Decrease of Telomeres and Increase of Interstitial Telomeric Sites in Chromosomes of Short-term Cultured Gastric Carcinoma Cells Detected by Fluorescence *In Situ* Hybridization

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Abstract. *Background: Deletion or shortening of chromosomal telomeres is associated with cellular aging and carcinogenesis. Telomeric sites are interstitially located in chromosomes. To clarify the frequency of telomerase abnormalities in cancer and their relationship with any characteristics of gastric carcinomas, telomeric aberrations in eleven cultured specimens of human gastric cancer were investigated by cytogenetic analysis. Materials and Methods: Chromosomal metaphase specimens, obtained by primary culture of cells from surgical specimens of eleven gastric cancer patients, were examined by fluorescent in situ hybridization (FISH) using all telomeric and chromosome 17 specific-telomeric DNA probes. The number of telomeric signals and interstitial telomeric signals (ITS) were counted. DNA ploidy was examined by flow cytometry. Results: The mean telomere signal per nucleus (MTS) observed in peripheral blood lymphocytes (PBLs) of normal volunteers (n=10) was 71.3±3.9%. The MTS in the carcinoma cells (46.9±2.6%) was significantly lower than in PBLs (p<0.01). Although ITS were not observed in PBLs, the mean rate of ITS was 41.2±22.0%, and the mean rate of ITS per chromosome was 2.1±2.1% in the cancer specimens. In DNA aneuploid carcinoma cells, the MTS was significantly lower, the mean rate of ITS tended to be higher (p=0.072), and the mean rate of ITS per chromosome was significantly higher (p<0.05), than in DNA diploid lymphocytes. Histologically, the mean rate of ITS per chromosome in carcinomas with venous infiltrations was significantly greater than in those without (p<0.05). Conclusion: Deletion and interstitial translocation of telomeric loci of chromosomes were frequent alterations in gastric carcinoma cells and increased numbers of interstitial telomeric signals were associated with venous invasion.*

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Gastric carcinoma is one of the most common malignancies in the world. Most gastric carcinomas show various genetic alterations that are associated with carcinogenesis and the process of development of distant metastasis (1-4). In addition to the classic chromosome banding techniques, two promising new cytogenetic analysis methods have been developed: fluorescence *in situ* hybridization (FISH) (5) and comparative genomic hybridization (CGH) (6). Such techniques have provided useful information on cancer genetics. During the past decade, our group has used FISH and CGH techniques to elucidate chromosomal alterations in digestive tract malignancies, including colorectal carcinoma (7-9). Terada *et al.* previously reported the relationships between specific chromosomal alterations and clinicopathological features of gastric carcinoma (9).

Telomeres are the terminal regions in the four arms (short (*p*) and long (*q*)) of chromosomes, which may play an important role in preventing fusions between chromosomes or replication (10). Telomeres consist of repeated sequences of 5-15kb pairs of (TTAGGG)n in humans and are shortened in each cell division cycle and aging until cell death (11). Telomerase, a type of reverse transcriptase containing ribonucleic acid (RNA), has been found to be expressed in cancer cells and induces immortality by telomeric DNA synthesis (12). The expression of telomerase has been used for cancer diagnosis or prediction of patient prognosis in various cancers including gastric carcinoma (13, 14). Interstitial locations in chromosomes of telomeric DNA, interstitial telomeric sequences (ITS), have been

reported in a variety of human cancer cells, which may behave as fragile sites (15). Therefore, the fragility or instability of chromosomes may be induced by telomeric abnormalities; however, the role in cancer development and the clinical significance of ITS have not been thoroughly clarified even at this stage. Although telomeric loss and ITS in human cancer cells have been detected by cytogenetic analysis (16, 17), such analysis of telomeric regions and ITS in gastric carcinoma has yet to be carried out. Sawai *et al.* (18) reported a relationship between chromosome instability and carcinogenesis in colorectal carcinoma by FISH analysis. Therefore, chromosomal instability due to telomerase abnormalities may also be associated with carcinogenesis or tumor progression in carcinomas of the digestive tract. Additional investigations with respect to telomere abnormalities by any analyses, including cytogenetic analysis, will be important.

We hypothesized that the number of deletions of telomeric regions or the existence of ITS may be associated with malignant behavior in gastric carcinoma. To test this hypothesis, telomeric loss and the presence of ITS were examined using FISH in primary cultured cells obtained from surgical specimens taken from eleven gastric cancer patients. The relationships between these chromosomal alterations and DNA ploidy or histological features were also investigated.

Materials and Methods

Patients and surgical specimens. Specimens were obtained from 45 patients with advanced gastric carcinoma who had undergone surgical resection at the Division of Surgical Oncology in the Nagasaki University Graduate School of Biomedical Sciences, Japan, between 1996 and 1998. Advanced gastric carcinoma specimens were prospectively and randomly selected. A representative section of each cancer tissue fixed in 10% formalin was used for hematoxylin and eosin staining for histological diagnosis. Definitions of the histological findings were based on the Japanese Classification of Gastric Carcinoma (19). Fresh cancer tissue from viable sections of the tumor were used for flow cytometric analysis and primary culture of cancer cells. The experimental protocol was approved by the Human Ethics Review Committee of our hospital and a signed consent for examination was obtained from each patient.

Primary culture. As a control sample, human peripheral blood lymphocytes (PBL) were separated by concentration gradient using Lymphoprep (Nycomed Pharma AS, Oslo, Norway) with centrifugation at 1000 rpm. The lymphocytes were cultured for 72 h following stimulation with phytohemagglutinin P (DIFCO Laboratories, Detroit, MI, USA) suspended in RPMI 1640 (Nissui Pharmaceutical, Tokyo, Japan) with 20% fetal bovine serum (Sanko Pharmaceutical, Tokyo, Japan) at 37˚C for 72 h. The cells were later treated with 0.01 μg/mL of colcemid solution (GIBCO Laboratories, Grand Island, NY, USA) for 3 h.

A total of 45 primary short-term cultures were attempted and eleven specimens (22%) were successfully used in the present study. The procedures described above were followed according to the method described by Nanashima *et al.* (20). In brief, 1 cm^3 of fresh tissue sample was washed with saline and transferred into minimum Eagle's medium (MEM; Nissui Pharmaceutical) with antibiotics. The samples were cut into small pieces with a scalpel and treated with an enzyme cocktail containing MEM with 2 mg/mL of collagenase type I (Sigma Chemical, St. Louis, MO, USA) and 10,000 protease units of dispase (Godo Shusei, Tokyo, Japan) in a water bath at 37˚C for 30 min. After washing, $1x10^6$/mL of cancer cells were placed in a collagen-coated flask (Corning, Medfield, MA, USA) and cultured in a humidified incubator at 37˚C with 5% CO_2 for 5 days. To obtain metaphase spreads, the resultant colonies were finally treated with 0.15 μg/mL of colcemid solution for 4 h.

Flow cytometry. The nuclear DNA content of 10,000 nuclei per specimen was determined using a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). Frozen tissues were cut into small pieces and treated with 0.1% Triton X-100 (Sigma). The paraffin-embedded tissue was deparaffinized, rehydrated and isolated by citrate-buffered trypsin using the method of Schutte *et al.* (21). The isolated cells were stained with 50 μg/mL propidium iodide (Sigma). All samples with a DNA index different from one were considered to exhibit DNA aneuploidy.

Fluorescence in situ hybridization (FISH). Fresh single cells with metaphase spreads were treated with hypotonic solution (75 mM KCl) for 30 min and were dropped onto a glass slide. The samples on the glass slide were fixed in a 3:1 mixture of ethanol-acetic acid solution at –20˚C (20). FISH was performed using the method described by Pinkel *et al.* (22). Briefly, the sample on the glass slide was washed using 2 x standard saline citrate (SSC), and the target DNA was denatured with 70% formamide/2 x SSC at 70˚C for 2 min. After dehydration in ethanol, 20 μL of hybridization mixture containing biotinylated DNA probes were added. The DNA probes used were all human telomere probes (Oncor Inc., Gaithersburg, MD, USA). The samples were incubated overnight in a humid chamber at 37˚C to induce hybridization. Hybridized samples were washed using 60% formamide and then conjugated with fluorescein-avidin DCS (green: Vector Laboratories, Burlingame, CA, USA). The nucleus and metaphase chromosomes were counterstained with propidium iodide (red). Fluorescein signals were observed and counted under a fluorescence microscope with a 400 x oil immersion lens (Olympus, Tokyo, Japan) or a confocal laser scanning microscope (LSM 10; Carl Zeiss Co., Oberkochen, Germany).

Evaluation of chromosome abnormality and scoring criteria. The number of FISH spots per nucleus was counted for 20 non-overlapping metaphase spreads in each specimen. Four parameters were evaluated with respect to telomeric signals. (i) First, four telomeric signals should be observed in a normal homologous chromosome (Figure 1a), which were deleted in the chromosomes of cancer cells (Figure 1b). Based on this definition, the incidence of telomeric signals (%) was counted as follow: the observed number of all telomeric signals / the number of all chromosomes x 4 in each metaphase spread. The mean rate of these incidences in 20 metaphase spreads was scored as "mean telomeric signals" (MTS) (%) per specimen. (ii) The rate of metaphase including telomeric signals interstitially located in the chromosome (ITS) (Figure 1c). (iii) The rate of ITS per

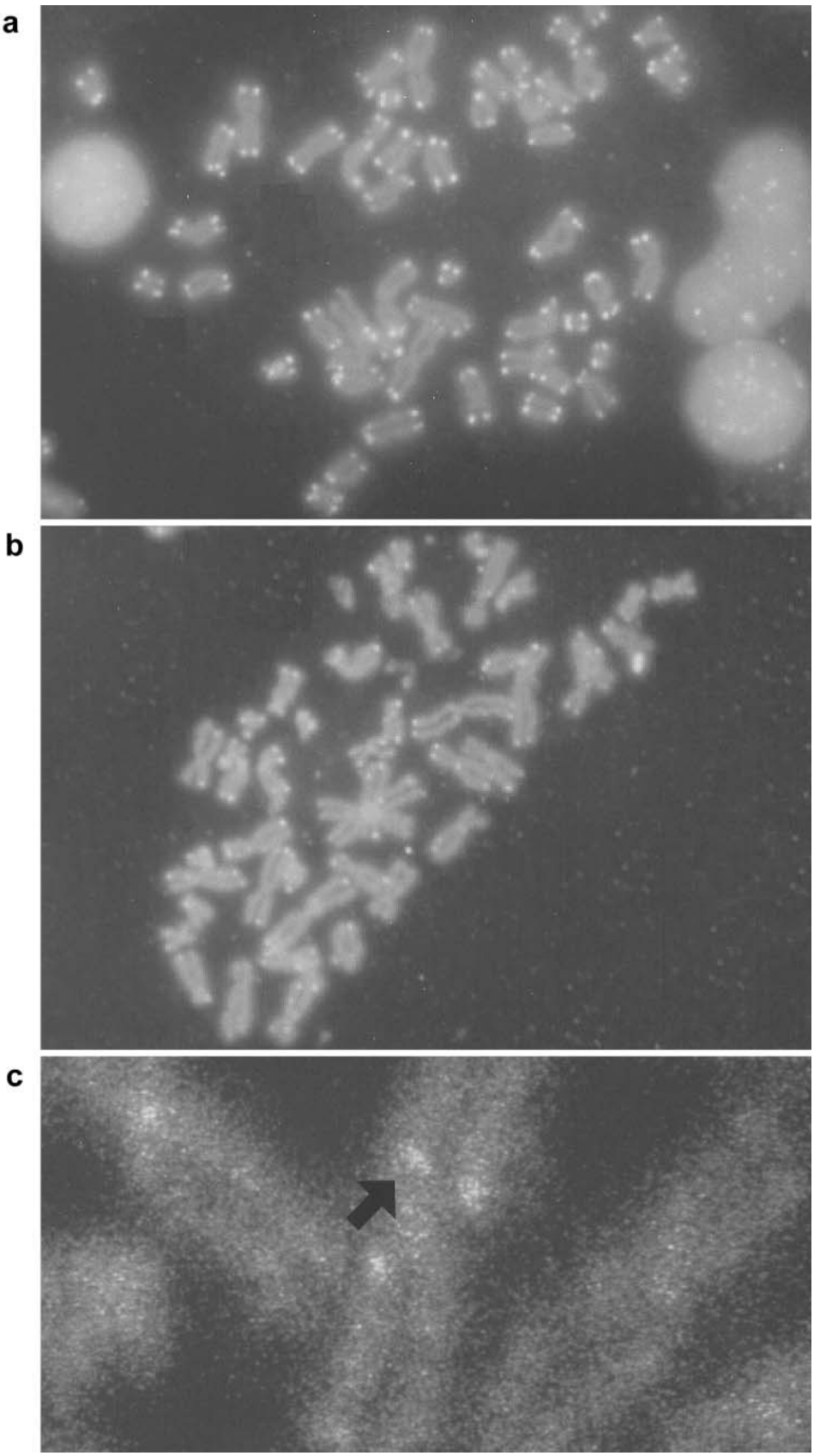

Figure 1. *Telomeric signals. (a) Normal chromosomes showing four telomeric signals on the edge of each chromosome arm; (b) deletion of telomeric signals in carcinoma cells; (c) interstitial telomeric signals in carcinoma cells (arrow).*

Table I. *Number of telomeric signals in peripheral blood lymphocytes of normal volunteers.*

Case	Age	Number of telomeric signals
1	32	74
2	38	75
3	61	68
4	71	75
5	73	66
6	74	70

chromosome was counted as the number of all ITS signals divided by the number of all chromosomes per specimen. Chromosomal aneuploidy was diagnosed in cases where the aneusomal population exceeded 20%.

Statistical analysis. Categorical data were analyzed by Fisher's exact test. Continuous data were expressed as mean ± standard deviation (SD). The data of different groups were compared using one-way analysis of variance (ANOVA) and examined by Student's *t*-test. A two-tailed *p*-value less than 0.05 was considered significant. Statistical analyses were performed using the computer software Stat View-J 5.0 (Abacus Concepts. Inc., Berkeley, CA, USA).

Results

Telomere signals in PBL. The MTS per nucleus observed in PBLs of ten normal volunteers was 71.3±3.9%. The MTS in six adult volunteers (>30 years) are provided in Table I. ITS was not observed in any PBLs.

Telomeric alterations in carcinoma cells. Deleted telomeric signals and ITS were observed. The MTS in carcinoma cells (46.9±2.6%) was significantly lower than in PBLs ($p<0.01$, Table II). The mean rate of ITS in carcinoma cells was 41.2±22.0% and the mean rate of ITS per chromosome was 2.1±2.1% in eleven cancer specimens.

The MTS in DNA aneuploid cells (44.0±3.2%) was significantly lower than in DNA diploid cells (48.3±1.3%, $p=0.042$). The mean rate of ITS (60.0±24.5%) in DNA aneuploid cells was generally greater than in DNA diploid cells (31.9±10.7%; $p=0.073$). The ITS per chromosome (3.8±3.0%) in DNA aneuploid cells were significantly greater than in DNA diploid cells (1.1±0.5%, $p=0.042$).

The significantly positive correlation between the number of chromosomes and the number of ITS ($p<0.05$) is shown in Figure 2.

Relationship between telomeric alterations and clinicohistological features. As seen in Table III, the MTS and the mean rate of ITS were not associated with any clinicohistological features in the eleven gastric carcinomas. The mean rate of ITS per chromosome in patients with venous infiltration of cancer cells (2.4±2.0%) was significantly greater than in patients without infiltration (0.5±0.2%, $p=0.036$).

Table II. *Frequency of telomeric alterations in surgical specimens of gastric cancer patients.*

Case	DNA ploidy pattern	Rate of telomeric signal (%)	Rate of ITS (%)	Rate of ITS per chromosome (%)
1	A	49.0±4.7	90	7.9
2	A	45.0±4.6	60	2.1
3	D	48.1±3.0	20	0.8
4	D	49.1±5.1	30	1.7
5	D	47.3±3.9	40	1.4
6	A	41.0±3.8	60	4.0
7	D	48.8±5.2	40	1.8
8	D	47.5±5.2	30	1.0
9	D	50.2±5.1	30	0.7
10	D	45.9±3.8	20	0.4
11	A	42.0±4.0	40	1.1

ITS: interstitial telomeric signals, D: diploid, A: aneuploid.

Table III. *Clinicopathological features of eleven gastric cancer patients.*

Case	Gender	Age	T	N	Stage	Histology	Ly	V
1	M	61	2	p	IV	tubular*	n	p
2	M	71	3	p	IV	poorly*	n	p
3	M	57	3	p	IV	tubular*	n	n
4	M	61	3	p	IV	poorly*	p	p
5	M	61	3	p	IV	poorly*	p	p
6	F	62	4	p	IV	–	–	–
7	M	34	3	p	IV	poorly*	p	p
8	M	34	3	p	IV	poorly*	p	p
9	F	70	2	n	II	tubular*	n	n
10	M	68	2	p	II	signet§	n	n
11	M	69	2	n	II	tubular*	n	p

M: male, F: female, T: tumor classification, N: node classification, Ly: lymphatic infiltration, V: venous infiltration, p: positive, n: negative.
*adenocarcinoma, §signet-ring cell carcinoma.
Stage: Japanese Classification of Gastric Carcinoma (19).

Discussion

Although loss of heterozygosity (LOH) of telomeric sites, telomere shortening or telomerase in human cancer cells have been reported previously using Southern blot analysis or other methods (8, 23-25), cytogenetic analyses using FISH or CGH and related studies using resected specimens are limited (16, 17, 26). In the present study, cells were successfully cultured for a short period and sufficient metaphase spreads prepared for analysis from the surgically

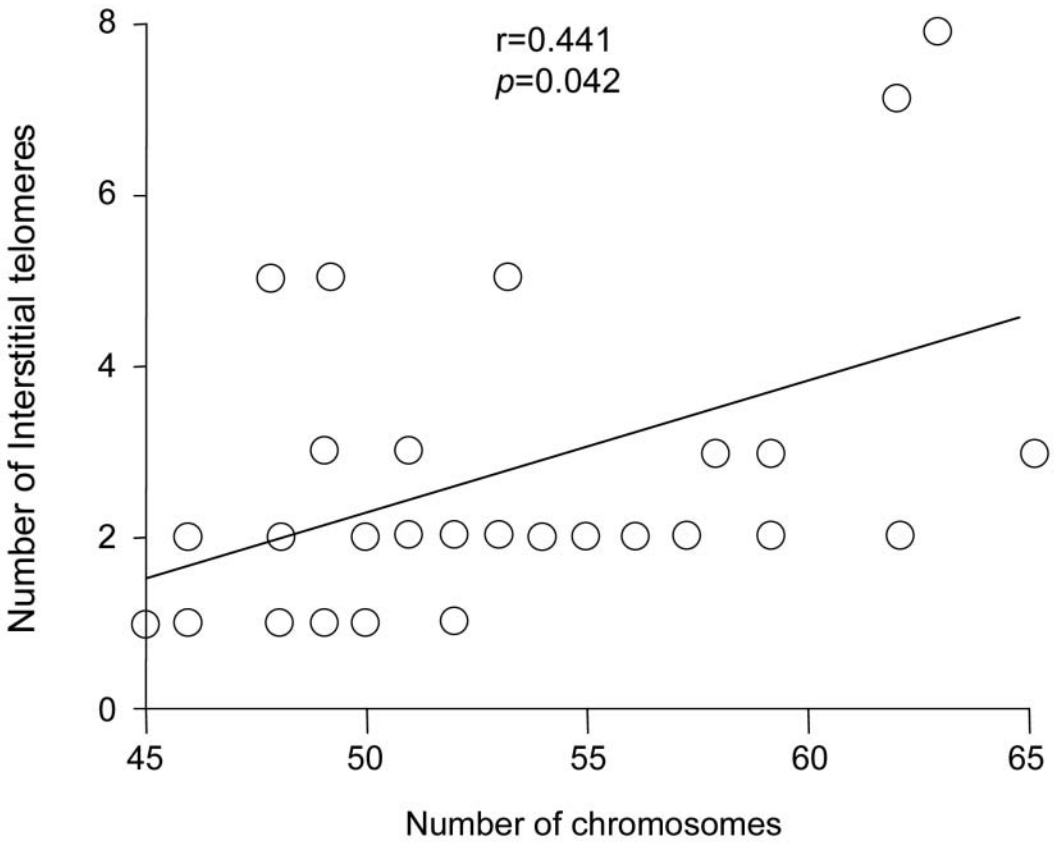

Figure 2. *Correlation between total chromosome number and the numbers of interstitial telomeric signals (ITS) per nucleus.*

resected specimens. Nanashima *et al.* (20, 27) previously reported FISH analysis in cultured colonic carcinomas and sufficient metaphase spreads were obtained from the main tumors in 63% of specimens. However, it was difficult to prepare such samples from primary gastric tumors in the present study. Therefore, specimens were obtained from tumors of peritoneal dissemination in five out of eleven patients (46%). If chromosomal heterogeneity between primary and metastatic tumors existed (28), it was possible that the present results did not sufficiently reflect the characteristics of the primary tumors. Using the present samples, it might have been difficult to perform karyotyping by the conventional banding method. However, the FISH method enabled the demonstration of numerous targeted and structured chromosome aberrations in such samples, because it was easy to visually show these aberrations using fluorescence (26).

In the present study, normal lymphocytes were examined as a control, as in our previous reports (7-9, 20). Theoretically, the terminal regions of all normal chromosomes should show telomeric signals (10, 11). However, approximately 30% of chromosomes in each normal volunteer did not show telomeric signals. If this result was not due to the technical limitations of FISH analysis using telomeric probes, telomere shortening might have occurred even in normal lymphocytes. Telomere shortening according to aging has previously been reported (10-12). However, there were no significant differences between young adults and elderly adults in the present study. Telomeric shortening in normal cells might thus be suppressed at some ranges in adults for cellular survival.

Here, the telomeric signals in gastric cancer cells were significantly decreased, an observation in agreement with previous reports (16, 17, 26). A case with interstitial telomeres detected by FISH was reported by Lin *et al.* (29) in 1990 and was considered as a tandem chromosome fusion or amplification of repeated arrangements of TTAGGG in chromosomes. Wiley *et al.* (30) reported that ITS resulted from chromosomal crossing or deletion and unusual amplifications. Furthermore, intra-chromosomal telomeric sites proved to be fragile sites in a study of exposure to irradiation (31). Therefore, interstitial telomeres might result from repair of fragile sites (30, 32). However, a function or significance of interstitial telomeres has yet to be fully clarified. In the present study, ITS were frequently observed in most specimens. The rate of ITS per

chromosome ranged between 0.4 and 7.9%. By comparison with the pattern of DNA ploidy, cancer cells with aneuploidy showed a decrease in telomeric signals and a higher rate of ITS. Previous reports showed that various DNA aneuploid cancer cells exhibited higher rates of chromosomal numerical aberrations (17, 33). It was shown that telomeric aberrations were also increased in DNA aneuploid cells, similar to the present results. ITS correlated with increased chromosome numbers. Therefore, telomere shortening and interstitial telomeres might be associated with chromosomal instability. Since chromosomal instability is closely associated with carcinogenesis (34), telomeric aberrations could also be related to carcinogenesis.

Although our samples were limited to eleven cases due to the difficulties associated with primary culture, the relationship between telomeric aberrations and some histological features was examined. Some chromosomal aberrations were closely associated with the histological features of gastric carcinomas in previous reports (2, 3, 35, 36). LOH at chromosome 17p was associated with poor differentiation (3). LOH at 17p13.3 including telomeric sites was also associated with histological findings in the mammary gland (25). However, Noguchi *et al.* (37) reported that any frequent chromosomal aberrations detected by CGH analysis were not related to any histological findings in gastric carcinoma. In gastric carcinoma, the expression of telomerase is considered an early event in cancer development because it is frequently observed in precancerous regions and early stage carcinoma (38). The relationship between the numerical aberrations of telomeric sites and clinicopathological features in gastric carcinomas has yet to be clarified. As precancerous adenomas or early stage carcinomas could not be cultured and many samples were stage IV carcinomas, the present study could not provide information as to whether or not telomeric aberrations were early events. Only the rate of ITS per chromosome was related to venous infiltration of cancer cells. To clarify the role or clinical significance of decreases of telomeric signals and increases of ITS, further examination of larger numbers of gastric cancer specimens will be necessary. As it is difficult to assemble a large number of samples by culture of cancer cells at this stage, analyses of interphase nuclei using dual color FISH (*i.e.,* telomere probe and centromere probe) should be performed as the next step (39).

In conclusion, numerical aberrations of telomeric regions in eleven cultured specimens of gastric cancer were investigated by FISH analysis. The MTS in normal lymphocytes in adults was approximately 70%. The MTS was significantly decreased and ITS were observed in carcinoma cells. In DNA aneuploid cancer cells, the MTS were still lower and the increase of ITS was higher than in DNA diploid cancer cells. The increase of ITS was associated with venous infiltration of the cancer cells. Deletions of telomere and interstitial telomeric loci were frequent alterations in gastric carcinoma cells; further cytogenetic analyses in a larger number of samples will be necessary to clarify the functional significance of these observations.

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