	+	3	244	GGC→GAC	Gly→Asp
		CAH	+	2	244	GGC→GAC	Gly→Asp						
8	sm	CAL	–	1	248	CGG→CAG	Arg→Gln	CAH	+	2	248	CGG→CAG	Arg→Gln
		CAL	+	5	248	CGG→CAG	Arg→Gln				158	CGC→CAC	Arg→His
		CAH	+	1	248	CGG→CAG	Arg→Gln						
		CAL	+	2	158	CGC→CAC	Arg→His						
					248	CGG→CAG	Arg→Gln						
9	ss	CAL	+	3	175	CGC→CAC	Arg→His	CAH	+	6	237–238 (6 bp)		Deletion
		CAH	+	1	237–238 (6 bp)		Deletion	CAH*	+	1	237–238 (6 bp)		Deletion
10	ss	CAL	–	1	213	CGA→TGA	Arg→Stop	CAH	–	6	196	CGA→TGA	Arg→Stop
		CAL	+	1	175	CGC→CAC	Arg→His						
		CAL	+	2	248	CGG→CAG	Arg→Gln						
		CAH	–	2	196	CGA→TGA	Arg→Stop						

m, mucosa; sm, submucosa; mp, muscularis propria; ss, subserosa; IHC, immunohistochemistry (mAb PAb-1801); Hist., histological diagnosis; CAL, carcinoma with low-grade atypia; CAH, carcinoma with high-grade atypia; CAH*, lymph node metastasis of CAH.

01 (Takara Shuzo Co., Ltd., Otsu), precipitated with ethanol, and dissolved in 50 μl of distilled water.

Labeling one of the second primers with biotin facilitates solid-phase DNA sequencing of PCR templates using an AutoLoad Solid Phase Sequencing Kit (Pharmacia, Uppsala, Sweden) with a fluorescently labeled primer. Samples were electrophoresed and analyzed on an automated laser fluorescent sequencer apparatus (A.L.F. DNA Sequencer; Pharmacia) equipped with A.L.F. Manager Version 2.5. The data generated show the wave and base initials (A, C, G and T). If an additional peak to the expected normal sequence was presented, we judged the peak as a point mutation (indicating a nucleotide change) only when the peak was unambiguously discriminated from other peaks. When the discrimination of an additional peak was ambiguous, we regarded the sequence as wild type. Thus, it is possible that tumors or areas with wild-type *p53* in this study may have contained a small number of tumor cells with mutated *p53*. All samples were examined at least twice (forward and reverse) to confirm the reproducibility of the results.

RESULTS

Table I shows the results for *p53* mutations within 10 colorectal carcinomas according to depth of invasion, p53 IHC and histological grade of atypia (whether high-grade or low-grade carcinoma). In the mucosal portions of carcinomas, 2 to 10 areas were examined for each case. Four cases contained an adenomatous component adjoining the carcinoma and evaluation was also done for these areas (Fig. 1, B–D). In the invasive portion of the carcinoma, 2 to 6 areas were also examined (Fig. 3).

In mucosal parts of carcinomas, the *p53* mutation pattern was homogeneous intratumorally in four cases (1 to 4) while six cases (5 to 10) showed heterogeneity in *p53* mutations. Although the identical mutation was observed within a tumor, two different wave patterns existed in sequence analysis (Fig. 4). Cases 5, 6 and 7 were composed of mixed areas of wild-type and mutant *p53* gene. In cases 8, 9 and 10, plural *p53* mutation patterns (different codons, or deletion in case 9) were seen. In the invasive part of the carcinoma, *p53* mutations were identical intratumorally in all cases. Examination of *p53* mutations in lymph-node metastases was carried out for cases 4 and 9, and these mutations proved to be identical to those found in the primary submucosal foci and deeper invasive portions of the carcinomas.

Among nine invasive carcinomas, the intramucosal parts and invasive parts of the carcinoma shared identical *p53* mutations. In three cases (2, 3 and 4) with homogeneous intratumoral intramucosal *p53* mutation patterns, the identical *p53* mutation was observed regardless of depth within the bowel wall. The remaining six cases (which showed heterogeneous intramucosal *p53* mutation patterns) had *p53* mutations in the invasive part of the carcinoma that were identical with one of the *p53* mutations in the mucosal part (Fig. 5).

There was no association between specific *p53* mutations and grade of atypia. A good correlation was found between *p53* mutation status and *p53* IHC results. Discordance was seen in three areas; CAL in case 8 and CAL, CAH in case 10. In case 10, areas without *p53* over-expression contained nonsense mutations in the *p53* gene.

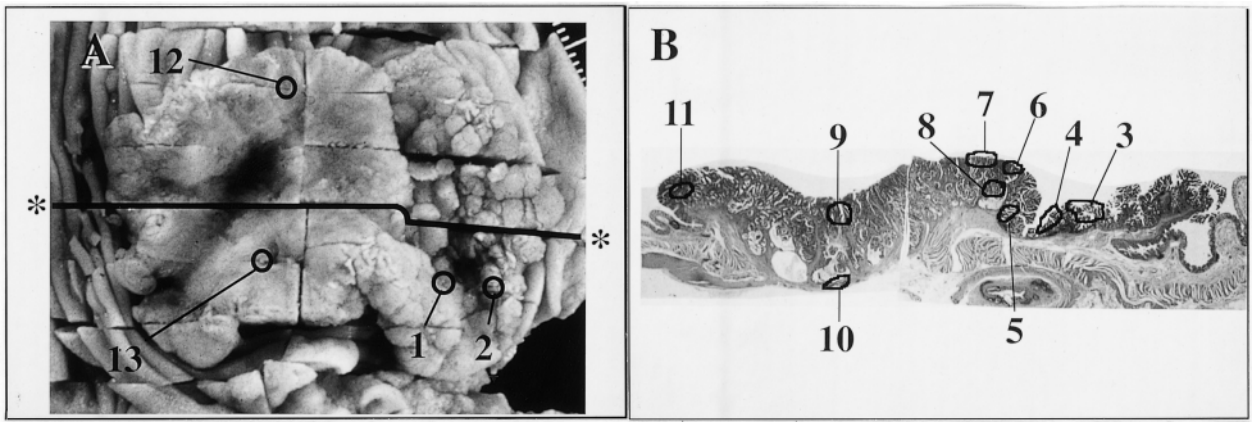

Fig. 3. A representative case (case 10) illustrating *p53* mutation site differences. A, Macroscopic figure. Type 2 carcinoma with mucosal spreading (IIa) at the cecum. B, Representative section from panel A. Numbers in figure correspond to analyzed *p53* sequences. 1: CAL with codon 175 (Arg→His) mutation. 2: adenoma with wild-type *p53* gene. 3 and 5: CAL with codon 248 (Arg→Gln) mutation. 4: CAL with codon 213 (Arg→Stop) mutation. 6–13: CAH with codon 196 (Arg→Stop) mutation.

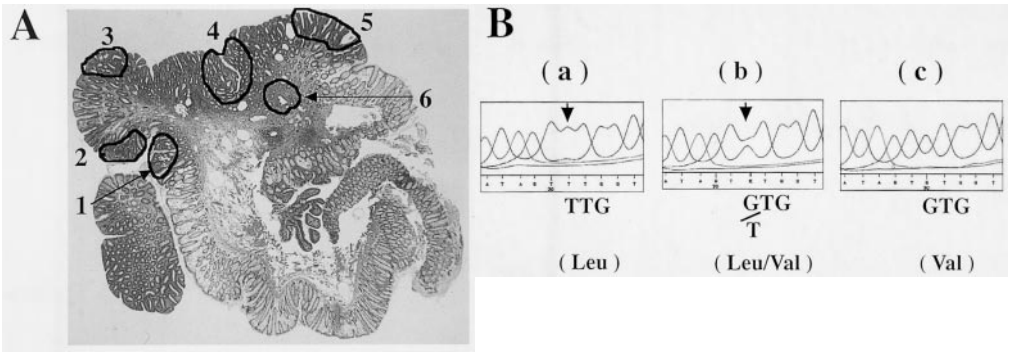

Fig. 4. Different sequence pattern in the intramucosal carcinoma at the sigmoid colon (case 1). A, This figure illustrates how microdomains were outlined in intramucosal carcinoma at the sigmoid colon (× 2.5). B, Microdissected samples (4, 5) show homozygous sequence in exon 6 at the first base position of codon 216, GTG→TTG; Val→Leu (a). Samples 1, 2, 3 and 6 show heterozygous sequence, GTG→T/GTG; Val→Leu/Val (code *k* indicates either G or T, because peaks of G and T appeared simultaneously (b). The wild-type sequence is present in normal mucosa from the same patient (c).

DISCUSSION

Tumor heterogeneity is thought to arise during tumor progression.[17] Although such heterogeneity[18] has been documented by cytogenic and DNA cytometric techniques in colorectal adenomas and carcinomas,[19–22] its exact relationship to tumor progression is still unclear. The current study was designed to examine *in vivo* tumor heterogeneity of the *p53* gene in the context of tumor progression.[23–25]

Six colorectal cancers in our study showed heterogeneity of *p53* mutations in the intramucosal portion. This finding supports our speculation that, in regard to *p53* gene alterations, colorectal cancers can be composed of various subclones when limited to the mucosa, but clonal selection occurs when one of the subclones commences invasion to the submucosa, generating a monoclonal invasive carcinoma. Similar observations in colorectal neoplasias indicated that c-K-*ras* mutations are heterogeneous in adenomas but homogeneous in adenocarcinomas.[22] However, few reports have described intramucosal heterogeneity of the *p53* gene.[23] Colorectal carcinoma has been regarded as a homogeneous mass. Recent reports[22, 26] also suggested a homogeneous distribution of p53 in colorectal carcinomas, except for one report on carcinoma in adenoma.[23] Most of the previous studies dealt only with advanced colorectal carcinomas, without considering the intramucosal part. With respect to the invasive portion of the tumors, our present data are consistent with the previous studies. The discrepancy with other studies is explained by the inclusion of data from the intramucosal portions as well.

There appeared to be two different sequence patterns of mutations in microdissected samples, homozygous with a single peak (Fig. 4B-a) and heterozygous with two peaks (Fig. 4B-b) on the automated sequencer. Heterozygous patterns always contained the wild-type sequence. Persistence of the wild-type allele would indicate that either the samples contained normal and mutated copies of the *p53* gene or that nonneoplastic tissues such as inflammatory infiltrates were present as contaminants. Microdissection can eliminate gross contamination by nonneoplastic tissues (we selected the area for DNA analysis to contain at least 80% of malignant cells), so it is most likely that the wild-type sequence reflected an undeleted normal allele of the cancers. However, analyses of loss of heterozygosity will be needed for further clarification of this matter.

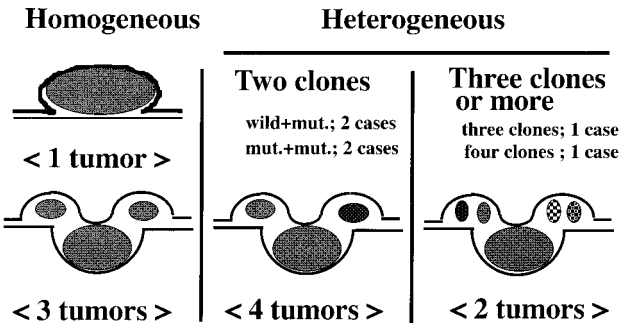

Fig. 5. Pattern of *p53* gene mutation distribution in colorectal carcinoma. Four cases show homogeneous distribution and six cases show heterogeneous distribution. *p53* heterogeneity is limited to the mucosa. Not only mutant plus wild-type, but also cases with more than two different *p53* alterations were found.

p53 protein is thought to play an important role in regulating DNA integrity.[27] Damage to DNA results in p53-mediated cell cycle arrest in the G1 phase to allow DNA repair, and *p53* can induce apoptosis.[28] In this manner,

mutations are prevented from being passed to daughter cells. Although the function of p53 has been determined in terms of molecular biology, the role and timing of *p53* alterations in tumorigenesis have not yet been clarified. In the Fearon and Vogelstein model of colorectal tumorigenesis,[2] *p53* alteration is thought to occur as a late event prior to the development of invasive properties. But this was based on a statistical comparison of *p53* alteration frequencies between intramucosal lesions and invasive carcinomas obtained independently from different individuals.

According to the model of stepwise accumulation of somatic mutations in tumor development, mutations acquired at an early stage of carcinogenesis would be shared by most tumor cells, while a heterogeneous distribution implies that the mutation occurred relatively late during tumor progression.[26] Our current investigation revealed homogeneity in *p53* mutations in the invasive portion of carcinomas and heterogeneity in pre-invasive (intramucosal) portions of the same tumor. This finding indicates that *p53* alterations are acquired, at least in some colorectal cancers, prior to cancer invasion.

Three possible explanations for the *p53* heterogeneity in the intramucosal portion of the colorectal carcinoma can be formulated: (1) multiple subclones could be generated in the intramucosal carcinoma, whatever its histogenesis, through the adenoma-carcinoma sequence or *de novo* cancerization,[29] (2) multiple subclones bearing different *p53* alterations could occur within a pre-malignant lesion (adenoma) and then merge, and (3) multiple clones bearing different *p53* alterations could be generated independently and merge to form an apparently single tumor. The former two explanations correspond to the clonal evolution theory,[17] and the last one to the field cancerization theory.[30, 31] Discordance of *p53* mutations in tumors of the upper aerodigestive tract and skin of the head and neck were presented as evidence of field cancerization.[30, 31] However, we believe that the field cancerization theory is unlikely to account for intramucosal *p53* heterogeneity in our study. It does not seem probable that the 10 tumors examined were made up by the merging of multiple tumors generated independently but closely enough to form an apparently single tumor within a large area of colorectal mucosa. Furthermore, recent reports have suggested a single clonal origin, based on studies of X-chromosome inactivation and microsatellite markers, for cancers regarded as arising through field cancerization.[32, 33] Colorectal cancer is thought to be a representative of tumors generated by clonal evolution. However, only a few reports have described intratumor *p53* gene heterogeneity. This may be because of insufficient analysis of intramucosal lesions, since our data revealed *p53* heterogeneity. The present data thus represent evidence strengthening the clonal evolution theory of colorectal cancers.

Finally, since only one clone among various subclones in intramucosal carcinoma apparently invaded the submucosa, not all clones in pre-invasive carcinomas may have the capacity to invade. There were no specific hot spots of *p53* mutation patterns in the invasive portion of the carcinomas in our study. In other words, *p53* inactivation may not be correlated with the invasive capacity of a carcinoma.

ACKNOWLEDGMENTS

The authors thank Satoru Nakagawa, MD, Jiro Hitomi, MD (First Department of Surgery, Third Department of Anatomy, Niigata University School of Medicine), and Yo Kato, MD (Department of Pathology, Cancer Institute, Tokyo) for their helpful advice, and our laboratory staff for excellent technical assistance. This work was supported in part by a Grant-in-Aid from the Ministry of Health and Welfare of Japan and a subsidy from the Niigata Medical Association.

(Received August 11, 1997 / Revised October 13, 1997 / Accepted October 22, 1997)

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