New Risk Classification of Gastrointestinal Stromal Tumors Based on Tumor Size and Three Indices Derived from Rapid Flow Cytometry of Fine-Needle Aspiration Specimens

TWMUJ 7: 34-41, 2023

Original

New Risk Classification of Gastrointestinal Stromal Tumors Based on Tumor Size and Three Indices Derived from Rapid Flow Cytometry of Fine-Needle Aspiration Specimens

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Background: Biopsies of suspected gastrointestinal stromal tumors in submucosal locations, which were once a challenge, are now easily performed with endoscopic ultrasound-guided fine-needle aspiration. However, fine-needle aspiration biopsy (FNAB) specimens may be inadequate for the diagnosis of malignancy using quantification of histological mitotic count.
Methods: We devised a procedure for rapid analysis with an algorithm for clinical use. DNA ploidy analysis conducted using flow cytometry determined the mitotic rate, which correlated with clinical prognostic factors (mitotic count and the Ki-67 labeling index). We then developed a risk classification system for gastrointestinal stromal tumors using flow cytometry parameters and tumor size.
Results: The combined flow cytometry and tumor size parameters correlated with the modified-Fletcher risk classification of gastrointestinal stromal tumors.
Conclusion: Rapid flow cytometry of endoscopic ultrasound-guided FNAB specimens can be used to diagnose gastrointestinal stromal tumors and assign a modified-Fletcher risk classification.

Keywords: biopsy, fine-needle aspiration, flow cytometry, gastrointestinal stromal tumors, risk

Introduction

Submucosal gastrointestinal stromal tumors (GIST) have been rendered accessible by biopsy with the development of endoscopic ultrasound-guided fine-needle aspiration biopsy (EUS-FNAB).[1-6]

However, the diagnosis of malignancy by histologic mitotic count (MC) is difficult using FNAB specimens.

We devised a flow cytometry algorithm for rapid analysis, which avoided many of the usual methodologi-

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Received August 24, 2022; Accepted November 14, 2022; Advance Publication by J-STAGE March 31, 2023
doi: 10.24488/twmuj.2022014

Table 1. Patient characteristics.

Characteristic	Quantity
Gender (M/F)	4/2
Age (Mean ± SD)	69.0 ± 12.6
Tumor size (cm)	5.0 ± 2.4
Mitosis count (HPF)	5.8 ± 5.0
Ki-67 labeling index (%)	10.3 ± 4.10
Risk classification with modified-Fletcher	
Low risk	2
Intermediate risk	3
High risk	1

Six formalin fixed gastrointestinal stromal tumor specimens obtained by laparoscopic partial gastrectomy were stratified using the modified-Fletcher risk classification for final diagnosis.
HPF, high-power fields.

cal difficulties that prevent flow cytometry use in more clinical settings.[7] This algorithm included DNA ploidy analysis, which reflects the mitotic rate and correlates with clinical prognostic factors (MC and Ki-67 labeling index [LI]). We investigated a risk classification for gastrointestinal stromal tumors that is based on flow cytometry parameters and tumor size.[8-10]

Materials and Methods

Materials

This study was conducted with the approval of the Institutional Review Board at Tokyo Women's Medical University (Approval No. 3257). The subset data was taken from 18 specimens mentioned in previous studies.[7] All procedures were performed at Tokyo Women's Medical University between 2014 and 2018. Patient characteristics are shown in **Table 1**.

Simulate a FNAB

Six GIST specimens obtained by laparoscopic partial gastrectomy were assessed using flow cytometry. For the flow cytometry analysis, a 22-gauge needle was used to simulate a FNAB in a fresh tissue specimen. The needle was moved back and forth five times while applying suction. This was performed for three punctures of each specimen. Subsequently, a tissue sample of approximately 3 mm was obtained from the fresh tissue specimen.

Flow cytometry analysis

The specimens were placed in a test tube and immersed in a solution (DNA Peak, FC-220 V; Nihon Kohden Corporation, Tokyo, Japan)[11, 12] that included ribonuclease A to remove RNA, TritonX-100 to render cell membranes permeable, and propidium iodide to stain DNA. Specimen disruption was performed with repetitive pipetting for 200 s using a prototype device (Nihon Kohden Corporation, Tokyo, Japan).[13, 14]

The punctured specimen was centrifuged in phosphate-buffered saline at 300 × g for 5 min. Subsequently, the supernatant was removed, and the above-mentioned reagent (FC-220V) was added to the precipitate.

The homogenized and stained samples were filtered through a 40 μm nylon mesh, and the DNA content was measured using a flow cytometer (EPICS-XL, Beckman Coulter, Miami FL, USA) to generate a DNA histogram over approximately 10 min. DNA aneuploidy (DA), DNA index (DI), and S-phase fraction (SPF) were derived from the histogram.

Methods

Flow cytometry of ploidy can reveal DNA heterogeneity of cells. The peak for diploidy is detected as a polyploid of the normal peak on the histogram produced from flow cytometry data, which is obtained using cell cycle analysis. DA was determined as cells with heteromerous DNA content, indicated by a separate peak from the diploidy peak on the histogram. DI was applied to determine whether a detected peak was DA. A cutoff value of DI = 1.00 was determined as a relative position based on the peak of normal cells on the histogram. Further, considering a significant correlation with the clinical prognostic factors, the cutoff value of DI showing DNA aneuploid was defined as DI = 1.30. If cells could not be distinguished from the G2/M phase of diploid cells, DNA aneuploid cells were not considered aneuploid ($1.90 < DI < 2.10$).[15] The SPF was defined as the area of the histogram between the peaks of G0/G1 and G2/M. The cutoff value of the SPF was set as 2 based on the correlation between the consecutive SPF values and the modified-Fletcher risk classification.[16]

Table 2. New risk classification by flow cytometry parameters.

	Tumor size (cm)	Flow cytometry parameters				
		DNA aneuploidy	DNA index		S-phase fraction	
Low risk	≤ 5.0	Absence	and	< 1.5	and	< 2
Intermediate risk	≤ 5.0	Presence	or	1.5 ≤	or	2 ≤
	5.1-10	Absence	and	< 1.5	and	< 2
High risk	5.1-10	Presence	or	1.5 ≤	or	2 ≤
	> 10	Regardless of the results of the flow cytometry				

Adapted from reference no. 7 with permission.

Statistical analysis

We initially investigated the accuracy of the flow cytometry parameter measurements (DA, DI, SPF) of the simulated FNAB specimens compared to those of the 3 mm-sized pieces of tissue. We also investigated the prognostic accuracy of these parameters compared to clinical prognostic factors, including MC and Ki-67 LI, using Pearson's chi-squared test. Tumor size and flow cytometry parameters from the simulated FNAB samples and the 3-mm tumor samples were further evaluated for correlation with the modified-Fletcher risk classification (**Table 2**).[9,10] All p values were calculated according to Pearson's chi-squared test. The histogram data analysis and statistical analysis were performed using the commercial software MATLAB (Version R2015b, Mathworks, Natick MA, USA) and JMP (ver. 14 SAS Institute, Cary NC, USA), respectively.

Results

The results of the analysis of the parameters measured using flow cytometry (DA, DI, SPF) were closely matched to the histogram pattern of the resection tissue specimen and the FNAB specimen in each case.

Figure 1-Case 1

The tissue isolate showed a peak DI = 1.23, but this was considered to include pseudo-DA as we used a cutoff value of DI = 1.30 for true DA. Therefore, the tissue and FNAB specimens had a judgment result of 0 in DA, DI, and SPF. Furthermore, there was an extremely low cell count in FNAB specimens as compared to that in the tissue, which may be due to differences in sample volume.

Figure 1-Case 2

For Case 2, the tissue isolate showed a peak DI = 1.18, but this was considered to include pseudo-DA as we used a cutoff value of DI = 1.30 for true DA. Therefore, both 0 each DA/DI/SPF judgment level neighbors, the tissue and FNAB results were the same.

Figure 1-Case 3

The results of the tissue and FNAB histogram patterns were extremely consistent.

Pseudo-DA was detected in the tissue, and the FNAB specimens had a slightly lower cell count than the tissue samples because of the difference in the sample volume.

As for DA of the tissue, DI = 1.53, whilst the DI = 1.54 in FNAB specimens.

Figure 1-Case 4

Very similar DA were detected for Case 4; for DA of the tissue, the DI = 1.47, whilst the DI = 1.42 for FNAB.

More debris was observed in the tissue than in the FNAB specimens.

Figure 1-Case 5

Pseudo-DA with a DI = 1.22 was detected in the tissue and an extremely small G2/M peak with DI = 1.95 in both the tissue and FNAB specimens was observed, but no other significant peaks were detected. Debris also appeared in the tissue. FNAB obtained higher cell counts than the tissue.

Figure 1-Case 6

Regarding DA of the tissue in case 6, the DI = 1.55, whilst for DA of FNAB specimens, the DI = 1.62. A mismatch in the DA peak position occurred between the tissue and FNAB specimen data. Only one slightly broad

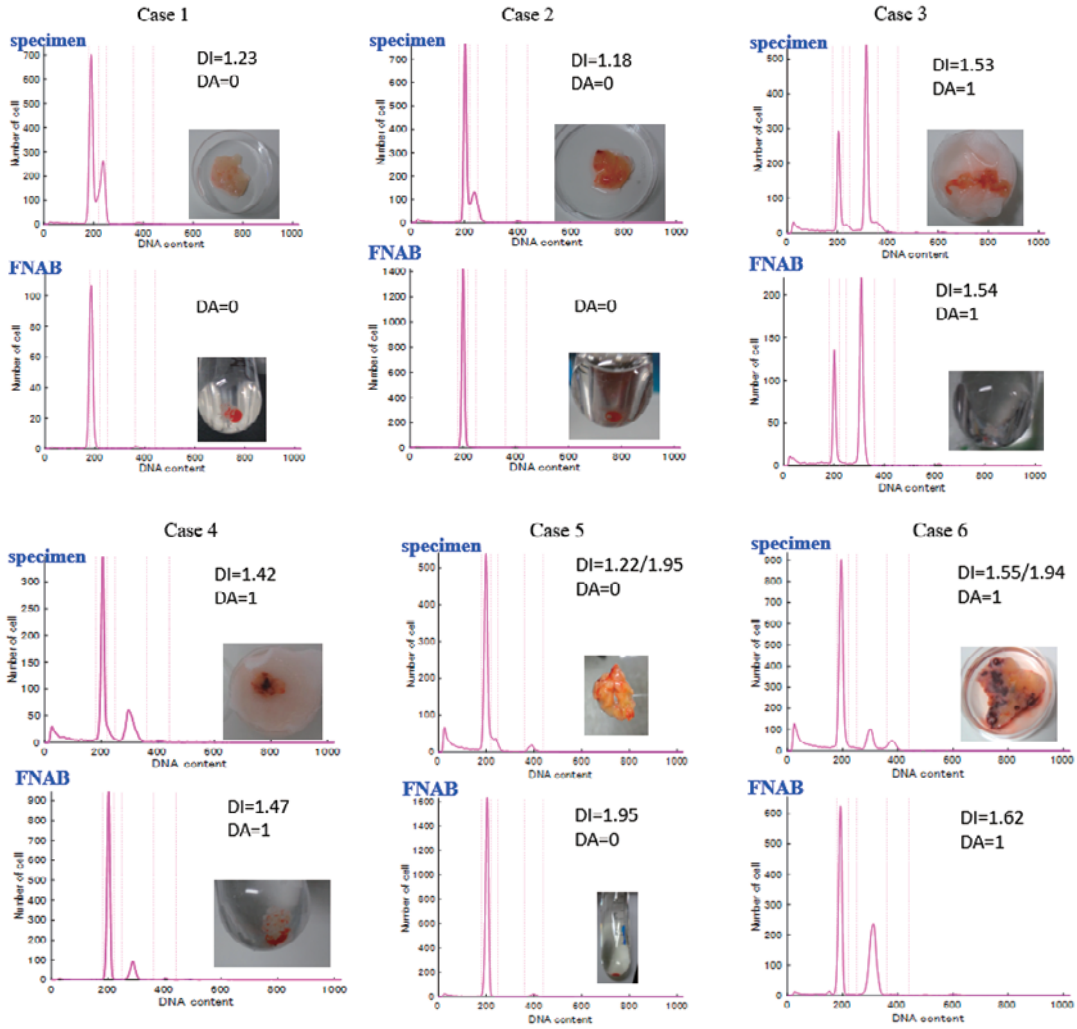

Figure 1. The histograms of resection specimens and simulated FNAB specimens contrasted for each case.

Case 1: A peak of DI = 1.23 appeared in the sample isolated from the tissue but was judged to be pseudo-DA because a cutoff value of DI = 1.30 was used for true DA. In addition, the FNAB sample had an extremely low cell count, possibly due to sampling deficiencies, however, the results for DA, DI, and SPF were 0 for the tissue and FNAB samples, and the overall results were in agreement.

Case 2: The tissue isolate showed a peak DI = 1.18, but this was considered to include pseudo-DA as we used a cutoff value of DI = 1.30 for true DA. Therefore, the tissue and FNAB samples had a judgment result of 0 in DA, DI, and SPF. The tissue and FNAB specimens' results were similar.

Case 3: Histograms with relatively consistent results were obtained from samples collected using the two methods. Pseudo-DA was detected in the tissue; although the histogram showed a lower cell count in fine-needle aspiration specimens, the judgment results were unaffected. For the DA of the tissue specimen, DI = 1.53 while for that of FNAB specimens, DI = 1.54.

Case 4: Very similar DA were detected for case 4; the DA of the tissue showed DI = 1.47 while that of the FNAB specimens showed DI = 1.42.

More debris was observed in the tissue specimen than in the FNAB specimens.

Case 5: Pseudo-DA with a DI = 1.22 was detected in the tissue specimen, and an extremely small G2/M peak with DI = 1.95 was observed in the tissue and FNAB specimens; however, no other significant peaks were detected. Debris also appeared in the tissue specimen. Moreover, FNAB specimens had higher cell count than the tissue specimen.

Case 6: In case 6, for the DA of the tissue specimen, the DI = 1.55 while for that of FNAB specimens, DI = 1.62. A mismatch in the DA peak position occurred between the tissue and FNAB specimen data. Only one slightly broad peak at DI = 1.62 was detected in the FNAB specimen, while DA (DI = 1.55) and G2/M (DI = 1.95) were detected in the tissue specimen.

DI, DNA index; DA, DNA aneuploidy; SPF, S-phase fraction; FNAB, fine-needle aspiration biopsy.

Table 3. Flow cytometry parameters with risk classification.

Case	Age	Gender	Tumor size (cm)	Mitotic count /50 HPF	Ki-67 labeling index (%)	FNAB			Specimen			FNAB	Specimen	
						DA	DI	SPF	DA	DI	SPF	Tumor size / flow cytometry	Tumor size / flow cytometry	Modified-Fletcher risk classification
1	79	F	28	4	10	0	2.03	0.29	0	1.23	0.28	low	low	low
2	43	M	28	1	7	0	NA	0.15	0	1.18	0.19	low	low	low
3	62	M	42	7	15	1	1.54	4.4	1	1.53	4.3	intermediate	intermediate	intermediate
4	69	M	43	8	10	1	1.42	1.1	1	1.47	2.9	intermediate	intermediate	intermediate
5	85	F	80	1	5	0	1.95	0.16	0	1.22/1.95	0.56	intermediate	intermediate	intermediate
6	63	M	80	14	15	1	1.62	2.2	1	1.55/1.94	4.9	high	high	high

F, female; M, men; HPF, high-power fields; FNAB, fine-needle aspiration biopsy; DA, DNA aneuploidy; DI, DNA index; SPF, S-phase fraction.

Table 4. Accuracy of the new risk classification.

Tumor size/flow cytometry parameters	Modified-Fletcher risk classification (tumor size/mitotic count)				p-value (*significant)
	Low risk	Intermediate risk	High risk	Total	
by simulated FNAB					
Low risk	2	0	0	2	
Intermediate risk	0	3	0	3	0.0164*
High risk	0	0	1	1	
by specimen					
Low risk	2	0	0	2	
Intermediate risk	0	3	0	3	0.0164*
High risk	0	0	1	1	

FNAB, fine-needle aspiration biopsy.

peak at DI = 1.62 was detected in the FNAB specimen while DA (DI = 1.55) and G2/M (DI = 1.95) were detected in the tissue (**Figure 1**).

Table 3 shows each association between flow cytometry parameters (DA, DI, SPF) and clinical prognosis parameters (MC and Ki-67 LI).

All included combined parameters were established from tumor size and flow cytometry parameters (DA, DI, SPF). Significant correlation was found between the modified-Fletcher risk classification and combined parameters such as tumor size, DA, DI, and SPF ($p = 0.0164$, $r = 1$) (**Table 4**).

Discussion

We devised a new risk classification for GIST using parameters measured by flow cytometry and demonstrated that combined parameters can correlate with the modified-Fletcher risk classification.

Various studies[1-6] have reported the effectiveness of FNAB for identifying pathological findings in GISTs and the possibility of evaluation by flow cytometry analysis of FNAB specimens. Despite this, risk classification for accurate diagnosis was thought to be impossible without pathological findings in surgical specimens.

Generally, obtaining GIST tissue from submucosal tumors (SMT) is challenging using normal endoscopic biopsy, and the diagnosis of GIST is difficult.

In recent years, the use of EUS-FNAB increased, enabling preoperative diagnosis of GIST. If sufficient tissue for flow cytometry and immunohistochemistry is obtained, a diagnosis may be reached, although the heterogeneity of these tumors still poses problems.[17]

The risk of GIST is stratified based on tumor size, location, mitotic rate, tumor location, and the presence or absence of tumor rupture. The MC is commonly used as an index of cell proliferation. Usually this is the number of mitotic figures per 50 high-power fields (HPF). A Ki-67 LI > 6% at 40× is another commonly used prognostic factor.[18, 19]

Cell cycle analysis combined with digital flow cytometry is another method that assesses cell division and

proliferation in tissue specimens without the time-consuming processes required in histopathology.[20-24] There have been reports on the correlation of flow cytometry histograms obtained from cell proliferation potency and the specific flow cytometry patterns of GIST.[18, 19, 25] Flow cytometry has not been evaluated in clinical studies because of the skill and technical requirements involved. Therefore, we developed a rapid and simple measurement procedure and quantitative analysis algorithm, using flow cytometry, that is suitable for clinical use. We showed that DNA ploidy analysis reflects mitotic rate and correlates with clinical prognostic factors (MC and Ki-67 LI) to evaluate GIST without histological diagnosis. We also set up a new risk classification of GIST using parameters measured by flow cytometry combined with tumor size and demonstrated that these combined parameters correlate with the modified-Fletcher risk classification.

It has been reported that specific flow cytometry patterns correlate with the MC of GIST,[26-28] suggesting that an accurate method of risk classification without histological diagnosis could be feasible.[7] In this study, we found that simulated FNAB samples could be closely matched with the modified-Fletcher risk classification (tumor size/MC), and a new risk classification (tumor size/flow cytometry parameters) could be developed using a tissue specimen and an FNAB specimen ($p = 0.0164$) (**Table 4**).

In the cases with high MC values (cases 3, 4, and 6), the SPF was also high in flow cytometry, indicating a correlation between MC and SPF; furthermore, the classification in these cases results also matched. The case with a large tumor diameter but low MC (case 5) was classified as intermediate by both the modified-Fletcher risk classification and flow cytometry (**Table 3**).

The analysis results obtained from each FNAB histogram and tissue specimen correlated with the flow cytometry parameters, although the shape of the histogram appeared to be different for some paired data. However, even though the histogram shape sometimes varied slightly in appearance, the FNAB and tissue specimens showed good correlation with flow cytometry parameters in all cases, suggesting that flow cytometry analysis of FNAB specimens is sufficient for risk assessment of GIST without using tissue specimens. Occasionally, a false-positive result is obtained for DA as a result of physical or chemical changes in a particular cell population that, for unknown reasons, alter dye binding during the cell preparation process. This DA result is referred to as pseudo-DA. The frequency of pseudo-DA depends on the target tissue and cell preparation method; however, pseudo-DA is particularly likely to appear when cells are isolated from tissues by surfactants. When pseudo-DA appears, it is generally excluded from the target area of histogram analysis.[1, 29]

Collecting specimens by FNAB is considered to have a lower risk of damaging cells than collecting them by forced isolation of cells from tissues with surfactant because the tissue is to some extent unbound when the specimen is collected. Thus, pseudo-DA is less likely to appear when collecting specimens by FNAB, which may be advantageous for flow cytometry histogram analysis. While FNAB provides more stable histograms than a tissue specimen, it may not provide correct analysis results if an insufficient amount of sample is collected from the appropriate location. This can be improved by optimizing the number of punctures and puncture points.

Thus, if the same amount of tissue can be collected each time by establishing a protocol for sample collection, the cell count information obtained by flow cytometry will reflect the cell density in the tissue and could become a new indicator for evaluating tissue malignancy.

EUS-FNAB has been suggested for the biopsy of gastric smooth muscle tumors, especially to differentiate GIST from other SMT.[1-5] Furthermore, the actual time required for flow cytometry was approximately 10 min to yield the diagnosis from a fresh specimen.

Our study has several limitations. First, our sample size of six cases was relatively small. Secondly, the six GIST specimens obtained by FNAB were only simulating EUS-FNAB.

However, a new risk classification of the six cases was closely matched to the modified-Fletcher risk classification. The possibility of the accuracy of this risk classification will be the subject of further studies.

Conclusions

Rapid measurements of tumor parameters by flow cytometry, avoiding histologic analysis, have been sug-

gested for endoscopic diagnosis by biopsy and EUS-FNAB. We describe a new risk classification for GIST that is based on flow cytometry parameters and tumor size and gives a reliable level of risk prediction. This can promote a future discussion of all possible surgical and diagnosis methods and evaluation of the optimum treatment strategy.

Sources of Funding: This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Conflicts of Interest: This study was conducted under a collaborative research agreement between Tokyo Women's Medical University and the Nihon Kohden Corporation for the voluntary lease of the pipetting device used for tissue preparation.

Author Contributions: KT made substantial contributions to the conception, design and data acquisition, analysis, and interpretation and participated in drafting the article. AS made substantial contributions to the analysis and interpretation of the data and participated in critically reviewing and revising the article for intellectual content. AS, SK, SI, KS, and SI made substantial contributions to data acquisition and participated in drafting the article. YM and MY made substantial contributions to the analysis and interpretation of data and participated in critically reviewing and revising the article for intellectual content. All authors approved the final version for publication.

Acknowledgments: The authors are grateful to all staff members of the Department of Gastroenterology and General Surgery, Tokyo Women's Medical University, and to all staff members of the Ogino Memorial Laboratory at Nihon Kohden Corporation for the help provided during this study. The authors also thank Libby Cone, MD, MA, and Jacquie Klesing, Board-certified Editor in the Life Sciences (ELS) of Yamada Translation Bureau, Inc., Tokyo, Japan (https://www.ytrans.com/home.html), for writing and editing assistance. We want to thank Editage (www.editage.com) for English language editing.

Ethical Approval: This study was conducted with the approval of the Institutional Review Board at Tokyo Women's Medical University (Approval No. 3257).

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