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Original article

Heterogeneity of *p53* mutational status in the superficial spreading type of early gastric carcinoma

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Abstract

Background. **The superficial spreading type of gastric carcinoma may originate from either a single cellular clone or from several different clones; this issue remains controversial. Indeed, the *p53* gene has been shown to play an important role in gastric carcinogenesis, but there have been only a few reports on the heterogeneity of gastric carcinoma with respect to the *p53* gene.**

Methods. **We analyzed seven cases of the superficial spreading type of gastric submucosal carcinomas (80 lesions; 10 to 17 per case) which showed different histological types and/or different p53 protein staining patterns. Direct sequences of polymerase chain reaction products were used for the analysis.**

Results. p53 **Gene heterogeneity in mucosal carcinoma lesions was detected in three cases. However, in all of the cases, the *p53* mutational pattern was identical to that found in the submucosal carcinoma lesions. In the heterogeneous cases, the mutation in the submucosal carcinoma was one of the mutation patterns found among the mucosal carcinoma lesions. More precisely, the mutational pattern of both submucosal carcinoma lesions and the mucosal lesions located just above them, was identical.**

Conclusion. **These data suggest that, with regard to the *p53* gene, in some superficial spreading types of gastric carcinomas, there are various subclones in the mucosal carcinoma; one of these subclones becomes predominant through clonal selection, and, thus, invades the submucosa.**

Key words Superficial spreading type of gastric carcinoma · *p53* Gene · Clonality · Heterogeneity · Clonal selection

Introduction

The superficial spreading type of early gastric carcinoma (SSEGC) is characterized by a wide, superficial spreading of carcinoma cells, with little vertical invasion in depth [1]. In Japan, this type of carcinoma is defined as an early gastric carcinoma when the affected area exceeds $5 \times 5\,cm^2$ [2]. The histogenesis of SSEGC is a controversial issue. The present discussion focuses on the origin of SSEGCs, namely, whether they are monoclonal or multiclonal in origin.

The *p53* gene plays an important role in regulating the cell cycle and apoptosis. Previous reports have recorded a 20% to 60% frequency for the *p53* mutational rate in gastric carcinoma [3–13]. Because it appears at an early stage in gastric carcinogenesis [13], *p53* mutation is highly important.

Heterogeneity of *p53* mutations has been observed in mucosal carcinomas; however, a monoclonal pattern was observed in invasive lesions (deeper than the muscularis mucosae) in both colorectal carcinomas [14] and esophageal carcinomas [15].

Some cases of SSEGC showed heterogenity as regards the intensity of p53 immunostaining. Overexpression, as observed by p53 immunostaining, is closely associated with *p53* gene alterations, especially missense mutations [11]. As far as we know, there have been no reports proving the heterogeneity of *p53* mutations in gastric carcinomas, including SSEGCs.

The aim of this study was to determine whether *p53* gene mutational status in SSEGCs is heterogeneous in mucosal carcinoma lesions, and homogeneous in submucosal lesions.

Materials and methods

Materials

We investigated seven cases of SSEGC (Table 1), based on the criteria used and defined by Kitamura et al. [2]. All of the patients included in the present study underwent surgical resection, without systematic adju-

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Table 1. Clinicopathological features of seven patients whose gastric cancer specimens were examined

Case no.	Age (years)	Sex	Gross type[a]	Histological type[b]	Depth[c]	Size (mm^2)
1	65	M	IIa + I	tub 1 (L and H)	sm 1	65 × 55
2	71	F	IIa + IIc	tub 1 (L and H)	sm 1	95 × 73
3	81	F	IIa + IIb	tub 1 (H), tub 2 (H), por 2	sm 1	70 × 58
4	75	M	IIc + IIa	tub 1 (H), tub 2 (H)	sm 2	69 × 50
5	86	M	IIa + IIc	tub 1 (H), tub 2 (H) > por 1	sm 3	105 × 69
6	52	M	IIc + IIb	tub 2 (H) > tub 1 (H), por 2	sm 3	70 × 50
7	67	F	IIb + IIa	tub 2 (H)	sm 1	60 × 50

L, Low-grade atypia; H, high-grade atypia; tub 1 (H), well differentiated adenocarcinoma with high-grade atypia; tub 1 (L), well differentiated adenocarcinoma with low-grade atypia; tub 2, moderately differentiated adenocarcinoma; por 1, poorly differentiated adenocarcinoma (solid type); por 2, poorly differentiated adenocarcinoma (nonsolid type)
[a,b,c] Gross type, histological, type, and depth are classified according to the *Japanese classification of gastric carcinoma* [16]

Table 2. Mutational analysis of *p53* in mucosal and Submucosal carcinoma lesions

	Mucosal carcinoma				Submucosal carcinoma			
				Mutated sequence				Mutated sequence
Case no.	No. of samples	IHC	Hist. type	Codon (sequence[amino acid])	No. of samples	IHC	Hist. type	Codon (sequence[amino acid])
1	2	(+++)	tub 1 (H)	248 ($CGG^{Arg} \rightarrow CAG^{Gln}$)	2	(+++)	tub 1 (H)	248 ($CGG^{Arg} \rightarrow CAG^{Gln}$)
	2	(+++)	tub 1 (L)	248 ($CGG^{Arg} \rightarrow CAG^{Gln}$)				
	1	(+++)	tub 1 (H)	273 ($CGT^{Arg} \rightarrow CAT^{His}$)				
	2	(−)	tub 1 (L)	ND				
	1	(−)	tub 1 (L)	258 ($CAA^{Gln} \rightarrow TAA^{Stop}$)				
2	3	(+++)	tub 1 (H)	282 ($CGG^{Arg} \rightarrow TGG^{Trp}$)	2	(+++)	tub 1 (H)	282 ($CGG^{Arg} \rightarrow TGG^{Trp}$)
	1	(+++)	tub 1 (L)	282 ($CGG^{Arg} \rightarrow TGG^{Trp}$)				
	1	(+++)	tub 1 (L)	282 ($CGG^{Arg} \rightarrow TGG^{Trp}$) 278 ($CCT^{Pro} \rightarrow TCT^{Ser}$) 300 ($CCC^{Pro} \rightarrow CTC^{Leu}$)				
	1	(+++)	tub 1 (L)	282 ($CGG^{Arg} \rightarrow TGG^{Trp}$) 153 ($CCC^{Pro} \rightarrow TCC^{Ser}$)				
	1	(+++)	tub 1 (H)	ND				
	1	(+)	tub 1 (H)	ND				
	2	(−)	tub 1 (L)	ND				
3	4	(+++)	tub 1 (H)	248 ($CGG^{Arg} \rightarrow CAG^{Gln}$)	1	(+++)	tub 2 (H)	248 ($CGG^{Arg} \rightarrow CAG^{Gln}$)
	2	(+++)	tub 2 (H)	248 ($CGG^{Arg} \rightarrow CAG^{Gln}$)				
	6	(+++)	por 2	248 ($CGG^{Arg} \rightarrow CAG^{Gln}$)	2	(+++)	por 2	248 ($CGG^{Arg} \rightarrow CAG^{Gln}$)
	2	(+++)	por 2	ND				
4	1	(+++)	tub 1 (H)	ND				
	2	(+++)	tub 2 (H)	ND				
	1	(−)	tub 1 (H)	ND				
	3	(−)	tub 2 (H)	ND	3	(−)	tub 2 (H)	ND
5[a]	2	(+++)	tub 1 (H)	ND				
	4	(+)	tub 2 (H)	ND				
					1	(−)	tub 2 (H)	ND
					4	(−)	por 1	ND
6	5	(+++)	tub 1 (H)	ND	2	(+++)	tub 1 (H)	ND
	2	(+++)	por 2	ND	1	(+++)	tub 2 (H)	ND
7[a]	8	(+++)	tub 2 (H)	273 ($CGT^{Arg} \rightarrow CAT^{His}$)	2	(+++)	tub 2 (H)	273 ($CGT^{Arg} \rightarrow \rightarrow CAT^{His}$)

Histological type is classified according to *Japanese classification of gastric carcinoma* [16]
IHC, Immunohistochemistry (monoclonal antibody) (PAb-1801); Hist. type, histological type; ND, mutation in exons 5 to 8 not detected
[a] Metastatic carcinomas to the lymph nodes were also available for immunohistochemistry and DNA analysis

vant therapy, in the affiliated hospitals of Niigata University between 1992 and 1998. The average age of the seven patients (four men; three women) at the time of surgery was 71.0 years. All resected specimens were fixed in 10% formalin immediately after resection. The entire lesion was serially cut into 4- to 5-mm-thick pieces. Samples were embedded in paraffin. In all cases, immunohistochemistry (IHC) and DNA analysis were conducted. Lymph node metastasis had occurred in two patients, and specimens were available for IHC and DNA analysis (Table 2). Histological examination was performed based on the Japanese Research Society for

Gastric Cancer classification of gastric carcinoma [16]. Using the system of classification of Watanabe et al. [17], we divided carcinomas into two groups, according to histological and cytological atypia: low-grade carcinoma (L), and high-grade carcinoma (H).

Immunohistochemical analysis of p53 protein

Three serial, 3-μm-thick sections were made from all blocks of each carcinoma. The first section was stained with hematoxylin and eosin (H&E), the second section was immunostained for p53, using the mouse monoclonal antibody PAb1801 (Oncogene Science I, Manhasset, NY, USA), and the third section was immunostained for Ki-67 (MIB1; Immunotech, Marseille, France). Immunohistochemical staining was performed using streptavidin-peroxidase complex. We used Ki-67 staining as the inner control to prove the adequacy of immunoreactivity, observing nonneoplastic proliferative cells in the neck zone or the germinal center cells of lymph follicles. In the total of seven cases, 80 lesions were analyzed for the p53 protein.

p53-Positive cells were defined as those with brown-stained nuclei, regardless of the staining intensity. Expression of p53 protein was classified into four categories: (1) (+++), positive cells distributed throughout most of the lesion; (2) (++), positive cells aggregated in focal area(s); (3) (+), a small number of isolated positive cells were scattered in the lesion; and (4) (−), negative. Overexpression of p53 was defined as (+++) or (++), according to a published study [18].

DNA preparation

We selected representative sections of carcinoma not only by depth but also by differences in p53 staining patterns and histological and cytological atypia. Six to 14 lesions in each tumor (60 in total) were sampled as mucosal carcinomas, and 2 to 5 lesions from each tumor (20 in total) were sampled as submucosal carcinomas.

DNA extraction from paraffin sections was performed as follows: tissue sections from five to ten serial, nonstained 10-μm-thick sections from each sample were dewaxed in xylene for 10 min and rehydrated in 99% ethanol for 5 min. The lesions were dissected under direct observation with a microscope to avoid contamination of noncarcinoma cells, using commercially available sterilized disposable 25-G × 1″ needles (Φ 0.5 mm) (Terumo, Tokyo, Japan). There were around 3500–7000 cells, including at least 80% carcinoma cells, used in each sample; every sample was selected using a DNA isolator PS kit (Wako, Osaka, Japan). DNA was precipitated successively with isopropyl alcohol and with 99% ethanol and was then dried. Samples were then dissolved in 30 μl of sterile water.

Polymerase chain reaction (PCR)

Oligonucleotide primers were synthesized by Takara Shuzo (Kyoto, Japan). Four fragments of DNA, including exons 5–8 of *p53*, were amplified, using nested PCR with two sets of primers for each exon. The first and second PCR amplification primers were described previously [19]. The first PCR was performed using a 50-μl reaction mixture containing 50 mM potassium chloride, 10 mM Tris-HCl (pH 8.0), 0.1% Triton X-100, 1.5 mM magnesium chloride, 200 μM of each nucleotide (dATP, dCTP, dGTP, dTTP (Takara Shuzo), 20 pM of each primer, 2.5 units of Taq DNA polymerase (Promega, Madison, WI, USA), and 1 or 2 μl of DNA template. One cycle was performed at 94 °C for 5 min, 55 °C for 1 min and 30 s, and 72 °C for 1 min; followed by 35 cycles at 94 °C for 1 min, 60 °C for 1 min 30 s, and 72 °C for 1 min; and finally, a 7-min extension at 72 °C, using the Program Temp Control System (ASTEC; Fukuoka, Japan). The product of the first PCR was used as the template for the second PCR, which was performed under the same conditions; however, during the second PCR, one primer in each set was biotinylated at the 5′ terminal (Takara Shuzo). In each experiment, control reactions containing no DNA samples or human placental DNA (Oncogene Science; Uniondale, NY, USA) were performed simultaneously with the sample reactions. The products of the second PCR were electrophoresed for 20 min on 4% agarose gel (NuSieve 3: 1 Agarose; FMC BioProducts, Rockland, ME, USA) using a Mermaid Kit from Bio101 (La Jolla, CA, USA). Informative cases were defined as those with amplified bands on the agarose gels.

Direct sequencing

All products of the second PCR were sequenced directly, using an Auto Load Solid Sequencing Kit (Pharmacia Biotech, Uppsala, Sweden) and an automated laser fluorescent sequencer (ALF DNA Sequencer II; Pharmacia). The same oligonucleotides as inner PCR primers were labeled fluorescently at their 5′ terminals (Takara Shuzo) and used as sequencing primers. To confirm the findings, we analyzed all samples at least twice, in both the sense and antisense directions, using different PCR products obtained from the same samples.

Results

We detected seven different kinds of *p53* mutations: one in exon 5, two in exon 7, and four in exon 8; no mutations were detected in exon 6. All the mutations were transitional mutations, and three occurred in CpG

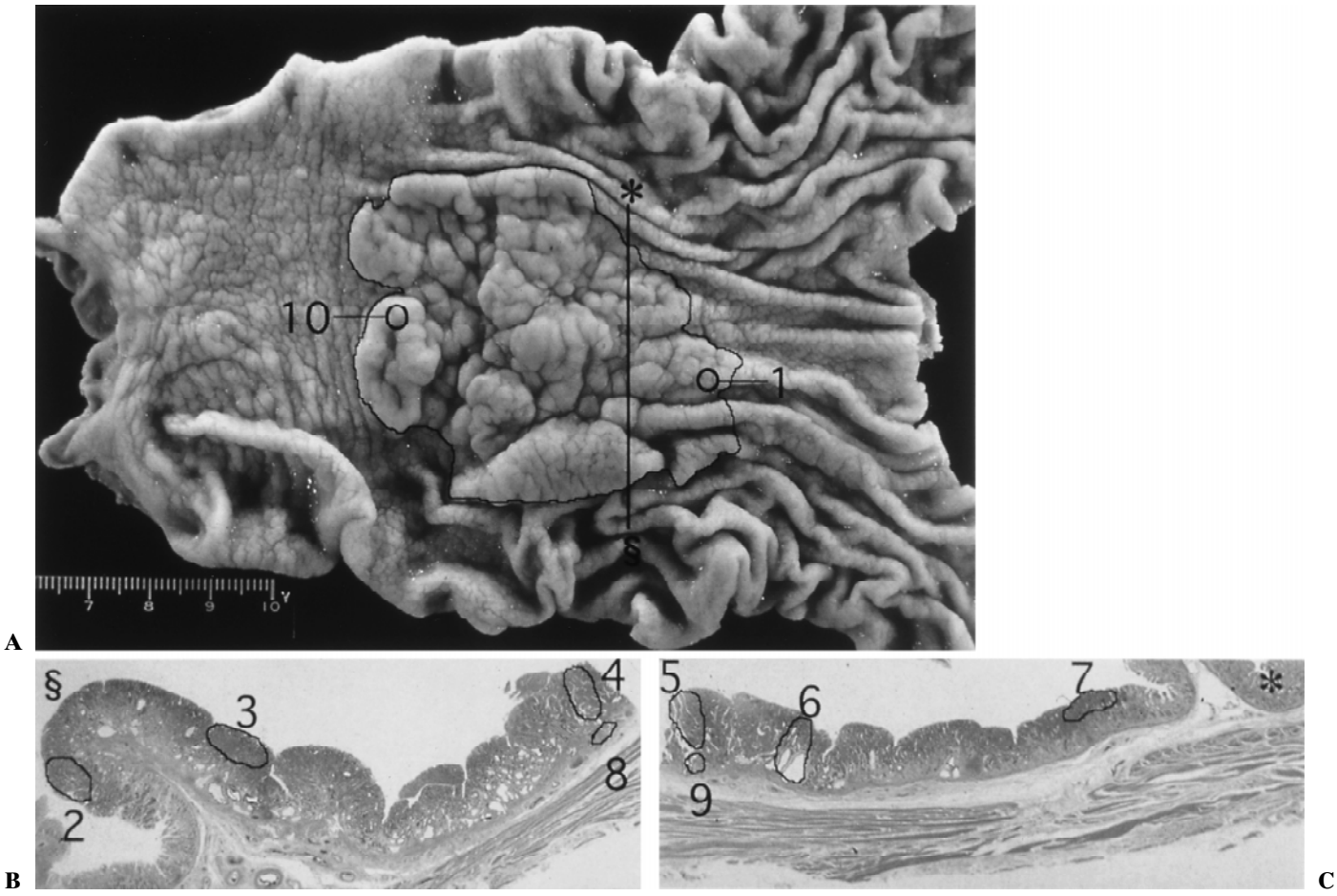

Fig. 1A–C. A representative case (case 1) showing heterogeneity for *p53* gene mutation. Specimens were sliced vertically. **A** *Asterisk* and § represent the upper and lower points that appear on the right and left, respectively, in the H&E sections (**B** and **C**). Topographic distributions of the mucosal carcinoma lesions (*1–7*, *10*) and submucosal carcinoma lesions (*8*, *9*) are shown. DNA was extracted from all of the lesions for *p53* mutational analysis

dinucleotide pairs. Point mutations of codon 248 (CGG to CAG) and codon 273 (CGT to CAT) were commonly observed in two different cases (Table 2).

We found heterogeneity of *p53* gene alterations in mucosal carcinoma lesions in three (cases 1 to 3) of the seven cases (Table 2). In case 1, we discovered 8 mucosal carcinoma lesions with four different *p53* mutational patterns: 6 lesions occurred with a single mutational pattern (codon 248 or 258, or 273) and 2 lesions appeared without mutations through exons 5 to 8 (Figs. 1, 2). In case 2, we identified 10 mucosal carcinoma lesions with four different *p53* mutational patterns: 4 lesions appeared with a single mutational pattern (codon 282), 1 with double mutations (codons 282 and 153), and 1 with triple mutations (codons 282, 278, and 300); 4 lesions without mutations through exons 5 to 8 were also observed. In case 3, there were 14 mucosal carcinoma lesions: 12 lesions with a same single mutation (codon 248) and 2 lesions were without mutations through exons 5 to 8.

Two other cases (cases 4 and 5) showed a heterogeneous p53 immunostaining pattern in the mucosal carcinomas, but no gene alteration was detected. There were three lesions with p53 protein overexpression and four without overexpression in case 4, and two lesions with overexpression and four without overexpression in case 5. Two other cases (cases 6 and 7) showed identical *p53* mutations in each tumor; both of them were immunohistochemical and gene alterations.

In all cases, the submucosal carcinoma showed a homogeneous mutational status that was identical to one of the mucosal mutations in the same tumor.

We also analyzed four lesions identified among the lymph node metastases, (one lesion from case 5, and three lesions from case 7). No mutations were found in exons 5 through 8 in case 5, and the same mutation (codon 273) as in the primary carcinoma was found in case 7.

Most lesions with a (−) or (+) pattern for p53 immunostaining (21 of 22; 95.5%) did not show any

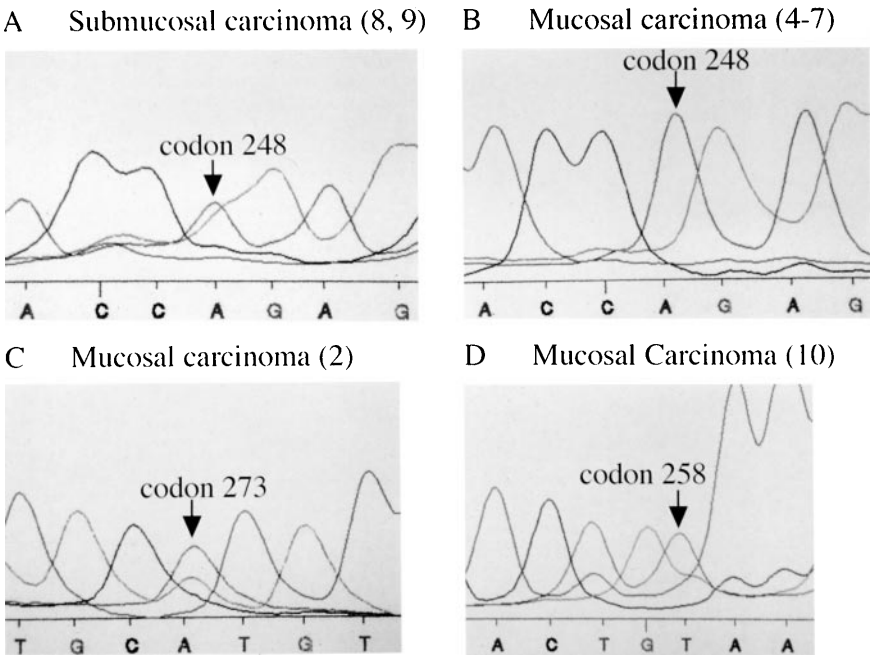

Fig. 2A–D. Mutation of *p53* detected by direct sequencing of DNA in case 1. **A** Both submucosal carcinoma lesions (*8*, *9*) showed the same mutation at codon 248 (CGG^{Arg} to CAG^{Gln}). **B** Mucosal carcinoma lesions (*4–7*) showed the same mutation at codon 248 (CGG^{Arg} to CAG^{Gln}). This mutation was identical to that of the submucosal carcinoma lesions. **C**, **D** Only one mucosal carcinoma lesion (*2*, *10*, respectively) showed a different mutation at 273 (CGT^{Arg} to CAT^{His}), and at 258 (CAA^{Gln} to TAA^{Stop}), respectively. The other two lesions (*1*, *3*) failed to demonstrate any mutations in exons 5 through 8. Numbers of lesions in parentheses refer to the numbers shown in Fig. 1

mutation in exons 5 through 8, although one negative lesion showed a nonsense mutation in codon 258 (case 1). Sixty-nine percent of lesions (40 of 58) with p53 protein overexpression revealed *p53* gene alterations, while the other 31.0% of lesions (18 of 58) did not show any mutations through exons 5 to 8. There was no specific correlation between either the various histological types or the mutational types among the tumors (Table 2).

Discussion

p53 Mutation is reported to be the most commonly implicated genetic event in gastric carcinogenesis [13]; it is also an early event that is often observed in differentiated types of gastric carcinoma [7,13]. Sequencing of the *p53* gene in mammals has revealed five highly conserved domains, four of which fall within exons 5 through 8, where most of the evolutionarily conserved amino acids are concentrated [20]. Most of the mutations occur within exons 5 through 8, which we have analyzed in this study. We assessed mutations in the *p53* gene by direct sequencing, because single-strand polymorphism analysis often fails to predict *p53* mutational status [21].

We found seven different kinds of mutations, all of which were transitional mutations; some of these mutations occurred at the CpG site. In gastric carcinoma, base transitions were thought to be the most frequent type of genetic change in the *p53* gene [6]. Our findings suggest that, even in SSEGCs, base transitions are common genetic alterations, as are those observed in other types of gastric carcinoma. CpG dinucleotides in the *p53* gene seem to be a primary target for mutations associated with dietary carcinogens such as the N-nitroso compounds [22].

Bamba et al. [23] have reported that most SSEGCs are monoclonal; their methodology included using polymorphisms of the X-linked human androgen receptor gene (HUMARA) methods. They examined eight informative cases, and only one case showed heterogeneity. It should be noted that their report did not clarify from which layers the carcinoma cells had been microdissected. In some cases, the number of the lesions examined was small. Thus there remains the possibility that the number of polyclonal cases could be higher than the number they reported. In addition, their study focused on gastric cancers that manifested at least some signet ring cells. In contrast, we focused on gastric cancers that mainly contained well differentiated carcinoma glands. Indeed, there are some differences between these two types of carcinoma. For example, the location of the proliferative zones is different [24], the premalignant lesions differ [25], and some genetic abnormalities differ as well [13,26]. Therefore, we inferred that the clonality of gastric cancinomas might differ according to the dominant histological types.

Three reasons are posited to explain why *p53* heterogeneity was found in mucosal carcinomas. The first is based on the "field cancerization" theory. SSEGCs may be derived from the collision of different small gastric carcinomas. In many SSEGCs, we found a number of histological types and various kinds of cytological

atypia; these findings support this reason for *p53* heterogeneity in the mucosal carcinomas. The second possible reason is that *p53* mutation occurred within a single tumor after clonal expansion. In case 2, we found the same mutation in codon 282 (CGG to TGG) in six mucosal carcinoma lesions; two of these lesions showed other additional mutation(s) in codon 282 (Table 2). The third reason is a combination of the two former explanations.

Kang et al. [27] reported 12 heterogeneous cases of synchronous multiple gastric carcinoma, using *p53*, *MCC*, and *APC* gene analysis. They also suggested that the theory of field cancerization could help to explain gastric carcinogenesis. Further research needs to be done to analyze other genes in order to clarify the clonality of gastric carcinoma. Cho et al. [28] analyzed heterogeneity by means of *p53* gene alterations, and showed that there was no correlation between histological type and *p53* mutations. In regard to this point, there is no discrepancy between their results and ours.

It should also be noted that there was no *p53* heterogeneity in layers deeper than that of the muscularis mucosae carcinomas. In all cases, the submucosal carcinoma showed a homogeneous mutational status that was identical to that of the mucosal mutations in the same tumor. The *p53* mutation must have occurred in the mucosal carcinoma, preceding the submucosal invasion, in some SSEGCs. In the mucosal carcinoma lesions, there was a combination of high- and low-grade carcinoma cells, while, in contrast, all of the submucosal carcinoma lesions showed high-grade atypia. We have reported that cells with high-grade atypia in carcinoma readily invade the submucosal layer. We suggest the possibility that a clone with high-grade atypia was selected from among multiple clones within the mucosal carcinoma, and subsequently invaded the submucosal layer.

We examined seven cases of SSEGCs because large carcinoma cells remained in the mucosal layer. But in almost all of the cases, except for cases 5 and 6, a small number of carcinoma cells were found in the invasive lesions. In the future, we hope to analyze more cases with a larger number of carcinoma cells from both mucosaosal and invasive lesions. In order to clarify the *p53* clonality of gastric carcinoma, similar analysis regarding early invasive carcinomas, other than SSEGC, needs to be done.

In conclusion, *p53* heterogeneity was determined in many cases of SSEGC. It is highly possible that gastric carcinoma cells arise polyclonally during the intramucosal carcinoma stage, and that they later develop into monoclonal populations, while invading the submucosal layer, through clonal selection, with respect to the *p53* gene alteration.

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