The Korean Journal of Internal Medicine
Vol. 12, No. 2, June, 1997

DNA Analysis by Flow Cytometry in Early Gastric Cancer

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Objectives : *Flow cytometric analysis of a paraffin-embedded block of tissue provides rapid and accurate means of analyzing the DNA content of a tumor. The aim of this study was to clarify the clinical significance of flow cytometric findings in early gastric cancer(EGC).Thus we conducted this study to investigate whether DNA contents of tumor cells can correlate with known prognostic indices in patients with EGC.*

Methods : *The flow cytometric DNA analysis was performed with paraffin-embedded specimens from tumors of 107 patients with EGC. Flow cytometric analysis was perfomed using a FACScan (Becton Dickinson). In constructing the histogram, 30,000 cells were scanned from each section and results were scored. The S-phase fraction was obtained according to the CellFit cell cycle analysis (Becton Dikinson). Frequencies of aneuploidy in tumors with various clinical and pathologic parameters were compared using the chi-square test. Mean SPF/PI valuse were compared by the student t-test.*

Results : *Diploidy pattern was observed in 80 (75%) cases while aneuploidy was seen in 27(25%) cases. Aneuploidy was more frequently detected in tumors with submucosal involvement (32.7%) and lymph node (+) group (30.8%) than in the mucosal tumor (17.3%) and lymph node (−) group (24.5%), but the differences were not significant. Frequency of aneuploidy was not affected by either the histologic type or morphologic clssification. On the other hand, high proliferative activities (SPF/PI) significantly correlated with the submucosal tumor invasion (66.7% vs. 45%; $p<0.05$) and lymph node metastasis (28.6% vs. 7.5%; $p<0.05$).*

Conclusion : *Tumor aggressiveness is not directly related to DNA aneuploidy but proliferative activities are responsible for the aggressive nature of early gastric cancer. The results of this study show that DNA analysis by flow cytometry in considered to be one method of determining the biological activity of gastric cancer cells.*

Key Words : *DNA ploidy, Early gastric cancer, Flow cytometry, Proliferative activity*

INTRODUCTION

Neoplasia is caused by a functional and structural disorder of cellular DNA, and the changes are reflected in the morphology of cells, oncogene expression, gene amplifications, chromosome number and nuclear DNA content etc. Recent advances of flow cytometric measurements have enabled us to determine the DNA ploidy pattern of cancer cells.

The prognostic influence of tumor cell DNA

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content was first demonstrated by Atkin and Kay[1] using a cytophotometric method. Since the development by Hedley et al.[2] of a technique allowing flow cytomeric DNA analysis of archival pathological meterial, DNA aneuploidy has been shown to be associated with a poorer prognosis in a variety of tumor types, including colon, breast, lung, esophagus, ovary and bladder cancers[3-6].

Nuclear DNA measurement is considered to be of value for clarifying the relationship between the malignancy and biological activity of cancer cells. Several clinical and pathologic factors have previously been identified as having prognostic significance in patients with gastric cancer, including the size of the tumors, age and sex of patients at operation, nodal and/or distant metastasis, histologic variability, anatomic location, histologic grade and treatment. Some of these factors are disputed as independent prognostic factors; nonetheless, they are thought to be very important. More recently, attention has focused on measuring DNA content and the proliferative activity of gastric cancer to determine if DNA content and proliferation influence the survival of patients with gastic cancer[7].

The flow cytometric DNA analysis by which the DNA index and its relationship with prognosis, examined by using paraffin-embedded cancer tissues from the stomach, was first reported by Kimura et al.[7] but many studies argue that the presence of aneuploidy is associated with a poor prognosis[8-12].

For the determination of DNA ploidy in a solid tumor, analysis of only a single specimen is said to be inadequate, since DNA ploidy heterogeneity in the same tumor has been identified in various organs[13-15]. Interestingly, a potentially narrower range of a ploidy pattern, as a result of smaller tumor size, may render early gastric cancer(EGC) as a better model to study aneuploidy abnormality in gastric cancer[16-19].

Our aims were to determine whether tumor cell DNA content was assoiated with known prognostic indices, such as the pathological stage and histological type in patients with EGC who underwent gastric resection.

MATERIALS AND METHODS

1. Patients

This study was performed on specimens from 107 patients who were diagnosed as EGC and treated at the Wonju Christian Hospital of Yonsei University during the ten years between January 1984 and August 1994. All patients underwent radical subtotal gastrectomy. Pathology and medical records were retrospectively reviewed for depth of invasion, size of tumor, pathologic types and lymph node metastasis.

2. Methods

To perform DNA flow cytometric analysis, three serial sections (80 μm thickness) were cut from a block of formalin-fixed, paraffin-embedded gastric tumor. In each case, the sample selected for the flow cytometric study featured the deepest neoplastic infiltration, excluding specimes with extensive necrosis and severe inflammation to minimize the obscuring effect of debris[20]. 80 μm sections were placed in polypropylene tubes, dewaxed in xylene, and rehydrated using a series of diluted alcohol (100%, 95%, 80%, 70% and 50%) and resuspended in phosphate-buffered saline for two periods of 10-15 minutes. The tissue was incubated in 2.5ml 0.5% pepsin solution (PH 1.5) in a 37℃ water bath with intermittent mixing for 30 minutes. After enzymatic digestion, 1ml of 0.025% peptostatin was added. The collected cells were washd with cold, phosphate-buffered saline and spun for 5 minutes at 3000 rpm. They were resuspended in phoshate-buffered saline and spun twice with intermittent vortexing, with 6 drops added of RNAse with at least 0.5ml of buffer in the tube. The pellets were incubated for 30 minutes at 37℃. Just before flow cytometric analysis, each sample was filtered through a 37 μm nylon mesh to remove cell clumps and improve cell dispersion. 50 μg/ml of propidium iodide was added. Flow cytometric analysis was performed using a FACScan (Becton Dickinson, USA). In constructing the histogram, 30,000 cells were scanned from each section and results were scored. The S-phase fraction (SPF) was obtained

according to the CellFit cell cycle analysis. The DNA index(DI) was the ratio of the relative content of the G0/G1 cells of the sample divided by the relative DNA measurement of the near-diploid standards. A DNA index of 1.0 indicates the presence of only diploid cell. DNA aneuplody was determined when at least two separate G0/G1 peaks were demonstrated[20-24]. Cell clones were divided into four ploidy classes on the basis of the DNA index(DI). DI equal to 1=DNA diploid, DI from 1 to 1.5=DNA hyperdiploid, DI from 1.5 to 2.0= DNA hypertriploid, and DI over 2=DNA hypertetraploid[24]. An estimate of the percentage of cells in (S+G2M) was taken as a measure of proliferative activity[9]. SPF and G2M were not obtained from 6 cases, so we excluded the 6 cases on the statistical analysis. A full peak-coefficient of variation for G0/G1 peak on each sample was calculated using the same software supplied by Becton Dickinson. In this study, we omitted some cases from the analysis where the coefficient of variance (CV) was greater than 8.0[7]. Frequencies of aneuploidy in tumors with various clinical and pathologic parameters were compared using the chi-squre test. Mean SPF/PI values were compared by the student t-test.

RESULTS

Among 107 tumors, 80 (75%) were found to contain diploid cell population and 27 (25%) contained aneuploid cell population. The mean DNA index of aneuploid tumor was 1.3. The mean DNA index of 107 tumors was 1.1 (Table 1). Mean SPF, G2M and PI of the aneuploid goup were higher than that of the diploid group ($p<0.05$) (Table 2).

Fig. 1 shows a typical DNA histogram recorded by flow cytometry. There is only one G1 peak resolved and this tumor was scored as diploid (DI=1.0). Fig. 2 shows an example of an aneuploid tumor (DI=1.45) from which there were two clusters observed in the histogram.

Table 1. DNA Ploidy of Early Gastric Cancer

	No. of Patients(%)	DNA Index
Diploid	80(75)	1.0
Aneuploid	27(25)	1.3
Total	107(100)	

Table 2. Results of DNA Analysis of Early Gastric Cancer

Variables	Diploid(%) (n=80)	Aneuploid(%) (n=21)	Total(%) (n=101)
S-phase fraction(SPF)	5.9±3.6	19.3±14.2*	8.7± 8.9
G_2M	3.5±1.9	7.2± 3.5*	4.3± 2.7
Proliferative Index(PI)	9.5±4.8	26.5±15.4*	13.0±10.7

* $p<0.05$

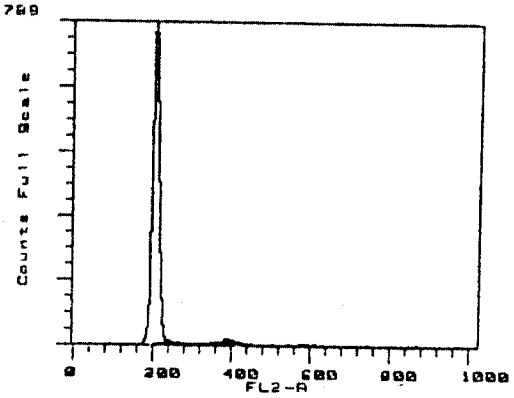

Fig. 1. Example of flow cytomerty histograms from diploid tumor.

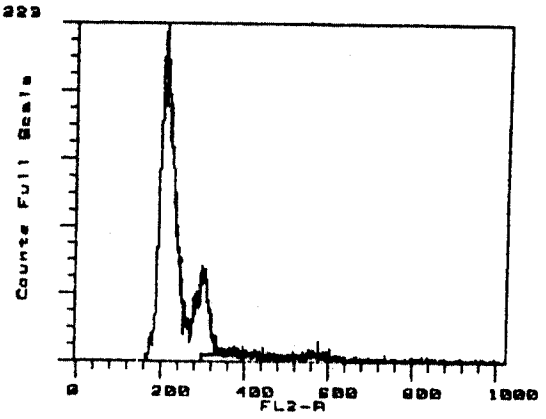

Fig. 2. Example of flow cytomerty histograms from aneuploid tumor.

Table 3. Clinico-Pathologic Features of Tumors and Ploidy (n=107)

Variables		Diploid(%)	Aneuploid(%)	p-value
Age	≤40	19(79.2)	5(20.8)	>0.05
	41-59	31(70.5)	13(29.5)	
	≥60	30(76.9)	9(23.1)	
Sex	male	52(74.3)	18(25.7)	>0.05
	femal	28(75.7)	9(24.3)	
Tumor Size	<2cm	44(81.5)	10(18.5)	>0.05
	>2cm	36(67.9)	17(32.1)	
Histologic Type	Well-differentiated	7(53.8)	6(46.2)	>0.05
	Mod-differentiated	20(71.4)	8(28.6)	
	Poorly-differentiated	53(80.3)	13(19.7)	
Depth of Invasion	mucosa	43(82.7)	9(17.3)	>0.05
	submucosa	37(67.3)	18(32.7)	
Lymph node Metastasis	negative	71(75.5)	23(24.5)	>0.05
	positive	9(69.2)	4(30.8)	

Table 4. Relationships Between Prognostic Parameters and Proliferative Activities(n=101)

	SPF			G2M			PI		
	Low (%)	High (%)	p-value	Low (%)	High (%)	p-value	Low (%)	High (%)	p-value
Depth of Invasion									
mucosa (n=51)	44(55.0	7(33.3)	<0.05	36(54.5)	15(42.9)	>0.05	43(57.3)	8(30.8)	<0.05
submucosa (n=50)	36(45.0)	14(66.7)		30(45.5)	20(57.1)		32(42.7)	18(69.2)	
Lymph Node Metastasis									
negative (n=89)	74(92.5)	15(71.4)	<0.05	59(89.4)	30(85.7)	>0.05	70(93.3)	19(73.1)	<0.05
positive (n=12)	6(7.5)	6(28.6)		7(10.6)	5(14.3)		5(6.7)	7(26.9)	
Ploidy									
diploid (n=80)	73(91.2)	7(33.3)	<0.05	62(93.9)	18(51.4)	>0.05	72(96.0)	8(30.8)	<0.05
aneuploid (n=21)	7(8.8)	14(66.7)		4(6.1)	17(48.6)		3(4.0)	18(69.2)	

SPF : S-phase fraction, PI : Proliferative index (SPF+G2M)
Low : patients with SPF/G2M/PI below mean value
High : patiens with SPF/G2M/PI over mean value

Aneuploidy was more frequently detected in the tumor with submucosal invation (32.7%) and in the lymph node positive group (30.8%) than in the mucosal tumor (17.3%) and the lymph node negative group (24.5%), but the findings were not significant. No significant difference was observed in the frequencies of DNA aneuploidy with respect to tumor size, pathologic type, depth of invasion and lymph node metastasis (Table 3).

A significant relationship was found between SPF/PI and the depth of invasion and lymph node metastasis. High and low SPF/G2M/PI goups were divided by mean value of SPF, G2M and PI. The high SPF/PI group had a higher incidence of submucosal invasion (66.7%) than the low SPF/PI goup (45.0%) ($p<0.05$). The high SPF/PI group had a higher incidence of lymph node metastsis (28.6%) than the low SPF/PI goup (7.5%) ($p<0.05$). However, SPF/PI did not correlate with the degree of tumor differentiation or size (Table 4).

DISCUSSION

Flow cytometry is a fast and accurate method for quantifying DNA content and the percnetage of cells in S, G2 and mitotic phases of a solid tumor. But interlaboratory technical differences in DNA staining, the handling of fresh or formalin-fixed paraffin-embedded material, enzymatic digestion, quality assurance and data analysis often result in major discrepancies in data from differnect laboratories.

Advanced gastric cancer has a different prognosis in individual cases even when the disease stage is the same[7]. In a retrospective study of patients with stomach cancer, many reports[7, 19-25] showed that DNA ploidy and S-phase fraction correlated with the survival of the patients. Compared to diploid tumors, aneuploid tumors had significantly more frequent invasion into serosa, as well as metastatic disease to the liver, peritoneum and lymph nodes.

The DNA ploidy patterns of early gastric cancers were similar to those of the advanced cancers; the occurrence of a diploid and an aneupoid cell line was similar between the early and advanced cancers, and even minute, intramucosal cancers consisted of similar DNA ploidy patterns[16-19]. Yonemura et al.[26] reported that the incidence of lymph node metastasis was higher in aneuploid early gastric cancer than in diploid cancers. Other reports[20, 23, 24] also show that DNA ploidy appears to be an independent predictor of recurrence, metastatic potenitial and survival.

But, unlike other studies, our study revealed no significant correlation between DNA ploidy and lymph node metasasis ($p>0.05$). There was, however, a tendency for a higher submucosal invasion and lymph node metastasis in aneuploid tumors (17.3% vs. 32.7%, 24.5% vs. 30.8%). Our finding of a 25% of DNA aneuploidy in EGC is slightly lower than that of others[10].

Recent studies[22, 27] show that proliferative activity is prognostically more notable than the DNA ploidy pattern and the proliferation rate was shown to be the most independent prognostic factor of the clinicopathologic factors. Filipe et al.[9], Yonemura et al.[22] and Ohyama et al.[27, 28] have reported that tumors with high S-phase fractions are associated with a higher incidence of lymph node metastasis and a higher risk of depth of invasion in comparison to tumors with low-S- phase fractions. The proliferative activity of the S-phase calculated in 101 of the EGC in this study has significantly correlated with submucosal invasion and lymph node metastasis (45% vs. 66.7%, 7.5% vs. 28.6%).

Ohyama et al.[28] and Yonemura et al.[29] showed that S-phase fraction measured by the BrdU labeling method and PCNA LR (labeling rate) is an important prognostic indicator of gastric cancer. They also thought that the proliferation rate was the most useful indicator for malignancy. Ohyama et al.[27, 28] have studies the S-phase fraction of the cell cycle by bivariate analysis using bromodeoxyuridine (BrdU). The SPF was higher in aneuploidy than diploidy tumors. The patients with SPF over 10% had a poorer prognosis than those showing SPF below 10% even in diploid tumor patients. Kamata et al.[18] reported that BrdU LI(labeling index) correlates well with lymph node metastasis in EGC. They concluded that in vitro BrdU LI of specimens obtained by endoscopic biopsy may be a useful indicator of lymph node status in an individual patient before surgery. Our findings of DNA study in EGC show similar results with that of the above studis. Although long term suvival data of our patients was not obtained, strong correlation was noted between the high proliferative activities with submucosal invasion and lymph node metastsis in EGC.

In conclusion, this study demonstrates that the proliferative activity has a strong correlation with submucosal invasion and lymph node metastasis in EGC. Proliferative activity (SPF/PI) is more useful than the DNA ploidy pattern in predicting submucosal invasion and lymph node metastasis in EGC. The results of this study show that DNA analysis by flow cytometry is considered to be one method of determining the biological activity of EGC. Further study is warranted to assess its significance on the long-term prognosis of affected patients.

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