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# Mutational Analyses of Multiple Target Genes in Histologically Heterogeneous Gastric Cancer with Microsatellite Instability

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**It has been recognized that gastric cancer often shows histological heterogeneity in a single tumor. Although microsatellite instability (MSI) has been reported in gastric cancer, the significance of genomic instability in gastric cancers with histological heterogeneity within a single tumor has never been addressed. We investigated MSI at 8 microsatellite loci in 40 normal/tumor DNA pairs from 20 gastric cancers with histological heterogeneity. Six of 20 patients (10 DNAs of 40 tumor DNAs) had severe MSI in more than 3 loci. Four of the MSI-positive cases had frameshift mutations in the poly(A)$_{10}$ tract of the *TGFβRII* gene. This mutation was found only in the MSI-positive component in the 2 cases (cases 4 and 5) in which only 1 component exhibited MSI. The other 4 cases demonstrated homozygous or heteroclonal mutations (1 and 2 base deletions) in the poly(A)$_8$ tract of the *hMSH3* gene; no mutation was detected in the poly(C)$_8$ tract of the *hMSH6* gene in any of the MSI-positive cases. The profile of alterations in multiple targets was different between the 2 components in most of the cases (5/6). These findings suggest that mismatch repair deficiency in MSI-positive tumors causes multiple gene inactivations through frameshift mutations in short repetitive sequences in a heterogeneous way within a histologically heterogeneous tumor.**

Key words: Gastric cancer — Histological heterogeneity — Microsatellite instability — Frameshift mutation — Target gene

MSI is well known to exist in various human cancers including gastric cancers,[1–10] and has become a frequently used criterion of genetic instability in human cancers.[1, 11, 12] Previous studies have demonstrated that MSI can be attributed to defects in several human MMR genes including *hMSH2*, *hMLH1*, *hPMS1* and *hPMS2* in hereditary and sporadic colorectal cancers,[13–17] and it is thought to be a mutator phenotype caused by MMR failure. Recently, genetic instability of gastrointestinal cancers was identified in the poly(A)$_{10}$ tract within the coding region of *TGFβRII*.[18–20] Similarly, frameshift mutations in other repetitive mononucleotide tracts of the coding regions of cancer-related genes, such as *IGFIIR*,[21, 22] and *BAX*,[23] might constitute targets of MSI in tumors. Moreover, small repetitive sequences in mismatch repair genes *hMSH3* and *hMSH6* themselves were reported to be hotspots for frameshift mutations in MSI-positive tumors.[24] These studies indicated that mutations of these target genes may play an important role in the development of MMR-deficient tumors in humans.

It is well recognized that the histology of carcinoma is often heterogeneous. In particular, gastric cancer frequently shows variable morphology within an individual tumor. This histological variation may reflect different genetic alterations. However, it remains unclear whether there are any specific histological changes of gastric cancer related to MSI and to frameshift mutations of MSI-associated target genes.

To evaluate different MSI profiles among different histological subtypes within a single gastric carcinoma, we studied MSI at 8 microsatellite loci in 40 different tumor areas from 20 gastric cancers showing histological heterogeneity. For the carcinomas with MSI, we further investigated the frameshift mutations in the 3 target genes (*TGFβRII*, *hMSH3* and *hMSH6*) to clarify the alterations of these genes and their relationship to histological appearance.

## MATERIALS AND METHODS

**Tissue samples** Archival formalin-fixed, paraffin-embedded specimens of surgically resected gastric cancers with histological heterogeneity were obtained from 20 Japanese patients at Hamamatsu Medical University Hospital (Hamamatsu, Shizuoka) and Kosai General Hospital (Kosai, Shizuoka) between 1993 and 1995. The subjects

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Abbreviations: MSI, microsatellite instability; RER, replication error; MMR, mismatch repair; PCR, polymerase chain reaction; SSCP, single strand conformational polymorphism; TGFβRII, transforming growth factor-β type II receptor; IGFIIR, insulin-like growth factor-II receptor.

consisted of 8 males and 12 females, ranging from 42 to 89 years of age at the time of surgery (average: 68 years). All of the cancers were in advanced stages (invasion below the proper muscle layer), and the size of the tumors ranged from 2.1×2.0 cm to 18.5×15.0 cm. The histological subtypes of these gastric cancers were grouped into the following 4 categories according to the criteria of the Japanese Research Society for Gastric Cancer: well to moderately differentiated adenocarcinomas (tub), poorly differentiated adenocarcinomas (por), signet-ring cell carcinomas (sig) and mucinous carcinomas (muc).[25] As for differentiated structure, we made no strict distinction between well and moderately differentiated types, because the differences were often gradual and sometimes ambiguous among tumors. When a single tumor showed 2 or more histologically distinct portions classified in different categories, it was described as a tumor with "histological heterogeneity." To estimate semiquantitatively the area of each histological subtype, the cut surface of the largest tangential view was thoroughly examined under a microscope and the geographical distribution of histological subtypes were mapped and roughly quantitated. In our cases, each of the histological components occupied more than 30% of the whole section.

Two different histological tumor areas including the corresponding normal tissue were individually microdissected for each case. A total of 40 normal/tumor DNA pairs were examined in this study. To extract DNA from all the tumor tissues, portions showing distinct histopathological characteristics were carefully taken under the microscope and the portions left were stained with hematoxylin and eosin to confirm the accuracy of dissection. The genomic DNAs were extracted as previously described[26] and used in the subsequent PCR amplifications.

**Microsatellite instability assay** Eight microsatellite loci containing dinucleotide repeat sequences and representing different chromosomes were studied in each case. The loci (chromosomal localization) were D1S116 (1p31-p21), D2S136 (2p), D3S1067 (3p14.3-p21), D5S82 (5q15-q23), MSX2 (5q34), D10S197 (10p), D17S261 (17p12-p11.2) and TP53 (17p13.1). The genomic DNAs were amplified by PCR with [$^{32}$P]ATP end-labeled primers and electrophoresed in 6% polyacrylamide/7.6 *M* urea gels as previously reported.[9] After electrophoresis, the gels were transferred on 3MM paper (Whatman, England), dried on a gel dryer, and exposed to X-ray film (Kodak, XAR-5) at −70°C for 24 to 48 h.

**Mutational analyses** To investigate mutations in the presumed target genes for MMR in histologically heterogeneous gastric cancers showing MSI, we amplified DNA sequences spanning the poly(A)$_{10}$, poly(A)$_8$ and poly(C)$_8$ tracts in the coding regions of *TGFβRII* (codons 125–128), *hMSH3* (codons 381–383) and *hMSH6* (codons 1116–1118), respectively. The sequences of PCR primers were previously described.[19, 24] PCR conditions consisted of 35 cycles of denaturing at 94°C for 1 min, annealing at 58°C for 1 min, and polymerizing at 72°C for 1 min, followed by a final elongation at 72°C for 10 min. PCR products labeled with [α-$^{32}$P]dCTP were denatured at 96°C for 10 min, electrophoresed on 8% polyacrylamide gels with or without 10% glycerol, and visualized by autoradiography. Extra bands were excised from the dried gels and reamplified by PCR using the corresponding set of primers and then cloned into the pBluescript vector. The insert was sequenced using the T7 Sequenase Version 2.0 Kit (Amersham/USB). Mutations were confirmed by sequencing 3 or more independent plasmid clones.

## RESULTS

**Histopathological features** We selected 20 gastric cancers with histological heterogeneity for microsatellite

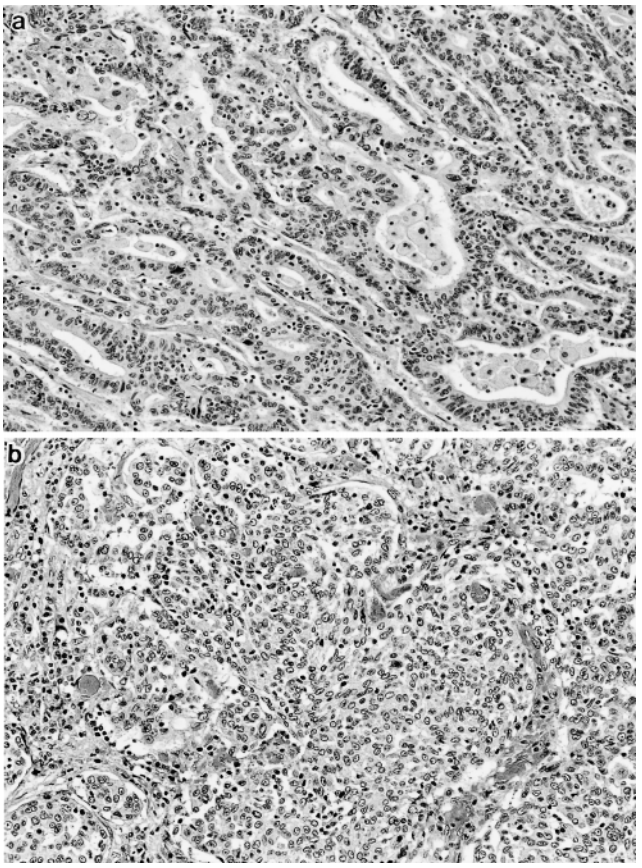


Fig. 1. Histopathological features of case 1 (hematoxylin and eosin, ×20). Gastric carcinoma with histological heterogeneity consists of a well differentiated adenocarcinoma area (a) and a poorly differentiated adenocarcinoma area (b).

Table I. MSI Frequency in Gastric Cancer with Histological Heterogeneity

| MSI | Histological subtype | | | Case group | | | |
|---|---|---|---|---|---|---|---|
| | Tub (*n*=17) | Por (*n*=18) | Sig (*n*=5) | Tub-Por (*n*=15) | Tub-Sig (*n*=2) | Por-Sig (*n*=3) | |
| 1 locus | 0 | 0 | 1 | 0 | 0 | 1 | |
| 2 loci | 0 | 0 | 0 | 0 | 0 | 0 | 7/20 |
| ≥3 loci | 5 | 4 | 1 | 4 | 2 | 0 | (35%) |
| | | | 11/40 (28%) | | | | |

Table II. MSI and MSI-associated Frameshift Mutations in Gastric Cancers with Histological Heterogeneity

| Case number | Sex | Age | Histo. | MSI instable markers /tested markers | MSI-positive loci | Mutational analysis | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | *hMSH3* ($A_8$) | *hMSH6* ($C_8$) | *TGFβRII* ($A_{10}$) |
| 1 | F | 82 | Por | 4/7 | D2S136, D3S1067, MSX2, D17S261 | A7, A6 | — | A9 |
| | | | W-M | 6/7 | D2S136, D3S1067, D5S82, MSX2, D10S197, D17S261 | A7, A6 | — | ND |
| 2 | M | 72 | Por | 3/7 | D3S1067, MSX2, TP53 | — | — | — |
| | | | W-M | 3/7 | D1S116, D3S1067, TP53 | — | — | — |
| 4 | F | 67 | Por | 8/8 | D1S116, D2S136, D3S1067, D5S82, MSX2 D10S197, D17S261, TP53 | A7, A6 | — | A9 |
| | | | W-M | 0/8 | | A7, A6 | — | — |
| 5 | F | 88 | Sig | 0/8 | | — | — | — |
| | | | W-M | 5/8 | D1S116, D2S136, D5S82, MSX2, TP53 | — | — | A9 |
| 10 | M | 77 | Por | 0/7 | | — | — | — |
| | | | Sig | 1/8 | D2S136 | — | — | — |
| 14 | M | 55 | Por | 4/8 | D1S116, D2S136, D5S82, MSX2 | A7 | — | — |
| | | | W-M | 5/8 | D1S116, D2S136, D3S1067, D5S82, MSX2 | A7, A6 | — | — |
| 20 | F | 61 | Sig | 5/5 | D1S116, D2S136, D3S1067, D5S82, D17S261 | A7, A6 | — | A9 |
| | | | W-M | 5/5 | D1S116, D2S136, D3S1067, D5S82, D17S261 | A7 | — | A9 |

Por, poorly differentiated adenocarcinoma; W-M, well to moderately differentiated adenocarcinoma; Sig, signet-ring cell carcinoma; ND, not determined.

analysis. In all 20 cases, two morphological features were recognized in a single tumor (Fig. 1). The macroscopic findings ruled out the possibility of multiple independent cancers. Fifteen cases had poorly differentiated portions (por) and well or moderately differentiated portions (tub) in a single tumor; 3 poorly differentiated and signet-ring cell subtypes (sig); and 2 cases that consisted of sig and tub components. Four portions showed scirrhous features among 18 "por" areas. These combinations and the MSI frequency are shown in Table I.

**Microsatellite analysis** A total of 40 normal/tumor DNA pairs obtained from 20 gastric cancers with histological heterogeneity were examined using 8 microsatellite markers. In 7 of 20 cases (35%), at least 1 of the DNAs taken from either portion of the tumor showed MSI at 1 or more loci. Four cases contained por and tub, 2 tub and sig, and another por and sig (Table I). The loci exhibiting MSI in each case are also shown in Table II.

The frequency of MSI with at least 1 microsatellite locus was 35% (7 out of 20 cases), or 28% (11 DNAs among 40 tumor DNAs). Six of the 7 MSI-positive cases showed severe MSI at 3 or more loci, and in the remaining case only a single locus was detected (case 10). The MSI prevalence in each component was not relevant to the histological subtypes; that is, 5 out of 17 tub components vs. 5 out of 23 por or sig components had MSI at more than 3 microsatellite loci (Table I). No MSI was detected in any of the scirrhous portions.

Four cases showed MSI in both components of the tumors while the other 3 cases showed MSI in only 1 of the components (Table II). MSI was found in all 8 microsatellite loci investigated in the poorly differentiated area (por) of case 4, but not at any loci in the well differentiated area (tub) of the same case (Fig. 2). In case 5, in contrast, MSI was detected at 5 loci in the well differentiated area (tub), but the signet-ring cell carcinoma area (sig) showed no change at any loci (Fig. 2). In case 10, only 1 component had MSI at one locus, but this "low frequency MSI" was not counted as a true mutator phenotype in this report.

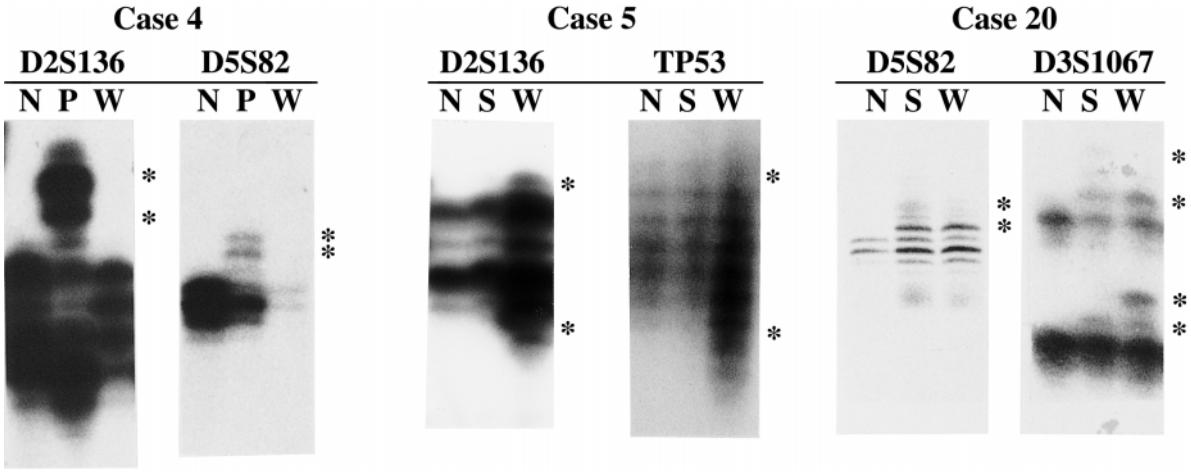


Fig. 2. Representative cases of the MSI$^+$ phenotype in a gastric cancer with histological heterogeneity. In case 4, abnormal patterns indicating RER were seen only in the DNA from the poorly differentiated area, whereas the well differentiated area showed no such changes. In case 5, only the well differentiated area was found to have MSI. No alterations were detected in the signet-ring cell carcinoma area. Both the signet-ring cell carcinoma and poorly differentiated areas exhibited MSI in case 20. N, corresponding normal tissue; P, poorly differentiated area; W, well differentiated area; S, signet-ring cell carcinoma area. A band representing an altered CA repeat is designated by an asterisk.

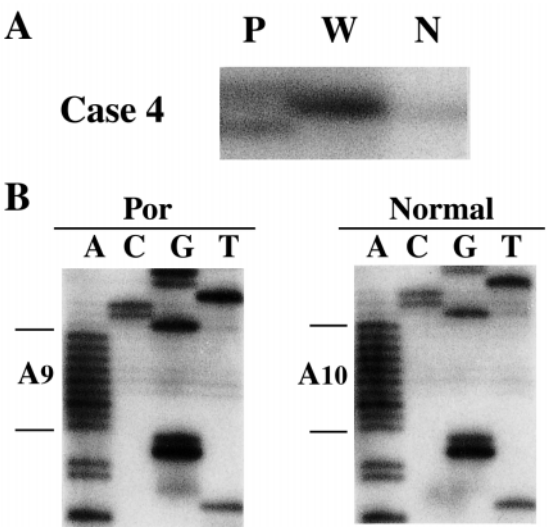


Fig. 3. Mutational analysis in the poly(A)$_{10}$ tract of the *TGFβRII* gene in case 4. A: An extra band is detected only in the poorly differentiated area by PCR-SSCP analysis. No alteration in the well differentiated area. B: A 1-base deletion in the poorly differentiated adenocarcinoma DNA of case 4 is confirmed by DNA sequencing. N, corresponding normal tissue; P, poorly differentiated area; W, well differentiated area.

**Mutational analyses of target genes** Seven MSI-positive cases (or 14 normal/tumor DNA pairs) were also screened for the presence of frameshift mutations in short repetitive sequences of 3 target genes. The results are listed in Table II and Figs. 3 and 4. Four (57.1%) of 7 MSI-positive cases exhibited frameshift mutations in the poly(A)$_{10}$ tract of *TGFβRII* and poly(A)$_8$ tract of *hMSH3*, respectively. Thus, 5 cases exhibited frameshift mutations of *TGFβRII* and/or *hMSH3*, including 3 cases (case 1, 4 and 20) with mutations in both genes. Analysis of 10 DNAs with MSI at more than 3 loci (severe mutator phenotype) showed that 8 DNAs (80%) had mutations in either *hMSH3* or *TGFβRII* (Table II). Mutations were also found in *hMSH3* in DNA which had no MSI in the component investigated (case 4). No mutation in the *hMSH6* gene was detected in any of our cases. In case 2 with the severe mutator phenotype, no alterations in any of these target genes were identified. No target gene alterations were detected in the low-frequency MSI case (case 10).

In Figs. 3 and 4, abnormal bands based on PCR-SSCP analysis in the poly(A)$_{10}$ tract of *TGFβRII* and the poly(A)$_8$ tract of *hMSH3* are shown. DNA sequence analysis confirmed 1 nucleotide deletion in the poly(A)$_{10}$ tract of the *TGFβRII* from 5 cancer areas of 4 MSI-positive cases. Interestingly, in cases 4 and 5, the frameshift mutations were only found in the tumor areas with MSI-positive phenotype and not in the MSI-negative areas of the cancer (Fig. 3). In case 1, the poly(A)$_{10}$ tract of *TGFβRII* gene in the well differentiated area of the tumor could not be characterized because the PCR reaction was unsuccessful. Homozygous or heteroclonal mutations (1 and 2 nucleotide deletions) in *hMSH3* were observed in 4 cases, with 6 components (Table II, Fig. 4). In 2 of these cases,

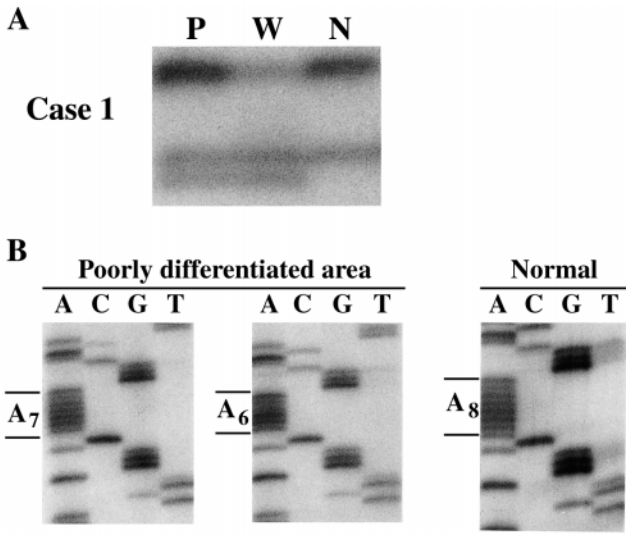


Fig. 4. Representative result in the poly(A)$_8$ tract of the *hMSH3* gene in case 1. A: Extra bands were found in both cancer areas by PCR-SSCP. B: 1- and 2-base deletions in either poorly or well differentiated area DNAs were determined by DNA sequencing. N, corresponding normal tissue; P, poorly differentiated area; W, well differentiated area.

the mutations were found in only 1 component. Furthermore, homozygous or heteroclonal mutations were also detected in the well differentiated area of case 4, which was MSI-negative (Table II).

## DISCUSSION

Previous studies have shown that MSI may be related to histological subtype and stage of gastric carcinoma. However, nothing was reported on its prevalence in different subtypes coexisting in a single tumor.[6, 27, 28] Our results show that the frequency of MSI in two separate sites from the same tumor is different in most MSI-positive cases, although the incidence of MSI (35%,7/20) was similar to those found in previous studies of MSI in gastric cancer (16–39%). Therefore, MSI represents heteroclonal progression at the molecular level in gastric cancer with histological heterogeneity. In the two MSI-positive cases 4 and 5, only one component showed severe mutator phenotype, depending on the histological subtype. Therefore, the mutator phenotype itself may be influenced by the clonal heterogeneity.

It is known that *TGFβRII* is a tumor suppressor gene whose mutational inactivation is important in human carcinogenesis.[29, 30] Mutations of this gene within the poly(A)$_{10}$ tract in MSI-positive colorectal cancer cell lines are thought to contribute to tumor development by allowing the cells to escape the TGF-β growth-inhibitory effects[18]. *TGFβRII* mutations have been reported to occur frequently, in 17 of 24 (71%) MSI-positive HNPCC tumors,[31] in 100 of 111 (90%) MSI-positive colorectal cancers,[20] in 5 of 7 (71%) and in 2 of 4 (50%) MSI-positive gastric cancers, but in only 4 of 24 (17%) MSI-positive endometrial cancers.[19, 32] In our study, 67% (4/6) of the MSI-positive gastric cancer cases with histological heterogeneity were found to carry one base deletion in the poly(A)$_{10}$ tract of *TGFβRII*. In particular, in cases 4 and 5, this frameshift mutation was only detected in the one histological subtype with MSI, not in another histological subtype without MSI in the same patient, suggesting that *TGFβRII* frameshift mutations are under strong positive pressure in MSI-positive gastric tumorigenesis. These results further support the hypothesis that the small repeat sequences in the *TGFβRII* gene make it a favorable target for the MSI-associated mutator mechanism.[18, 20]

Recently, two MMR genes, *hMSH3* and *hMSH6* have been identified as components of the *hMSH2*-dependent MMR pathway and are thought to contribute to the stabilization of microsatellite sequences.[33] Mononucleotide tracts of *hMSH3* and *hMSH6* are known to be targeted in mutator phenotype gastrointestinal cancers.[24] In our MSI-positive cases, two cases showed two kinds of deletions in only one component of a tumor, suggesting a stepwise alteration of *hMSH3* alleles or heteroclonality in the tumor. The accumulation of *hMSH3* gene mutations also seems to be involved in the progression of gastric cancers with histological heterogeneity. Furthermore, the *hMSH3* gene mutation was found in the well differentiated area of case 4, where no alteration in the 8 microsatellite loci was detected. Since this was contrary to our expectations, that is, *MSH3* mutation had always been found in the MSI-positive cases, we did the same procedure in this case twice more to exclude sampling error and obtained consistent results. We have no explanation for the status of tumor DNA in this portion. The molecular basis for the progression of each component from poorly to well differentiated adenocarcinoma may be different in case 4.

We did not find any mutations in the poly(C)$_8$ tract of the *hMSH6* gene in our MSI-positive cases. Shinmura *et al*. also did not observe any mutations in *hMSH6* in familial gastric cancers with the MSI-positive phenotype.[34] A very recent report by Edelmann *et al.*, showed that *MSH6* mutation causes cancer suspectibility in mice and suggested that mutations in this gene may be involved in hereditary cancer predisposition syndromes as well as in some sporadic tumors that do not show MSI.[35] According to our results and the previous data, we presume that MSI-positive cancers show few or no mutations of the *MSH6* gene.

In the present study, 3 cases (cases 1, 4 and 20) containing double mutations of the *TGFβRII* and *hMSH3* genes were identified. Our results and previous data both support the notion that defective MMR may cause MSI at

multiple loci in sporadic cancers, and frameshift mutations in multiple targets can actually be a consequence of MSI. Generally in our studies, histological heterogeneity, MSI, and target alterations were consistent with each other. It is interesting that histological "instability" seems to reflect molecular instability. However, we should be aware that the heterogeneity in alterations of target genes in different portions of a single tumor described here is not necessarily related to histological appearance in light microscopy. Heterogeneity in target gene alterations in multiple portions of tumors has been observed regardless of identifiable morphological differences.[36] Each histological area we examined was small, and we could not extract DNA from multiple smaller areas within the tumor. When a 1:1 mixture of two inconsistently mutated components (e.g. case 5, *MSH3*) was detected (data not shown), we did not perform a more detailed assessment.

In terms of the chronological relationship of generation of heteroclonalities and acquisition of MSI, our observations can be interpreted in two ways. Defects in MMR may cause multiple gene defects in various ways, thus generating heteroclonalities. Alternatively, heterogeneous tumor cells may exhibit various molecular profiles within the tumor. For elucidation of this issue, heterogeneous tumors at an early stage should be studied.

There may be additional target genes other than the genes we investigated here, and the alterations in those genes caused by MSI may be more biologically significant for gastric cancer progression. However, Simms *et al*. surveyed repeat sequences in 17 regions and found no alterations,[37] suggesting that preferential and multiple inactivations of *hMSH3* and *TGFβRII* represent a significant involvement of these genes in gastric carcinogenesis and progression. It is necessary to elucidate the actual inactivation pathways of these genes in gastric carcinogenesis.

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