Loss of Heterozygosity on Chromosome 18q in Cohesive-type Gastric Cancer Is Associated with Tumor Progression and Poor Prognosis[1]

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ABSTRACT

Although loss of heterozygosity (LOH) on chromosome 18q is frequently found in gastric cancer, the clinical significance of this abnormality has not been well documented. We examined LOH on chromosome 18q22–23 in DNA extracted from the tissues of gastric cancer patients using the PCR-based dinucleotide repeat assay with two microsatellite markers, *D18S61* and *D18S58*. We investigated LOH in 100 samples of DNA extracted from formalin-fixed, paraffin-embedded tissues of cohesive-type gastric cancer patients operated on between 1984 and 1993. Thirty-two of 83 informative cases (39%) showed LOH on chromosome 18q22–23 at one or two loci. The LOH correlated significantly with serosal invasion of the tumor ($P = 0.004$) and hematogenous recurrence ($P = 0.035$). In 60 cases who were cured, the 5-year survival rate in patients with LOH (54%) was lower than that in patients without LOH (81%; $P = 0.019$). These results suggest that 18q22–23 LOH in cohesive gastric cancer is associated with tumor progression and a patient's poor prognosis.

INTRODUCTION

Recent studies have helped to increase our understanding of the genetic alterations in the alleles of oncogenes (1) and tumor suppressor genes (2) involved in the carcinogenesis or progression of cancers in various organs. Gastric cancer is one of the most common cancers in the world, and the prognosis of patients with tumor invading the serosa or neighboring structures is extremely poor. However, in contrast to colorectal cancer, oncogenes and tumor suppressor genes in gastric cancer are poorly understood (3–6).

LOH[3] on the long arm of chromosome 18 is frequently found in tissues of colorectal cancer (3–8). The *DCC* gene was isolated from the commonly deleted region of chromosome 18q21 (9). This gene is thought to be one of the tumor suppressor genes and encodes a deduced protein with an immunoglobulin-like C2 domain and a fibronectin type III domain. The predicted amino acid sequence shows homology to the IgG superfamily of cell adhesion molecules, including the neural cell adhesion molecule (9). The expression of mRNA was extremely decreased in colorectal cancer tissues with the deletion of the *DCC* gene. Furthermore, LOH at the *DCC* locus was found in all metastatic liver tissues of colorectal cancer, although the incidence of 18q LOH in primary site was about 70% (8). Additionally, LOH on chromosome 18q or reduced expression of the *DCC* protein in colorectal cancer is reported to be a prognostic marker in patients with stage II or III colorectal cancer (7, 10). This evidence suggests that the loss of *DCC* protein may be associated with tumor invasion or metastasis. Moreover, *DPC4*, a candidate tumor suppressor gene, has recently been identified at 18q21.1 (11).

We previously reported that the long arm of chromosome 18, which includes the *DCC* locus, was also frequently deleted in gastric cancer (12). The implication of LOH on chromosome 18q in the development or progression of gastric cancer has not yet been elucidated. To investigate the relationship between LOH on chromosome 18q and the clinicopathological features of gastric cancer, we examined LOH on chromosome 18q22–23 with special reference to tumor histological type using the PCR-based dinucleotide repeat assay.

MATERIALS AND METHODS

Patients. Histopathological classification was based on the General Rules for the Gastric Cancer Study outlined by the Japanese Research Society for Gastric Cancer (13). In addition to these criteria, we subdivided gastric cancers into two types as described previously (12, 14–18). One was cohesive type (papillary adenocarcinoma, well and moderately differentiated tubular adenocarcinoma, and poorly differentiated adenocarcinoma with solid nests or focal tubular structures) and the other was scattered type (signet ring cell carcinoma and poorly differentiated adenocarcinoma growing in a scattered manner). First, fresh frozen tumor tissues and corresponding normal gastric mucosae were randomly collected from 16 patients with solitary gastric cancer who underwent gastrectomy at Oita Medical University between 1992 and 1993. Five cases were diagnosed

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[3] The abbreviations used are: LOH, loss of heterozygosity; DCC, deleted in colorectal cancer; DPC4, deleted in pancreatic carcinoma, locus 4.

as well to moderately differentiated tubular adenocarcinoma, six as poorly differentiated adenocarcinoma of solid type, three as poorly differentiated adenocarcinoma of diffuse type, and two as signet ring cell carcinoma. In the alternative classification, 11 cases were diagnosed as cohesive type and 5 as scattered type.

To investigate the correlation between LOH on chromosome 18q22–23 and the clinicopathological parameters of the cohesive type of gastric cancer, we also examined formalin-fixed, paraffin-embedded tumor tissues and corresponding normal gastric mucosae from patients who underwent gastrectomy for solitary gastric cancer at Department of Surgery I, Oita Medical University, between January 1984 and December 1993. All cases underwent surgery during this period, and the cases that had a cancer cell-rich portion were selected. Twenty-five cases were diagnosed as papillary adenocarcinoma, 46 as well to moderately differentiated tubular adenocarcinoma, and 29 as poorly differentiated adenocarcinoma. The total number of cases was 100. Curative operation was performed in 76 patients and palliative operation in 24. The reasons of palliative operation were hepatic metastasis in 10 cases, peritoneal dissemination in 2, both hepatic metastasis and peritoneal dissemination in 3, and residual primary tumor with nonresectable para-aortic lymph node metastasis in 9. Clinicopathological evaluations were made according to the General Rules for the Gastric Cancer Study (13).

DNA Extraction and the PCR-based Dinucleotide Repeat Assay. Because gastric cancer tissue often has numerous nonneoplastic cells, we used a microscopic dissection method as described previously (12). Briefly, to ascertain the area where the cancer cells were relatively dominant, 5-μm sections of fresh frozen materials immersed in OCT compound (Miles Scientific) or formalin-fixed, paraffin-embedded materials were stained with H&E. Carcinomas that contained 40% more cancer cells than nonneoplastic cells were judged to be cancer cell rich, and only these cases were selected in this study. The cancer cell-rich portion was then cut with a scalpel from successive 50-μm sections. These sections, including the predominant cancer cells, were subjected to DNA extraction. DNAs were extracted from primary gastric tumor tissues and corresponding normal gastric mucosa as described previously (12). The following two microsatellite markers on chromosome 18q22–23 were synthesized as described by Jen *et al.* (7): 5′-GCTCCCG-GCTGGTTTT-3′ as the forward primer and 5′-GCAG-GAAATCGCAGGAACTT-3′ as the reverse primer for *D18S58*, and 5′-ATTTCTAAGAGGACTCCCAAACT-3′ as the forward primer and 5′-ATATTTTGAAACTCAGGAG-CAT-3′ as the reverse primer for *D18S61*. Each reverse primer was end labeled with [γ-^{32}P]ATP, and PCR was carried out for 30 cycles, each consisting of amplification for 30 s at 95°C, 1 min at 50°C, and 1 min at 70°C. The amplified PCR products were diluted 10-fold with 0.1% SDS, 20 mM EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol and electrophoresed on a 6% neutral polyacrylamide gel at 15 W for 30 min. The gel was dried on the filter paper using a gel drier and then subjected to autoradiography. The estimated size of both PCR products was less than 180 bp each. If two defined bands smaller than 180 bp in noncancerous tissues were detected, that case was judged to be informative. A sample was judged to be LOH when the intensity of one allele in cancerous tissue was less than 40% of the other allele compared with the ratio of the intensity of the two bands in the corresponding noncancerous tissue measured by a BAS 1000 bio-imaging analyzer (Fuji Photo Film Co., Tokyo, Japan). When LOH was found in at least one marker, the case was judged to be LOH positive.

Statistical Analysis. The statistical significance of the differences were analyzed by the χ^2 and Fishers' exact tests. Survival curves were drawn by the Kaplan-Meier method and tested by the log-rank test.

RESULTS

Among 16 randomly selected cases of DNAs extracted from fresh frozen samples of gastric cancer, 10 cases (63%) were informative at the *D18S61* locus, and of these, 5 (50%) showed LOH. In addition, 11 cases (69%) were informative at the *D18S58* locus, and of these, 4 (36%) showed LOH. With the combined results of the two loci, LOH was specifically found in 5 of 10 informative cases of the cohesive type (50%) but was not found in 2 informative cases of the scattered type (0%). From this results and our previous investigation, there is a possibility that 18q LOH may occur selectively in gastric cancer of cohesive type, so we investigated an additional 100 cases of cohesive type of gastric cancer.

PCR products of some tumors [1 of the 16 fresh frozen samples and 5 of the 100 (5%) formalin-fixed, paraffin-embedded samples] showed a ladder band in addition to the standard band, and these were considered to be cases of replication error (19–21). The replication error cases were excluded from this LOH analysis, because they may have a different genetic mechanism from that of the LOH cases.

Fifty eight cases (58%) were informative at the *D18S61* locus, and 19 of 58 (33%) showed LOH. Seventy cases (70%) were informative at the *D18S58* locus, and 24 of 70 (34%) showed LOH (Fig. 1). With the combined results of the two loci, 83 cases (83%) were informative at least at one locus, and 32 of 83 (39%) had LOH on chromosome 18q22–23 (Table 1). Two cases showed LOH at the *D18S58* locus but not at the *D18S61* locus.

The relationship between LOH on chromosome 18q22–23 and the clinicopathological parameters are shown in Table 2. LOH was significantly correlated with serosal invasion of the tumor ($P = 0.004$). However, there was no significant difference between LOH-positive and LOH-negative cases in tumor size, lymphatic invasion, vascular invasion, lymph node metastasis, or postoperative chemotherapy. Of 83 informative cases, 60 underwent curative surgery. LOH was also significantly correlated with serosal invasion of the tumor in cases who were cured ($P = 0.031$; Table 3). Among these, three died of postoperative complication and were excluded from the follow-up analysis. As for recurrence, liver metastasis occurred in 10 (18%), peritoneal dissemination in 4 (7%), lymph node recurrence in 4 (7%), and local recurrence in 1 (2%). Liver metastasis was significantly higher in patients with LOH (33%) than in those without LOH (10%; $P = 0.035$; Table 4). In the 57 cases who were cured, the five-year survival rate of LOH-positive group (54%) was significantly lower than that of the LOH-negative group (81%; $P = 0.019$; Fig. 2).

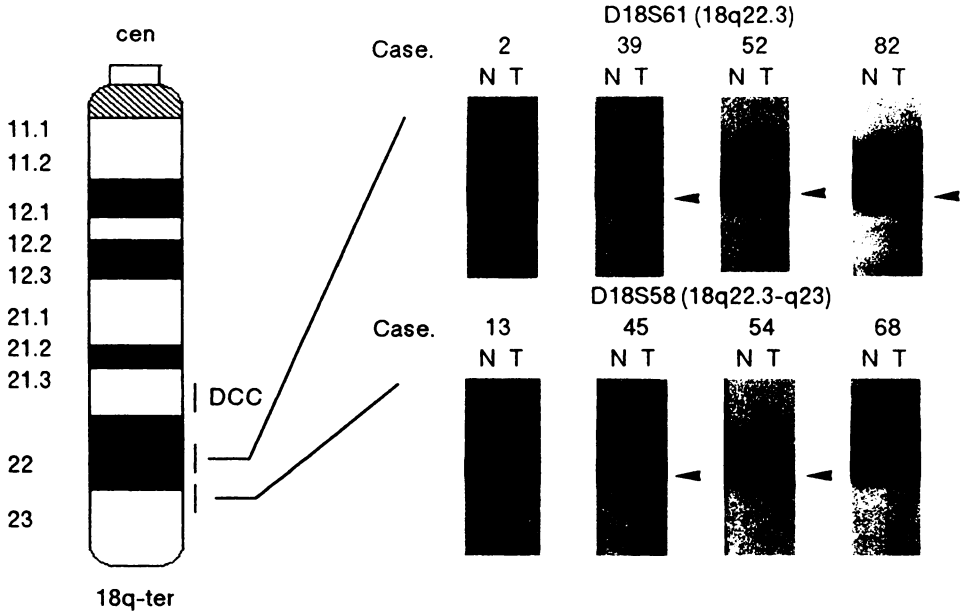

Fig. 1 Examples of LOH on chromosome 18q22–23 at the two markers of cognate in primary gastric tumor tissue and corresponding normal gastric mucosa. Case numbers are shown at the *top* of each lane. *N*, nomal DNA; *T*, tumor DNA. The tumors in panels 39, 45, 52, 54, and 82 showed LOH (*arrowheads*), whereas those in panels 2, 13, and 68 did not.

Table 1 Chromosome 18q LOH in cohesive-type gastric cancer

Locus	Localization	No. of cases tested	No. of Informative cases	No. of LOH-positive cases (%)
D18S61	18q22.3	100	58	19 (33)
D18S58	18q22.3–23	100	70	24 (34)
Combined		100	83	32 (39)

Table 2 Clinicopathological characteristics with regard to chromosome 18q status

	18q LOH-negative (n = 51)	18q LOH-positive (n = 32)	P
Sex ratio (M:F)	2.6:1	2.6:1	NS[a]
Mean age (yr)	67.4 ± 10.2	69.9 ± 10.6	NS
Mean tumor size (cm)	6.4 ± 2.7	7.4 ± 2.7	NS
Serosal invasion			
Absent	41 (80)[b]	16 (50)	
Present	10 (20)	16 (50)	0.004
Lymphatic invasion			
Absent	6 (12)	4 (13)	
Present	45 (88)	28 (87)	NS
Vascular invasion			
Absent	21 (41)	17 (53)	
Present	30 (59)	15 (47)	NS
Lymph node metastasis			
Absent	14 (27)	5 (16)	
Present	37 (73)	27 (84)	NS
Operation			
Curative	40 (78)	20 (62)	
Noncurative	11 (22)	12 (38)	NS
Postoperative chemotherapy			
Absent	18 (35)	7 (22)	
Present	33 (65)	25 (78)	NS
Median follow-up time (months)	38.8	27.4	NS

[a] NS, not significant.
[b] Numbers in parentheses are percentages.

DISCUSSION

A high incidence of LOH on chromosome 18q22–23 occurred selectively in gastric cancers of the cohesive type but not in those of the scattered type. Our previous study also revealed histopathological specificity concerning genetic alterations (12, 14–18). Histologically, the two main types of gastric carcinoma, cohesive and scattered, display a different spectra of genetic alterations. Point mutations in the c-Ki-*ras* (22) and *p53* genes (14, 15, 23) and amplification of c-*erb*B-2 gene (16, 24, 25) are associated with the cohesive type. On the other hand, K-*sam* amplification (26, 27) and abnormalities of E-*cadherin* and catenins (17, 28) are associated with the scattered type. These two types of cancer are considered to develop through independent genetic pathways, and the difference of genetic abnormalities is directly reflected in the different histological features.

We also previously reported that LOH on chromosome 18q was frequently detected in gastric cancers of the cohesive type using RFLP analysis, and a putative common region showing LOH was 18q21.3-qter, which includes the *DCC* locus (12). Gastric cancers were examined for LOH on chromosome 18q by another several researchers (29, 30); however, a significantly high incidence of LOH was not demonstrated. Numerous nonneoplastic cells are often intermingled with the cancer stroma, and tissues with predominantly cancer cells are difficult to collect, even in the cohesive type. Therefore, the selection of patients and collection of cancer cells from the gastric cancers with consideration of histological type are very important in studying the molecular biology of gastric cancer. Jen *et al.* (7) recommended the use of two dinucleotide repeat markers, *D18S58* and *D18S61*, because these markers from chromosome 18q22–23 were sufficient to determine the status of its chromosome. We confirmed a high informative rate on chromosome 18q22–23 using these two markers.

Table 3 Clinicopathological characteristics with regard to chromosome 18q status in cases who were cured

	18q LOH-negative (n = 40)	18q LOH-positive (n = 20)	P
Sex ratio (M:F)	2.3:1	2.8:1	NS[a]
Mean age (yr)	68.3 ± 9.5	69.9 ± 12.5	NS
Mean tumor size (cm)	6.2 ± 2.5	7.0 ± 2.9	NS
Serosal invasion			
Absent	34 (85)[b]	12 (60)	
Present	6 (15)	8 (40)	0.031
Lymphatic invasion			
Absent	6 (15)	3 (15)	
Present	34 (85)	17 (85)	NS
Vascular invasion			
Absent	21 (55)	11 (55)	
Present	19 (45)	9 (45)	NS
Lymph node metastasis			
Absent	13 (32)	5 (25)	
Present	27 (68)	15 (75)	NS
Postoperative chemotherapy			
Absent	18 (45)	5 (25)	
Present	22 (55)	15 (75)	NS
Median follow up time (months)	48.8	37.1	NS

[a] NS, not significant.
[b] Numbers in parentheses are percentages.

Table 4 Relationship between chromosome 18q LOH and postoperative recurrence

Recurrence	18q LOH-negative (n = 39)	18q LOH-positive (n = 18)	P
Liver metastasis			
Absent	35 (90)[a]	12 (67)	0.035
Present	4 (10)	6 (33)	
Peritoneal dissemination			
Absent	38 (97)	15 (83)	NS[b]
Present	1 (3)	3 (17)	
Local recurrence			
Absent	39 (100)	17 (94)	NS
Present	0	1 (6)	
Lymph node recurrence			
Absent	38 (97)	15 (83)	NS
Present	1 (3)	3 (17)	

[a] Numbers in parentheses are percentages.
[b] NS, not significant.

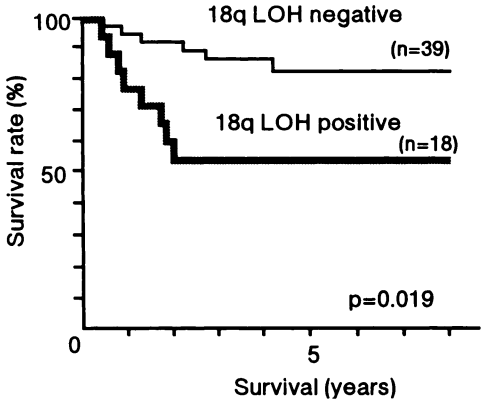

Fig. 2 Survival curves for 57 patients who underwent radical gastrectomy with or without LOH on chromosome 18q22–23. There was a significant difference between the two curves by log-rank test (P = 0.019).

Certain specific genetic alterations have been studied as potential prognostic markers in gastric cancer. The c-Ki-*ras* point mutation has an important role in the development from adenoma to carcinomas in the stomach, but it has no prognostic importance (22). We reported that overexpression of c-*erb*B-2 protein was found selectively in the cohesive type of gastric cancer and this correlated to the clinical prognosis (16). The *p53* tumor suppressor gene, which is one of the most commonly affected genes in various human cancers, is also frequently inactivated in gastric cancer (14, 15). Our data from an examination of the *p53* mutation and immunohistochemical analysis suggest that it is useless for determining patient's prognosis (14, 15). In gastric cancer of the cohesive type, a significant association was found between LOH on chromosome 18q22–23 and the serosal tumor invasion, and in addition, the survival rate for patients with LOH on chromosome 18q22–23 who underwent curative surgery was significantly poorer than those of patients without LOH. Moreover, hematogenous recurrence was found significantly more frequently in the group of patients with LOH on chromosome 18q22–23 than in those without LOH. Therefore, LOH on chromosome 18q22–23 may be associated with the grade of malignancy, tumor progression, and high metastatic potential through the vessel.

In colorectal cancer, Jen *et al.* (7) reported that LOH on chromosome 18q was an important prognostic factor in stage II disease, and O'Connell *et al.* (31) reported from analysis of disease-free survival after surgical resection in colorectal carcinoma patients that a poorer prognosis was associated with LOH on chromosome 18q. In the studies of Vogelstein and coworkers (5, 7), distant metastasis in colorectal cancer was significantly associated with LOH on chromosome 18q. In addition, Ookawa *et al.* (8) reported that 18q LOH was found in almost all of the liver metastases from colorectal carcinoma. The association of LOH on chromosome 18q22–23 with patient's prognosis, serosal tumor invasion, and metastasis in gastric cancer suggests the possibility that the abnormalities of the gene present on chromosome 18q22–23 or nearby may lead to a decrease in the ability of cell to cell contacts, thereby contributing to tumor growth, invasion, and metastasis.

Although the specific gene(s) on chromosome 18q22–23 in gastric and colorectal cancer has not yet been identified, the *DCC* and *DPC4* genes are candidates. Using RFLP analysis with several *DCC* probes, we noted allele loss at the *DCC* locus in 61% of cohesive type gastric cancers (12). Barletta *et al.* (29) also reported *DCC* allele loss in 4 of 4 gastric carcinomas, whereas Kataoka *et al.* (32) reported that *DCC* mRNA was decreased in 40% of gastric cancers. Recently, *DPC4*, a candidate tumor suppressor gene, has been identified at 18q21.1 (11). This gene is inactivated in nearly one-half of pancreatic carcinoma cases. However, Powell *et al.* (33) reported that only one case of apparent biallelic inactivation of *DPC4* was found in the study of 35 gastric carcinoma cases, and Nishizuka *et. al.* (34) reported that no *DPC4* mutations was found in 30 primary

gastric carcinoma cases and 5 gastric carcinoma cell line. Therefore, there is a possibility that an additional gene(s) on chromosome 18q may be the gene responsible for the progression of gastric cancer, and further genetic and biological testing is necessary to resolve this question. In the near future, tests for the status of chromosome 18q may be combined with other genetic and biochemical assays to improve the prognostic evaluation of patients with gastric cancer.

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