Relationship between Chromosomal Instability and Intratumoral Regional DNA Ploidy Heterogeneity in Primary Gastric Cancers

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ABSTRACT

The purpose of this study was to elucidate the relationship between intratumoral regional heterogeneity in DNA ploidy and chromosomal instability (CIN) in primary gastric adenocarcinomas. In 45 sporadic gastric adenocarcinomas, we measured DNA ploidy and numerical aberrations for chromosomes 7, 11, 17, and 18 by laser scanning cytometry and fluorescence in situ hybridization, respectively, in small tissue specimens taken from 2 to 6 (on the average 4) different portions of the same tumor. A total of 231 specimens including 45 normal control specimens were examined. All 98 tumor specimens with DNA aneuploidy (DNA index > 1.2) showed large intercellular variations in chromosome copy number, indicating CIN. In contrast, 85 tumor specimens with (near) diploidy (1.0 < DNA index < 1.2) exhibited much small intercellular variations in chromosome copy number as compared with aneuploid specimens (P < 0.0001). The relationship between DNA ploidy and intercellular variation in chromosome copy number was true for tumors consisting of a mixture of (near) diploid and aneuploid subpopulations. These data indicate that DNA aneuploidy is associated with CIN but that (near) diploidy is not. Intratumoral regional DNA ploidy heterogeneity was conspicuous in 33 (92%) of 36 tumors with regions of DNA aneuploidy, and all aneuploid specimens showed great intercellular variation in chromosome copy number. Diploid regions were predominant in early stage cancers (intramusosal and submucosal cancers), and five of eight early cancers contained only diploid population. In contrast, all tumors without (near) diploid regions were advanced cancers. These observations suggest that CIN is a necessary prerequisite for developing intratumoral DNA ploidy heterogeneity with DNA aneuploidy.

INTRODUCTION

Although the mortality rate of patients with gastric cancer has decreased due to early detection coupled with improvements in therapeutic procedures, many patients still die of the disease. In general, the prognosis for patients with early cancer is excellent, and prognosis for patients with advanced cancers is poor. However, there are exceptions; some early cancers progress rapidly, and the prognosis in advanced cancer patients is not always grave. The biological characteristics of the cancer depend primarily on genetic alterations in cancer cells. In this context, there is an important issue which we have to face concerning a common genetic feature for all malignancies. Malignant tumors intrinsically manifest genetic instability, and consequently genetic aberrations successively accumulate in tumor cells as the tumor progresses. Recently, genetic instability in colon cancer cell lines was conceptually classified into two distinct types: MIN and CIN (1–4). MIN, which is a replication error phenotype found in hereditary nonpolyposis colorectal cancer, results from abnormalities in the DNA mismatch repair pathway. The mechanism of MIN has been partially elucidated (5, 6). Mutations in mismatch repair genes such as hMSH2 and hMLH1 have been found in some colorectal cancers and are thought to be a cause of MIN. In contrast, defects in chromosome segregation induced by aberrations of mitosis-regulating genes result in a gain and/or loss of chromosomes in tumor cells (4, 7, 8), which gives rise to aneuploidy (3, 4). MIN has been studied intensively in various malignant tumors. The incidence of MIN was reported to be 16–39% in gastric cancer (9–11). However, reports concerning CIN in primary cancers including gastric cancers is scarce (12). This is due in part to methodological difficulties, because CIN, which is defined as a rate, cannot be assessed from a single experiment. Recent investigations have demonstrated that the extent of chromosome copy number variations can be used as a surrogate marker for CIN (1–4). In vitro studies of colon cancer cell lines suggested that whereas MIN generates subtle changes in nuclear DNA content, CIN causes aneuploidy that is detectable by cytometry (1–3).

To examine whether the relationship between CIN and DNA aneuploidy found in colon cancer cell lines holds for...
primary gastric cancers, we measured variations in chromosome copy number and DNA ploidy in tissue specimens of surgically removed gastric carcinomas using FISH and LSC, respectively. This study reveals close relationships between intercellular variation in chromosome copy number and DNA ploidy and provides insight into the evolution of intratumoral heterogeneity in ploidy in primary gastric cancers. This is the first report that CIN causes intratumoral regional heterogeneity in DNA ploidy in primary gastric cancers.

MATERIALS AND METHODS

Specimens. We used 45 consecutive surgically removed gastric cancers: 8 early (3 mucosal and 5 submucosal tumors) and 37 advanced cancers (Table 1). There were 32 male patients and 13 female patients with an average age of 67.4 years (range, 44–86 years). Family histories were noncontributory for all patients. Usually, tumor tissue specimens were taken from five different parts of the same tumor, and as a control, an additional specimen was taken from the mucosa distal to the tumor. A total of 231 tissue specimens were examined, 186 tumor specimens and 45 normal mucosal tissue specimens. Tissue specimens were stored at −80°C until use.

Touch Smear Preparations for FISH and LSC. At least five touch smears were prepared by touching thawed tissue specimens to glass slides after wiping blood from the cut surface of the specimens with a paper towel. One touch sample was dipped in 70% ethanol for fixation for DNA measurement by LSC (13). The others were dried well and fixed with 100% ethanol for FISH analysis (14).

FISH. Touch smears fixed in 100% ethanol were refixed in 0.2% paraformaldehyde-PBS at 4°C for 10 min as previously described (14). We examined numerical aberrations in chromosomes 7, 11, 17, and 18 using biotinylated alphoid satellite DNA probes specific for the pericentromeric region of each chromosome (D7Z1, D11Z1, D17Z1, and D18Z1, respectively; Oncor, Inc., Gaithersburg, MD), as described elsewhere (13). Briefly, 10 μl of a hybridization mixture containing 1 μg/ml salmon sperm DNA (Sigma Chemical Co., St. Louis, MO), 55% formamide, 2 × SSC (1 × SSC is 0.15 M NaCl and 15 M sodium citrate), and 10% dextran sulfate was heated in a water bath at 70°C for 5 min. The DNA mixture was applied to the slides, which were denatured at 70°C for 2 min. Incubation for hybridization was performed overnight at 37°C in a moist chamber. The slides were rinsed in washing solution containing 50% formamide and 2 × SSC at 45°C and processed to stain the hybridized probe using FITC-avidin (Vector Laboratory, Burlingame, CA). Nuclei were counterstained by adding glycerol with PI (2 μg/ml, Sigma) and p-phenylenediamine dihydrochloride (1 μg/ml, Sigma).

Scoring of Hybridization Signals. The number of hybridization signals in each nucleus was determined by observing more than 200 nuclei on each slide with an epifluorescence microscope equipped with a ×100 oil immersion objective (Olympus Co., Tokyo, Japan).

DNA Measurement by LSC. DNA ploidy was determined as described previously (13, 15–17). Touch preparations fixed in 70% ethanol were dipped in PI solution (25 μg/ml in PBS) containing 0.1% RNase (Sigma). A coverslip was placed on the slide and sealed with nail polish. DNA content was measured with a laser scanning cytometer (LSC 101; Olympus Co.). Usually, at least 5000 cells were examined for each sample. DNA histograms were generated, and DNA ploidy was

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a Histological type: por1, poorly differentiated adenocarcinoma, solid type; por2, poorly differentiated adenocarcinoma, nonsolid type; tub1, well differentiated tubular adenocarcinoma; tub2, moderately differentiated tubular adenocarcinoma; sig, signet ring cell carcinoma; muc, mucinous carcinoma.

b Depth of tumor invasion: m, mucosa; sm, submucosa; ss, subserosa; se, serosal exposure; si, invasion to the pancreas.

c LNM, lymph node metastasis. Figures indicate the number of lymph nodes involved in carcinoma.

d L.M, liver metastasis: +, positive; −, negative.

Table 1 Pathological data and DNA ploidy in gastric cancers

1. Downloaded from clincancerres.aacjournals.org on April 14, 2017. © 2000 American Association for Cancer Research.
were separated from DNA aneuploid tumors (DI $\leq 1.2$). DNA ploidy heterogeneity was conspicuous in 11 of these tumors, but in the remaining aneuploid cases it was difficult to find significant heterogeneity. Intratumoral regional DNA ploidy heterogeneity was considered to have intratumoral heterogeneity becomes conspicuous. Intratumoral regional heterogeneity was conspicuous in 11 of these tumors, but in the remaining aneuploid cases it was difficult to find significant differences in DI among the specimens. Mixed tumors consisted of both (near) diploid and aneuploid populations, and 22 (49%) of 45 tumors were placed within this category. All tumors included in this group showed DNA ploidy heterogeneity. In summary, intratumoral regional heterogeneity in DNA ploidy was detected in 36 (80%) of 45 tumors (Table 2).

Five of eight early gastric cancers were included in the diploid tumor group. In the remaining three early cancers, aneuploid regions were found in the limited parts of a tumor. All cases in which every specimen represented DNA aneuploidy within a tumor were advanced cancers (Table 1).

Intercellular variation in the chromosome copy number detected by FISH. In normal mucosa, $\sim$90% of cells had two signal spots for all chromosomes examined, and polysomic ($>4$ signals) cells were virtually never observed. In diploid tumors (1.0 $\leq$ DI $< 1.2$), although there was a small population of polysomic cells, disomic cancer cells were apparently predominant for all chromosomes examined (Fig. 1). In contrast, DNA aneuploid specimens (DI $\geq 1.2$) exhibited great intercellular variations in chromosome copy numbers as compared with diploid tumor specimens (1.0 $\leq$ DI $< 1.2$) (Fig. 1). Occasionally, polysomic nuclei were frequent in aneuploid tumors. In 10 of 14 aneuploid tumors, the modal chromosome copy number was 3 or 4. The modal chromosome number was 2 for all chromosomes in the remaining four aneuploid tumors, but the variation in chromosomal number was great. The intercellular variation in the chromosome copy number was not largely affected by the modal chromosome copy number in the aneuploid tumor group.

The percentage of cells with signal spots equivalent to the modal chromosome number was significantly smaller in aneuploid tissue specimens (DI $\geq 1.2$) than in (near) diploid ones (1.0 $\leq$ DI $< 1.2$) ($P < 0.0001$; Table 3). The relationship between DNA ploidy and intercellular variation in chromosome copy number was also true in mixed tumors ($P < 0.0001$). Namely, disomic cells were predominant in DNA diploid regions, whereas cells in DNA aneuploid regions exhibited great intercellular variation in chromosome copy numbers (Fig. 1).

There were foci with the great intercellular variation in the chromosome copy number in 33 of 36 cases showing intratumoral DNA ploidy heterogeneity (Table 4). These 33 tumors were aneuploid or mixed tumors, but the remaining 3 cases were diploid tumors consisting of diploid and near-diploid populations. In contrast, intercellular variations in chromosome copy numbers were obvious in only three of nine tumors without DNA ploidy heterogeneity (Table 4). These three cases were aneuploid tumors, whereas the remaining six were diploid tumors. All tumor specimens with great intercellular variation in chromosome number showed DNA aneuploidy and vice versa (Table 5).

### Table 2

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<th>Diploid tumors (9)</th>
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<td>WOH (6)</td>
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$^a$ Numbers in parentheses, number of cases.

$^b$ WH, with heterogeneity; WOH, without heterogeneity.

### RESULTS

Cells isolated from the normal mucosa invariably showed DNA diploidy (DI $= 1.0$). However, DNA indices ranged from 1.0 to 2.64 in gastric cancers. In this series, 88 of 186 tumor samples were judged to be (near) diploid (1.0 $\leq$ DI $< 1.2$), and the remaining 98 were classified as aneuploid (DI $\geq 1.2$).

#### Intratumoral Regional DNA Ploidy Heterogeneity

A tumor consisting of heterogeneous subpopulations with different DNA ploidies within the tumor was considered to have intratumoral regional DNA ploidy heterogeneity.

#### Histological Diagnosis

Histological diagnoses were made via routine 5-μm sections stained with H&E and categorized according to the Japanese Classification of Gastric Carcinoma System (19).

#### Statistical Analysis

Student’s $t$ test was used to compare the population size of tumor cells with signal spots equivalent to the modal chromosome number between two tumor groups (diploid versus aneuploid tumor specimens) in gastric cancers based on DNA indices. Statistical significance was set at $P \leq 0.05$.

### DISCUSSION

Tumors are thought to develop along a multistep pathway in tissues exposed to carcinogens that accumulate genetic alterations in functional targets relevant to tumor evolution. Once a tumor is established, genetic instability, which is one characteristic of cancer cells, leads to successive genetic abnormalities in tumor cells. Cells with different genetic alterations successively emerge in parts of the tumor; some expand within the tumor after clonal selection, and eventually intratumoral regional heterogeneity becomes conspicuous. Intratumoral regional heterogeneity coupled with genetic instability is an important issue not only from the standpoint of DNA ploidy measurement but also with respect to cancer treatment (20–23). Our results indicate that analysis of a single tissue specimen may lead to an errone
ous interpretation. Fortunately, in this study, the possibility that we overlooked a major subpopulation is considered low, because most stemlines are detected cytometrically when four samples taken from different regions of a tumor are examined (24).

Cytogenetically cancers containing both diploid (DI = 1.0) and near-diploid (1.0 < DI < 1.2) populations were included in the diploid tumor group, because tumors in this group were characterized by disomic cells with a small population of aneucistic cells for all chromosomes examined. Namely, the intercellular variations in chromosome copy numbers were smaller in DNA (near) diploid cell populations than in aneuploid cell populations (P < 0.0001). This was also true of DNA (near) diploid regions in tumors consisting of a mixture of (near) diploid and aneuploid populations, i.e., mixed tumors. These observations indicate that near-diploid tumors are different from aneuploid tumors and that they should therefore be included in the diploid group. Gastric cancers confined to the mucosal layer have been reported to exhibit DNA diploidy more frequently than advanced cancers (20, 25). The present study also revealed that diploid cells occupied most of or entire parts of a tumor in cases of intramucosal cancer and that no early gastric cancers (mucosal and submucosal tumors) were included in the aneuploid group. Although aneuploid foci within a tumor were found in three of eight early gastric cancers, tumors in which every specimen was aneuploid were always advanced cancers. On the basis of these observations, we hypothesize that although some

Fig. 1. Intercellular copy number variation for chromosomes 7, 11, 17, and 18 in diploid (A) and aneuploid (B) primary gastric cancers. In a diploid tumor, most of cells (>80%) are disomic for all chromosomes examined in all regions, whereas aneucistic cells are rare. In contrast, the percentage of cells with the modal chromosome number is lower in an aneuploid tumor than in a diploid tumor (P < 0.0001); furthermore, intercellular variation in chromosome copy number is conspicuous in aneuploid tumors. These are seen in all regions within a tumor. Ordinate, percentage of cells with different chromosome copy numbers; abscissa, circled numbers, part of the tumor from where a tissue specimen is taken. N, normal control.
Cancers for all chromosomes examined in this study (of cells with modal chromosome number between diploid and aneuploid results in intratumoral DNA ploidy heterogeneity. CIN is critical cause CIN is a dominant phenotype, genetic instability during tumor progression, however, mutations in cell division checkpoints may occur in cases with MIN, and eventually, CIN in cancer cells with MIN also have properties of CIN. Be-
ding imperceptible alterations in nuclear DNA content. During tumor progression, however, mutations in cell division checkpoint genes may occur in cases with MIN, and eventually, tumor cells with MIN also have properties of CIN. Because CIN is a dominant phenotype, genetic instability results in intratumoral DNA ploidy heterogeneity. CIN is critical for the development of intratumoral heterogeneity. CIN is induced by aberrations in genes relevant to mitosis, but the affected genes are thought to be different among tumors.

DNA ploidy analysis coupled with FISH examination elucidates the relationship between DNA ploidy and genetic instability; furthermore, it provides insight into the evolutionary mechanisms of intratumoral heterogeneity in gastric adenocarcinomas. To our knowledge, this is the first report that CIN causes intratumoral regional DNA ploidy heterogeneity in primary gastric cancers.

**REFERENCES**

Relationship between Chromosomal Instability and Intratumoral Regional DNA Ploidy Heterogeneity in Primary Gastric Cancers

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