Heterogeneity of p53 Mutational Status in Intramucosal Carcinoma of the Colorectum

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The aim of this study was to elucidate whether or not p53 genetic heterogeneity would occur while colorectal carcinoma was limited to the mucosa. Eight cases of endoscopically resected colorectal intramucosal carcinomas were analyzed to determine the p53 gene sequence (exons 5 to 8). Six out of 8 cases showed p53 gene mutations, and in all of them, the mutational status was heterogeneous. In 4 cases, mutated codons were heterogeneous as well. These data indicate that p53 gene alterations in colorectal carcinomas occur and diverge at the stage of intramucosal carcinoma, supporting our previously proposed hypothesis that colorectal carcinomas can be composed of various subclones as regards p53 gene mutation, while the carcinoma is limited to the mucosa, and one of these subclones commences invasion to the submucosa after clonal selection, thus generating a monoclonal invasive carcinoma.

Key words: Colorectal carcinoma — p53 gene — Heterogeneity — Intramucosal carcinoma

It is widely accepted that colorectal carcinomas arise through a multistep accumulation of genetic alterations.1) Mutations of the K-ras gene2) and alterations of the p53 gene,3) APC gene,4) and DCC gene5) are most frequently observed, and they play a crucial role in the development of colorectal carcinomas. Among these genetic alterations, p53 gene mutations are the most common genetic change observed in human malignancies.6, 7) During colorectal carcinogenesis, inactivation of the p53 gene is thought to play a role in the transition from benign (adenoma) to malignant growth.8) This hypothesis is based on data that suggest that p53 gene alteration occurs in 49–70% of colorectal carcinomas, but that it is rare in adenomas.3, 8) As regards p53 mutational status in colorectal carcinomas, most earlier studies have found that it was homogeneous.9–13) However, one report on carcinoma in adenoma14) has reported otherwise. In a previous report, we revealed that p53 mutational status was heterogeneous as regards the mucosal component and was homogeneous in the invasive portion. In that study, invasive colorectal carcinomas with a remnant mucosal component were studied.15) The variety of findings from earlier studies can be accounted for because they examined invasive carcinomas which had no remnant mucosal components, or else only the invasive part was analyzed. Our previous study was the first to examine the variety in mutational status of the p53 gene in the context of tumor progression. We hypothesized that colorectal carcinomas can be composed of various populations of subclones when the tumor is limited to the mucosa. Then, one of these subclones begins to invade the submucosa after clonal selection, generating a monoclonal invasive carcinoma.15) It remained unclear whether or not intramucosal heterogeneity of the p53 gene mutation was generated after, and not before, the submucosal invasion of the carcinoma.

The purpose of this study was to elucidate whether or not p53 genetic heterogeneity would occur at a stage when colorectal cancer was limited to the mucosa. p53 mutation in various portions of identical intramucosal carcinomas was analyzed. The results showed intratumoral p53 mutational heterogeneity in intramucosal colorectal carcinomas. This finding supports our previous hypothesis and suggests the possibility that genetic evolution of colorectal carcinoma occurs and that it is completed at an early stage of its development.

MATERIALS AND METHODS

Samples The samples consisted of 8 cases of endoscopically resected human polypoid intramucosal colorectal carcinomas with p53 protein overexpression. The sizes of the tumors were less than 20 mm and all of them were accompanied by an adenomatous component. Samples were immediately fixed in 10% formalin and embedded in paraffin. Two serial 3 µm-thick sections and seven to eight 6
µm-thick sections from all blocks of each sample were prepared. Three micrometer-thick sections were stained with hematoxylin and eosin (HE) for histologic examination and p53 immunostaining. Six micrometer-thick sections were used for DNA extraction.

**Histologic and immunohistochemical evaluations**  Histologic diagnosis was done according to the criteria of Watanabe, classifying the carcinoma into low-grade atypia (CAL) and high-grade atypia (CAH).16–18) p53-immunostaining was done using monoclonal antibody PAb1801 (Oncogene Science, Manhasset, NY) by the streptavidin-peroxidase complex method (SAB method). The p53 staining pattern was classified into the following four types: 1) diffuse, 2) nested, 3) scattered, or 4) negative, and 1) and 2) were regarded as involving an overexpression of p53 protein.19–21)

**DNA extraction**  Four to 10 areas of carcinomatous and adenomatous component from each sample were selected for DNA extraction by means of a microdissection technique. Before the extraction, 6 µm-thick serial sections were p53-immunostained in order to obtain DNA from areas with and without p53 protein overexpression, separately. Two to 5 µl of 70% ethanol was dropped on the tissue sections to visualize immunostained areas and samples were scraped out by hand with a sterile needle under microscopic observation. Five microliters of 1× TK Buffer

### Table I.  p53 Mutational Status of Intramucosal Carcinoma

| Case | Macroscopic typing | Location | Size (mm) | Hist. | p53 IHC overexpression | No. of foci | Codon | Base change | Amino acid change |
|------|--------------------|----------|-----------|-------|------------------------|------------|-------|-------------|------------------|
| 1    | Isp                | Sigmoid  | 15×6×9    | CAH   | +                      | 2          | 208   | GAC→GTC    | Asp→Val          |
|      |                    |          |           | CAL   | –                      | 2          |        |             |                  |
| 2    | Ip                 | Sigmoid  | 15×8×8    | CAH   | +                      | 1          | 248   | CGG→CAG    | Arg→Gln          |
|      |                    |          |           | CAL   | –                      | 4          |        |             |                  |
|      |                    |          |           | CAL   | –                      | 1          |        |             |                  |
| 3    | Ip                 | Sigmoid  | 11×8×6    | CAH   | +                      | 1          | 273   | CGT→CAT    | Arg→His          |
|      |                    |          |           | CAL   | +                      | 2          | 273   | CGT→CAT    | Arg→His          |
|      |                    |          |           | CAL   | +                      | 1          | 273   | CGT→CAT    | Arg→His          |
|      |                    |          |           | CAL   | +                      | 2          | 273   | CGT→CAT    | Arg→His          |
|      |                    |          |           | CAL   | –                      | 1          |        |             |                  |
| 4    | Ip                 | Sigmoid  | 10        | CAL   | +                      | 1          | 306   | CGA→TGA    | Arg→Stop         |
|      |                    |          |           | CAL   | +                      | 1          | 306   | CGA→TGA    | Arg→Stop         |
|      |                    |          |           | CAL   | –                      | 1          |        |             |                  |
| 5    | Ip                 | Sigmoid  | 14×8×6    | CAH   | +                      | 1          | 237   | ATG→ATA    | Met→Ile          |
|      |                    |          |           | CAL   | +                      | 1          | 237   | CTG→TTG    | Leu→Leu          |
|      |                    |          |           | CAL   | +                      | 1          | 243   | ATG→ATA    | Met→Ile          |
|      |                    |          |           | CAL   | –                      | 1          |        |             |                  |
| 6    | Ip                 | Sigmoid  | 16×14×10  | CAH   | +                      | 1          | 220   | TAT→TGT    | Tyr→Cys          |
|      |                    |          |           | CAL   | +                      | 1          | 134   | TTT→TTG    | Phe→Leu          |
|      |                    |          |           | CAL   | –                      | 2          |        |             |                  |
| 7    | Isp                | Sigmoid  | 20×12×10  | CAH   | +                      | 1          | 220   | TAT→TGT    | Tyr→Cys          |
|      |                    |          |           | CAL   | –                      | 4          |        |             |                  |
| 8    | Isp                | Sigmoid  | 18×15     | CAH   | +                      | 2          |        |             |                  |

IHC, immunohistochemistry (mAb PAB-1801); Hist., histologic diagnosis; CAL, carcinoma with low-grade atypia; CAH, carcinoma with high-grade atypia.

a) Macroscopic typing: according to the classification of the Japanese Research Society for Cancer of Colon and Rectum.31)
(10× TK Buffer: 5% Tween 20, 2 mg/ml of proteinase K, 1x TE9) was added and the samples were incubated overnight at 56°C, and at 97°C for 12 min. DNA sequencing of the PCR products was performed using an AutoLoad Solid Phase Sequencing Kit (Pharmacia, Uppsala, Sweden) with a fluorescent sequencer apparatus (A.L.F. DNA sequencer; Pharmacia) equipped with A.L.F. Manager Version 2.6. Each PCR product was sequenced in both directions (forward and reverse) at least twice to confirm the reproducibility of the results.

RESULTS

Table I shows the results of p53 immunohistochemistry and mutational analysis of 8 intramucosal colorectal carcinomas by histologic diagnosis. In total, 47 DNA samples were analyzed according to the histologic diagnosis and p53 immunoreactivity was analyzed in the carcinomatous and adenomatous areas. All adenomatous areas showed neither p53 protein overexpression nor gene mutation (data not shown in Table I). Twelve different mutational codons were detected in the carcinomas, and all mutations were single base-pair substitutions. Missense mutation resulting in an amino acid substitution was observed in 11 of 12 mutations, and the final one was a nonsense mutation in codon 306 in exon 8, resulting in a stop codon. p53 mutational heterogeneity was present in 6 cases (75%). In 4 cases (50%), mutated codons were heterogeneous as well. Two cases showed wild-type p53 gene throughout.

Intratumoral p53 mutational heterogeneity was classified into three patterns. 1) Type A: the tumor consisted of two populations of subclones, either of which carried a single common mutation or wild-type genes (cases 1, 2). 2) Type B: the tumor consisted of a population of subclones carrying a single common mutation with additional differing mutations in some foci, or carrying wild-type genes (cases 3, 4). In case 4, for example, one of three areas examined showed wild-type genes and the other two areas presented with identical mutations (codon 306); one of the two areas carried an additional mutation at codon 175 (Fig. 1). 3) Type C: the tumor consisted of a population of subclones which carried different mutations at different sites or had wild-type genes (cases 5, 6). In case 6, for example, two different point mutations (codon 220 or 134) were detected in three of five areas and the remaining two areas exhibited wild-type genes (Fig. 2).

Correlations among histologic diagnosis, p53 protein overexpression, and gene mutation are shown in Table II. As regards association with the histologic grade of atypia in the carcinomas, CAH revealed a significantly higher incidence of both p53 protein overexpression and p53 gene mutation compared to CAL: protein overexpression and mutation were observed in 87% (13/15) vs. 38% (10/26) of the areas and 67% (10/15) vs. 23% (6/26) of the areas.
DISCUSSION

Intratumoral genetic heterogeneity is thought to arise during progression. In colorectal neoplasms, intratumoral genetic heterogeneity of the K-ras gene, changes of DNA ploidy, loss of heterozygosity (LOH), microsatellite instability and appearance of TGF-β, BAX, and other molecular markers have been demonstrated. Whether or not a certain genetic alteration shows intratumoral heterogeneity depends on the stage in the tumor progression at which the examination was conducted. During colorectal carcinogenesis, in the so-called “adenoma-carcinoma sequence,” K-ras mutations are reported to be heterogeneous when the focus is adenoma, a pre-malignant proliferation, and homogeneous when the focus is post-malignant transition (adenocarcinoma). The interpretation of the K-ras mutational status in the context of tumor progression suggests that K-ras mutation occurs at the stage of adenoma. An adenoma cell that carries the mutation gains an advantage as regards growth and transforms into a carcinoma consisting of a monoclonal population with identical K-ras mutation.

As regards the p53 mutational status, most earlier studies have suggested that colorectal carcinomas are homogeneous masses, but it has not been determined at which stage of tumor progression p53 alterations occur. Previous studies have investigated the invasive portion of advanced-stage colorectal carcinomas alone. However, p53 mutational status has not yet been investigated in detail as regards the various stages of development. In the present study, 6 (75%) out of 8 cases of intramucosal carcinoma showed p53 mutational heterogeneity, which indicates that p53 alteration in colorectal carcinomas would occur at a stage of growth limited to the mucosa. Furthermore, 4 (50%) out of 8 cases of intramucosal carcinoma showed p53 mutational codons. This finding supports our previous hypothesis that at least some colorectal carcinomas are composed of various populations of subclones carrying various p53 mutations in the mucosa. One of the subclones may commence invasion into the submucosa after clonal selection, ultimately generating a monoclonal invasive carcinoma.

In the present study, a heterozygous sequence pattern with two peaks (Fig. 1b) appeared on the automated sequencer. The heterozygous pattern always contained the wild-type sequence. Persistence of the wild-type allele may indicate either that the samples contained normal undelated normal allele of the cancers. However, analyses of LOH will be needed for further clarification of this matter. In Table II, we classified carcinomas as either CAH or CAL, according to the grade of cytological atypia. This cytological phenotype classification corresponds to the biological behavior of the tumor: CAH shows greater cell proliferative activity than does CAL. The capacity for vascular invasion and lymphnodal metastasis of CAH was comparable to that of CAL. Although there was no association between specific p53 mutations, the incidence of
mutations, as well as protein overexpression, significantly differed according to the grade of atypia. Sixty-seven percent (10/15) of CAH tumors revealed p53 mutation, whereas only 23% (6/26) of CAL tumors showed p53 mutation. These results indicate that p53 gene alteration may not be responsible for transition to malignancy in adenomas. Instead, p53 gene alteration may play a role in the acceleration of the malignant potential of colorectal carcinomas that progress from CAL to CAH. p53 mutational heterogeneity was classified into three types. Type A (single common mutation or wild-type) and type B (a single common mutation with additional different mutations or wild-type) may represent different stages of a single clonal evolution process in which a single mutant subclone is added to other mutations (type A proceeds to type B). In type C (different mutations at different sites or wild-type), a variety of subclones for p53 could be generated at the stage of intramucosal carcinoma.

Previously, we have considered two possible explanations for p53 heterogeneity in the intramucosal component of invasive colorectal carcinoma,15) namely, 1) multiple subclones for p53 could be generated in the intramucosal carcinoma, or 2) multiple subclones bearing different p53 alterations could occur within pre-malignant lesions (adenoma). The present results support the first explanation, since the adjoining adenoma tissue showed neither p53 protein overexpression nor genetic alteration. In addition, heterogeneity was observed in p53 mutational status in intramucosal carcinomas. Combining the evidence that p53 alteration occurs in and varies among intramucosal carcinomas and the evidence that p53 mutational status was homogeneous in the invasive portions (as demonstrated by our previous study),15) it may now be speculated that genetic evolution of colorectal carcinoma as regards p53 gene mutation could be generated and completed at a stage in which the tumor is limited to the mucosa.

Although the present investigation is confined to p53 mutational status, it is possible that several other genetic evolutions occur and are completed while the tumor is limited to the mucosa. We would like to stress the possibility that genetic evolution in colorectal carcinoma, which is regarded as representative of tumors generated through a multistep accumulation of genetic alterations,15) may not occur according to its stage of development, but rather, such tumors may undergo transition to malignancy at the earliest stage of cancer, and furthermore, transition may be completed at this early stage. The progenitor for the more advanced forms may already be complete, with the full accumulation of necessary genetic alterations that determine terminal biological behavior, during the stage of intramucosal carcinoma.

A more comprehensive study using multiple markers over several chromosomes (for example, APC41 and DCC40) will be necessary in order to draw further conclusions. The key to any such investigation will be to focus on intramucosal carcinomas.

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