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Intratumor Heterogeneity of Centromere Numerical Abnormality in Multiple Primary Gastric Cancers: Application of Fluorescence *in situ* Hybridization with Intermittent Microwave Irradiation on Paraffin-embedded Tissue

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Our recent success in retrieving distinct fluorescence signals in response to centromere specific probing of paraffin-embedded tissues after intermittent microwave (MW) treatment provided the opportunity to analyze chromosome numbers or centromere abnormality *in situ* in human tumors in various clinicopathological settings. In this study, centromere numerical abnormality (CNA) was investigated by fluorescence *in situ* hybridization (FISH) in a case of multiple gastric cancer having intratumor histological heterogeneity. The different profiles as determined using a total of 20 specific probes on 4 multifocal lesions in the stomach confirmed the multi-clonality of these tumors. FISH with probes specific for chromosomes 10, 11, 16 and 18 revealed intratumor heterogeneity of the CNA, which corresponded to the histological heterogeneity. Our report clearly demonstrates, for the first time, intratumor heterogeneity of CNA and its association with the histological picture, and substantiates the applicability of the MW-assisted FISH protocol to paraffin-embedded pathological specimens.

Key words: Centromere (chromosomal) numerical abnormality — FISH — Histological heterogeneity — Gastric cancer — Hyperploidal progression

Fluorescence *in situ* hybridization (FISH) is a powerful method to identify single-copy DNA in human tissues, but distinct hybridization signals in formalin-fixed paraffin-embedded tissue sections have been difficult to obtain using the conventional FISH protocol. On the other hand, analysis of the ploidy pattern of tumors through measurement of the DNA content has been extensively described, but DNA spectrophotometry has insufficient sensitivity to detect subtle DNA changes. Thus, FISH should be superior to spectrophotometry in terms of the detection of subtle DNA changes. Currently, FISH using chromosome-specific centromeric α-satellite DNA probes is available for the detection of minute cytogenetic alterations in the nuclei of tumor cells. In this communication, we report on the application of the microwave (MW)-assisted FISH protocol recently developed by us,[1,2] to a case with multiple primary gastric cancers showing histological heterogeneity in order to obtain information on centromere numerical abnormality (CNA) in early-stage gastric cancer and on the relation of early CNA to the heterogeneous histological components. Eighteen chromosome-specific centromere probes and 2 other locus-specific gene probes were employed to evaluate the CNA profile and its intratumor heterogeneity.

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METHODS

Pathological profile of the case A 51-year-old man who had had multiple, metachronous colorectal cancer and survived 5 operations, developed gastric cancer, diagnosed by routine endoscopy in 1997. The details of this patient have been reported elsewhere.[3] Endoscopic and preoperative biopsy diagnosis of three early gastric carcinomas was made. Subtotal gastrectomy was performed on June 17, 1997. Macroscopically, four lesions, two type-IIc early gastric carcinomas on the anterior (C1) and posterior (C3) walls of the antrum, a type-IIc-like advanced gastric carcinoma on the posterior wall of the antrum (C2), and one type-IIa early gastric carcinoma on the posterior wall (C4) of the antrum were recognized as independent tumors (Fig. 1).

Interestingly, postoperative microscopic examination of the four lesions revealed further intratumor morphological heterogeneity in three of them as described in the results section. The gross and microscopic pathological profiles are summarized in Table I.

Under the microscope, each portion of the tumors with distinct histological features had a front separating two different areas, which enabled us to dissect each region from the sections.

FISH with intermittent MW irradiation

Validation of the protocol for normal lymphocytes and

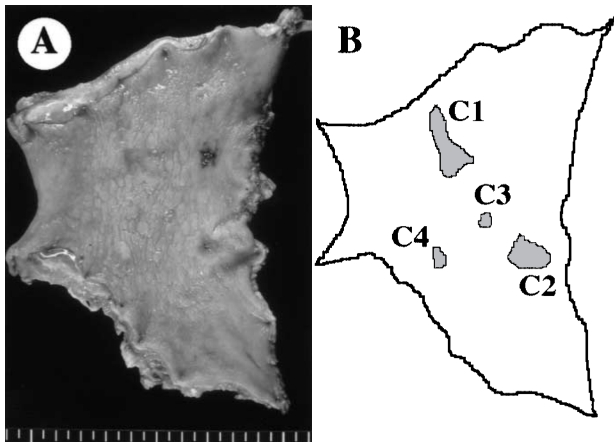

Fig. 1. Macroscopic view of the resected stomach. A: Resected stomach, distal gastrectomy, formalin-fixed. Four arrows indicate multiple lesions. B: Schematic representation of the four mucosal lesions, C1–C4, identified on the surface of the stomach mucosa.

Table I. Clinicopathological Findings of Multiple Gastric Carcinoma

	Macroscopic[a] classification	Histological[a] classification	Depth[b]	Size (mm)
C1	0-IIc	tub1–tub2	m	12×10
C2	0-IIc adv.	tub1–tub2	mp	17×15
C3	0-IIc	tub1	sm1	6×6
C4	0-IIa	pap	m	13×9

a) Macroscopic and histological classification are according to the Japanese Classification System of Gastric Cancer.[7]
b) The depth of invasion, early cancers: m, sm, advanced cancers: mp, also according to Japanese Classification System of Gastric Cancer.

foveolar epithelium of this case: The tissue sample was fixed in a 4% neutral formaldehyde buffer at room temperature for a day or two, followed by routine processing; embedding in paraffin wax, and storage at room temperature. The formalin-fixed paraffin-embedded tissue sections (5 μm thick) of noncancerous gastric mucosa and the carcinoma were investigated histologically. Normal lymphocytes and normal epithelial cells were examined by dual-color FISH analysis after hybridization with Centromeric Enumeration Probes (CEPs) 10 and 8 with initial intermittent MW treatment,[1] to test the quality of the blocks. Normal lymphocytes and normal gastric mucosa served as controls, and signals were counted by two independent investigators. To obtain the cut-off value, 20 probes (described later) were eventually tested on the paraffin blocks of this case.

FISH using centromere probes for multiple primary gastric cancers: CNA in the 4 primary tumors in the resected stomach was investigated by MW-assisted FISH as reported previously.[1] As mentioned above, histological heterogeneity within the tumor was noted in three of them. We determined the area for counting FISH signals considering the histological appearance. At the same time, we dissected the tissues for DNA extraction from each of the histologically heterogeneous areas under the microscope as per the method described elsewhere.[4] Thus, a total of 7 areas was evaluated for centromere numbers in the tumor cells and for microsatellite instability.

A panel of 18 centromeric α-satellite DNA probes (D1Z5, D2Z, D3Z1, D4Z1, D6Z1, D7Z1, D8Z2, D9Z1, D10Z1, D11Z1, D12Z3, D15Z1, D16Z1, D17Z1, D18Z1, D20Z1, DXZ1, DYZ3) and two locus-specific identifier probes (c-myc and p53) were purchased from Vysis Inc. (Downers Grove, IL). All of the probes were labeled either orange (Cy 3) or green (FITC) using digoxigenin-11-dUTP and nick translation. The sections were deparaffinized by five successive 3-min washes in xylene followed by five washes in ethanol, and MW heating for 10 min in a 0.01 *M* citrate buffer (pH 6.0). After treatment in 0.2% pepsin/0.01 *N* HCl for 10 min at 37°C, the samples were aged in 0.1% NP-40/2× SSC for 30 min at 37°C and the DNA was denatured by treatment in 70% formamide/2× SSC for 5 min at 85°C. Ten microliter aliquots of the probe solution (hybridization buffer 7 μl, probe 1 μl, DW 2 μl) were placed on a glass slide and coverslipped. The sample slides with the hybridization mixture were then placed in a MW processor (MI-77, Azumaya Co., Tokyo) and irradiated for 3 s-on and 2 s-off cycles (2.45 GHz, 300 W), with the temperature set at 42°C, for 1 h during the initial 16 h of hybridization. DAPI-II (4,6-diamidino-2-phenylindole, 125 ng/ml, Vysis Inc.) was used for counterstaining of the nuclei.

The samples were immediately observed under a fluorescence microscope equipped with epifluorescence filters and a photometric CCD camera (Quantix 1400, Olympus Optical Co., Ltd., Tokyo). The captured images were digitized and stored by an image analysis program (CytoVision; Applied Imaging, Olympus).

For each probe and each of the tumor areas, 100 to 200 intact and non-overlapping nuclei were counted, and the number of signals per nucleus was scored.

Scoring of all of the areas by two independent investigators (KK, YK) yielded almost identical counts. Chromosome aberrations exceeding a certain cut-off value were considered as reflecting monosomy, trisomy, or tetrasomy (cut-off value against normal control lymphocytes, 20%) of the corresponding chromosomes. The 20% cut-off value was empirically adopted based on the possible (maximum) percentage of pseudo-triploidy due to overlapping of normal cells and other reasons as experienced in the previous paper[1] and in the normal epithelial cells of our case (data not shown).

Mirror section study: Mirror-image sections stained with hematoxylin & eosin (HE) were also examined to allow evaluation of the FISH signals in relation to the morphological features revealed by the HE staining. Two 4-μm sections were made. One of the sections was stained with HE, and the other was hybridized with centromeric α-satellite DNA probes, which showed heterogeneous profiles in the first pilot screening using adjacent sections.

Table II. FISH of Normal Lymphocytes[a)]

Signal numbers	1	2	3	4
CEP10	0%	98%	2%	0%
CEP11	2	95	3	0
CEP16	5	93	1	1
CEP18	4	96	0	0

a) Percentage among 100–200 cells which have nearly “full diameter” of the nuclei. A complete data set including the other chromosomes is available on request.

DNA extraction and genetic instability analysis: DNA was extracted from these different-appearing areas of the tumor by dissection under a microscope in adjacent sections. The DNA extraction procedure from the pathology archives was performed according to a previously described procedure.[4, 5)]

Five recommended loci (BAT-26, BAT-25, D5S346, D2S123, D17S250) were tested for microsatellite instability as described previously.[6)] As a positive control, we used a sample of DNA extracted from a human cancer showing marked microsatellite instability.

RESULTS

Evaluation of the protocol for normal lymphocytes
Two pairs of different-colored signals were detected in

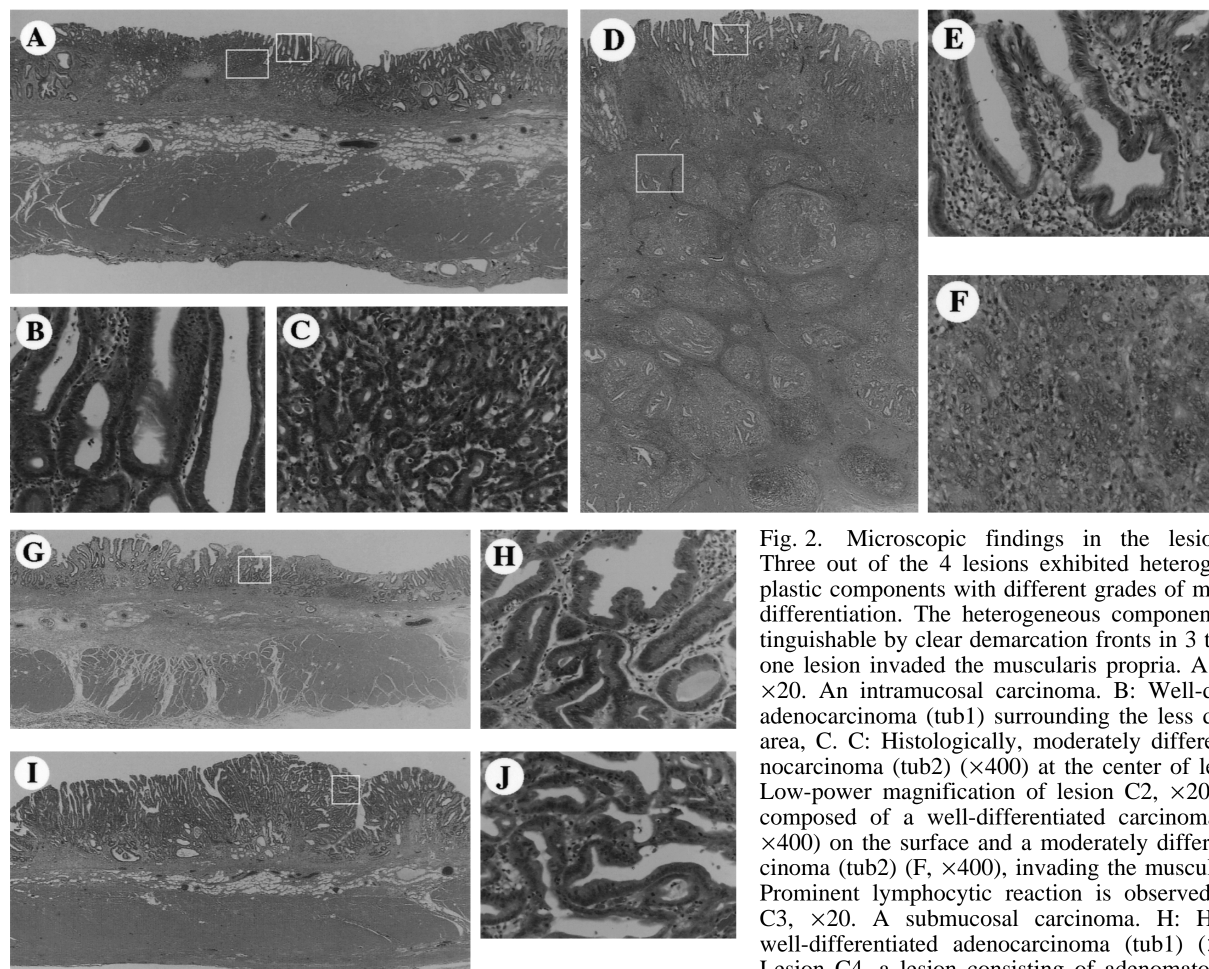

Fig. 2. Microscopic findings in the lesions C1–C4. Three out of the 4 lesions exhibited heterogeneous neoplastic components with different grades of morphological differentiation. The heterogeneous components were distinguishable by clear demarcation fronts in 3 tumors. Only one lesion invaded the muscularis propria. A: Lesion C1, ×20. An intramucosal carcinoma. B: Well-differentiated adenocarcinoma (tub1) surrounding the less differentiated area, C. C: Histologically, moderately differentiated adenocarcinoma (tub2) (×400) at the center of lesion C1. D: Low-power magnification of lesion C2, ×20. Carcinoma composed of a well-differentiated carcinoma (tub1) (E, ×400) on the surface and a moderately differentiated carcinoma (tub2) (F, ×400), invading the muscularis propria. Prominent lymphocytic reaction is observed. G: Lesion C3, ×20. A submucosal carcinoma. H: Histologically well-differentiated adenocarcinoma (tub1) (×400). I, J: Lesion C4, a lesion consisting of adenomatous proliferation (I, ×20) with very well-differentiated carcinoma (pap) adjacent to it (J, ×400).

Table III. Profile of Aberrations of Chromosome Numbers in Multiple Primary Gastric Cancers Having Histological Heterogeneity in Tumors (in Part)

		Centromere ploidy fraction (percentage among 100–200 cells)					
		Histological subtype[a]	1	2	3	4	5
C1	CEP18	tub1	9%	85%	4%	2%	0%
		tub2	4	39	54	3	0
C2	CEP10	tub1	25	70	5	0	0
		tub2	75	22	3	0	0
C2	CEP11	tub1	2	95	3	0	0
		tub2	6	53	35	6	0
C2	CEP16	tub1	33	62	5	0	0
		tub2	79	19	2	0	0

a) Histological subtypes are according to the Japanese Classified System of Gastric Cancer. [C1], [C2]: are the lesions described in Table I. CEP10, 11, 16 and 18 are all commercially available, centromere enumeration probes specific for each chromosome (Vysis). Both lesions C1 and C2 had two components with different histological features and CNA detected by 4 probes, CEP10, 11, 16 and 18, are shown. A complete data set for 4 lesions (7 areas) with 19 different probes is available on request.

almost all of the lymphocytes and normal epithelial cells having intact (almost a full diameter identified) nuclei in the paraffin sections and an example with 4 probes is shown in Table II. This confirmed that the blocks were suitable for the subsequent analysis by FISH with initial, intermittent MW treatment.

Multiple gastric cancers with histological heterogeneity The histological features of the 4 lesions containing 6 heterogeneous areas are shown in Fig. 2, A–F. Fig. 2, A–C are micrographs of lesion C1. This lesion has two components, a moderately differentiated adenocarcinoma (tub2) (Fig. 2B) at the center, surrounded by well-differentiated adenocarcinoma (tub1) (Fig. 2C). Fig. 2, D–F shows advanced carcinoma invading the muscularis propria (lesion C2). Well-differentiated adenocarcinoma (tub1) (Fig. 2E) is seen on the surface of the moderately differentiated adenocarcinoma (tub2) (Fig. 2F). Fig. 2, G and H shows a submucosal carcinoma (lesion C3) with the histological features of a well-differentiated adenocarcinoma (tub1). Fig. 2, I and J shows a mucosal carcinoma (lesion C4) consisting adenomatous proliferation and a very well-differentiated (papillotubular) adenocarcinoma (pap).

Centromere numerical abnormality Marked CNA was found in all of the lesions, with various profiles in terms of the chromosomal specificities. These 4 lesions in the stomach had different profiles of CNA. There were commonly changed chromosomes among the 4 lesions and chromosomes specifically altered in some of them (a complete data set as a supplementary table is available on request). CEP3, 4, 16, 17, and 18 are altered in both C1 and C2, but c-myc and CEPY alterations are merkmals of C3 and C4, respectively. Generally, numerical abnormalities were found in chromosomes 3, 4, 10, 11, 16, 17, 18, c-myc and p53. Gain of chromosomes 3, 4 and 17 appeared most prominently in the early-stage lesion among the 4 tumors. In the early-stage cancer within the submucosal layer, gain of chromosome 18 and c-myc was found, while loss of chromosome 16 was found in all of the tumors. In lesion C2 which invaded the muscularis propria, gain of chromosome 11 and loss of chromosome 10 and p53 were detected. A part of the numerical abnormality profile determined with a total of 20 probes and FISH signals from chromosomes 10, 11, 16 and 18 in the heterogeneous components of lesions C1 and C2 is shown in Table III.

Intratumor morphological heterogeneity as compared with CNA status With the aid of the MW irradiation technique, we could compare exactly matched FISH profiles and histological images, using adjacent or mirror-image paraffin sections (Figs. 3 and 4). As shown in Table III and as mentioned above, a heterogeneous profile of FISH signals from chromosomes 10, 11, 16 and 18 was found in the heterogeneous components of lesions C1 and C2.

In lesion C1, which was a mucosal carcinoma, CEP18 showed diploidy in the well-differentiated carcinoma (tub1) portion, while triploidy of this chromosome was identified in a considerable percentage of the cancer cells in the moderately differentiated carcinoma area (tub2) (Table III). Lesion C2 also showed a heterogeneous profile within the tumor with three specific probes (CEP10, 11, 16); CEP10 and 16 showed diploidy in the well-differentiated adenocarcinoma (tub1), while monosomy was found in the moderately differentiated adenocarcinoma (tub2) (Table III).

Representative FISH images probed with CEP3 (orange) and CEP10 (green) in two portions of lesion C2 and the HE-stained image of a mirror-image section are shown in Fig. 3, A–F. It is clearly evident that the CNA profiles in the areas with different histological features are different. CEP10 detected two signals in the area of the well-differentiated carcinoma (Fig. 3, A–C), while in the moderately differentiated carcinoma in the same tumor, C2, most of the cancer cells had a single signal (loss) of CEP10 (Fig. 3, D–F). As for CEP3, three signals per cancer cell were noted in each component of this heterogeneous tumor C2. On the other hand, triploidy of CEP11 was only found in one portion, in the moderately differentiated carcinoma area, of the tumor C2 (Fig. 4).

Genomic instability DNAs for genomic analysis of all the dissected carcinomas were shown to have altered mononucleotide and dinucleotide repeats in 2 or more loci among 5 loci tested (Table IV). These tumors were considered to be MSI-H.

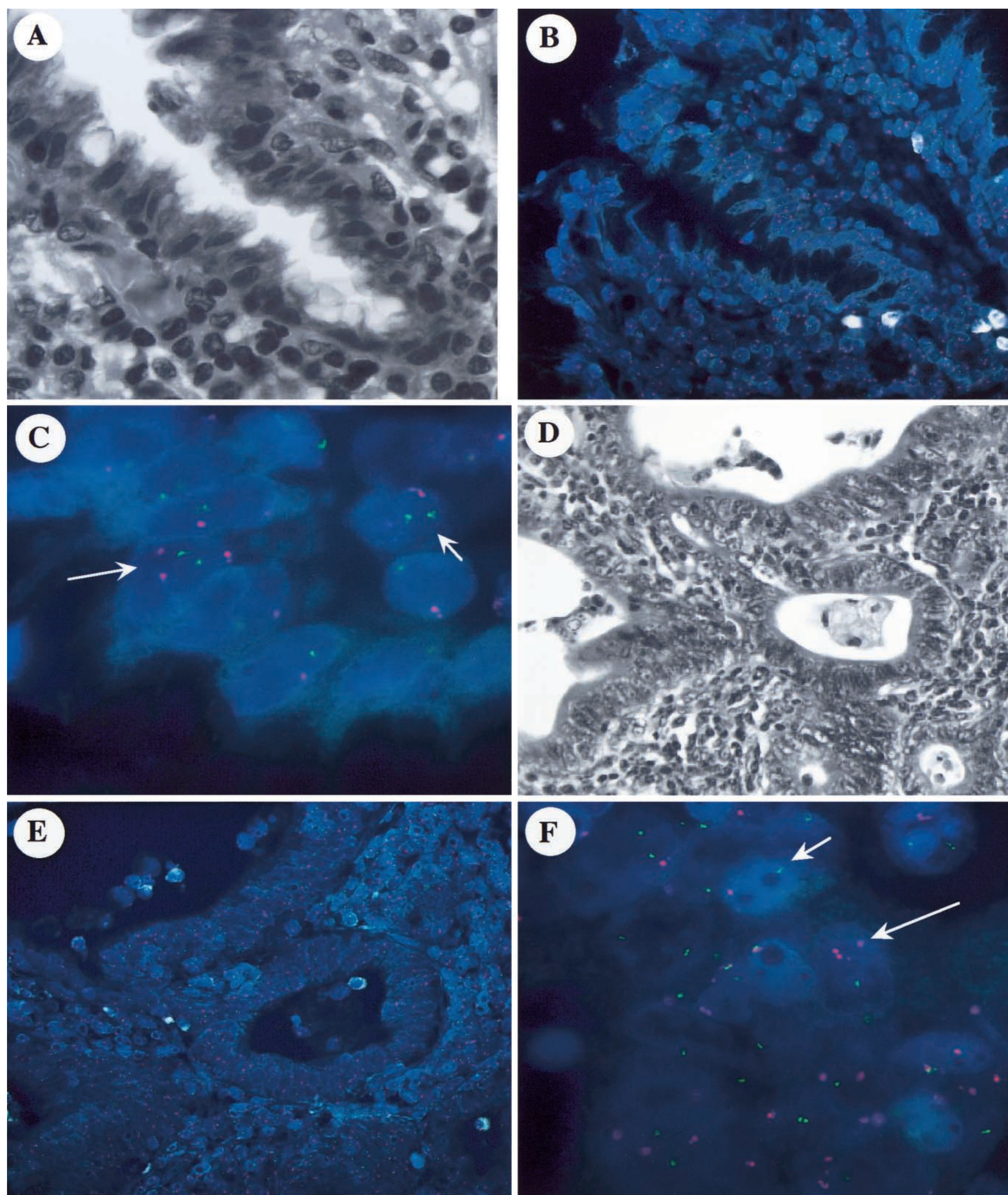

Fig. 3. Fluorescence *in situ* hybridization (FISH) analysis on a formalin-fixed, paraffin-embedded section of carcinoma and adjacent or mirror-image sections by hematoxylin & eosin (HE) staining. A: High-power photomicrograph (×1000) of an HE-stained section in a component of well-differentiated adenocarcinoma of the tumor C2 (×400). B: FISH with CEP3 (orange colored Cy3) and CEP10 (green colored FITC) (Vysis). Equivalent hybridization of the probes covering all the areas is noted. C: Higher magnification of B. Almost all of the nuclei of the cancer cells show two FITC signals (green) for CEP10 (a short arrow) and three Cy 3 signals (orange) for CEP3 (a long arrow). D to F: All three images are from the moderately differentiated adenocarcinoma portion in lesion C2. D (HE stain) and E (FISH) are mirror-image sections. F: Higher magnification of E. A considerable proportion of the nuclei of the cancer cells shows one FITC signal (a short arrow) and three Cy 3 signals (a long arrow) (×1000).

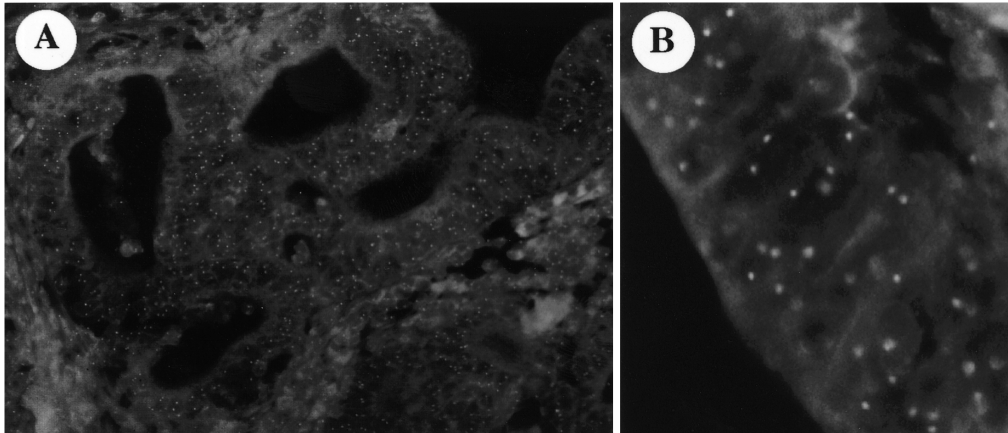

Fig. 4. FISH for CEP11 in lesion C2. A: FISH with CEP11 in the moderately differentiated adenocarcinoma component (tub2) (×400). B: Three FITC signals for chromosome 11 are seen (higher magnification, ×1000), while two FITC signals (photograph not shown) are seen in the well-differentiated adenocarcinoma portion (tub1) of this lesion, as described in Table III.

Table IV. Microsatellite Instability at 5 Loci of Multiple Gastric Carcinoma

Sample		BAT26	BAT25	D5S346	D17S250	D2S123
Non-cancerous tissue		−	−	−	−	−
C1	tub1	+	+	−	+	−
	tub2	+	+	−	−	+
C2	tub1	+	+	−	−	−
	tub2	+	+	−	−	+
C3	tub1	+	+	−	−	+
C4	pap	+	−	+	+	−

+, positive; −, negative.

DISCUSSION

Recently, we successfully obtained clear and reproducible fluorescence signals in FISH with centromeric α-satellite DNA probes in formalin-fixed, paraffin-embedded tissues using a new protocol which included a MW irradiation step.[1] In this study, we analyzed the centromere instability in a case with multiple gastric carcinoma having four synchronous, independent lesions using this technique.

We could confirm the different clonality of these 4 lesions in the stomach by demonstrating the different CNA profile of each lesion (a supplementary table is available on request). This observation implies multiple primary occurrence of these four cancers, though identical profiles would not exclude independent occurrences. Since three of the lesions were intramucosal and the fourth, with invasion of the muscularis propria, did not show any detectable lymph vessel invasion, our chromosomal data are consistent with the preoperative, first-look interpretation of this case, in terms of the tumors being multicentric.

Furthermore, we demonstrated, for the first time, a heterogeneous CNA pattern in a single tumor containing regions with different morphological features, such as the type of tubular structures, e.g. the so-called tub1 and tub2 according to the Japanese Classification System (JCS).[7]

The results of FISH analysis with 2 and more chromosome-specific centromeric α-satellite DNA probes have been reported only recently. Gomyo *et al.*[8] examined 31 gastric carcinoma specimens and detected numerical aberrations of chromosome 17 and the *p53* gene in well-differentiated carcinoma, while no numerical aberrations of these loci were found in the normal mucosa or in regions with intestinal metaplasia. Rao *et al.* also previously reported the numerical change of centromeres in gastric and esophageal adenocarcinomas using a panel of 15 specific centromeric α-satellite DNA probes.[9] Recently, we reported on the hyperploidal progression profile of gastric cancer using 17 centromere FISH probes in various stages of sporadic, solitary gastric carcinoma.[10] All the previous investigations, including ours, used fresh biopsy or resected specimens. In the present study, we examined the CNA profile in multiple gastric cancers in paraffin-embedded archives, by FISH using 18 specific centromeric α-satellite DNA probes and 2 locus-specific probes, in order to elucidate the characteristics of multiple gastric cancer and to compare them with those of sporadic gastric cancer. The newly developed protocol for paraffin archives also facilitated the investigation of possible CNA heterogeneity in morphologically heterogeneous tumor components. In this study, we detected gain of chromosome 17 in all the lesions, mostly in early-stage gastric cancer, but no numerical aberrations were detected with any probes tested here in the adenomatous portion in lesion C4.

Terada *et al.* reported that numerical aberrations of chromosome 17, especially gains, were associated with tumor progression (depth of invasion, lymphatic invasion and venous invasion),[11] and demonstrated that the prevalence of polysomy increased with the depth of cancer invasion, while monosomy was more prevalent in mucosal cancer. Gomyo *et al.*[8] reported that no numerical aberrations of chromosome 17 or mutations in the *p53* gene were detected in either normal mucosa or in regions of intestinal metaplasia. In regard to the numerical aberrations of chromosome 17, our case exhibited them at an earlier stage than that in Terada's series, but this could be a peculiar feature of our case.

According to previous documentation by Kitayama *et al.*, the centromeric number of chromosomes 3 and 18 was exceptionally stable in solitary gastric cancer, even after the tumor had progressed to the advanced stage.[10] In contrast, our case showed gain of chromosomes 3 and 18 from the early stage of gastric cancer.

Loss of chromosome 10 has previously been reported in a cytogenetic study of gastric cancer.[9] Chromosome 10 is also postulated to harbor tumor suppressor genes,[12] but the accumulated data on the various tumors are ambiguous.[13] Amplification of DNA on chromosome 10 has also been reported in gastric cancer.[14] In our case, only one carcinoma, with invasion of the muscularis propria showed loss of chromosome 10, especially in tub2 (JCS).

Further investigation is required to elucidate whether these observations in our case, namely, the early loss of chromosomes 3 and 18, and early gain of chromosome 17, which are different from the CNA profile of gastric cancer generally reported previously, is related to multiplicity or a peculiar genetic and individual background of this subject. Lengauer *et al.*[15, 16] suggested that centromeric instability, represented by temporal changes in CNA, can occur due to mechanisms other than microsatellite instability caused by a mismatched repair deficiency. They further argued that the processes of carcinogenesis and cancer progression can arise through these two distinct pathways. The lesions in which the CNA profile was investigated in this study exhibited microsatellite instability at the same time, which is intriguing.

Concerning the independence in occurrence of cancers in the stomach, neither a different nor a shared profile of CNA would exclusively support or preclude multifocal development. Furthermore, we cannot generalize the reasons for the CNA profile heterogeneity in multiple gastric cancer based on the observations in one case, but this is the first documentation of intratumor heterogeneity of CNA *in situ* and should encourage further study. We have validated the usefulness of the initial intermittent MW irradiation-assisted FISH protocol to characterize carcinogenesis in terms of the progression, multiplicity and histological heterogeneity of gastric cancer.

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