The Relationship of DNA Ploidy to Chromosomal Instability in Primary Human Colorectal Cancers

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ABSTRACT

The aim of this investigation was to corroborate the relationship between DNA ploidy and chromosomal variation in surgically removed colorectal cancers. For 101 specimens from 21 advanced colorectal cancers, the numerical variations in chromosomes 7, 17, and 18 among cells were measured by fluorescence in situ hybridization using DNA probes specific for centromere of each chromosome, and DNA ploidy was determined by laser scanning cytometry or flow cytometry. DNA diploidy (DNA index = 1.0) was linked with minor variation in copy number of chromosomes 7, 17, and 18, whereas DNA aneuploidy (DNA index ≥ 1.2) was found exclusively in tumors with large variations in centromere copy number for all chromosomes. There was a significant difference in the degree of intercellular variations in chromosome copy number between diploid and aneuploid clones for all chromosomes examined ($P < 0.001$). In near-diploid clones (1.0 $< \text{DNA index} < 1.2$), the numerical variation of chromosome 18 was significantly different from that in diploid clones ($P < 0.002$), but it was not different from that in aneuploid clones. These observations support the hypothesis that chromosomal instability is associated with DNA aneuploidy in colorectal cancers. Additionally, they suggest that near-diploid tumors are also unstable at a lower level than classic aneuploid tumors and that all chromosomes are not affected equally in near-diploid cases.

INTRODUCTION

It is widely accepted that malignant tumors intrinsically exhibit genetic instability and that, consequently, genetic aberrations successively accumulate with tumor progression. Recently, genetic instability has been shown to be divided into two types: MIN and CIN (1–3). MIN, which is known as a genetic phenotype of nonpolyposis colorectal cancer, results from abnormalities in the DNA mismatch repair pathway. The mechanism of MIN has been partially elucidated by molecular investigations (4, 5). Mutations of mismatch repair genes such as hMSH2 and hMLH1 are italicized. Please check throughout manuscript that all genes, alleles, and loci are italicized. It is widely accepted that malignant tumors intrinsically exhibit genetic instability and that, consequently, genetic aberrations successively accumulate with tumor progression. Recently, genetic instability has been shown to be divided into two types: MIN ($^3$) and CIN (1–3). MIN, which is known as a genetic phenotype of nonpolyposis colorectal cancer, results from abnormalities in the DNA mismatch repair pathway. The mechanism of MIN has been partially elucidated by molecular investigations (4, 5). Mutations of mismatch repair genes such as hMSH2 and hMLH1 are italicized. Please check throughout manuscript that all genes, alleles, and loci are italicized.

Received 1/27/99; accepted 8/19/99.

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Specimens. We used 21 surgically removed advanced colorectal cancers that were histologically well-differentiated adenocarcinoma. The patients consisted of 11 males and 10 females with a mean age of 63.0 years (range, 44–86 years). Family history was noncontributory for all patients. Usually, tumor tissue specimens were taken from five different parts of the same tumor, and as the control, an additional specimen was also obtained from the intact mucosa far from the tumor. Totally, 101 samples were examined in this study. The tissue specimens were stored at −80°C until use.

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

FISH. The touch smears fixed in 100% ethanol were rehydrated in 0.2% paraformaldehyde/PBS at 4°C for 10 min as described previously (11, 12). We examined numerical aberrations of chromosomes 7, 17, and 18 using biotinylated alpha satellite DNA probes specific for the pericentromeric region of each chromosome (D7Z1, D17Z1, and D18Z1; Oncor Inc., Gaithersburg, MD), as described elsewhere (11, 12). 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, 0.15 M NaCl and 0.015 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 for 2 min. Incubation for hybridization was performed overnight at 37°C in a moist chamber. The slides were rinsed in a washing solution containing 50% formamide and 2× SSC at 45°C, and then processed immunologically to stain the hybridized probe with FITC-avidin (Vector Laboratory, Burlingame, CA). The nuclei were counterstained by the addition of glycerol with propidium iodide (Sigma) and $p$-phenylenediamine dihydrochloride (Sigma).

Scoring of Hybridization Signals. Only those cells having a malignant cytological appearance (especially large nuclei) were scored. Small, round lymphocyte-like cells and overlapping or damaged nuclei were disregarded. The number of hybridization signals in each nucleus was determined by $\text{in vitro}$ experiments using colorectal cell lines (1). As far as we know, however, there are no published reports that focus on the relationship between DNA ploidy and CIN in surgically removed tissue specimens. In this brief report, we validated the view that CIN is associated with DNA aneuploidy but not with DNA diploidy even in surgically removed colorectal cancers. Furthermore, we also show that near-diploid clones manifest a characteristic pattern of numerical chromosome alterations, suggesting an intermediate pattern between diploid and aneuploid populations.

MATERIALS AND METHODS

Specimens. We used 21 surgically removed advanced colorectal cancers that were histologically well-differentiated adenocarcinoma. The patients consisted of 11 males and 10 females with a mean age of 63.0 years (range, 44–86 years). Family history was noncontributory for all patients. Usually, tumor tissue specimens were taken from five different parts of the same tumor, and as the control, an additional specimen was also obtained from the intact mucosa far from the tumor. Totally, 101 samples were examined in this study. The tissue specimens were stored at −80°C until use.

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Scoring of Hybridization Signals. Only those cells having a malignant cytological appearance (especially large nuclei) were scored. Small, round lymphocyte-like cells and overlapping or damaged nuclei were disregarded. The number of hybridization signals in each nucleus was determined by observing more than 200 nuclei on a slide, using an epifluorescence microscope equipped with a ×100 oil immersion objective ( Olympus Co., Tokyo, Japan). The percentages of cell populations with different signal counts were determined for each slide, as shown in Fig. 2. The mode number of individual chromosome represented the number of chromatids of the tumor.

DNA Measurement by LSC. Samples were made and DNA ploidy was determined by the procedures described previously (13–16). Briefly, the slides fixed in 70% ethanol were dipped in a propidium iodide solution (25 μg/ml in PBS) containing 0.1% RNase (Sigma). A coverslip was put on the slide and sealed with fingernail polish. DNA content was measured by a laser scanning cytometer (LSC 101; Olympus). Usually, more than 5,000 cells were examined in each sample. A DNA histogram was generated, and DNA ploidy was determined.

DNA Measurement by FCM. Tumors in which nuclear DNA content was not measured by LSC were subjected to FCM analysis. The remaining tissue specimens were used for flow cytometric DNA ploidy analysis, which was carried out according to the method reported previously (17–19). Briefly, the tissue specimens were minced with scissors and suspended in a PBS solution containing 0.2% Triton X-100. Single nuclear suspensions were prepared by
Intratumoral Heterogeneity. A tumor consisting of heterogeneous subpopulations with different DNA ploidies and/or different number of chromosomes among parts of a tumor was regarded as showing intratumoral regional heterogeneity. The difference in the DI value between two peaks was more than 10% of the diploid DNA content, the tumor was considered to consist of heterogeneous subpopulations with different DNA ploidy.

RESULTS

In the mucosa with normal appearance, no aneuploid peaks were detected by cytometric analysis, i.e., DI = 1.0. FISH studies revealed that 90.8–96.4% (mean, 93.6%) of cells were disomic for each chromosome and that virtually no cells had more than three spots.

DNA Ploidy Analysis. DNA indices ranged from 1.0 to 2.01 in this series of colorectal cancers. There were two cases (10% of tumors) in which neither DNA aneuploid (DI ≥ 1.2) nor near-diploid (1.0 < DI < 1.2) clones were detected, these tumors consisting of DNA diploid (DI = 1.0) clones alone. In other tumors (90%), DNA aneuploid and/or near-diploid clones were found in at least one region within a tumor. One of the remaining five tumors consisted of only near-diploid clones, and four tumors were composed of a mixture of near-diploid and diploid clones. Four tumors were devoid of diploid and near-diploid clones, i.e., these tumors consisted of only aneuploid clones, and 10 tumors consisted of a mixture of aneuploid and (near)diploid clones (Fig. 1). From the viewpoint of DNA ploidy, intratumoral regional heterogeneity was observed in 13 tumors (62% of cases).

With respect to individual sample, 39, 16, and 49 specimens were in diploid, near-diploid and aneuploid groups, respectively.

FISH Analysis. There were great difference in the population sizes of cells with spots equivalent to modal chromosome number among samples. In DNA diploid clones, however, most tumor cells had two signals for each chromosome, and cells with more than three spots were occasionally detected. On average, 83.0, 83.0, and 90.6% of tumor cells in DNA diploid clones were disomic for chromosomes 7, 17, and 18, respectively (Table 1). In contrast, DNA aneuploid clones showed a great intercellular variation in the copy number of chromosomes (Fig. 2). The

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**Table 1** Population sizes (mean ± SD) of cells with modal chromosome number for three different DNA ploidy groups in colorectal cancers

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Normal</th>
<th>Diploid</th>
<th>Near-diploid</th>
<th>Aneuploid</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>94.1 ± 2.8</td>
<td>83.0 ± 12.1</td>
<td>78.6 ± 18.9</td>
<td>63.2 ± 15.2</td>
</tr>
<tr>
<td>17</td>
<td>93.5 ± 2.9</td>
<td>83.0 ± 12.3</td>
<td>84.8 ± 8.8</td>
<td>65.5 ± 15.9</td>
</tr>
<tr>
<td>18</td>
<td>92.9 ± 2.7</td>
<td>90.6 ± 8.4</td>
<td>74.2 ± 13.1</td>
<td>79.6 ± 17.7</td>
</tr>
</tbody>
</table>

* Figures indicate the areas within tumors from which tissue specimens were taken:

- **a** Normal mucosa.
- **b** DNA diploid clones (DI = 1.0).
- **c** DNA near-diploid clones (1.0 < DI < 1.2).
- **d** Aneuploid clones (DI ≥ 1.2).

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**Fig. 2** Intercellular numerical variation of chromosomes 7, 17 and 18 in representative cases of diploid (A), near-diploid (B), and aneuploid (C) colorectal cancer. Most cells were disomic for all chromosomes examined, and aneusomy cells were seen occasionally in diploid tumors. In contrast, the fraction of cells with modal chromosomal number was smaller in aneuploid tumors than in diploid tumors (P < 0.0001), and intercellular variation in chromosome number was distinct in aneuploid tumors. Near-diploid tumors, however, showed an intermediate pattern between diploid and aneuploid tumors. Monosomy 18 was prominent in a near-diploid tumor shown here, but chromosomes 7 and 17 were still disomic. **Ordinate,** the percentage of cells with different chromosome numbers. **Abscissa,** numbers indicate the areas within tumors from which tissue specimens were taken: 1, oral part of a tumor; 2, anal part of a tumor; 3, anterior part of a tumor; 4, posterior part of a tumor.
mean percentage of cells with modal chromosomal number was significantly smaller in aneuploid tumors than in diploid tumors ($P < 0.0001$; Table 1). This was also true of tumors consisting of a mixture of (near) diploid and aneuploid populations. Polysomic nuclei that were incidental in diploid clone were frequent in DNA aneuploid clones.

Near-diploid clones (1.0 < DI < 1.2) showed a different pattern from diploid and aneuploid clones. The intercellular variation in the copy number of chromosomes 7 and 17 was not different from that in diploid clone, whereas for chromosome 18 it was significantly different between diploid and near-diploid clones ($P < 0.0002$; Table 1). The modal number of chromosome 18 was frequently monosomic in a near-diploid tumor. The intercellular variation in the copy number of chromosomes 7 and 17 was significantly different between near-diploid and aneuploid clones as well as between diploid and aneuploid clones ($P < 0.0001$). However, it was not significant for chromosome 18 between near-diploid and aneuploid clones.

**DISCUSSION**

Recently, an interesting and attractive hypothesis has been proposed for genetic instability in colorectal cancers (1–3). The *in vitro* experiments using established cell lines revealed that, whereas MIN was linked with (near) diploidy, CIN was associated with aneuploidy (1–3). Although instability is defined as a rate and cannot be assessed from a single experiment (1–3), chromosome number variation can be used as a surrogate marker for instability. This is a practical strategy for elucidating CIN in surgically removed tissue specimens. In this study, DNA aneuploid clones showed numerous variations in chromosomes in surgically removed cancer specimens as well as in cell lines, whereas diploid clones showed fewer variations. DNA diploid clone exhibited that on average 83.0, 83.0, and 90.6% of tumor cells stained with PI. Cytometry, 37: 63–69,

MIN involves successive aberrations in many regions of the genome with tumor progression (1–3). Some of them are genes relevant to control of chromosomal division, e.g., mitosis checkpoint genes, and aberrations in such genes result in CIN (1–3). Defective repair of mismatched bases may provoke aberrations of genes checking mitosis in cells with intact mitotic checkpoints, and eventually CIN is established. For example, the expression of *hBUB1* mutants converts the normal checkpoint status of MIN cells to the defective type characteristic of CIN cells (10). Taking into account recent studies concerning the mitotic checkpoint in the cell cycle, the present investigation suggests that a transient phenotype in which some genes that check mitosis are involved may change diploid cells into near-diploid cells in which intercellular numerical variation is detected in only some of chromosomes. Subsequently, other mitotic checkpoint genes are hit successively, and eventually extensive involvement of these genes results in distinct aneuploidy (11–23). In this series, approximately half of tumors showed intratumoral heterogeneity consisting of a mixture of aneuploid and (near) diploid clones (Fig. 1D). Provided that virtually aneuploid clones cannot change into diploid clones, it is natural to consider that aneuploid clones evolve in preexisting diploid clones. The hypothesis offered here is compatible with the phenomenon that MIN is a dominant phenotype in a fusion cell of CIN and MIN (1–3).

It is likely that near-diploid clones are in the transient state between two distinct types of genetic instability.

**REFERENCES**


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