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Fully Automatic Rapid DNA Ploidy Analyzer for Intraoperative Rapid Diagnosis Support

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Abstract— **Frozen section studies are a useful method to rapidly define tumor malignancy and identify the extent of surgical resection. However, diagnosis with a frozen section is qualitative and sometimes difficult. Therefore a quantitative method for grading tumors is desired. We have already reported a technique of intraoperative flow cytometry (iFC) that supports intraoperative histopathological examination of frozen sections. In this study, we report an advanced system named "Fully Automatic Rapid DNA Ploidy Analyzer" with a tissue pretreatment function and a freeze-dried reagent kit for cell staining. To evaluate our system, we analyzed samples from glioma patients who underwent open surgery for brain tumors. We observed obvious difference of the Malignancy Index (MI) between neoplastic and perilesional brain tissue (26.0 ±22.1% and 4.1 ±2.5%, respectively, P<0.001). Cut-off level for identification of the tumor in the biopsy specimen was 6.8% which provided 86% sensitivity and 81% specificity. We also obtained a good correlation between the MI and histological grade (WHO grading). Our new system also enabled finishing the process from sample preparation to the end of analysis in ten minutes or less. These results demonstrate that our fully automatic rapid DNA ploidy analyzer is feasible for rapid determination of glioma presence in a surgical biopsy sample.**

I. INTRODUCTION

Glioma infiltrates the normal surrounding tissues as tumor malignancy increases. At the time of resection, surgeons have to identify the boundary between the tumor and its surrounding healthy tissues so as to not damage the healthy tissue. Recent studies have shown that higher resection rate corresponds with higher survival rate and lower recurrence rate[1][2]. To increase resection rate, neuronavigation[3][4] and photodynamic diagnosis using 5-aminolevulinic acid (5-ALA)[5][6] have been used. On the other hand, intraoperative histopathological examination on frozen sections is a standard method for acquiring histopathological information and making decisions about the resection area. Therefore, there have been many studies on direct microscopic investigation of the tissue specimen with the stained frozen or smear sections[7-10]. However, compared to paraffin embedded section diagnosis, frozen section diagnosis is easily influenced by artifact in the process of making frozen section.

Therefore, we presumed that if extracted tissues could be rapidly analyzed and quantitative data provided to the surgeons, it would help them determine the extent of resection. It would be also possible to remove the tumor more accurately in conjunction with diagnostic image information and our analysis result.

We have already reported a technique of intraoperative flow cytometry (iFC) that can support intraoperative histopathological examination on frozen sections[11]. Building on that previous report, we developed a fully automatic rapid DNA ploidy analyzer with tissue pretreatment function and a freeze-dried reagent kit for cell staining. This system will support the conventional diagnosis method with frozen specimen and smear as a new intraoperative rapid tissue analysis system with quantitative data for brain tumors.

II. MATERIALS AND METHOD

A. Fully automatic rapid DNA ploidy analyzing system

Figure 1 shows our fully automatic rapid DNA ploidy analyzer (a) and its dedicated reagent kit (b).

This analyzer is a flow cytometer (equipped with a 488 nm laser) with an automatic sample pretreatment unit and data analyzing function. Flow cytometry is a powerful technique for analysis of individual cells with multiple parameters. In this study, samples can be prepared using the original reagent kit. The kit is composed of three ingredients listed in Table 1. Propidium iodide (PI) is a nucleic acid staining reagent, and

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has a maximum fluorescence wavelength at 610 nm when excited with a 488 nm laser. These ingredients are mixed together in an aqueous solution, dispensed into a test-tube, and lyophilized in a vacuum freeze dryer (Kyowa Vacuum Engineering Co., Ltd., Tokyo, Japan) to turn it into a pellet. Our preliminary experiments showed that cells can be stained in six minutes in the dark at room temperature. We also developed specialized pipette tips with a filtration mechanism for this system. Figure 2 shows the operating procedure of the analyzer using the reagent and the pipette tip. The investigated tissue was placed into the reagent tube, and the pipette tip was inserted into the tube. After the tube was set into the analyzer, the specimen was automatically pretreated by pipetting then analyzed.

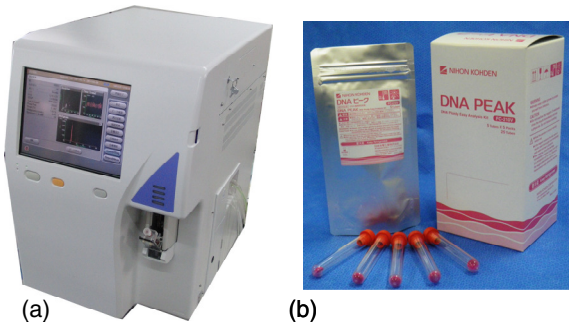

Figure 1. Fully automatic rapid DNA analyzing system

Rapid DNA ploidy analyzer (a) and cell staining reagent kit (b)

Table 1. Ingredients of the reagent kit

Reagents	Concentration
Propidium Iodide (Sigma-Aldrich Japan)	0.1 mg/mL
RNase A (Wako Pure Chemical Industries, ltd.)	0.05 mg/mL
TritonX-100 (Kishida Chemical Co., ltd.)	0.20%

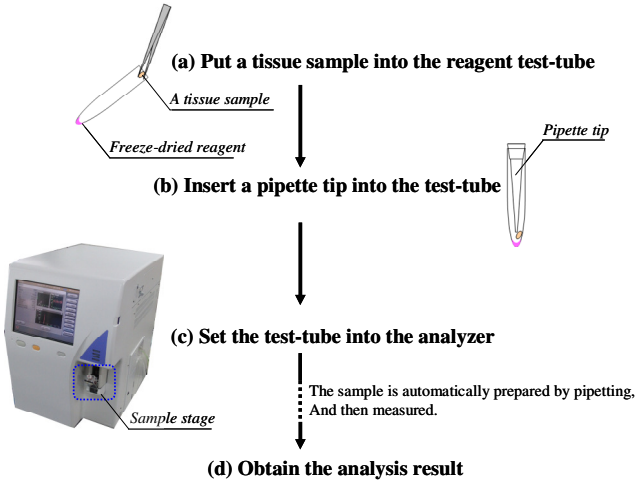

Figure 2. Schematic view of the operating procedure with the analyzer

Specimen and protocols for DNA ploidy analysis

Clinical evaluation of this system was carried out during resection of 41 patients with well-defined glial neoplasms. Every patient agreed to participate in the study and provided written informed consent. The requirements for enrollment were negative testing for hepatitis B, hepatitis C, HIV, lues, parvo virus and HTLV. Research protocol was approved by the local Ethics Committee.

Patient and case information is shown in Table 2 (a) and (b). All procedures were performed at the Department of Neurosurgery of Tokyo Women's Medical University from June to December in 2012.

Table 2.

(a) Patient information

gender	case	age
man	26	38.1±12.6
woman	15	43.7±11.7

(b) Case information

type of tumor	number	total
grade II		14
Diffuse astrocytoma	5	
Oligodendroglioma	4	
Oligoastrocytoma	5	
grade III		12
Anaplastic astrocytoma	4	
Anaplastic oligodendroglioma	5	
Anaplastic oligoastrocytoma	3	
grade IV		15
Glioblastoma	15	
		41

Analyzed pathological tissue was sampled with a forceps from the specified area of the operative field. In total, 121 separate biopsy specimens were taken during resection of 41 patients with gliomas. Each specimen was separated into two equal parts of 1-2 mm^3 for analysis with our system and permanent histopathological investigation on the formalin-fixed paraffin-embedded tissue section stained with Hematoxylin and Eosin (H&E). They were defined according to the WHO brain tumor grading.[12] Perilesional brain tissue without histopathologically identifiable tumor was defined as "Normal." Figure 3 shows the flowchart of the evaluation procedure.

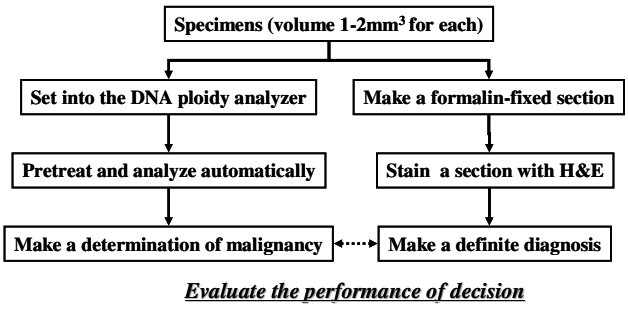

Figure 3. Process flowchart of the evaluation method

B. *Method for analysis of DNA histograms*

Figure 4 shows a typical DNA histogram of DNA contents versus the number of cells. Before drawing a histogram, gating was performed by using a scattergram to eliminate doublet factor for this analysis. In our method, malignancy of tumor was determined with malignancy index (MI) that was defined as follows.

$$\mathrm{MI}\,[\%] = \frac{(\text{the number of proliferating and abnormal cells})}{(\text{the number of full analysis set of cells})} \times 100$$

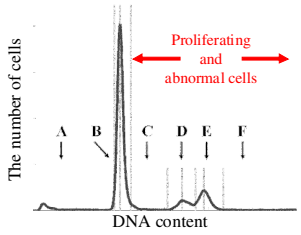

Figure 4. Example of DNA histogram with graphic display

In figure 4, each area (A – F) suggests the existence of the following cells. A: apoptotic cells and debris, B: G0G1-phase (diploid) cells, C: S-phase cells, D: aneuploid cells with abnormal number of chromosomes, E: G2/M-phase cells, F: cells containing more DNA than G2/M-phase cells.

The group of area from C to F can be collectively called "proliferating and abnormal cells."

C. *Statistical analysis*

The student's t-test was applied for comparison of MI between the normal and all the tumor tissues. The cut-off value of MI to discriminate tumor tissues was set to 6.8% which is defined in our previous study[11], and the sensitivity and specificity to identify the normal and the tumor were obtained. Next, analysis of variance for one-way layout was performed to determine the significant difference of MI of each malignant group (WHO classification). Then Tukey's HSD was conducted to find significant difference between each group.

III. Results

In our system, it took 1 minute or less for sample preparation before setting a sample to the analyzer (Figure 2a, b), 8 minutes for sample preparation and measurement with the analyzer (Figure 2c) and 1 minute for finishing the analysis and displaying the result (Figure 2d). It only took a total of 10 minutes or less for each sample.

As shown in Table 3, there is a significant difference between the normal tissue MI and all the tumor tissue MIs (4.1±2.5% and 26.0±22.1%, respectively, $p<0.001$). When the cut-off value of MI=6.8% was applied, the values of sensitivity and specificity were 86% and 81%, respectively.

MI increased as the histological grade increased (Figure 5). MI values in grade II, III, and IV gliomas were 17.2±16.0%, 34.1±24.6%, and 41.9±21.9% respectively. Significant difference was observed in the value of MI of each malignant tissue group (ANOVA $p<0.001$). In addition, the results of statistic tests of MI indicated that there were significant differences at all selected combinations of the groups ($p<0.001$) except the cases between grade III and grade IV. Association between the pattern of DNA histograms and the histological grade was also observed (Figure 6).

Table 3. MI of the normal tissues and tumor tissues

	Number of sample	Malignancy Index mean	SD	
Normal	35	4.1	2.5	]*
Tumor	86	26.0	22.1	

* $p < 0.001$

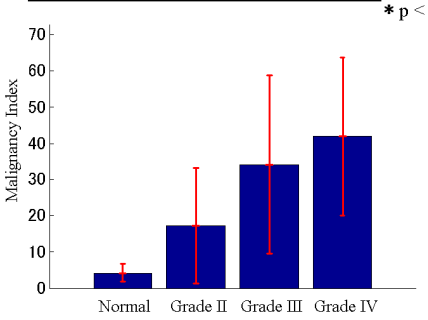

Figure 5. Dependence of MI detected by intraoperative flow cytometry analysis on presence of glioma and its WHO histopathological grade. The data are presented as mean ± standard deviation. Perilesional brain tissue without histopathologically identifiable tumor is defined as "Normal."

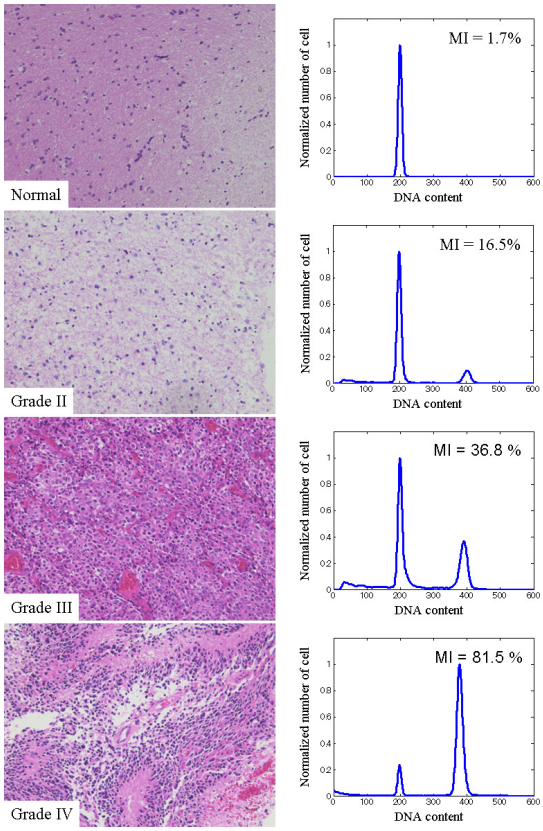

Figure 6. Examples of the histopathological characteristics of the tissue samples (left column) evaluated on permanent sections (H&E; x200) and corresponding DNA histograms obtained with intraoperative flow cytometry analysis (right column): perilesional edematous brain tissue without histopathologically identifiable tumor (A), WHO grade II oligoastrocytoma (B), WHO grade III anaplastic oligodendroglioma (C), WHO grade IV glioblastoma (D). MI, malignancy index.

IV. Discussion

We evaluated our system with specimens of gliomas. Human error and processing time in the sample preparation procedure can be significantly reduced when the reagent kit is used. Fully automatic analysis was also achieved using the original pipette tip, and we obtained highly reproducible data. These features of the system enabled us to reduce the throughput time to less than 10 minutes to analyze tissue samples. These results indicated that our new system could be useful to rapidly analyze tissue samples during glioma surgery and greatly help physicians by providing quantitative information for the diagnosis in combination with the conventional frozen specimen or smear method which provides qualitative information.

In this study, the accuracy of distinction between tumor and normal tissues was 86% of sensitivity and 81% of specificity. In our previous study, both sensitivity and specificity were 88%[11]. Therefore, we considered the result of this study to be almost equivalent to the previous one and acceptable for the surgery of glioma. We believe that the reduction in specificity observed in this study was due to the small number of samples (Normal: 35 samples, Tumor: 86 samples). Also, the results of statistic tests of MI indicated that there was not a significant difference between grade III and grade IV because the number of samples of grade IV was 10. We will improve the system by analyzing many more samples and continue evaluating the system.

The value of MI tended to increase as the histological grade increased. However, the high standard deviation of MI in each group makes it difficult to clearly classify tissues into WHO grades with the cutoff value of MI.

Although these problems remain to be solved, we believe that the accuracy of diagnosis is improved using our system. Since our system can offer a perspective different from histopathological investigation with quantitative data to pathologists and surgeons, they can evaluate tissues with quantitative information obtained with our new system. In addition, even if the judgment of tissue grading with frozen section during an operation is difficult for pathologists because of its faint image, this system can provide numerical data and support operative decision-making. Moreover, we suspect that there are some relationships between MI value and prognosis. The prognosis might be affected by greatly different MIs of two cases whose pathological examination suggests the same grade.

It is often clinically observed that grade II glioma becomes a higher-grade glioma at recurrence. Since DNA content is examined for each cell in our method, MI value can indicate the possibility of malignant transformation when grade II tissues indicate large MI. We will continue our research and follow up on treated patient prognosis.

V. Conclusion

Our fully automatic system of iFC with determination of MI was technically feasible and reliable for rapid determination of glioma presence in surgical biopsy samples. Our system enabled completion of tissue analysis within 10 minutes and requires a minimal amount of pathological tissue. It can identify the type of lesion at an earlier phase of surgery and provide surgeons sufficient time to determine surgical sites.

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