Stage-dependent Evaluation of Microsatellite Instability in Gastric Carcinoma with Familial Clustering[1]

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Abstract

Familial clustering of gastric cancer is probably caused by multifactorial processes, both environmental and genetic. In this report, the incidence of microsatellite instability (MSI) in 31 cases of gastric cancer in Japanese (33 lesions) with familial clustering (two or more gastric cancers within second-degree relatives) was compared to MSI in Japanese cases without a family history of any cancer in an age (±10 years)-, stage-, and histological subtype-matched case-control study. Although the difference noted was not significant, we noted a strong trend for MSI at any of up to seven loci of CA repeats to occur more frequently in the patients with a family history of gastric cancer than in the control patients in early cancer (intramucosal and submucosal), whereas the prevalence of MSI was similar in both groups in more advanced cases, in which the tumor invaded beyond the proper muscle layer of the gastric wall. Because the contribution of a family history of gastric cancer to MSI apparently differs in early and advanced gastric cancer, interpretation of MSI in familial gastric cancer cases published previously requires reevaluation in terms of stage and proper controls. An acquisition of CA repeat alterations in the early stage rather than in the late stage of gastric carcinogenesis may have in common etiological factors, at least in some cases, with the familial clustering of gastric cancer.

Introduction

Familial clustering of gastric cancer has been well documented (1), and various reasons, including shared environmental carcinogens such as *Helicobacter pylori* and food mutagens, have been proposed, as well as genetic factors such as blood type and cancer family syndrome, but analysis of such cases has been limited to date. In contrast, studies of familial colorectal cancer have revealed many markers that provide evidence of a genetic predisposition. MSI[3] detected by PCR spanning CA repeats was identified originally in tumors of HNPCC, which is associated with defects in mismatch repair genes (2–4). MSI has been found in many human cancers, including gastric cancer, without regard to familial clustering. Although MSI in gastric cancers with familial clustering suggests a "familial" gastric cancer situation analogous to that of HNPCC (5–7), it is difficult to interpret this as evidence for genetic predisposition, because of the frequent occurrence of MSI in gastric cancers without any evidence of family history (8–11). Clustering of gastric cancer has been observed in familial cancers such as Li-Fraumeni syndrome caused by germ-line p53 mutations (12), and defects other than mismatch repair gene defects may be the cause of familial clustering of gastric cancer. Therefore, the significance of MSI in gastric cancer in terms of its association with familial history requires further evaluation.

Because previous reports indicate that histopathological categories and stages may influence the detectability of MSI in gastric tumors (11, 13, 14), we evaluated the contribution of family history to detectability of MSI in gastric cancer by matching histopathological subtypes and stages of gastric cancer invasion in the stomach wall.

Subjects and Methods

Pathological Examination. Resected stomachs were examined independently by two or more pathologists, and the findings were reported in standardized form by one of the authors (I. K.). The subject of pathological examination included the entire area covered by the cancer, the deepest layer of the gastric wall into which the tumor had invaded, and all regional lymph nodes.

All lesions were resected stomach cancers in which the deepest layer of the gastric wall involved was identified by studying multiple sections covering the entire mucosal tumor area.

All sections were classified according to the General Rules of the Japanese Research Society for Gastric Cancer (15). This classification system was converted to the Lauren classification system (16), in which each category, diffuse or intestinal, corresponded to one or more categories in the Japanese classification system. The papillary structure of gastric cancer was used to determine the category "papillary carcinoma of the stomach" (pap), and well and moderately differentiated adenocarcinomas were subtyped "tub1" and "tub2" based on the

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[3] The abbreviations used are: MSI, microsatellite instability; HNPCC, hereditary nonpolyposis colorectal cancer.

Table 1 MSI of gastric cancer with and without family history: early gastric cancer

No. of pairs	With family history of gastric cancer		Matched parameters				Age and sex	Without family history of any cancer	
	MSI[a]	LOH[b]	Subtype[c]	Subtype[d]	Depth[e]	Stage[f]		MSI[a]	LOH[b]
1	0/5	−	Intestinal	pap	m/sm	t_1	72/69F	2/7	−
2	1/7	−	Intestinal	pap	m	t_1	69/66F	0/7	−
3	4/7	+	Intestinal	pap	m	t_1	62F/M	0/7	−
4	0/3	−	Intestinal	pap	m	t_1	60M	0/4	−
5	0/3	−	Intestinal	pap	m	t_1	60M	0/7	−
6	0/3	−	Intestinal	tub1	m	t_1	64F	0/7	−
7	2/6	−	Intestinal	tub1	m	t_1	69M	1/6	−
8	0/3	+	Intestinal	tub1	m	t_1	64M	0/7	−
9	2/6	−	Intestinal	tub1	m	t_1	78/77F	0/5	−
10	2/6	−	Intestinal	tub1	m	t_1	58M	0/7	−
11	0/6	−	Intestinal	tub2	m	t_1	56M	0/1	−
12	0/7	−	Diffuse	sig	m	t_1	62M	0/7	−
13	0/7	−	Diffuse	sig	m	t_1	44M	0/6	−
14	0/5	−	Diffuse	por	m	t_1	55M	0/7	−
15	1/5	−	Diffuse	por	sm	t_1	75F	0/1	−
16	0/6	−	Diffuse	por	sm	t_1	56M	0/3	−
17	0/7	−	Diffuse	por	sm	t_1	77M	0/3	−
18	2/7	−	Diffuse	sig	sm	t_1	70M	0/7	−

[a] Number of loci showing MSI/number of loci for which data were available.
[b] Presence (+) or absence (−) of the loss of heterozygosity at any locus studied.
[c] Subtypes according to the Lauren classification.
[d] Subtypes according to the Japanese classification; see "Subjects and Methods."
[e] The deepest layer of gastric wall involved by tumor, according to the Japanese classification; see "Subjects and Methods."
[f] The tnf classification (pathologically verified TNM) according to the Japanese system.

formation of well differentiated and moderately differentiated glands. Poorly differentiated carcinoma and signet ring cell carcinoma are categorized separately in the Japanese classification system. In this report, we did not separately classify "por1" and "por2," *i.e.*, solid and scirrhous subtype of poorly differentiated adenocarcinoma, respectively. The first three categories by the Japanese classification system (pap, tub1, and tub2) correspond to the intestinal subtype in the Lauren classification system, and the Japanese categories "por" and "sig" correspond to Lauren's "diffuse" subtype.

The depth of tumors was recorded according to the rules in the Japanese classification system, which state that "depth" should be the deepest layer of gastric wall involved by the tumor. Identification of the deepest layer affected was achieved by examining multiple sections of the whole tumor and recorded as invasion to the lamina muscularis mucosae (m), submucosal loose connective tissue (sm), proper muscle layer (mp), subserosa (ss), invasion through the serosa with the tumor exposed to the abdominal coelom (se), or direct infiltration to adjacent organs through adherent serosa (si).

The "t" parameters of the staging of gastric cancer were also classified based on the Japanese system.

Gastric Cancer with Familial Clustering. Thirty-one cases of gastric cancer with familial clustering were retrieved from the pathological and clinical records for 1985–1995, at Hamamatsu University Hospital, Fujieda Municipal Hospital, and Jichi Medical School. The retrieval of the cases was based on family records in which at least two members within second-degree relatives had gastric cancer in addition to the proband. The patients consisted of 22 males and 9 females, and their ages ranged from 44 to 77 years old. Two of the 31 patients had double primary cancers pathologically identified in the resected stomach, and thus 33 gastric cancer tissues were investigated. The 33 lesions consisted of 5 cases of papillary adenocarcinoma (pap), 14 tubular adenocarcinomas [8 well differentiated (tub1) and 6 moderately differentiated tubular carcinomas (tub2)], 10 poorly differentiated (por) adenocarcinomas, and 4 signet ring cell carcinomas (sig). As for depth, the deepest involvement of the tumor was to the mucosal layer (m), including muscularis mucosae, in 14 lesions, and 4 were to the submucosal layer (sm); the other cases were in advanced stage with the tumor in the proper muscle (mp) of the gastric wall in 2 cases, tumor invasion rupturing through the muscularis propria to subserosa (ss) in 3 cases, and tumor invasion observed on the serosal surface (se) or in adjacent structures (si) in the remaining cases. This classification system is described in detail elsewhere (15).

Matched Control Group. We selected control cases with no family history of any cancer from the pathological and clinical records of Hamamatsu University School of Medicine and Fujieda Municipal Hospital (1985–1995). The areas from which the cases and controls were collected were within 200 km of Tokyo. No endemic gastric cancer is known in any of the areas from which the cases and controls were collected. All of the subjects were Japanese and neither consanguinity nor particular exposure to chemical carcinogens was recorded in any of the cases or the controls. No record of occupational history of exposure to high levels of carcinogens was found for the subjects of this study. We matched age (±10 years old), depth (early *versus* advanced: m, sm, mp, ss, se, and si), and histopathological subtypes (pap, tub1, tub2, por, and sig) in the control group, as explained above. When matching histological subtypes, we first collected cases so that the histological subtypes according to the Japanese classification system matched, and then we included cases that belonged to the same type according to the Lauren classification system, whenever a strict match based on the Japanese classification system was impossible. In collecting depth-matched controls, the deepest layers involved were matched with equivalent layers of involvement, or, where this was not possible, with the closest more advanced case. The family records of the controls as well as the patients

Table 2 MSI of gastric cancer with and without family history: advanced cancer

No. of pairs	With family history of gastric cancer		Matched parameters				Age and sex	Without family history of any cancer	
	MSI[a]	LOH[b]	Subtype[c]	Subtype[d]	Depth[e]	Stage[f]		MSI[a]	LOH[b]
19	1/7	−	Intestinal	tub1	mp/ss	t_2	60F/66M	1/7	−
20	0/7	−	Intestinal	tub1	mp/se	t_2/t_3	66/76M	1/6	−
21	0/6	+	Intestinal	tub1	se/ss	t_2	72M	0/7	−
22	0/7	−	Intestinal	tub2	ss	t_2	64M/F	2/7	−
23	2/4	+	Intestinal	tub2	ss	t_2	57F/M	0/3	−
24	3/7	−	Intestinal	tub2	ss/se	t_2/t_3	71F/68M	2/6	−
25	1/5	−	Intestinal	tub2	si	t_4	54M	5/7	−
26	0/6	+	Intestinal	tub2	si	t_4	78M/76F	3/6	−
27	3/5	−	Diffuse	por	se	t_3	56F/66M	0/6	−
28	0/7	−	Diffuse	por	se	t_3	77M	1/6	−
29	0/7	−	Diffuse	por	se	t_3	76/69M	4/7	−
30	1/7	−	Diffuse	por	se	t_3	77/69M	2/7	−
31	0/5	−	Diffuse	por	se	t_3	77M/71F	1/6	−
32	3/7	−	Diffuse	por/sig	se	t_3	60/58M	0/7	−
33	0/6	+	Diffuse	sig	se	t_3	76M/73F	0/7	−

[a] Number of loci showing MSI/number of loci for which data were available.
[b] Presence (+) or absence (−) of the loss of heterozygosity at any locus studied.
[c] Subtypes according to the Lauren classification.
[d] Subtypes according to the Japanese classification; see "Subjects and Methods."
[e] The deepest layer of gastric wall involved by tumor, according to the Japanese classification; see "Subjects and Methods."
[f] The tnf classification according to the Japanese system.

were written by nurses and doctors. All of the information from clinical, pathological, and family history records for both patients and controls was reviewed and standardized.

MSI and Germ-Line p53 Mutations. DNA was extracted from a tumorous portion dissected from paraffin-embedded tissue, as reported previously (9). Primer sets covered loci *D1S116, D2S136, D3S1067, D6S87, D10S197, D17S261,* and *TP53* (17, 18). ^{32}P-labeled ATP was used for end labeling of primers with a Megalabel kit (Takara, Kyoto, Japan), PCR products were electrophoresed on a 6% polyacrylamide gel, and Kodak XAR film was then exposed to the gel. Identification of compressed and expanded bands was performed blindly by two independent investigators. Because this assay could produce artifactual bands when we used DNA from paraffin-embedded tissues, we repeated the experiments more than twice under several different conditions, including magnesium chloride concentrations in the PCR buffer and annealing temperatures. Ambiguous results were excluded from the comparative analysis.

The procedures for detection of germ-line p53 mutations have been reported elsewhere (12, 19). Briefly, PCR-single-strand conformational polymorphism was performed to detect p53 mutations covering exons 5–9 in DNA from normal portions of the tissue blocks in each case.

Statistical Analysis. The prevalence of MSI at one or more loci was compared in the cases and controls, and prevalence in each case-control subgroup, such as depth of cancer invasion and histopathological subcategory, was also compared. χ^2 analysis and Fisher's exact test were performed, and the two groups were compared with 1 degree of freedom.

Results

The clinical and pathological profiles of all of the cases and the controls are listed in Tables 1 and 2. Changes in microsatellite markers (instability and loss of heterozygosity) in the cases and controls are also summarized in Tables 1 and 2. Examples of MSI at four loci are shown in Fig. 1. The numbers of available

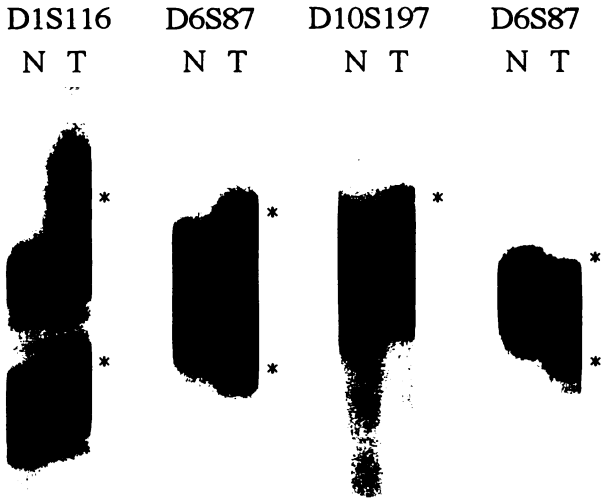

Fig. 1. MSI found in the tumorous DNA (*T*, tumor; *N*, normal) at four loci, *D1S116, D6S87, D10S197,* and *D6S87*. *, extra bands of CA repeats.

data were approximately the same in both groups. The difference in prevalence of MSI at one or more loci in the cases and controls was not statistically significant (14 of 33 *versus* 12 of 33; $P = 0.45$); however, separation into early stage (depth of m or sm, corresponding to t_1) and advanced stage (t_2, t_3, or t_4) revealed a trend for the prevalence of MSI in familial gastric cancer to be greater than in gastric cancer without a family history of any cancer (7 of 18 *versus* 2 of 18; $P = 0.054$; Table 3). This trend increased when we compared microsatellite alterations, including replication errors and loss of heterozygosity (9 of 18 *versus* 2 of 18; $P < 0.05$), and it was still present when cases in which two or more loci exhibiting microsatellite markers, *i.e.,* the cases of more likely mutator phenotype were

Table 3 Prevalence of MSI in case and control according to stage of disease

Group	MSI[a]	With family history of gastric cancer	*P*	Without family history of any cancer	Total
Early	−	11 of 18		16 of 18	27 of 36
	≥1	7 of 18	0.05[b]	2 of 18	9 of 36[c]
	≥2	5 of 18	0.07[b]	1 of 18	6 of 36[d]
Advanced	−	8 of 15		5 of 15	13 of 30
	≥1	7 of 15	0.27[b]	10 of 15	17 of 30[c]
	≥2	4 of 15	0.43[b]	6 of 15	10 of 30[d]
Total	−	19 of 33		21 of 33	
	≥1	14 of 33	0.45[b]	12 of 33	
	≥2	9 of 33	0.57[b]	7 of 33	

[a] Number of loci with MSI. −, no MSI at any loci.
[b] *P*s are the result of χ^2 analysis (without Yate's correction). The values become greater when Fisher's exact test is applied.
[c] Frequency of MSI at one or more loci between early and advanced cancer is significantly different ($P = 0.009$).
[d] Frequency of MSI at two or more loci between early and advanced cancer is not statistically different ($P = 0.11$).

Table 4 Prevalence of MSI in case and control according to histological subtype

Group	MSI[a]	With family history of gastric cancer	*P*[b]	Without family history of any cancer	Total
Intestinal	−	10 of 19		11 of 19	21 of 38
	≥1	9 of 19	0.74[b]	8 of 19	17 of 38[c]
	≥2	6 of 19	0.72[b]	5 of 19	11 of 38[d]
Diffuse	−	8 of 14		9 of 14	17 of 28
	≥1	6 of 14	0.90[b]	4 of 13	10 of 28[c]
	(or LOH ≥1)	(8 of 14)	(0.25)[b]	(4 of 14)	
	≥2	3 of 14	1.00[b]	2 of 14	5 of 28[d]

[a] Number of loci with MSI. −, no MSI at any loci. LOH, loss of heterozygosity.
[b] *P*s are the result of χ^2 analysis (without Yate's correction). The values become greater when Fisher's exact test is applied.
[c] Frequency of MSI at one or more loci between intestinal and diffuse type is not statistically different ($P = 0.46$).
[d] Frequency of MSI at two or more loci between intestinal and diffuse type is not statistically different ($P = 0.29$).

compared (5 of 18 *versus* 1 of 18). In contrast, prevalence was the same in the study cases and controls in advanced gastric cancer (8 of 13 *versus* 10 of 13; $P = 0.6$). The overall prevalence of MSI was greater in advanced-stage cancer (17 of 30) than early-stage cancer (9 of 36; $P = 0.009$), which is consistent with previous reports (10, 20). Furthermore, when divided according to histological subtype, no differences between the two groups in prevalence of MSI were seen (Table 4). In addition, overall prevalence of MSI did not differ in the intestinal and diffuse histological subtypes (17 of 38 *versus* 10 of 28; $P = 0.46$; Table 4).

No p53 mutations in exons 5–9 were detected in the DNA from normal portions of any of the cases or controls when single-strand conformational polymorphism screening was performed.

Discussion

Because MSI in colorectal tumors in HNPCC patients reflects mismatch repair gene defects in the germ line of these patients, genomic instability detected as MSI in tumors is believed to be associated with individual genetic predisposition to cancer (4, 21). However, not all MSI found in tumors represents germ-line mismatch repair gene defects, and somatic mutations of those genes have also been reported. Gastric cancer exhibits a variety of histological findings and is one of the common epithelial tumors in which MSI is observed frequently. MSI in gastric tumors has frequently been correlated with stage, location, prognosis, and erbB-2 expression (4, 10, 13, 14, 22–25). It is also associated with multiple tumors and familial history (8, 9, 26). Recently, Akiyama *et al.* (5) reported MSI in the gastric cancer tissue of six patients with a family history of gastric cancer, two of which were early-stage cancers. On the basis of their own data on the four families and data on three probands reported by others (27), they speculated that MSI is more frequent in gastric cancer with familial clustering and that the frequent MSI in the tumor may mean that its pathogenesis might be similar to that of HNPCC. However, none of these previous reports have examined MSI in sporadic gastric cancers, and little is known about the association of MSI in gastric tumors and a family history of gastric cancer (22). Our report is the largest study of MSI in gastric cancer with a family history and addresses the importance of stage-dependent comparison for the first time. We adopted a histological subclassification system that included more specific morphological characteristics than a simple dichotomy such as "diffuse" and "intestinal" in selecting controls. Furthermore, taking advantage of information on tumor depth, which is standardized by the Japanese classification system, we matched each case to the control on the basis of depth of invasion. We believe that this strictly matched evaluation is necessary to assess the association between familial clustering and the presence of MSI in gastric cancer. Actually, a trend for increased prevalence of MSI in familial cases had only been noted in early cancer, whereas our data clearly showed that the prevalence of MSI was similar in advanced gastric cancer, with or without familial clustering of gastric cancer. This MSI, more frequently found in advanced cancer, appears to be acquired during the progression of gastric cancer. It is more probable that the MSI in early-stage cancer is associated with familial clustering, but the numbers of cases and controls were small, and these results should be

interpreted with caution. In particular, cases exhibiting MSI at more than two loci, potentially mutator phenotype tumors, which are too small in number to allow a statistically significant conclusion in this data set, are the most important candidates for further investigation for genetic predisposition to gastric cancer. Possible targets such as the transforming growth factor β receptor type II gene (*TGFβRII*) or the *MSH3* gene must also be examined to confirm that the MSI in these familial gastric cancers represents the mutator phenotype of the tumor. Naturally, the germ-line alterations of mismatch repair genes in our cases are of interest, but because most of the patients in this study were older than 50 years old, the situation differs from that of HNPCCs, which have germ-line mutations of mismatch repair genes. Akiyama *et al.* (5) found a somatic hMSH2 mutation in tumor DNA but not in the germ-line DNA of the family members, suggesting a contribution of somatic changes to MSI in gastric cancer with familial clustering. Although we have not yet examined germ-line changes in our cases, the causal factors in familial gastric cancer, including environmental and genetic factors, may also possibly be associated with CA repeat alterations in early stages of carcinogenesis. Among the cases of gastric cancer with familial clustering, which are obviously heterogeneous entities, the cases that exhibited MSI from the early stage would be good candidates in which to pursue the genetic predisposition of gastric cancer. Early acquisition of MSI in HNPCC has been reported very recently (28). The mechanism of early acquisition of MSI in familial gastric cancer is intriguing from the standpoint of genetic defects in the mismatch repair system. Target molecule changes now under investigation will resolve this issue.

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