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Flow cytometry as a diagnostic method for colorectal cancer

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Abstract—

Intraoperative histopathological investigation plays an important role during surgery. Since the pathologist performs a diagnosis with a limited level of specimen, it may sometimes be difficult to reach a correct diagnosis. To improve the accuracy of the diagnosis, quantitative data from the whole specimen are helpful. It said that the detection capability for DNA aneuploidy (aneuploidy) is low for solid cancer compared with hematopoietic organ cancer. A new method that includes fresh tissue is introduced, the histogram from cancer tissue (cancer) and normal tissue (normal) is compared, new classification criteria are introduced, the Fast Fourier Transform (FFT) pattern (FFT pattern) obtained from FFT on the histogram is analyzed, and the area under the FFT pattern of the histogram (AUC) is compared. This method, named the "FFT-AUC method", which includes comparisons of AUC and the FFT pattern, shows good results.

I. Introduction

Intra-operative histopathological investigation plays an important role during surgery. Since the pathologist performs the diagnosis with a limited specimen, correct diagnosis may sometimes be difficult. To improve the accuracy of diagnosis, quantitative data from the entire specimen are helpful. In the past, paraffin-embedded samples were analyzed for cancer using a flow cytometer (FCM)), and the detection rate of aneuploidy ranged from 35% to 75 %[(1)(2)(3)], but this method is not yet in practical use. Akao[(4)] et.al. reported that the use of fresh tissues gives a better aneuploidy detection rate. This paper presents a new method to analyze the histograms and shows that this is an effective method for cancer diagnosis using FCM.

II. MATERIALS AND METHODS

A. Materials

The specimen samples from 26 cases of tissues surgically removed from patients with advanced colorectal cancer at the Cancer Institute Hospital were divided into two pieces, one for HE and the other for histogram analysis. The specimen for histogram analysis was isolated, stained, and then measured.

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This research received the approval of the Ethics Committee of the Cancer Institute Hospital, Tokyo, Japan.

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B. Cell Isolation Procedure

1. Preparation of reagent solution

2.5 mL of buffer solution were added into the test tube containing freeze-dried reagent. The reagent had to be stirred and dissolved well.

2. Placement of the tissue in the test tube.

A piece of tissue of around 2 mm^2 was placed into the test tube filled with reagent.

3. Cell isolation & staining

The test tube was set at a sample stage of the cell isolation device. Cell isolation and staining were carried out for 6 minutes in the dark. Table 1 shows the detailed settings for cell isolation.

4. Filtration

The cell suspension was filtered with a 40-μm nylon mesh.

5. Measurement & analysis

The filtered suspension was put on an FCM to obtain the histograms. Fast Fourier transform (FFT) processing was applied to the histogram.

Table 1 Detailed condition for cell isolation

Parameters	Setting	Unit
Pipetting flow	800	μL
Aspiration speed	2000	μL/sec
Aspiration interval	0.5	Sec
Discharge speed	4000	μL/sec
Discharge interval	1	Sec
Number of pipettings	170	Times

C. Detailed Description of Reagent

The reagent consisted of 0.1 mg/mL propidium iodide (Sigma-Aldrich Japan, Tokyo, Japan), 0.05 mg/mL RNaseA (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and 0.2% tritonX-100 (Kishida Chemical Co., Ltd., Osaka, Japan). First, these components were mixed together in water and solution, dispensed into test tubes, and lyophilized in a vacuum freeze dryer (Kyowa Vacuum Engineering CO., Ltd., Tokyo, Japan). The mixed reagent was finally formed into a pellet.

D. Cell Isolator

Pipetting was done by the cell isolator with the special pipette that has two small holes which are in 4 mm position from the tip and which faced each other. The cell isolator repeats suction, pause, discharge and pause for the constant time and the volume**.** The detailed procedure is in table 1.

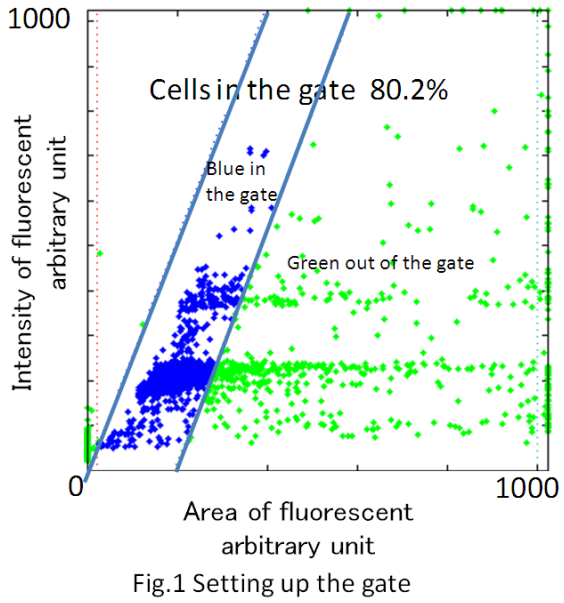

Fig.1 Setting up the gate

E. Setting for flow cytometer

Sensitivity was set up so that the fluorescence intensity of G_0/G_1 phase might become 200 addresses. As shown in Fig. 1, the gate was set up in order to remove doublets, triplets, etc.

F Histogram Categorization

The histograms were categorized into two categories, diploidy and aneuploidy, and there were histograms that could not be categorized. A new category named non-aneuploidy, which is categorized into three sub-categories (high S, high G_2/M, and atypical histogram) was introduced. The definitions of high S histogram, high G_2/M histogram, and atypical histogram were "the histogram has a relatively large number of cells in S period", "the histogram has a relatively large number of cells in G_2/M period", and "the histogram has a G_0/G_1 peak that has a different shape from the diploidy histogram", respectively. Furthermore, because of the characteristics of FFT, the spread histogram was defined as combining the high S and high G_2/M histograms.

G. Function of FFT

The normal, aneuploidy, spread, and atypical histograms were simulated, and FFT was applied to these histograms. In Fig.2, the upper green is the histogram of the normal, red is that of aneuploidy, the lower green is the FFT pattern of normal, and the red is the FFT pattern of aneuploidy. By comparing the red and green, the ripples of the FFT pattern of aneuploidy are obvious, and by comparing the AUC of the FFT patterns, the AUC of aneuploidy is smaller than that of the normal. In Fig.3, the upper green is the histogram of normal, red is that of the spread, the lower green is the FFT pattern of normal, and the red is the FFT pattern of spread. By comparing the red and green, the FFT pattern of spread shows a steep down at the left side of the figure and a smaller AUC than that of normal. In Fig.4, the upper green is the histogram of normal, red is that of atypical, the lower green is the FFT pattern of normal, and the red is the FFT pattern of atypical. Similarly, the FFT pattern of the atypical histogram has a smaller AUC than that of the normal.

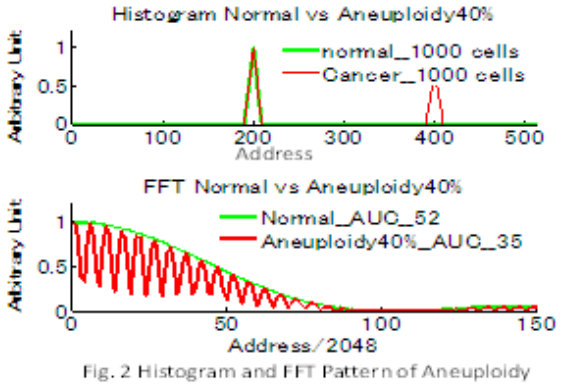

Fig. 2 Histogram and FFT Pattern of Aneuploidy

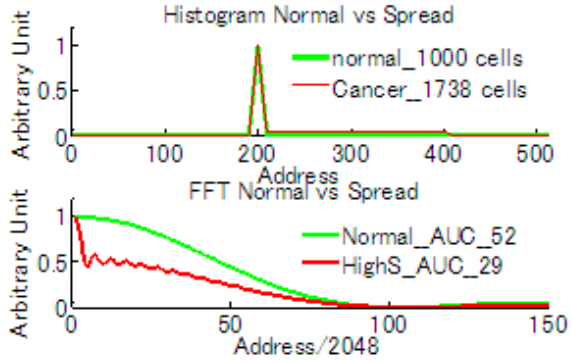

Fig 3 Histogram and FFT pattern of spread

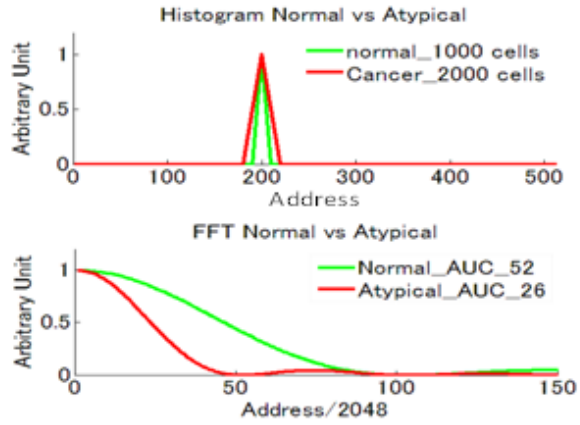

Fig.4 Histogram and FFT Pattern of Atypical

H. Analysis

The histology and the histogram, as well as the histogram and the FFT pattern, were compared and are depicted in the two figures per case. The AUC was calculated for every FFT pattern, and the AUCs of the same case were compared. Classification of diagnosis was done according to the logic described below. The tissues with a larger AUC than the largest area of cancer were normal. The tissue with a narrower AUC than the narrowest AUC of normal was cancer. For the tissues with intermediate AUCs between the maximum AUC of cancer and the minimum AUC of normal, the FFT pattern was observed. Tissues with a monotonically decreasing FFT

pattern were considered normal, with one minimal value suspicious of cancer, and with multiple minimal values considered cancer.

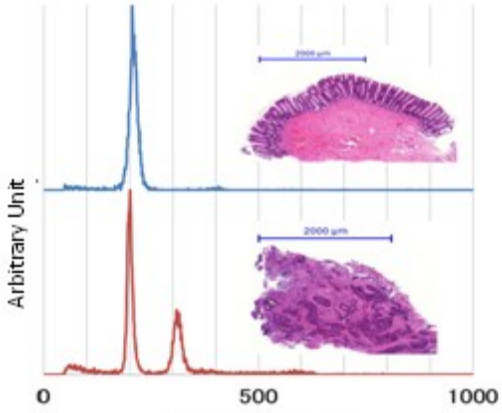

Fig. 5 Histogram and Histology of aneuploidy

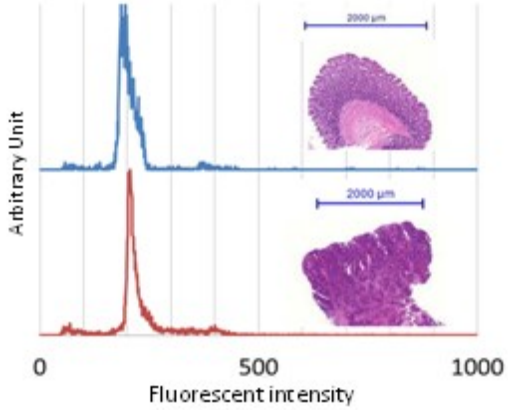

Fig.6 Histogram and Histology of aneuploidy

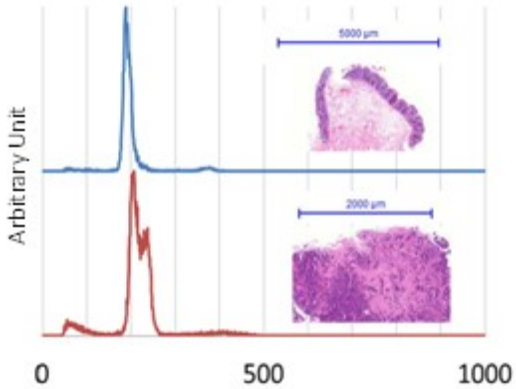

Fig 7 Histogram and Histology of atypical

III. RESULTS

The histograms and histology from the patients shown in Fig.5 to Fig.7 show the contrast between normal and cancer in the upper and lower panels, respectively. From Fig.8 to Fig.10, the histograms and FFT patterns of cancers and normals are shown in the upper and lower panels, respectively. Figs. 4 and 8 show typical aneuploidy, Figs. 5 and 9 show spread, and Figs. 6 and 10 show atypical, respectively. An example of aneuploidy (Fig.8) shows ripples and a smaller AUC. An example of spread (Fig.9) shows the steep down FFT pattern at the left side of the figure. An example of atypical (Fig.10) shows a smaller AUC. Hence, all patterns appear as predicted.

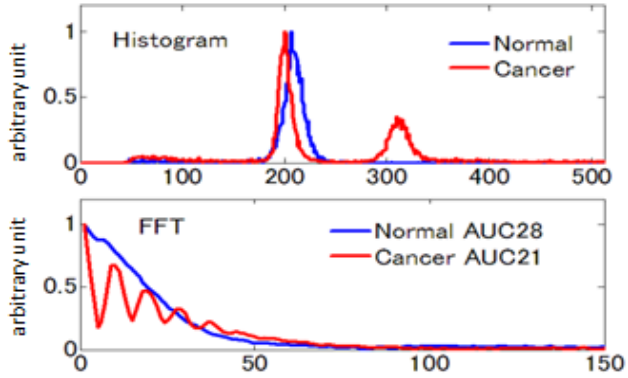

Fig. 8 FFT pattern and histogram of aneuploidy

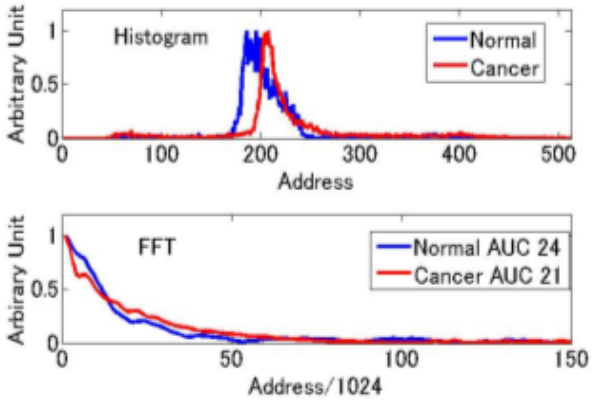

Fig. 9 FFT pattern and histogram of spread

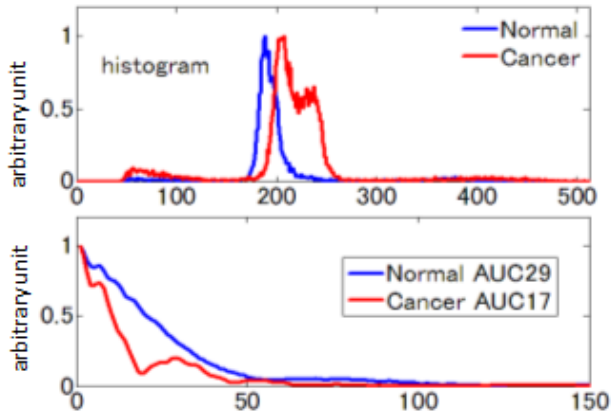

Fig. 10 FFT pattern and histogram of atypical

Table 2 shows the AUC (Normal and Cancer) in the 2nd and 3rd column. The 4 and 5th columns show the classification result of a FFT pattern. If AUC was over 32, "-" was entered. If AUC was between 15 to 32, then the ripples were counted, and if there were no ripples, "○" was entered. If there was one minimal then "1" was entered "1". If there were multiple minimal values then "×"was entered. If AUC was under 15,

then "v" was entered. Table 3 shows the sensitivity and specificity of the FFT-AUC method.

A. Comparing Normal and Cancer

By obtaining both normal and cancer specimens and comparing their AUCs, a 100% correct diagnosis was obtained. However, obtaining both specimens in clinical practice cannot be expected because of ethical issues, except when normal tissue is excised as part of treatment.

B. In the case of Cannot Obtain Normal

For the FFT-AUC diagnosis of normal, AUC was >32 in 15/26, and when AUC<32, the FFT pattern was normal in 5/26, showed 1 minimal value in 5/26, and showed multiple minimal values in 1/26. Thus, a false diagnosis was given in only 1/26.

For cancer cases, 6/26 showed AUC values <14, while for those with AUC≥15, multiple minimal values were seen in 14/26, 1 minimal value was seen in 4/26, and no minimal value was seen in 2/26. Based on these results, no cancer tissue was diagnosed as normal; 24/26 was diagnosed as cancer, and 2/26 were diagnosed as suspicious of cancer.

IV. DISCUSSION

There is a possibility that the ratio of cancer cells and healthy cells is changed through the isolation. If it arouse, sensitivity and specificity are affected by this. It shall be confirmed in the future using image-cytometer or the other way

Even for normal tissue they do not show diploidy. The causes need to be investigated. The isolation process is the most doubtful. It requires for investigation.

The ability to make a diagnosis of cancer as the number of cancer cells decreases will be examined using mixed samples of two cancer cell lines with different DNA; this investigation is expected to be completed by the end of this year.

V. CONCLUSION

Rapid diagnosis is done within 10 minutes; this method can provide a diagnosis within 10 minutes because it needs 6 minutes for isolation and staining and 4 minutes to obtain the histogram and FFT pattern.

Because of the small number of cases (26) and the fact that all subjects had colorectal cancers, it cannot be concluded that this method is established, but it has a high potential for clinical use.

In the future, we hope to answer the questions raised in the discussion by increasing the numbers of subjects and cases, especially breast cancer, lymph node, bile duct and adenoma, etc.

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Table 2

Case	AUC		Ripple	
	Normal	Cancer	Normal	Cancer
1	21	20	1	×
2	38	20	−	×
3	32	17	○	×
4	42	25	−	○
5	35	27	−	×
6	24	21	○	1
7	53	23	−	×
8	29	17	○	×
9	34	16	−	×
10	25	13	1	V
11	29	12	1	V
12	51	14	−	V
13	33	19	−	×
14	30	21	1	○
15	41	24	−	×
16	29	10	1	×
17	47	26	−	1
18	40	32	−	1
19	45	30	−	×
20	36	25	−	1
21	33	22	−	×
22	37	29	—	×
23	51	18	−	×
24	28	21	○	×
25	19	11	○	V
26	15	12	×	V
max	53	32		
min	15	10		

Table 3 Results

Diagnosis	N vs C	Normal	Cancer
TRUE	26/26	20/26	20/26
Error	0	1/26	0/26
suspicion	0	5/26	6/26

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