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# Numerical Aberrations of Chromosomes 8, 11, 12, 17, X, and Y on Esophageal Squamous Cell Carcinomas by Fluorescence in situ Hybridization

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Numerical aberrations of chromosomes 8, 11, 12, 17, X, and Y were investigated on fifteen cases of esophageal squamous cell carcinoma using fluorescence in situ hybridization (: FISH) with chromosome specific DNA probes. There were various aberrations in autosomal chromosomes. Trisomy 12 and trisomy 17 were the most common numerical aberrations (found in six cases, respectively), followed by trisomy 11 and monosomy 17 (in five cases, respectively), trisomy 8 (in four cases), tetrasomy 8 and monosomy 11 (in three cases, respectively). In regard to sex chromosomes, all cases except for one showed extra copy number of X chromosome, two signals were found in male and three signals were encountered in female. Loss of Y was found in six cases and gain of Y was shown in two cases. Quantification of nuclear DNA content by flow cytometry was performed using the same materials. Three of fifteen (20%) revealed DNA diploidy on DNA histogram, but several numerical aberrations were found in all DNA diploid cases by FISH. Comparing the results with clinicopathologic parameters, there is a good correlation between the number of chromosome 8 and lymph node metastasis ( $p = 0.0089$ ). Numerical chromosome aberrations of esophageal cancer can be detected easily in preoperative status using endoscopic biopsy specimens. FISH analysis correlates with the extent of disease and may be helpful to determine methods of surgical procedure for the patients with esophageal squamous cell carcinoma.

## INTRODUCTION

The prognosis of patients with esophageal carcinoma is poor even if surgical resection is followed by chemotherapy and radiation therapy<sup>1)</sup>. To permit more effective treatment and to determine the etiology, more study concerning the biological characteristics of this aggressive cancer is necessary. In the current study, loss of heterozygosity was observed for regions of 17p<sup>2)</sup> and for the p53<sup>3)</sup>, RB1 (: retinoblastoma)<sup>4)</sup>, APC (: adenomatous polyposis coli)<sup>5)</sup>, and MCC (: mutated in colon cancer)<sup>6)</sup> genetic loci in esophageal cancer. Chromosomal aberrations may reflect oncogenic changes and may relate to tumor progression

and aggressiveness. However, very few cytogenetic studies on esophageal cancer have been reported because of difficulties in obtaining good metaphase spreads<sup>6-9)</sup>. Recently, technological advances in the field of molecular cytogenetics have made it possible to detect chromosomal aberrations in interphase cells by using specific DNA probes for different centromeric regions together with fluorescence in situ hybridization (: FISH)<sup>10-12)</sup>. In this study, numerical aberrations of chromosomes 8, 11, 12, 17, X, and Y were analyzed on fifteen cases of esophageal cancers by FISH using chromosome specific repetitive DNA probes. As a result, a close correlation was shown between numerical chromosome aberrations and clinicopathologic features in esophageal cancer. In addition, this paper showed that preoperative determination of numerical chromosome aberrations was feasible by using endoscopic biopsy specimens for carcinomas of the esophagus.

## MATERIALS AND METHODS

### Materials:

Pathological specimens for this study were obtained from patients who underwent surgical resection at the First Department of Surgery in Nagasaki University School of Medicine from 1989 to 1990. None of the patients had received neither chemotherapy nor radiation therapy until surgical resection was performed. All patients underwent a subtotal esophagectomy with regional lymph node dissection. Relationship between numerical chromosome aberrations and clinicopathologic parameters (patient's age, sex, tumor site, grade, depth of microscopic invasion, lymph node status, histologic stage, and tumor size) was analyzed.

### Sample preparation and flow cytometric analysis:

50  $\mu$ m sections from formalin-fixed paraffin-embedded tissue were deparaffinized with xylene for 60 min. They were rehydrated through an ethanol series and then incubated overnight with citrate buffer trypsin. After

centrifugation the pellets were resuspended in 0.3 ml DW 3 and a half of nuclei were refixed by addition of 0.7 ml cold ethanol on vortex (final concentration: 70 %). These nuclei were stored at  $-20^{\circ}\text{C}$  before FISH. The remainder was centrifuged and nuclear DNA was stained by Vindelov's method. The fluorescence of the propidium iodide stained nuclei was analyzed in a FACS can (Becton Dickinson) and displayed as histograms of DNA content per nucleus versus number of nuclei.

#### DNA probes:

Cloned centromeric alpha satellite DNAs from chromosome 8 (D8Z1), 11 (pLC11A), 12 (pSP12-1), 17 (p17H8), X (pBamX7), and Y (DYZ1) were used in this study. These probes were labeled with biotin-11-dUTP by standard nick translation method.

#### Fluorescence in situ hybridization :

The cells were dropped on poly-L-lysine coated slides and air-dried. The slides were incubated with 0.1 % pepsin (Sigma Co)/0.1N HCl for 10 minutes at  $37^{\circ}\text{C}$  to improve target accessibility. After washing with PBS (-), the slides were immersed in 70% acetic acid for 30 seconds at room temperature to remove residual cytoplasm. After washing and dehydration, the slides were immersed for 10 minutes in 0.25 % acetic anhydride/Tris HCl to block any residual aldehyde groups. After washing twice in  $2\times\text{SSC}$  (: 0.3 M NaCl, 30 mM sodium citrate, pH 7.0), the slides were denatured for 2 minutes at  $70^{\circ}\text{C}$  formamide,  $2\times\text{SSC}$ . Formamide was deionized by treatment with a mixed-bed ion-exchange resin. The slides were dehydrated and air-dried before applying the probe. The probe was denatured for 10 minutes at  $70^{\circ}\text{C}$  in the hybridization mixture (containing 0.5  $\mu\text{g}/\text{ml}$  DNA probe, 10 % dextran sulphate,  $2\times\text{SSC}$  and 500  $\mu\text{g}/\text{ml}$  herring sperm DNA in 50 % formamide). The hybridization mixture (20  $\mu\text{l}$ ) was put under a  $24\times 24$  mm glass coverslip and placed in a humidified chamber.

After overnight hybridization at  $37^{\circ}\text{C}$  the slides were washed twice for 10 minutes in  $2\times\text{SSC}$  and subsequently once for 10 minutes in 60 % formamide,  $2\times\text{SSC}$  at  $45^{\circ}\text{C}$ . The slides were incubated with 5  $\mu\text{g}/\text{ml}$  FITC conjugated avidin DCS (Vector Labo. Inc.) in BST buffer (: 2 % BSA,  $4\times\text{SSC}$ , and 0.1 % Tween 20) for 30 minutes at  $37^{\circ}\text{C}$  and washed in 4SSCT (:  $4\times\text{SSC}$ , 0.1 % Tween 20). If necessary, the probe-linked fluorescence was amplified by additional treatment with 5  $\mu\text{g}/\text{ml}$  biotin-labeled goat-anti-avidin antibody (Vector Labo, Inc.) in BST buffer and 5  $\mu\text{g}/\text{ml}$  FITC avidin DCS. After washing in 4SSCT, the nuclei were counterstained with 1  $\mu\text{g}/\text{ml}$  propidium iodide (Sigma Co) in an antifade solution containing p-phenylenediamine dihydrochloride.

#### FISH using endoscopic biopsy specimens:

Using flow cytometric quantification, nuclear DNA

contents from endoscopic biopsy specimen were in accordance with those from paraffin-embedded tissue<sup>13)</sup>. To ascertain whether endoscopic biopsy specimens are suitable to FISH for preoperative detection of numerical chromosome aberrations, FISH was also applied to tumor implants from biopsy specimens (case 3 and case 12). The same manner was performed for FISH, but pretreatment was modified. Briefly, the slides were incubated with 0.01 % pepsin/0.2N HCl instead of 0.1 % pepsin/0.1 N HCl and 70 % acetic acid.

#### Evaluation:

Hybridization results were analysed using fluorescence microscopy (BH-2; Olympus). The number of hybridization signals were counted in 100-200 interphase nuclei from each slides. Results were interpreted as significant for polysomy when greater than 15 % of the nuclei had more signals than diploid for a specific chromosome probe, while determination of hypodiploidy was based on finding that more than 20 % of the cells had fewer signals than expected for a diploid cell.

Fisher's exact probability test was used for statistical analysis between the numerical chromosome aberrations and clinicopathologic parameters. Differences causing P values less than 0.05 were accepted as statistically significant.

## RESULTS

#### Clinicopathologic parameters:

Clinicopathologic data of the fifteen patients are summarized in Table 1<sup>14)</sup>. All tumors were squamous cell carcinomas. The mean patients age was 64.2 years (range, 43-78 years), there were two women and thirteen men. Lymph node metastasis was found in eight patients (53.3 %). There were no cases with distant metastasis.

#### Interphase cytogenetics:

Numerical chromosome aberrations were easily detectable using biopsy specimens. Detail of nuclear DNA contents and numerical chromosome aberrations were shown in Table 2. Numerical aberrations of chromosome 8 were found in nine cases, monosomy in two, trisomy in four, and tetrasomy in three cases. In six of seven cases (86 %) with gain of chromosome 8 had no lymph node metastasis. By contrast, in seven of eight cases (88 %) without gain of chromosome 8 had several positive lymph node involvement. There was a good correlation between copy number of chromosome 8 had lymph node status in patients with esophageal cancer ( $p = 0.0089$ ). Of six cases which did not invade to adventitia (mp), all three cases with gain of chromosome 8 had no lymph node involvement and all three cases without gain had several lymph node metastases. The copy number of chromosome 8 did

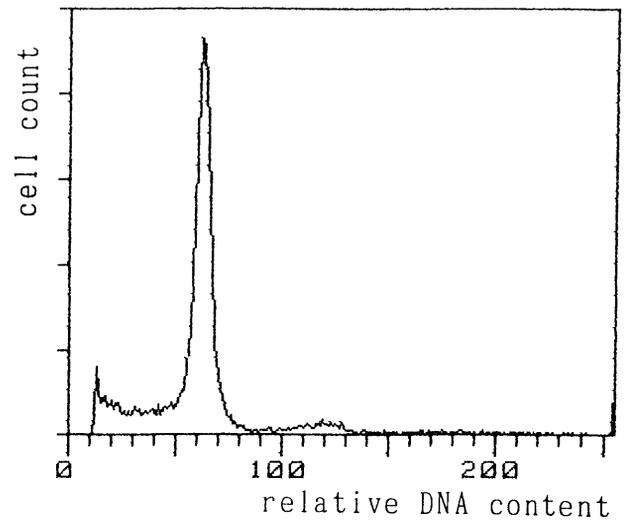
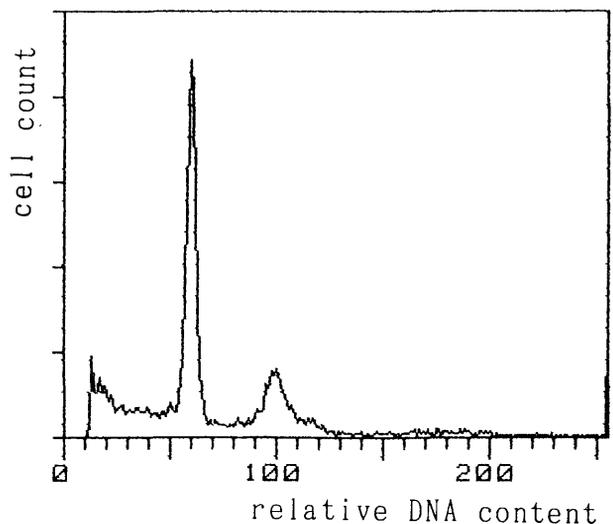
**Table 1.** Clinicopathologic features of 15 cases of esophageal squamous cell carcinomas.

Characteristic	No.(%) of patients
All patients	15
Sex	
Male	13 (87%)
Female	2 (13%)
Age	
<65 yr	7 (47%)
≥65 yr	8 (53%)
Tumor location	
Ce/Iu	5 (33%)
Im	7 (47%)
Ei	3 (20%)
Grade	
well	1 (7%)
moderate	10 (67%)
poor	4 (27%)
Depth of invasion	
mp	6 (40%)
≥ a <sub>1</sub>	9 (60%)
Lymph node status	
negative	7 (47%)
positive	8 (53%)
Histological stage	
stage I / II	7 (47%)
stage III / IV	8 (53%)

**Table 2.** Detail of clinicopathologic parameters, DNA contents, and numerical chromosome aberrations in 15 cases of esophageal squamous cell carcinomas.

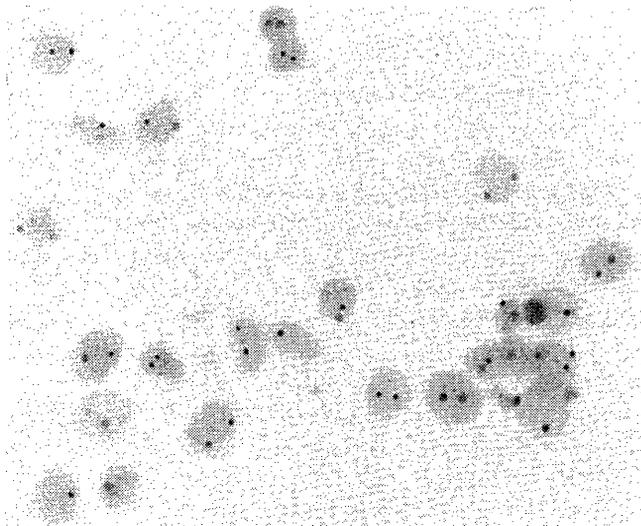
Pt.	site	size (mm)	grade	stage	DI	(copy number)							
						8	11	12	17	X	Y		
1.	50M	Im	30	poor	mpn <sub>0</sub> M <sub>0</sub> PI <sub>0</sub> , I	1.00	3	3	3	3	1	2	2
2.	66M	Im	45	mode	mpn <sub>0</sub> M <sub>0</sub> PI <sub>0</sub> , I	1.64	4	3	3	3	2	0	
3.	77M	Iu	40	mode	mpn <sub>0</sub> M <sub>0</sub> PI <sub>0</sub> , I	1.66	3	-	-	3	-	-	
4.	78F	Iu	29	mode	mpn <sub>0</sub> M <sub>0</sub> PI <sub>0</sub> , II	1.83	1	2	2	2	3	-	
5.	63M	Iu	27	mode	mpn <sub>0</sub> M <sub>0</sub> PI <sub>0</sub> , II	1.92	2	1	2	1	2	1	
6.	71M	Ei	35	mode	mpn <sub>0</sub> M <sub>0</sub> PI <sub>0</sub> , II	1.60	2	2	2	1	2	1	
7.	76M	Iu	42	mode	a <sub>1</sub> n <sub>0</sub> M <sub>0</sub> PI <sub>0</sub> , II	1.63	4	1	3	1	2	0	
8.	71M	Ei	71	mode	a <sub>2</sub> n <sub>0</sub> M <sub>0</sub> PI <sub>0</sub> , III	1.64	4	2	2	3	2	-	
9.	43M	Ei	60	poor	a <sub>2</sub> n <sub>0</sub> M <sub>0</sub> PI <sub>0</sub> , III	1.00	2	2	2	1	2	1	
10.	64M	Im	43	mode	a <sub>2</sub> n <sub>0</sub> M <sub>0</sub> PI <sub>0</sub> , III	1.66	3	3	3	3	2	0	
11.	56M	Im	45	mode	a <sub>2</sub> n <sub>2</sub> M <sub>0</sub> PI <sub>0</sub> , III	1.47	2	4	3	3	2	0	
12.	51F	Im	115	well	a <sub>2</sub> n <sub>2</sub> M <sub>0</sub> PI <sub>0</sub> , III	1.00	1	-	-	2	-	-	
13.	73M	Ce	80	poor	a <sub>3</sub> n <sub>2</sub> M <sub>0</sub> PI <sub>0</sub> , IV	1.67	2	1	3	3	2	0	
14.	65M	Im	65	poor	a <sub>2</sub> n <sub>3</sub> M <sub>0</sub> PI <sub>0</sub> , IV	1.48	2	3	-	2	2	0	
15.	59M	Im	65	mode	a <sub>2</sub> n <sub>4</sub> M <sub>0</sub> PI <sub>0</sub> , IV	1.38	3	3	1	2	1	2	

M: male, F: female, Ce: cervical esophagus, Iu: upper intra-thoracic esophagus, Im: middle intra-thoracic esophagus, Ei: lower intrathoracic esophagus, well: well differentiated, mode: moderately differentiated, mp: muscularis propria, a<sub>1</sub>: invasion reaching the adventitia, a<sub>2</sub>: definite invasion, a<sub>3</sub>: invasion into the neighboring structures, n<sub>0</sub>: no lymph node metastasis, n<sub>1</sub>: metastasis to group 1, n<sub>2</sub>: metastasis to group 2, n<sub>3</sub>: metastasis to group 3, n<sub>4</sub>: metastasis to group 4, M<sub>0</sub>: no distant metastasis, PI<sub>0</sub>: no pleural dissemination, DI: DNA index

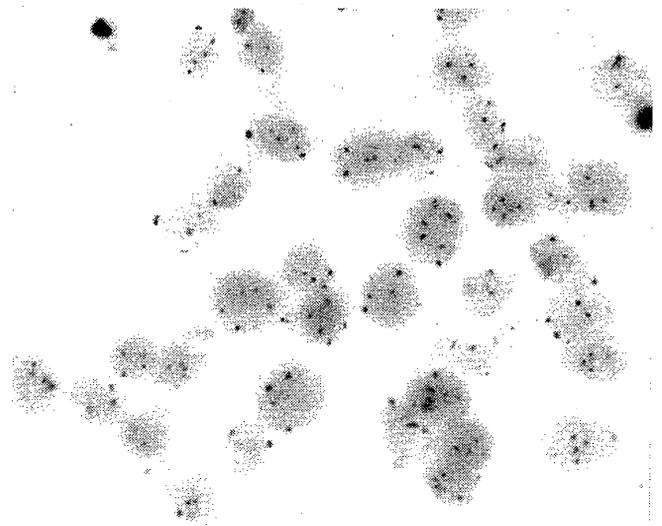
**Fig. 1** Flow cytometric DNA histogram of case 1 reveals DNA diploid pattern (DI = 1.00), the coefficient of variation (CV) is 6.2.**Fig. 2** Flow cytometric DNA histogram of case 13 shows DNA aneuploid pattern (DI = 1.67), the CV is 4.1.

not show a significant correlation with the other clinicopathologic parameters such as depth of microscopic invasion, distant metastasis, and histological stage.

There were nine cases with abnormal copy number of chromosome 11, three cases of monosomy, five of trisomy, and one of tetrasomy. As for chromosome 12, there were six cases with abnormal copy number, one was monosomy and remaining five were trisomy. No relationship were found between chromosome 11 and 12 copy number and clinicopathologic parameters.



**Fig. 3** Fluorescence microscopic findings of FISH with chromosome 17 specific DNA probe in case 15, 81 % of the interphase nuclei exhibit two signals.



**Fig. 4** Fluorescence microscopic findings of FISH with chromosome 11 specific DNA probe in case 11, 29 % of the interphase nuclei show four signals, indicated as a tetrasomy.

**Table 3.** Correlation with copy number of chromosome 8 and extension of esophageal squamous cell carcinoma.

Parameters	No. (%) of patients with monosomy 8	No. (%) of patients with gain of # 8
Depth of invasion		
mp	1/6 (17%)	3/6 (50%)
≥ a <sub>1</sub>	1/9 (11%)	4/9 (44%)
Lymph node status		
negative	0/7 (0%)	6/7 (86%)
positive	2/8 (25%)	1/8 (13%)
Histological stage		
stage I / II	1/7 (14%)	4/7 (57%)
stage III / IV	1/8 (13%)	3/8 (38%)

\*\* p = 0.0089

**Table 5.** Correlation with copy number of chromosome 17 and extension of esophageal squamous cell carcinoma.

Parameters	No. (%) of patients with monosomy 17	No. (%) of patients with gain of # 17
Depth of invasion		
mp	3/6 (50%)	2/6 (33%)
≥ a <sub>1</sub>	2/9 (22%)	4/9 (44%)
Lymph node status		
negative	3/7 (43%)	4/7 (57%)
positive	2/8 (25%)	2/8 (25%)
Histological stage		
stage I / II	4/7 (57%)	2/7 (29%)
stage III / IV	1/8 (13%)	4/8 (50%)

\*\* p = 0.1002

**Table 4.** Correlation with gain of # 8 and lymph node status in mp-cases.

Lymph node status	No. (%) of patients
negative	3/3 (100%)
positive	0/3 (0%)

\*\* p = 0.05

**Table 6.** Correlation with copy number of chromosome Y and extension of esophageal squamous cell carcinoma.

Parameters	No. (%) of patients with monosomy # Y	No. (%) of patients with gain of # Y
Depth of invasion		
mp	1/4 (25%)	1/4 (25%)
≥ a <sub>1</sub>	5/7 (71%)	1/7 (14%)
Lymph node status		
negative	3/5 (60%)	1/5 (20%)
positive	3/6 (50%)	1/6 (17%)
Histological stage		
stage I / II	2/5 (40%)	1/5 (20%)
stage III / IV	4/6 (67%)	1/6 (17%)

\*\* p = 0.1970

Numerical aberrations of chromosome 17 were found in eleven cases (73%), monosomy in five and trisomy in six. Monosomy 17 was seen in four cases (57%) of stage I and II disease, but only in one case (13%) of stage III and IV. Four cases (50%) of eight stage III/IV disease showed trisomy 17, but the difference was not statistically significant. There were no correlation with number of chromosome 17 and the other clinicopathologic parameters such as grade, lymph node status, and depth of microscopic invasion.

Gain of chromosome X was seen in all cases except for one case. Two signals were countable in male cases and three signals were encountered in female cases.

Loss of chromosome Y was seen in six cases (55%) and disomy Y in two cases (18%). In four cases without invasion to the adventitia ( $a_0$ ), only one case (25%) revealed loss of Y. By contrast, loss of Y was shown in five of seven cases (71%) which had invasion to the adventitia, but the difference was not statistically significant. The number of chromosome Y did not show a significant correlation with the other clinicopathologic parameters.

Comparison with interphase cytogenetics and DNA content:

The DNA indices ranged from 1.00-1.92. The frequency of DNA aneuploidy was 80% of the thirteen cases, and only three cases (20%) revealed DNA diploidy. Two of three DNA diploid tumors had no lymph node metastases. Three cases with DNA aneuploidy by flow cytometry did not accompany any extra copy number in a set of six chromosomes except for X chromosome. In all these three patients, several positive lymph nodes were involved.

## DISCUSSION

Cytogenetic studies using karyotyping on the basis of chromosome banding techniques have shown several characteristic abnormalities in human neoplasms, but it is still uncertain whether the chromosomal aberration in solid tumors is correlated as closely with clinicopathologic features as it is in hematologic malignancies. The interpretation of the chromosome banding patterns is often difficult due to the small number of recognizable metaphases, minimal chromosome spreading, poor banding quality, and condensed or fuzzy appearance of the chromosomes<sup>15</sup>. Technological advances in the field of molecular cytogenetics have made it possible to detect chromosomal aberrations in interphase cells by using specific DNA probes for different centromeric regions together with fluorescence in situ hybridization<sup>10-12</sup>. Therefore, culturing of tumor cells is not required and there is no risk of selection of a certain highly proliferative subpopulation of tumor cells. Its application for interphase cytogenetic analysis has increased in recent years because of those

advantages.

There are a few reports of FISH applied to gastrointestinal malignancies. In gastric cancer, Dekken et al.<sup>16</sup> showed a good correlation between FISH results and the acridine orange DNA histograms, and reported that six of eight male patients revealed loss of Y chromosome. They suggested that loss of Y may be a tumor specific abnormality. Yamaguchi<sup>17</sup> reported that gastric cancers with a gain in chromosome 7 and/or 17 were often accompanied with a high level of metastasis in lymph nodes and/or distant metastasis. In colorectal cancers, numerical chromosome aberrations were also found in DNA diploid tumors<sup>18</sup> and monosomy 11 was frequent in early stage<sup>19</sup>. On the other hand, in 23 cases of Astler-Coller stage C colon cancers, a good correlation was indicated between chromosome copy number and tumor proliferative activity (S-phase fraction) and DNA ploidy<sup>20</sup>. Previous study on proliferative cell nuclear antigen (: PCNA) expressions and numerical chromosome aberrations had also demonstrated that colorectal carcinomas with high PCNA labeling index showed a tendency of trisomy 17<sup>21</sup>.

In this study, monosomy 17 was frequently found in stage I/II esophageal cancer, and trisomy 17 in stage III/IV. The cells with trisomy 17 fail to derive from monosomy 17 cell even if mitotic nondisjunction or endoreduplication occurs to monosomy 17 cells. Therefore, it was taken into consideration that trisomy 17 cells were minor subpopulation in early disease. However, these showed a potent proliferative activity than the other subpopulations, escaping from cell death. This explanation contradicts my estimate that subpopulation with monosomy 17 is predominant in late stage due to allelic loss of p53/nm23 tumor/metastasis suppressor gene on chromosome 17. Interestingly, the same findings were also seen in gastric cancer and colorectal cancer. One possible interpretation is that extra copies of chromosome 17 may result in a significantly selective growth advantage due to high expression of the c-erbB-2 gene coded on the long arm of chromosome 17 (17q).

Trisomy 8 is a common numerical aberration found in hematological disorders<sup>22</sup>, Ewing's sarcoma<sup>23</sup>, myxoid liposarcoma<sup>24</sup>, pleomorphic adenoma of the parotid gland<sup>25</sup>, uveal melanoma<sup>26,27</sup>, benign nevi<sup>28</sup>, colon adenoma<sup>29</sup>, breast cancer<sup>30</sup>, and benign mature teratoma<sup>31</sup>. A number of oncogenes have been mapped to 8q, including *mos* and *c-myc*. In chronic and acute myeloid leukemias, it is well known that a trisomy 8 is indicative of poor prognosis. Furthermore, it was reported that loss of chromosome 3 alleles and multiplication of 8q alleles may define a subgroup of uveal melanoma with poor prognosis<sup>32</sup>. In this study, however, six of seven cases (86%) with gain of chromosome 8 had no lymph node metastases. By contrast, seven of eight cases (88%) without gain of chromosome 8 had positive lymph node involvement. There was a good correlation between copy number of chromosome 8 and

lymph node status in patients with esophageal cancer ( $p = 0.0089$ ). Of six cases who did not involve the adventitia (mp), three cases with gain of chromosome 8 had no nodal involvement and three cases without gain had several lymph node metastases. This suggests that FISH using chromosome 8 specific DNA probe may be helpful to determine the method of surgical procedures to the patients with esophageal cancer.

Since esophageal squamous cell carcinomas showed a striking male predominance, sex chromosomes were also examined in this study. Loss of Y chromosome has been reported in various malignancies such as prostatic cancer<sup>33</sup>, gastric adenocarcinoma<sup>34</sup>, meningioma<sup>35</sup>, renal cell carcinoma<sup>36</sup>, and leukemia<sup>37</sup>. In this series, loss of Y was found in six cases (55%) of esophageal cancer. In four cases without invasion to the adventitia (an), only one case (25%) revealed loss of Y. By contrast, loss of Y was shown in five of seven cases (71%) who involved the adventitia. Although loss of Y has been shown in nonneoplastic tissues, including brain<sup>38</sup>, bone marrow<sup>39</sup>, and kidney<sup>40</sup>, it is assumed that there would exist an unidentified gene which suppress invasiveness to the adventitia of esophageal cancer on chromosome Y.

Combination analysis with DNA content and numerical chromosome aberration revealed that all three cases with DNA aneuploidy which did not show any extra copy number of a set of six chromosomes except for chromosome X had several positive lymph nodes. One possible explanation is that if more probes were used in these cases, the frequency of numerical aberrations would increase rather than that in our results. Alternatively, double minutes or homogenous staining lesion which may be associated with proliferation and progression<sup>41,42</sup> would be increased in those cases. Because these fragments have no centromeric regions that it is impossible to detect it by FISH using centromeric DNA probes.

Because of diagnostic progression, it has become possible to detect the early stage of esophageal cancers which are applicable for not only trans-hiatal esophagectomy but also endoscopic mucosectomy. If determination of accurate staging, detection of the depth of invasion and the presence of lymph node involvement are preoperatively feasible, an indication of minimal surgical access would be increased. Although endoscopic ultrasonography is an important tool, micrometastases to lymph nodes are difficult to distinguish from inflammatory changes.

In conclusion, the results of this study suggests that FISH using endoscopic biopsy specimens is available for the judgement of minimal surgical approach. Further prospective study is necessary to confirm these findings as well as the possible adjunctive prognostic value of chromosome copy number in esophageal squamous cell carcinoma.

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