Possible alternative carcinogenesis pathway featuring microsatellite instability in colorectal cancer stroma

Differential microsatellite instability (MSI) in tumour epithelial and stromal compartments has not been well examined for colorectal cancers. Using laser-captured microdissection, separate specimens of these compartments of 40 sporadic colorectal cancers were sampled and MSI was tested with four markers. To examine the relation between the MSI phenotype in the stroma and other genetic events and histopathological features, p53 and K-ras gene mutations were analysed, and the expression of p53, hMLH1, and hMSH2 protein was determined by immunohistochemistry. Microsatellite instability positive results were obtained for both epithelium (34%) and stromal tissue (41%). While MSI in epithelium correlated with differentiation and Duke's stage, that in stroma demonstrated an inverse relation, being particularly frequent in well-differentiated adenocarcinomas (54%) and Duke's A lesions (55%). Further, a significant inverse correlation between p53 protein overexpression in the epithelium and MSI in the stroma was found ($P = 0.02475$). The results suggest an alternative pathway of carcinogenesis involving stromal genetic instability in the development of colorectal cancers.

**Keywords:** microsatellite instability; stroma; epithelium; colorectal cancer; p53

**MATERIALS AND METHODS**

**Samples and preparation**

In total, 40 surgically resected sporadic colorectal adenocarcinomas from patients, 24–89 years old, undergoing treatment at Kitasato University Hospital and Kitasato University East Hospital, were randomly selected. Histological typing was performed according to the criteria of the Japanese Society for Cancer of the Colon and Rectum (Jinnai, 1983) and also Duke's classification. On surgical removal, tissues were immediately frozen with liquid nitrogen in OCT compound for storage at $-80°C$. Frozen sections 10 ($10 \mu m$ thick) were fixed in 70% alcohol and stained with Mayer’s haematoxylin. Neoplastic epithelial and adjacent stromal tissues in the lamina propria were carefully microdissected with a laser-captured microdissection system (LM200, Arcturus, Mountain View, USA) to avoid contamination (Figure 1A). Tissues from whole sections, including both epithelial and stromal elements (mixed), were also manually dissected. Tumour tissues with strong inflammatory cell infiltration were excluded from study. Likewise, normal mucosa from the colorectum of the same patients was sampled and processed as an internal control. Tissues were lysed in sodium dodecyl sulphate-lysis buffer with proteinase

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Microsatellite analysis

The polymerase chain reaction (PCR) was performed for four microsatellite markers, D17S796, TP53 (forward: 5'-ACTGCACTCTTGGCCATCGTC-3', reverse: 5'-CACTGGGGCTGAA-TAGTATCCTC-3'), D17S786, and D17S579, selected for analysing allelic instability in chromosome 17. Microdissected DNA (5–10ng) was amplified with a Rapid Cycler (Idaho Technology, Idaho falls, ID, USA) and Takara DNA polymerase (Takara, Kyoto, Japan) under conditions as follows: 94°C for 3 min as the initial step, 35 cycles of 0 s at 94°C, 0 s at an appropriate temperature for each marker amplification, 6 s at 74°C, and a final step of 74°C for 3 min. The PCR products were fractionated by 3.6% polyacrylamide gel electrophoresis, fixed with 10% formalin, and stained for 30 min. The PCR products were detected as abnormally shifted bands.

RESULTS

MSI status

Microsatellite instability was frequently detected in epithelial and stromal areas of sporadic colorectal cancers (Table 1). Of the 40 colorectal cancers studied, 13 out of 38 informative cases (34%) were MSI+ for one or more of the markers in tumour epithelium, 16 out of 39 cases (41%) in adjacent stromal areas, and nine of 40 (23%) in mixed tissue. MSI+ in two or more markers was found in three cases in the epithelium and two in stroma. While MSI was slightly more common in the stroma than epithelium, the difference did not reach significance (Table 1). For each component, only three cases had MSI in both epithelium and stroma for the same markers (two for TP53 and one for D17S796), suggesting appropriate microdissection without contamination. Others showed MSI specific to the epithelium alone, stroma alone, epithelium and mixed tissue (Figure 1B; D17S786), or stroma and mixed tissue. All the tumours with MSI in mixed tissue also demonstrated MSI in epithelium and stroma. A comparison of MSI frequency for each marker between the epithelium and stroma revealed stromal MSI+ to be less frequent (1/36 = 3%) than epithelial MSI+ (6/37 = 16%) for D17S786 (P = 0.0512) (Table 1). With D17S796, MSI was more often found in the stroma (8/33 = 24%) than in the epithelium (4/36 = 11%), with significant difference (Table 1). No differences were found for D17S579 and TP53. Concerning the low MSI frequency for mixed tissue as compared with the epithelium or stroma alone, MSI+ DNA might disturb the positivity with MSI-PCR due to lowered sensitivity.

Immunohistochemistry and p53 and K-ras gene analysis

To analyse p53, hMLH1, and hMSH2 protein expression and for assessment of p53 and K-ras gene mutations, tissues were fixed routinely in 10% buffered formalin and embedded in paraffin. Serial sections (3μm thick) were applied for haematoxylin and eosin staining, immunohistochemistry, and mutation analyses. Immunohistochemical staining was performed with monoclonal anti-p53 (DO7, × 300 dilution, Novocastra Lab., Newcastle, UK), monoclonal anti-hMLH1 (Clone; G168-15, × 200 dilution, BD PharMingen, San Diego, CA, USA) and monoclonal anti-hMSH2 (Clone; G219-1129, × 500 dilution, BD PharMingen) antibodies, using the standard labelled streptavidin–biotin–peroxidase complex method described in our previous report (Yamashita et al, 2001). The amounts of positive cells were expressed as the percentage of the total number of epithelial cells and assigned to one of three categories for p53: ++, > 50%; +, 0.5–50%; −, < 0.5% (Figure 3I–K), for hMLH1 and hMSH2: ++, > 50%; +, 10–50%; −, < 10% (Figure 3A–F). We also examined the expression of hMLH1 and hMSH2 proteins in stromal cells, with classification into: ++, strong staining; +, focal staining; −, negative staining. Definite nuclear staining of adjacent non-neoplastic epithelial and stroma cells or lymphocytes served as internal positive controls.

K, and DNA was extracted with the standard phenol–chloroform–ethanol precipitation method.
Histopathological features of epithelial and stromal MSI status

Histopathological and molecular features of the sporadic colorectal cancers are detailed in Table 2. MSI frequencies differed between epithelium and stroma in well-differentiated (P-value = 0.0393) and poorly differentiated (P-value = 0.0510) (Figure 2A) adenocarcinomas. Stromal MSI+ was more often detected in well-differentiated adenocarcinomas (7/13 = 54%) than in poorly differentiated cancers (1/10 = 10%) (P-value = 0.0286), whereas the frequency of epithelial MSI+ correlated with progression (Figure 2A). With analysis of Dukes’ stage, although there was a tendency for an inverse relation with stromal MSI+ (Figure 2B), it did not reach significance (P-value = 0.4807). In contrast, epithelial MSI+ showed significant variation with the Dukes’ stage (P-value = 0.0277) (Figure 2B).

A significant correlation was also detected between epithelial MSI+ and stromal MSI+ and Dukes’ stage (P-value = 0.0455). It is notable that in stage A lesions, stromal MSI+ (6/11 = 55%) was more frequent than epithelial MSI+ (0/10 = 0%) (P-value = 0.0057) (Figure 2B).

**Table 1** MSI frequencies for each microsatellite marker

| Marker | MSI+ samples/informative samples | MSI+ samples/informative samples |
|--------|----------------------------------|----------------------------------|
|        | Epithelium                      | Stroma                           | P-value | Mixed tissue |
| D17S796| 4/36 (11%)                      | 8/33 (24%)                       | 0.1506  | 1/38 (3%)    |
| TP53   | 6/35 (17%)                      | 6/35 (17%)                       | 0.9999  | 5/37 (14%)   |
| D17S786| 6/37 (16%)                      | 1/36 (3%)                        | 0.0512  | 3/40 (8%)    |
| D17S779| 4/36 (11%)                      | 3/37 (8%)                        | 0.6631  | 4/39 (10%)   |
| Total  | 13/38 (34%)                     | 16/39 (41%)                      | 0.5372  | 9/40 (23%)   |

Significance was determined by the χ² test (epithelium vs stroma). Total refers to informative cases that showed MSI+ for at least one marker.

**Table 2** MSI findings for the epithelium and stroma, with reference to tumour features

| Variable      | Total cases | MSI+ cases/informative cases | P-value | MSI+ cases/informative cases |
|---------------|-------------|------------------------------|---------|------------------------------|
| Location      |             | Epithelium                   | Stroma  | P-value | Mixed tissue |
| Right side    | 14          | 6/14 (43%)                   | 7/14 (50%) | 0.3549 | 4/14 (29%)    |
| Left side     | 26          | 7/24 (29%)                   | 9/25 (36%) | 0.1388 | 5/26 (19%)    |
| Differentiation|             |                              |         |         |              |
| Well          | 14          | 2/13 (15%)                   | 7/13 (54%) | 0.0393* | 2/14 (14%)    |
| Moderate      | 16          | 6/15 (40%)                   | 8/16 (50%) | 0.5761 | 5/16 (31%)    |
| Poor          | 10          | 5/10 (50%)                   | 1/10 (10%) | 0.0510** | 2/10 (20%)    |
| Dukes' stage  |             |                              |         |         |              |
| A             | 11          | 0/10 (0%)                    | 6/11 (55%) | 0.0057* | 1/11 (9%)     |
| B             | 12          | 5/12 (50%)                   | 5/12 (42%) | 0.6820 | 4/12 (33%)    |
| C             | 17          | 7/16 (44%)                   | 5/16 (31%) | 0.4652 | 4/17 (24%)    |
| p53 mutation  |             |                              |         |         |              |
| +             | 15          | 4/13 (31%)                   | 5/14 (36%) | 0.7854 | 4/15 (27%)    |
| −             | 25          | 9/25 (36%)                   | 11/25 (44%) | 0.5637 | 5/25 (20%)    |
| K-ras mutation|             |                              |         |         |              |
| +             | 12          | 5/11 (45%)                   | 6/11 (55%) | 0.6698 | 4/12 (33%)    |
| −             | 28          | 8/27 (30%)                   | 10/27 (37%) | 0.6307 | 5/28 (18%)    |
| p53 protein   |             |                              |         |         |              |
| −             | 7           | 1/7 (14%)                    | 3/7 (43%)  | 0.5594 | 1/7 (14%)     |
| +             | 10          | 3/10 (30%)                   | 7/10 (70%) | 0.0943** | 3/10 (30%)    |
| ++            | 23          | 9/21 (43%)                   | 6/22 (27%) | 0.5127 | 5/23 (22%)    |
| hMLH1 protein |             |                              |         |         |              |
| −             | 2/4 (50%)*  | 1/1 (100%)                   |         |         |              |
| +             | 0/6 (0%)    | 12/30 (40%)                  |         |         |              |
| ++            | 1/11 (27%)  | 3/7 (43%)                    |         |         |              |
| hMSH2 protein |             |                              |         |         |              |
| −             | 0/1 (0%)*   | 0/0 (0%)                     |         |         |              |
| +             | 3/9 (33%)   | 16/35 (46%)                  |         |         |              |
| ++            | 10/25 (40%) | 0/2 (50%)                    |         |         |              |

Significance was determined by the χ² test and Fisher’s exact test (epithelium vs stroma). *MSI+ cases’ refer to cases that showed MSI+ for at least one marker. **P < 0.05. ***P < 0.1. *n = The cases of hMLH1 or hMSH2 protein expression in epithelial cells. **n = The cases of hMLH1 or hMSH2 protein expression in stromal cells.
DISCUSSION

Regarding interactions between the epithelium and stroma, several hypotheses have been proposed to explain fibroblast-promoting effects on tumour growth. Most of the intercellular material, the extracellular matrix (ECM) molecules that are required for tumour growth and progression, is produced by stromal cells (Noel et al., 1998). It has been demonstrated that neoplastic breast stroma drives alteration in gene expression as compared with normal tissue (Leygue et al., 1998). In fact, it is generally believed that the epithelium is the neoplastic element in most tumours and that altered gene expression in stroma occurs as the secondary reaction. However, the recent finding of frequent genetic changes in mammary stromal tissue in breast cancer patients (Moinfar et al., 2000), and the demonstration that inflammation-associated stroma promotes conversion of colonic adenoma cells to adenocarcinoma cells in nude mice (Okada et al., 2000) suggest a more complex scenario.

Our present study showed that MSI in stromal and epithelial elements can occur independently in sporadic colorectal cancers, in line with the previous findings for breast carcinomas (Kurose et al., 2001). Further, while MSI in the epithelium tended to correlate with differentiation and the Dukes’ stage, the inverse was the case for MSI in stroma. These interesting results strongly suggest that there are alternative mechanisms involving stromal MSI operating in colorectal carcinogenesis and progression. According to Young et al., methylation of CpG island occurs both in the epithelium and stroma (Young et al., 2001). Stromal MSI presented in this study might be due to the methylation of mismatch repair enzymes in stromal cells, although the identification of the enzyme remains unclear. Previously, it was shown that high-level MSI (MSI-H) tumours are more likely to be right sided than their low-level MSI (MSI-L) or MSI stable counterparts (Michael-Robinson et al., 2001; Ward et al., 2001). In the present study, while MSI frequencies in both the epithelium and stroma were high in right-side (43, 50%) as compared to left-side lesions (29, 36%), the difference did not reach statistical significance. This might be due to relatively small numbers of examined cases or inclusion of both MSI-L and MSI-H results in our analysis. Additionally, we examined the expression of DNA mismatch repair enzymes, hMLH1 and hMSH2 protein, in epithelial and stromal cells, respectively, as the MSI-H phenotype has been suggested to be of importance for the DNA mismatch repair system in sporadic colorectal cancers (Dietmaier et al., 1997; Ward et al., 2001). In the present study, epithelial MSI + for two or more markers was found in three cases, two of which showed loss of hMLH1 protein expression. In stroma, MSI + for two or more markers was found in two cases, one of which showed loss of MLH1 in stromal cells (Figure 3G and H). Although we have not used the standard markers that were recommended for MSI analysis on the basis of a National Cancer Institute Workshop (Boland et al., 1998), the results of hMLH1 and hMSH2 protein expression provide support for the validity of our study of MSI in epithelial and stromal cells. NCI-recommended standard markers are two mononucleotide repeat markers and three dinucleotide markers, but all of the markers we used in this study are dinucleotide repeat markers ((CA)n, repeat). Therefore, it is difficult to compare MSI-H entity with our MSI + for two or more markers. Additionally, discordance of the loss of hMLH1 or hMSH2 and our MSI + tumours could occur in the present study.

Although no significant links were found between MSI and p53 or K-ras gene mutations, MSI in the epithelium, but not stroma, tended to correlate positively with p53 protein overexpression. Recently, Kapiteijn et al. (2001) reported that p53 gene mutation corresponds more often to p53 overexpression in left- than in right-sided tumours, suggesting that mechanisms of oncogenesis may differ between the two cases. This is in line with our results for a greater prevalence of p53 overexpression with p53 gene mutations in left-sided tumours (9/11, 81%), than in those found on the right side (1/4, 25%) (P = 0.0390, data not shown). Furthermore, we found a difference in the relation between p53 mutations and stromal MSI (stromal MSI + p53 mutation, 3/11 (27%) in the left-side; 3/4 (75%) in the right-side, P = 0.0952).
These results support the conclusion of Kapiteijn et al. (2001) and suggest a relation of p53 mutation to stromal MSI+.

From the available data, contrary to the general belief that abnormalities in stroma occur as reactions to epithelial tumour cells, we propose the hypothesis that an alternative pathway may exist, with stromal genetic instability influencing epithelial cells in carcinogenesis.

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