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DNA Sequence Copy Number Aberrations Associated with Histological Subtypes and DNA Ploidy in Gastric Carcinoma

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We have analyzed DNA sequence copy number aberrations (DSCNAs) and DNA ploidy by using comparative genomic hybridization and laser scanning cytometer in gastric carcinomas (GCs) to elucidate the genomic aberrations in relation to clinicopathological parameters. Thirty-two out of 33 cases showed one or more DSCNAs with a mean number of 11.7 per tumor. High-level gains were detected at 2p, 3q, 6p, 7p, 7q, 8q, 12p, 13q, 19q, and 20q. Frequency of gross genomic abnormalities and chromosome regions that have genomic aberrations were similar in both intestinal- and diffuse-type GCs, except aberrations at 8p, 9p, 12q, and 20q. The overall number of DSCNAs was significantly greater in DNA aneuploid tumors than that in DNA diploid tumors. We detected genomic aberrations characterized by histological subtype, tumor location, and DNA ploidy status: gain of 20q and losses of 8p and 9p in intestinal-type GCs, gains of 8p and 12q in diffuse-type GCs, gain of 20q in the lower third GCs, and loss of 5q, 9p, 10q, 16q, and 18q in DNA aneuploid GCs. Furthermore, 5q loss is associated with DNA aneuploidy (*P*=0.0001) or the total number of losses (*P*=0.001), gain+losses (*P*=0.004), and high-level gains (*P*=0.001) in GCs. Among these loci, chromosome 8p was unique. Gain of 8p was more common in diffuse-type GC, whereas loss of 8p was more frequently detected in intestinal-type GC. In conclusion, we describe chromosomal regions of 5q, 8p, and 20q, which are of interest for further investigation of GCs.

Key words: Comparative genomic hybridization — Data base — Gastric cancer — Genomic aberration — Laser scanning cytometry

Despite recent advances in early diagnosis and treatment of gastric carcinomas (GCs), GC still continues to be a major health problem worldwide. Much evidence indicates that the development of GC is associated with the accumulation of multiple genetic alterations through a process of multi-step carcinogenesis and progression. These genetic alterations include activation of proto-oncogenes and inactivation of tumor suppressor genes. During the last two decades, about 70 oncogenes and a dozen tumor suppressor genes have been found by cytogenetic and molecular biologic methods. Several studies have revealed that genetic changes of these oncogenes and tumor suppressor genes are frequent in the development of primary GC.[1,2)]

GC is divided into two major histological types: the intestinal type and diffuse type.[3)] Carcinomas of intestinal and diffuse types are characterized, at least partially, by different genetic alterations. Some of these alterations, including p53 mutation or allelic loss of 17p13 and *APC* gene mutation or loss of 5q21, are more frequent in intestinal-type GC than in diffuse-type GC. On the other hand, *E-cadherin* gene mutation and overexpression of c-met are peculiar to diffuse-type GC.[2,4–6)] However, the relationship of the difference of genetic alterations to histologic subtypes is largely unknown.

Comparative genomic hybridization (CGH) is a newly developed, powerful method for molecular and cytogenetic analysis of solid tumors.[7)] It enables the screening of the entire genome for chromosomal aberrations, DNA sequence copy number aberrations (DSCNAs). We have analyzed various tumors including intestinal-type GC by CGH.[8–10)] Within 2 years of the first report of CGH analysis of GC,[11)] recurrent DSCNAs of some chromosomal regions were revealed in GC[12–15)]; gains of 8q and 20q and losses of 5q and 18q. A few DSCNAs have been associated with histological subtypes,[11,16)] and tumor location.[15)] Recently, we have found that 5q loss is characteristic of DNA aneuploidy in intestinal-type GC.[17)] However, little is known about the cytogenetic aberrations in relation to clinicopathological parameters. In the present study, we have analyzed the genomic aberrations of GC using the CGH method to elucidate their clinicopathologic significance.

MATERIALS AND METHODS

Tumor samples Thirty-three fresh tumor tissues from surgically resected adenocarcinomas of the stomach were obtained from the Department of Pathology, Hanyang Uni-

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versity (Seoul, Korea). Samples were immediately frozen in liquid nitrogen and stored in −80°C. According to Lauren's classification, 17 tumors were diffuse type and 16 tumors were intestinal type. The specimens of the intestinal-type tumors were used in our previous report.[17] The tumors were located in the upper third of the stomach (2 cases), the middle third of the stomach (11 cases) or the lower third of the stomach (20 cases). All tumors were advanced cancer. The clinicopathological data are summarized in Table I. To confirm that samples were rich in cancer cells, we performed morphological analysis following Giemsa staining using one slide before DNA extraction. The DNA extraction method was described elsewhere.[17]

CGH and digital image analysis CGH was performed according to a method based on that reported by Nishizaki *et al.*[18] Briefly, DNA extracts from tumor cells and lymphocytes were labeled with SpectrumGreen-dUTP and SpectrumRed-dUTP (VYSIS Inc., Downers Grove, IL), respectively, by nick translation. Each labeled DNA sample (200 ng) and Cot-1 DNA (10 μg, Gibco Life Technologies, Gaithersburg, MD) were cohybridized onto normal denatured metaphase chromosomes for 72 h at 37°C. The specimens were mounted in antifade solution (DAPI-II, VYSIS). Images were captured with an epifluorescence microscope (BX 50, Olympus Co., Tokyo) equipped with a plan apochromatic objective ("UplanApo" ×100, Olympus) and a CCD camera (SenSys 1400, Photometrics Ltd., Tucson, AZ). Digital image analysis was done with a CGH system (QUIPS XL, VYSIS). At least five representative images were analyzed, and the results were combined to produce an average fluorescence ratio profile for each chromosome. Increases (gains) and decreases (losses) in DNA sequence copy number were defined by green-red ratios of >1.2 and <0.8, respectively. High-level copy number increases (amplifications) were defined by a green-red ratio of >1.4.[19]

Nuclear DNA measurement by laser scanning cytometry (LSC) LSC[20] was carried out as described previously.[21] Briefly, touch preparations were made and fixed in 70% ethanol at room temperature. The cells were treated with 0.1% RNase (Sigma Chemical Co., St. Louis, MS), stained with 50 μg/ml propidium iodide (Sigma), and analyzed with an LSC (LSC 101, Olympus). When the peak on the DNA histogram differed from the DNA diploid peak by more than 10%, the tumor was considered to be a DNA aneuploid tumor. In LSC, a normal diploid peak or DNA aneuploid peak or both were determined by observing lymphocytes or cancer cells morphologically on the computer display of the instrument and/or under the microscope.

Statistical analyses Differences in the frequency of copy number changes at chromosomal loci in relation to histological subtype, location, and DNA ploidy status were analyzed using Fisher's exact test. For some special regions, copy number aberrations were divided into 4 ranks (loss, no change, gain, and high-level gain) and analyzed by Mann-Whitney's *U* test. Differences in the total number of DSCNAs (gains, losses, gains+losses, and high-level gains) were analyzed using Mann-Whitney's *U* test.

RESULTS

Overview of genetic changes in GC Thirty-two out of 33 cases showed one or more DSCNAs, with a mean number of 11.7 per tumor (gains, 7.2; losses, 4.5; Table II). The most common gain occurred at 8q (63.6%) with two minimal common regions (MCRs) at 8q12–22 and 8q22–ter. Other frequent gains were at 3q (39%; MCR, 3q26),

Table I. Clinical Data of 33 Gastric Cancer Patients

Case	Sex/age (years)	Stage	Site	Histology	Size (cm)
22	M/62	III	A	I	12.5×8.5
23	M/40	III	B	I	7.0×6.5
24	M/44	III	A	I	5.0×5.0
25	M/63	III	A	I	5.5×4.5
26	F/39	III	B	D	8.5×8.5
27	F/68	III	B	D	6.5×4.0
28	F/69	III	B	D	4.5×4.0
30	M/66	III	A	I	8.0×4.5
34	M/62	II	A	I	6.0×3.0
36	M/66	III	A	D	8.0×5.2
38	M/64	III	A	I	5.0×4.5
43	M/43	II	A	I	9.0×5.0
44	M/44	III	B	D	9.0×8.5
45	M/61	III	A	I	8.0×2.5
47	M/53	III	A	D	6.0×5.0
48	M/58	III	A	I	7.5×7.0
49	M/54	III	A	D	11.0×7.0
56	M/64	III	A	I	7.6×6.5
60	M/63	II	C	I	12.0×9.0
61	M/44	III	A	D	7.0×6.5
62	M/57	III	B	D	10.0×9.0
63	M/63	III	A	I	8.0×6.5
65	M/67	III	B	D	10.0×5.0
67	F/42	IV	B	D	6.5×6.5
68	M/72	III	A	D	4.3×3.5
69	F/57	III	A	D	15.0×12.0
70	M/65	IV	B	D	5.0×4.0
71	M/43	III	C	D	4.5×3.5
72	F/62	III	A	I	7.0×6.5
73	M/55	III	A	I	6.5×6.0
78	M/57	III	A	D	11.0×7.0
79	F/47	III	B	D	5.0×3.5
83	M/60	III	B	I	7.0×6.0

A, antrum (the lower third); B, body (the middle third); C, cardia (the upper third); D, diffuse type; I, intestinal type.

Table II. DNA Sequence Copy Number Aberrations

Case	Gain	Loss	Ampli.[a]	Total[b]	Ploidy[c]	Case	Gain	Loss	Ampli.	Total	Ploidy
22	8	3	1	11	D	60	13	15	6	28	A
23	3	1	2	4	D	61	2	1	0	3	A
24	8	13	2	21	A	62	18	9	0	27	A
25	5	2	0	7	A	63	5	9	1	14	A
26	4	0	0	4	D	65	15	5	0	20	D
27	6	8	0	14	A	67	3	0	0	3	D
28	1	0	0	1	D	68	13	2	0	15	A
30	9	3	1	12	D	69	8	5	0	13	D
34	12	10	0	22	A	70	6	1	0	7	D
36	20	6	0	26	A	71	0	1	0	1	D
38	12	11	3	23	A	72	3	1	0	4	D
43	2	4	0	6	D	73	2	3	0	5	D
44	20	6	0	26	D	78	8	3	0	11	D
45	3	5	0	8	A	79	0	0	0	0	D
47	12	8	3	20	A	83	5	1	0	6	A
48	1	0	0	1	D	Total	238	148	21	386	
49	11	11	2	22	A	Mean	7.2	4.5	0.6	11.7	
56	0	1	0	1	D	SD	5.8	4.2	1.3	9.1	

a) Amplification.
b) Gain+loss.
c) D, DNA diploidy; A, DNA aneuploidy.

20q (36%; MCR, 20q13.1), 13q (36%; MCR, 13q21), and 7p (27%; MCR, 7cen-p15). Frequent losses were observed at 17p (36%; MCR, 17p13), 19q (36%), 19p (33%), 16p (30%), and 5q (27%) (Fig. 1, a, b). Nine cases showed a total of 21 high-level gains (amplifications, which were detected three times at 6p12–21.2 and 7p15, twice at 13q32–34, 19q13.1, and 20q13, and once at 2p25, 3q26.1–26.3, 5p, 8q22, 8q12–ter, 12p11–12, and Xp21–qter.

Histological subtypes of GC

Intestinal type: CGH disclosed DSCNA(s) in all cases (average, 10.8/tumor; gain, 5.7/tumor; loss, 5.1/tumor; Table III). Gain of 20q13, 8q12–22, 8q22–ter was detected in more than half of the tumors (56%, respectively). Other frequent gains were at 7p (38%), and Xq25–26 (38%). Frequent losses were observed at 19q (44%), 19p (44%), 17p (38%), and 5q (38%) (Fig. 1a). Seven cases revealed 14 high-level gains, which were observed three times at 6p12–21.2, twice at 7p15 and 13q32–34, and once at 3q26.1–26.3, 5p, 8q12–ter, 12p11–12, 19q13.1, 20q13, and Xp21–qter.

Diffuse type: Sixteen of 17 cases in this subgroup had DNA copy number changes (average, 12.5/tumor; gain, 8.6/tumor; loss, 3.9/tumor; Table III). The most frequent gain was detected at 8q (65%). Other frequent gains were at 3q (47%; MCR, 3q24–25.3 and 3q26.1–26.3) and 13q (47%; MCR, 13q21–22). The most frequent loss was detected at 17p (35%). Other frequent losses were at 16p, 19q, and 4q (29%, respectively) (Fig. 1b). High-level gain was observed in 2 cases with 5 loci. Gains of 8p and 12q were more common in diffuse-type GC ($P<0.05$).

Differences of genetic alterations between intestinal- and diffuse-type GC: As shown in Table IV, significant differences of DNA copy number changes between intestinal- and diffuse-type GC were found at 8p, 9p, 12q, and 20q ($P<0.05$) by Fisher's exact test, and more than half of intestinal-type GC showed 20q gain, in particular. Furthermore, 8p was a remarkable region; gain of 8p frequently occurred in diffuse-type GC, whereas loss of 8p frequently occurred in intestinal-type GC. Significant differences of DNA copy number change between intestinal- and diffuse-type GC were found at 8p ($P<0.01$) by Mann-Whitney's *U* test.

DNA ploidy GC specimens examined were classified as 18 DNA diploid and 15 DNA aneuploid tumors. In total, genomic aberrations were more common in both intestinal- and diffuse-type tumors characterized with DNA aneuploidy than in tumors characterized with DNA diploidy ($P<0.05$, Table III). Loss of 5q was found exclusively in DNA aneuploid tumors ($P=0.0001$), and losses of 16q ($P=0.002$), 18q21.3–ter ($P=0.005$), and 9p21–24 and 10q21–22 ($P=0.033$, Table V), were also significant. Furthermore, the total number of DNA copy number aberrations or amplifications in GCs with 5q loss was greater than in GCs without 5q loss (Table III).

Tumor location Gain of 20q was more frequently detected in the lower third stomach tumors than in the middle third stomach tumors ($P=0.002$, Table VI).

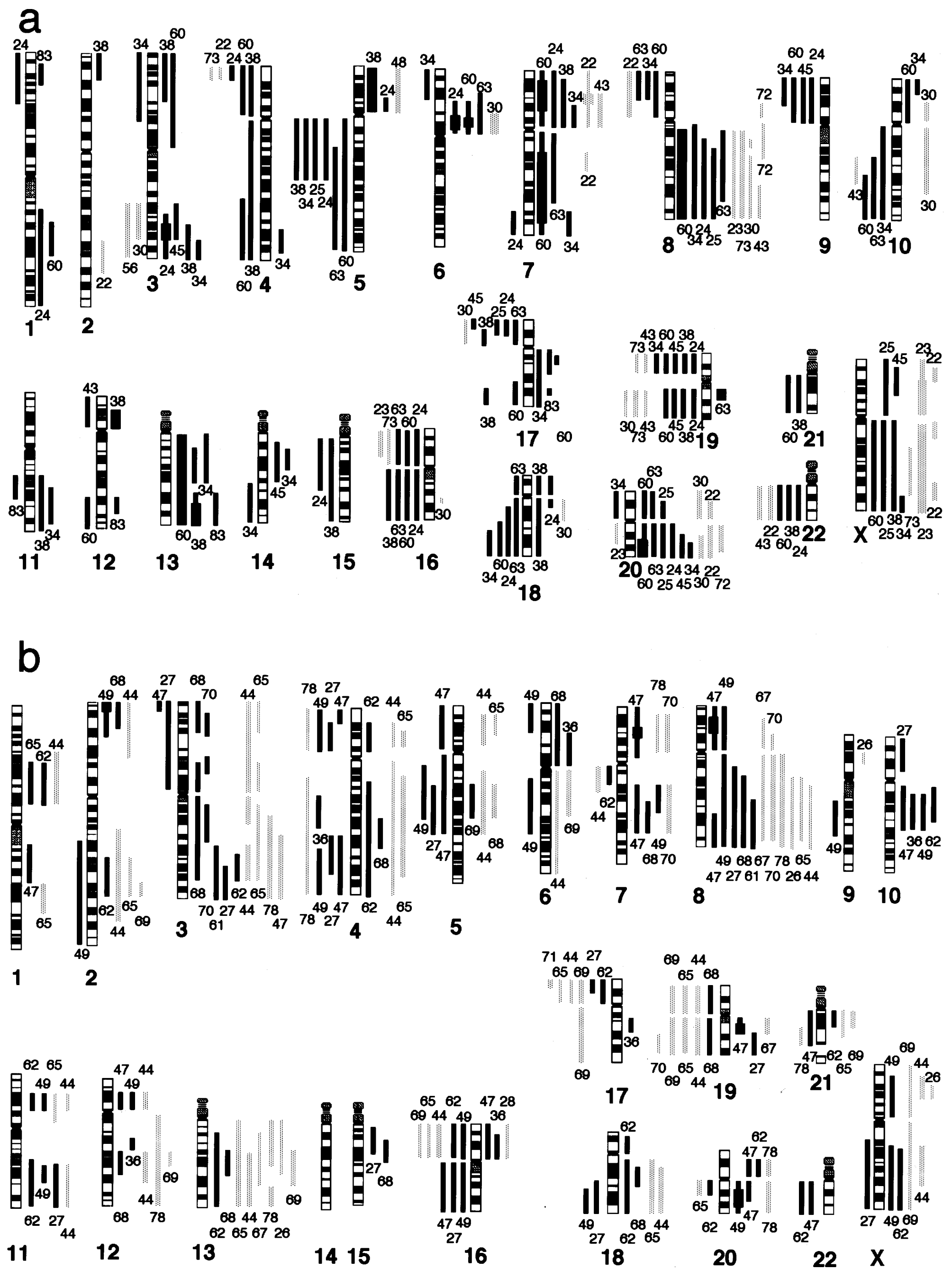

Fig. 1. DNA copy number changes in 33 cases of advanced gastric carcinomas (GC). Losses are shown on the left of the chromosome and gains are shown on the right of the chromosome. Amplifications are displayed in bold. Black lines and gray lines indicate DNA aneuploidy (black) and DNA diploidy (dots), respectively. Case numbers are marked. a: CGH results of 16 cases of intestinal-type GC. b: CGH results of 17 cases of diffuse-type GC.

DISCUSSION

We performed a total genomic analysis of gastric carcinoma by CGH in order to identify the differences of genomic aberrations in clinicopathological parameters. Frequent genomic aberrations detected in the present study include gains and amplifications in 8q, 3q, 20q, 13q, and 7p and losses in 17p, 19q, 19p, 16p, and 5q. Most of these findings are in agreement with previous CGH findings.[12–16] This study clearly confirms that these genetic alterations are frequently involved in the development of GC. On comparison of genomic aberrations in both histological types of GC, it was remarkable that the frequency of gross genomic abnormalities (gains and losses of copy number

Table III. Relationship between Number of DNA Copy Aberrations and Histology, Location and DNA Ploidy

	Gain	Loss	Ampli.[a]	Gain+loss
Histology				
Intestinal	5.7	5.1	1.0	10.8
Diffuse	8.6	3.9	0.3	12.5
P value	0.27	0.42	0.06	0.80
Location				
Middle third	7.3	2.8	0.2	10.2
Lower third	7.2	5.1	0.7	12.3
P value	0.82	0.06	0.13	0.38
Ploidy				
DA[b]	9.7	7.4	1.1	17.1
DD[c]	5.2	2.1	0.2	7.2
P value	0.1	<0.001*	0.08	0.02*
Histology+ploidy				
Intestinal				
DA	7.9	8.3	1.5	16.1
DD	3.5	2.0	0.5	5.5
P value	0.02*	<0.01*	0.26	<0.01*
Diffuse				
DA	11.7	6.4	0.9	18.1
DD	6.5	2.1	0	8.6
P value	0.11	0.01*	0.08	0.04*
5q loss				
+	9.3	9.6	1.9	19
−	6.4	2.5	0.2	10.5
P value	0.07	0.0001*	0.001*	0.004*

a) Amplification.
b) DNA aneuploidy.
c) DNA diploidy.
* Statistical significance.

Table IV. Differences in DNA Copy Number between Intestinal- and Diffuse-type GCs

Loci	Type		*P* value
	Intestinal (*n*=16)	Diffuse (*n*=17)	
+3q26	3	8	0.087
−5q14–21	6	3	0.217
+7p15.1–15.3	6	3	0.217
+8p11–12	0	4	0.044*
−8p21–23	4	0	0.044*
+8q12–22	9	8	0.429
+8q23–24	9	11	0.739
−9p21–24	4	0	0.044*
+12q21	0	4	0.044*
+13q21	3	8	0.087
+20p	5	3	0.343
+20q	9	3	0.033*

+, gain; −, loss.
* Statistical significance.

Table V. Differences in DNA Copy Number between DNA Aneuploidy and Diploid Types of GCs

Loci	DNA ploidy		*P* value
	DA[a] (*n*=15)	DD[b] (*n*=18)	
−5q14–21	9	0	0.0001*
+8q12–22	8	7	0.316
+8q23–24	10	10	0.386
−9p21–24	4	0	0.033*
−10q21–22	4	0	0.033*
−16q	7	0	0.002*
−18q21.3–ter	6	0	0.006*
+20q	9	3	0.068

a) DNA aneuploidy.
b) DNA diploidy.
* Statistical significance.

Table VI. Differences in DNA Copy Number between the Middle and Lower Third GCs

Loci	Location		*P* value
	Middle (*n*=11)	Lower (*n*=20)	
−5q14–21	1	7	0.092
+8q12–22	5	12	0.343
+8q23–24	7	12	0.577
+20q	0	11	0.002*

* Statistical significance.

changes) was similar in both diffuse- and intestinal-type GC. Furthermore, frequencies of chromosome regions with genomic aberrations were similar, except DNA copy number aberrations of 8p, 9p, 12q, and 20q.

The present study has demonstrated that gain at 20q occurs frequently in intestinal-type GC ($P<0.05$). Trisomy 20 has been detected frequently in intestinal-type GC by karyotyping analyses.[22] El-Rifai *et al.*[14] reported the 20q gain to be a consistent aberration in intestinal-type adenocarcinoma of the gastro-esophageal region. Although the biological significance is not clear, 20q gain of GC is more common in the lower third stomach than in the middle third stomach ($P=0.002$). All diffuse-type GCs with 20q gain (3 cases) were located at the lower third stomach. This chromosomal region contains several genes with oncogenic potentiality that have a role in the development of various human cancers. These genes include AIB1, 3, 4 (steroid receptor coactivator), PTP1B/PTPN1 (nonreceptor tyrosine phosphatase), MYBL2 (nuclear transcription factor), and *CAS* (human cellular apoptosis susceptible gene).[23–27] Since those genes in 20q12–13 have been reported to be amplified in breast, ovary, pancreas, and bladder cancers, they are likely to be candidate oncogenes involved in the tumorigenesis of GC. So far, none of these

genes has been reported to have a role in gastric carcinogenesis. The finding observed in the present study that gain at 20q exclusively occurred in intestinal-type GC gives a motive to conduct further molecular and/or cytogenetic investigations of this region, especially in intestinal-type GC.

The copy number aberration of 8p is remarkable, although the frequency is less than that of 20q gain. Gain of this chromosomal region was more common in diffuse-type GC, while loss of this region was more frequently detected in intestinal-type GC. Amplification of 8p has sometimes been detected in GCs,[13–17] but is rare in other carcinomas.[28, 29] It is likely that there are two different pathways for GC in tumors linked with 8p gain or 8p loss. Sakabe *et al.*[30] reported that expression of a gene within an amplicon at 8p23.1, *MASL1*, was enhanced significantly in malignant fibrous histiocytoma. Several tentative tumor suppressor genes were located in 8p, i.e., putative prostate cancer tumor suppressor (N33; 8p22) and deleted in liver cancer 1 (DLC1; 8p21–22).

The most frequent genomic aberration was a gain on chromosome 8q detected in 21 of 33 cases. This gain was common regardless of the histological subtype (diffuse or intestinal), location (the middle third or lower third), and DNA ploidy (DNA diploidy or DNA aneuploidy). Based on analysis of our CGH data, there are two minimal common regions, 8q12–22 and 8q22–qter. The 8q22–qter contains the *c-myc* oncogene, for which a critical role has been demonstrated in the development of solid tumors, including stomach, breast, lung, prostate and bladder cancers.[7, 12, 31–33] Of particular interest is the fact that frequent gains at 8q12–22 were detected in this study. Recently, an increased copy number in 8q12–22 independent of c-myc at 8q24 has been detected in breast cancer.[32] Furthermore, such change of 8q12–22 has been correlated with poor prognosis and node metastasis in breast cancer[34] and esophageal cancer.[9] However, in respect of stomach cancer, there has been no remarkable finding in previous studies. Thus, our results suggest that the chromosome region of 8q12–22 could contain candidate oncogenes that are involved in the development of GC.

Amplifications were also noted in 6p and 7p. Several oncogenes involved in cell proliferation and regulation of growth factors have been assigned to the chromosome region of 6pcen–21 or 7pcen–7p15. Of these, the best potential candidate gene is vascular endothelial growth factor (VEGF) or epidermal growth factor receptor (EGFR). Maeda *et al.*[35] reported that VEGF expression in patients with early gastric carcinoma was observed more frequently in patients with disease recurrence. Amplification of *EGFR* gene (harbored in chromosome 7p12) has been reported in 5–10% of GC in a Japanese population.[36, 37] The present study disclosed amplifications in three cases (9.1%) at 7p, as well as 7p gains with 27% of the cases, in line with the results of previous studies.[12–15, 28] Gains and amplifications of 17q were detected in four cases (12%) with lower frequency than in previous CGH studies.[12, 13] Kokkola *et al.*[12] reported gains at 17q in 36% of intestinal-type GC and further noted this genetic change was uncommon in diffuse-type GC. This discrepancy between present and previous studies may originate from the ethnic difference in samples studied. Further study will be needed to elucidate this inconsistency.

The chromosome regions with recurrent losses in primary GC were 17p, 19q, 19p, 16p, 18q and 5q. About 36% of the GC cases in the present study showed losses on 17p with a minimal common region at 17p13. This 17p13 is well-known as the site of p53 tumor suppressor gene. Loss of heterozygosity (LOH) and inactivation of *p53* gene frequently occur in most human primary malignancies. Allele loss and mutation of *p53* gene are detected in more than 60% of GC regardless of histologic type.[2] Recently, we have demonstrated that 5q loss is associated with DNA aneuploidy in intestinal-type GC.[17] The present study reveals that 5q loss is associated with DNA aneuploidy in both intestinal- and diffuse-type GC. Furthermore, amplifications detected by CGH in GCs were associated with 5q loss. These results indicate that 5q loss is related to acquisition of DNA aneuploidy and DNA copy number aberrations (gain, loss, and amplification) in GCs. Chromosome 5q harbors the *APC* and *MCC* genes, both of which are putative tumor suppressor genes that are frequently mutated, not only in colorectal cancer, but also in primary GC.[38] In addition, LOH analysis of 5q indicated that there is at least one more candidate tumor suppressor gene, besides *APC* or *MCC*, which can be inactivated in primary GC.[39, 40] These regions might harbor as-yet unknown tumor suppressor gene(s). Loss of 9p detected by CGH is common in various tumors. A possible tumor suppressor gene *p16* (*INK4*; 9p21) may be involved in the genomic aberration. Myung *et al.*[41] reported that losses of p16 and p27 seem to play a significant role during gastric carcinogenesis.

In conclusion, the present CGH analysis confirms chromosomal aberrations in gastric carcinomas that have previously been described in several reports. In addition, we demonstrated that chromosomal regions 5q, 8p, and 20q would repay further investigation.

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