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Rapid Flow Cytometry of Gastrointestinal Stromal Tumours Closely Matches the Modified Fletcher Classification

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Abstract. *Aim: We aimed to develop a rapid, simple procedure and an algorithm for quantitative analysis and classification of the metastatic risk of gastrointestinal stromal tumours (GIST) for clinical use. Materials and Methods: Eighteen specimens from laparoscopic local gastrectomy were assessed by flow cytometry. We devised a new risk classification for GIST by combining flow cytometry parameters with tumour size and evaluated whether the combined parameters correlated with the modified Fletcher risk classification. Results: We found a significant correlation between clinical prognostic factors (mitotic count and Ki-67 labelling index) and the flow cytometry parameters DNA ploidy, DNA index and S-phase fraction. The combined parameters established from tumour size and the flow cytometry parameters showed a high correlation with the modified Fletcher risk classification (p=0.0064). Flow cytometry had to be performed for approximately 10 minutes to determine the metastatic risk. Conclusion: Rapid flow cytometry parameters can classify risk without the need for histological analysis.*

Currently, the metastatic risk of gastrointestinal stromal tumours (GIST) is predicted by tumour size, mitotic count, tumour location, and presence or absence of tumour rupture (1-3). The number of mitotic figures per 50 high-power fields (HPF) and a positive rate of the Ki-67 labelling index (Ki-67 LI) >6%, assessed with a 40× objective lens, are commonly used to estimate cell proliferation potential. However, the pathological diagnostic process requires many time-consuming procedures. Cell cycle analysis with digital flow cytometry is another method to assess cell division and proliferation in tissue specimens (4-8). The correlation of flow cytometry histograms obtained from cell proliferation potency with the specific flow cytometry patterns of GIST have previously been reported (9-11). However, flow cytometry has not been adopted in clinical studies because of the skilled preparation and technical requirements involved.

Here, we propose a rapid, simple procedure and an algorithm for quantitative analysis for clinical use that correlates with clinical prognostic factors: DNA ploidy analysis by rapid flow cytometry reflects the mitotic rate, which allows risk to be classified on the basis of flow cytometry parameters without the need for histological analysis.

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Key Words: Gastrointestinal stromal tumour, flow cytometry, risk classification, DNA ploidy.

Materials and Methods

Patient characteristics and flow cytometry assay. This study was conducted with the approval of the institutional review board at Tokyo Women's Medical University (approval no. 3257). All procedures were performed at Tokyo Women's Medical University between 2014 and 2018. Patient characteristics are shown in Table I.

Eighteen specimens taken after laparoscopic local gastrectomy for GIST were measured by flow cytometry with the methodology used in previous studies (12, 13). Briefly, a ~3 mm-sized piece of tissue was cut from a fresh surgical specimen. All specimens were collected from the centre of the tumour, placed in a microtube and immersed in a staining reagent kit (FC-220V; Nihon Kohden Corporation, Tokyo, Japan) (12, 13) that included ribonuclease A, TritonX-100 and propidium iodide. The specimen was then disrupted by repetitive pipetting for 200 s with an automatic cell isolation system for flow cytometry consisting of a cell isolation unit and a staining reagent kit prototype device (Nihon Kohden Corporation, Tokyo, Japan) (14). DNA aneuploidy (DA), DNA index (DI) and S-phase fraction (SPF) were obtained from the flow cytometry histogram. The actual time required for flow cytometry was ~10 min.

Flow cytometry parameters (DA, DI and SPF). Ploidy analysis with flow cytometry can reveal the DNA heterogeneity of cells. The peaks detected in the histograms represent the number of chromosomes in the analyzed cells. DA was seen on the histogram as a different peak from the diploidy peak. The DI was applied to determine whether a detected peak was DA. A DI value of 1.0 was determined as the position relative to the diploid peak of normal cells on the histogram. Next, to evaluate the presence of a significant correlation with the clinical prognostic factors we defined a DI cut-off value of 1.3 as indicating DA. If cells could not be distinguished from the G_2/M phase of diploid cells, DNA was not considered to be aneuploid (*i.e.*, if 1.90<DI<2.10) (15). The SPF was defined as the mean of the cell counts in the area of the flat part of the histogram between the G_0/G_1 and G_2/M peaks (16).

Clinical prognostic factor grading. Clinical factors were investigated as potential prognostic factors that obeyed the Fletcher and Miettinen classifications, including tumour diameter (≥5 cm *vs.* ≥2 cm and <5 cm *vs.* <2 cm), mitotic count (≥5/50 HPF *vs.* <5/50 HPF) and Ki-67 LI (≥6% *vs.* <6%) (17-20).

Statistical analysis. First, we compared the accuracy of the flow cytometry factors with that of the clinical prognostic factors (mitotic count and Ki-67 LI) by Pearson's chi-squared test. Next, we used the flow cytometry parameters that showed significant accuracy in the first analysis (*i.e.*, DA, DI and SPF) and tumour size to develop a new risk classification. Subsequently, we compared the accuracy of this new risk classification with that of the modified Fletcher risk classification with Pearson's chi-squared test. Histograms were analysed with MATLAB (version R2015b, Mathworks, Natick, MA, USA) and statistical analyses were performed with JMP software (version 14, SAS Institute, Cary, NC, USA).

Results

Correlation of flow cytometry parameters and clinical prognostic factors. Table II shows the association of clinical prognostic factors (tumour size, Ki-67 LI and mitotic count) and flow cytometry parameters (DA, DI and SPF) with the modified Fletcher classification in the individual participants. All correlations were low or intermediate.

The accuracy of flow cytometry parameters for identifying clinical prognostic factors. The cut-off values for the mitotic count and Ki-67 LI were chosen on the basis of the Fletcher and Miettinen classifications. The accuracy of the flow cytometry parameters DA, DI and SPF for identifying a mitotic count ≤5 were 88.9% (95% CI=69.6-96.9; p=0.0022), 83.3% (95% CI=65.4-92.2, p=0.0168) and 94.4% (95% CI=76.8-94.4, p=0.0003), respectively. The accuracy of the flow cytometry parameters DA, DI and SPF for identifying a Ki-67 LI ≤6 were 83.3% (95% CI=63.3-92.2, p=0.0092), 77.8% (95% CI=59.6-86.7, p=0.0045) and 88.9% (95% CI=70.5-88.9, p=0.0013), respectively. However, the tumour size did not significantly correlate with any of the flow cytometry parameters (Table III).

Table I. *Patient characteristics.*

Gender (M/F)	8/10
Age (Mean±SD)	63.6±12.0
Tumor size (cm)	4.27±1.84
Mitosis count (HPF)	1.2±1.2
Ki-67 LI (%)	3.9±3.2
Risk classification with modified-Fletcher	
Low risk	10
Intermediate risk	6
High risk	2

New risk classification for GIST on the basis of flow cytometry parameters. In our new risk classification of GIST, we replaced mitotic count with the flow cytometry parameters in the modified Fletcher classification. We defined the 3 risk levels as follows (see Table IV): low risk=tumour size ≤5 cm, absence of DA and DI <1.5 and SPF <2; intermediate risk=tumour size ≤5 cm, presence of DA or DI ≥1.5 or SPF ≥2 or tumour size between 5.1 and 10 cm, absence of DA and DI <1.5 and SPF <2; high risk=tumour size between 5.1 and 10 cm and presence of DA or DI ≥1.5 or SPF ≥2 or tumour size >10 cm.

Correlation of tumour size with flow cytometry parameters and risk classification. We found a significant correlation between the modified Fletcher classification and the combined parameters established from tumour size and flow cytometry parameters (p=0.0064).

When we compared our risk classification of low-risk GIST with the modified Fletcher classification, we found a value of 94.4% for accuracy, 100% for sensitivity, 87.5% for specificity, 90.9% for positive predictive value and 100% for negative predictive value. In the comparison of intermediate-risk GIST, the values were as follows: accuracy of 88.9%, sensitivity of 71.4%, specificity of 100%, positive predictive value of 100% and negative predictive value of 84.6%. The values for high-risk GIST were as follows: accuracy of 94.4%, sensitivity of 100%, specificity of 94.1%, positive predictive value of 50% and negative predictive value of 100% (Table V).

Discussion

In this study, we were able to devise a new risk classification of GIST by combining flow cytometry parameters with tumour size and to demonstrate that the combined parameters correlate with the modified Fletcher risk classification. Previous reports showed that specific flow cytometry patterns correlate with the cell proliferation potency of GIST (21-23), suggesting that they could be used as an accurate method for classifying risk without a need for histological diagnosis.

Table II. *Clinical and flow cytometry parameters*.

		Clinical parameters			Flow cytometry parameters			
Age	Gender	Tumor size (cm)	Mitotic count (number)	Ki-67 LI (%)	DA	DI	SPF	Modified-Fletcher classification
57	F	34	0	3	0	2.00	0.31	Low
72	F	38	0	3	0	1.12/1.26	0.32	Low
67	F	60	0	2	0	1.17	0.73	Intermediate
65	M	30	0	0	1	2.01/3.15	0.31	Low
75	F	30	1	0	0	1.99	1.58	Low
67	M	35	0	7	1	1.55/2.76	17.9	Intermediate
52	M	30	2	1	0	NA	1.95	Low
45	F	47	3	3	0	1.18/1.97	0.22	Intermediate
60	M	72	7	5	0	1.99	0.84	High
52	F	32	2	5	0	NA	0.25	Low
69	F	42	7	15	1	1.53	4.25	Intermediate
79	F	28	4	10	0	1.23/2.03	0.19	Low
49	F	28	1	7	0	1.18	0.28	Low
69	M	43	8	10	1	1.42	2.89	Intermediate
85	F	80	1	5	0	1.22/1.95	0.56	Intermediate
63	M	80	14	15	1	1.54/1.94	4.94	High
44	M	44	0	5	0	1.24	0.79	Low
75	M	17	1	5	0	1.98	0.65	Low

Table III. *Accuracy of flow cytometry parameters for identifying clinical prognostic factors*.

Clinical prognostic factors		Flow cytometry parameters	Accuracy % (95% CI)	*p*-Value
Tumor size	*vs*.	DNA aneuploidy	38.9% (21.6-47.9)	0.8882
		DNA index	66.7% (57.7-82.9)	0.8796
		S phase fraction	5.6% (5.6-23.2)	0.6230
Mitotic count	*vs*.	DNA aneuploidy	88.9% (69.6-96.9)	0.0022*
		DNA index	83.3% (65.4-92.2)	0.0168*
		S phase fraction	94.4% (76.8-94.4)	0.0003*
Ki-67 LI	*vs*.	DNA aneuploidy	83.3% (63.3-92.2)	0.0092*
		DNA index	77.8% (59.6-86.7)	0.0045*
		S phase fraction	88.9% (70.5-88.9)	0.0013*

*$p<0.05$.

Currently, GIST risk is stratified on the basis of tumour size, mitotic count, tumour location and the presence or absence of tumour rupture. The mitotic count is commonly used as an index of the cell proliferation potential, which is estimated from the number of mitotic figures per 50 HPF and a Ki-67–positive rate >6% with a 40× objective lens (24-26).

Fletcher *et al*. suggested that the mitotic count should be standardised according to the surface area examined (based on the size of the HPF). However, no agreed-upon definitions exist (1), even though such mitotic counts may still prove useful (1, 27, 28). We chose to focus on current flow cytometry parameters because cell cycle analysis with flow cytometry is a common method for analysing ploidy and proliferation in clinical specimens. We confirmed a significant correlation between clinical prognostic factors (MC, Ki-67 LI and SPF) and flow cytometry values and therefore propose a rapid, simple procedure and an algorithm for quantitative analysis for clinical use.

Flow cytometry is a technology that can identify the abnormal division of malignant cells, in which cellular DNA

Table IV. *New risk classification by flow cytometry parameters.*

		Flow cytometry parameters				
	Tumor size (cm)	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
	5.1~10.0	Absence	and	<1.5	and	<2
High risk	5.1~10.0	Presence	or	≥1.5	or	≥2
	>10.0			Any results		

Table V. *Accuracy of new risk classification.*

	Tumor size/flow cytometry parameters				
	Low risk	Intermediate risk	High risk	Total	*p*-Value
Modified-Fletcher Classification (tumor size/mitotic count)					
Low risk	10	1	0	11	
Intermediate risk	0	5	0	5	0.0064*
High risk	0	1	1	2	

Modified-Fletcher classification	Accuracy	Sensitivity	Specificity	Positive Predictive value	Negative Predictive value	*p*-Value
Low risk	94.4 (74.9-94.4)	100 (82.4-100)	87.5 (65.5-87.5)	90.9 (74.9-90.9)	100 (74.9-100)	0.0001*
Intermediate risk	88.9 (69.4-88.9)	71.4 (46.4-71.4)	100 (84.1-100)	100 (84.1-100)	84.6 (71.2-84.6)	0.001*
High risk	94.4 (22.4-100)	100 (22.4-100)	94.1 (89.6-94.1)	50.0 (11.2-50.0)	100 (95.2-100)	0.004*

*$p<0.05$.

is stained with fluorescent dyes (9-11). To date, flow cytometry is not in clinical use because preparation of the chemical reagent is troublesome, pre-treatment of the sample needs time and use of the flow cytometer is operator dependent, *i.e.*, the results are not reproducible. We previously reported on a grading system for malignant brain tumours, in which Shioyama *et al.* used flow cytometry during an operation (12). In this report, pre-treatment was simplified by using a commercial preparation (FC-220V) for the chemical reagent mixture. The pipetting method, which used an automatic cell isolation system and staining reagent kit to separate the cell nuclei from the cells, further shortened the required time. This technology allowed a measurement to be obtained within 10 minutes. The information obtained by intraoperative flow cytometry correlated closely with the pathological diagnosis and enabled the diagnosis of GIST (29).

Cells with heteromerous DNA content show DA, which is equivalent to the peak that is distinct from the diploid peak on a DNA histogram. DI is applied to determine whether a detected peak corresponds to a polyploidy profile. Furthermore, the correlation between SPF and Ki-67 LI is reported. The SPF corresponds to DNA replication, which occurs between the G_1 and G_2 phases, and the SPF ratio is correlated to tumour aggressiveness. In this study, we compared SPF distribution in the group with a Ki-67 LI value ≥ 6% and the group with a Ki-67 LI value < 6%. The SPF ratio was significantly higher in the former group (29). It is noteworthy that we identified a significant correlation between the modified Fletcher classification, tumour size and the combined parameters.

Most gastrointestinal tumours are diagnosed by endoscopic biopsy. However, submucosal tumours, such as GIST and leiomyoma, are difficult to diagnose because the results of biopsies are frequently negative. Endoscopic ultrasonography-guided fine-needle aspiration (EUS-FNA) has been widely performed to aid in diagnosing these tumours and was suggested for the differential diagnosis of gastric submucosal tumours, especially to differentiate GIST from other submucosal tumours (30-33).

Flow cytometry lasting approximately 10 minutes was required to classify risk from an unfixed specimen. Rapid flow cytometry parameters have been suggested for evaluating samples obtained by biopsy and EUS-FNA and this study showed that these parameters are useful for the risk classification of GIST without having to perform a histological diagnosis.

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.

Authors' Contributions

T.K. made substantial contributions to the conception, design and data acquisition, analysis and interpretation and participated in drafting the article. S.A. made substantial contributions to the analysis and interpretation of the data and participated in critically reviewing and revising the article for intellectual content. Y.T., S.A., N.T., A.K., K.S., I.S. and S.K. made substantial contributions to data acquisition and participated in drafting the article. O.A. made substantial contributions to data acquisition and participated in critically reviewing and revising the article for intellectual content. M.Y. and Y.M. 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.

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