Medical Oncology (2019) 36:54
https://doi.org/10.1007/s12032-019-1277-x

ORIGINAL PAPER

Performance of a new system using a one-step nucleic acid amplification assay for detecting lymph node metastases in breast cancer

Kenzo Shimazu[1] · Tomonori Tanei[1] · Yasuhiro Tamaki[2] · Toshiaki Saeki[3] · Akihiko Osaki[3] · Takahiro Hasebe[3] · Yasuhiko Tomita[4] · Motonari Daito[5] · Mayuko Kobayashi[5] · Shinzaburo Noguchi[1]

Received: 21 February 2019 / Accepted: 26 April 2019 / Published online: 6 May 2019

Abstract

One-step nucleic acid amplification (OSNA) for *CK19* mRNA is an intraoperative diagnostic procedure for detection of lymph node metastasis. Automated Gene Amplification Detector RD-200 and the LYNOAMP *CK19* gene amplification reagent as components of a new OSNA system have been developed. As an improvement over a conventional system, the new system can analyze 14 samples per run, evaluate two lymph nodes in ~ 17 min, and reduce inhibition of reactions. This study was aimed at evaluating clinical performance of the new system by comparing it with performance of histopathological analysis and a conventional OSNA system. A total of 150 lymph nodes in 63 breast cancer patients (T1–3) who underwent sentinel lymph node biopsy or axillary lymph node dissection were examined intraoperatively with the new OSNA system, the conventional system, and histopathological analysis. In comparison with histopathological analysis, sensitivity, specificity, and concordance rate of the new system were 93.9, 98.8, and 96.7%, respectively. In comparison with the conventional system, similar corresponding values were obtained: 96.9, 98.8, and 98.0%, respectively. The results show that clinical performance of the new OSNA system is equivalent to that of histopathological diagnosis and the conventional OSNA system. The new system is superior to the conventional one because of processing of a greater number of samples, shorter testing time, and the absence of inhibited reactions.

Keywords Breast cancer · Axillary lymph node metastasis · One-step nucleic acid amplification assay · CK19 mRNA

Introduction

Axillary lymph node status is still the most important prognostic factor for patients with breast cancer. Lately, sentinel lymph node (SLN) biopsy became the standard procedure for staging axillary nodal status of patients with early invasive breast cancer [1–4]. During SLN biopsy, intraoperative analysis (frozen section, touch imprint cytology) plays an important role in the decision making as to whether immediate axillary lymph node dissection should be performed. Even in the era of Z0011, intraoperative examination of SLNs is still important because a significant percentage of patients do not meet the inclusion criteria of the American College of Surgeons Oncology Group Z0011 study [5].

One-step nucleic acid amplification (OSNA) has been developed as a quick and semiautomatic procedure for detection of lymph node (LN) metastasis (LNM) by targeting cytokeratin 19 (*CK19*) mRNA. OSNA can be completed within 30–40 min, making it suitable as an intraoperative procedure for detecting SLN metastases [6–10]. Diagnostic accuracy of OSNA involving automated Gene Amplification Detector RD-100i and the LYNOAMP BC gene amplification reagent (Sysmex Corporation, Kobe, Japan) has repeatedly been shown to be equivalent to that of routine

✉ Kenzo Shimazu
kshimazu@onsurg.med.osaka-u.ac.jp

1 Department of Breast and Endocrine Surgery, Osaka University Graduate School of Medicine, 2-2-E10 Yamadaoka, Suita-shi, Osaka 565-0871, Japan

2 Department of Breast and Endocrine Surgery, Osaka International Cancer Institute, Osaka, Japan

3 Department of Breast Oncology, Saitama Medical University International Medical Center, Saitama, Japan

4 Department of Pathology, International University of Health and Welfare, Chiba, Japan

5 Gene Testing Business, Sysmex Corporation, Kobe, Japan

histopathological analysis, including an immunohistochemical assay (IHC) using formalin-fixed paraffin-embedded LNs, and better than the accuracy of intraoperative frozen section examination [11–14].

Although the OSNA assay system has been used in many countries, there is a need to decrease measurement time, to increase the number of samples analyzed per run, and to improve device operability. Moreover, it has been difficult to diagnose LNM in some patients with strong inhibition of gene amplification; thus, such tissue samples have required additional processing with a high dilution rate as a reference. To overcome the shortcomings of the conventional system, the Sysmex Corporation recently developed automated Gene Amplification Detector RD-200 and the LYNOAMP CK19 gene amplification reagent. This new system is an improvement increasing the number of analyzed LN specimens from four to 14 and enabling automatic reagent management (of remaining amounts of reagents) and detection of an expiration date via a barcode. In addition, the improved reaction efficiency of the new reagents means a new cut-off value, shorter operation time (for two LNs, 24 min 38 s to 17 min 9 s between the start of measurement and the result), and improvement of diagnostic accuracy without high dilution of samples. Despite the improvement of reaction efficiency, the results on *CK19* mRNA expression levels measured by the new system are consistent with those of the conventional system. Analytical performance of the new system has already been confirmed. This study was aimed at evaluating for the first time clinical performance of the new RD-200/LYNOAMP CK19 system for the detection of LNM in comparison to histopathological analysis and the conventional RD-100i/LYNOAMP BC system.

Patients and methods

Patients

A total of 63 patients aged 18 years or older with T1–2 N0 breast cancer who underwent surgical resection at Osaka University Hospital, Osaka International Cancer Institute, or Saitama Medical University International Medical Center between October 2015 and February 2016 were enrolled in the study. The patients were ineligible if they had a history of other metastatic cancers, had been treated with neoadjuvant therapy (except for anthracycline, fluorouracil [5-FU], taxane, trastuzumab, cyclophosphamide, and carboplatin), or were deemed to be incapable of participating in the study by an investigator. LN specimens were selected randomly, at fewer than five LNs per patient at each center. LN specimens weighing ≥ 50 mg were collected from patients who underwent an SLN biopsy or axillary lymph node dissection: LNs weighting > 600 mg were cut into smaller portions, each weighing between 50 and 600 mg. All the patients were informed about the study and provided written consent. The study protocol was approved by the institutional review boards of all the participating institutions.

Methods

The metastasis detection accuracy of RD-200/LYNOAMP CK19 (henceforth referred to as the new system) was compared to that of histopathological analysis and of RD-100i/LYNOAMP BC (henceforth referred to as the conventional system).

LN processing for OSNA and histopathological analysis

To evaluate clinical performance of the new system, it was compared with clinical performance of histopathological analysis, the gold standard of LNM diagnosis. The LNs were cut into four slices (Fig. 1), two of which were used for the OSNA assay and the other slices were subjected to histopathological analysis on three surfaces (two surfaces of slice B and one surface of slice D). LNs serving as tissue slices were embedded in paraffin and subjected to histopathological analysis via an IHC with the FLEX Monoclonal Mouse Anti-Human Cytokeratin Clone AE1/AE3 Ready-to-Use Antibody (Agilent Technologies Inc.; Santa Clara, USA). The immunostaining was performed using the Envision FLEX kit on an Autostainer Link 48 automated staining platform (Agilent Technologies Inc.). Classification of LNMs was conducted by histopathological analysis according to the Cancer Staging Manual of the American Joint Committee on Cancers, 8th edition [15] (Table 1).

Analysis of LNs by the conventional system and new system

LN sections weighing 50–600 mg were homogenized in 4 mL of lysis buffer LYNORHAG (Sysmex Corporation) with NS-360D (Microtech Nichion; Funabashi, Japan) and a LYNOPREP blade set (Sysmex Corporation) at 10,000 rpm for 60 s. Each LN homogenate was centrifuged at 10,000×*g* for 60 s at room temperature. The intermediate aqueous layer was regarded as the "LN lysate" and was diluted 10-fold with LYNORHAG in a sample container on ice (0–4 °C) with mixing by vortexing. This solution was regarded as the "analysis sample." The analysis sample was further diluted 10-fold with LYNORHAG in a sample container on ice (0–4 °C) with vortexing. This solution was regarded as the "diluted sample." Analysis samples and diluted samples were analyzed by means of the conventional system, whereas only analysis samples were analyzed using the new system following the manufacturer's

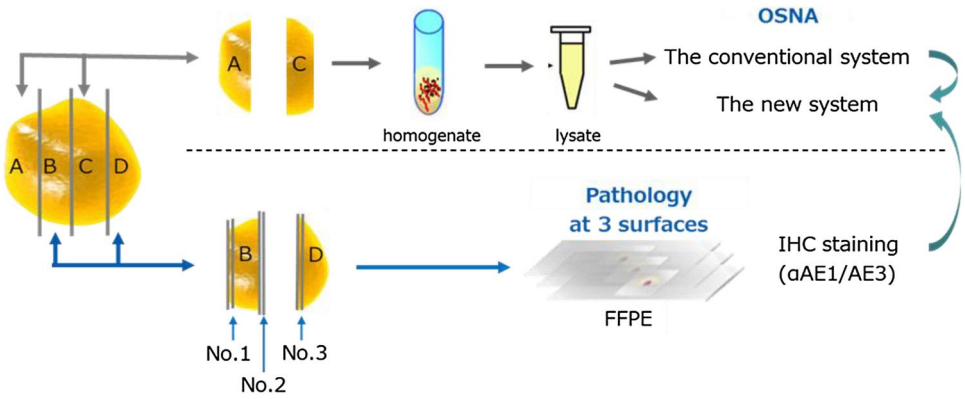

Fig. 1 Schematic representation of lymph node slicing preparation for the study protocol. Study design: OSNA (the new system) versus OSNA (the conventional system) or histopathological analysis. LN specimens were cut into four pieces. Slices "A" and "C" were subjected to the OSNA assay, slices "B" and "C" to histopathological examination of three surfaces (two surfaces of slice B and one surface of slice D) with hematoxylin and eosin staining and an IHC. OSNA, one-step nucleic acid amplification; IHC, immunohistochemical assay; FFPE: formalin-fixed paraffin-embedded

Table 1 Diagnostic characteristics of histopathological analysis, of the conventional system, and of the new system

Pathological diagnosis gold standard [15]		OSNA		Existing system	New system[a]
Positive	Macro-metastasis: size of metastatic lesions in the greatest dimension > 2 mm	Positive	(++)	[*CK19* mRNA] ≥ 5000 copies/μL	[*CK19* mRNA] ≥ 5000 cCP/μL
	Micro-metastasis: size of metastatic lesions in the greatest dimension ≤ 2 mm and > 0.2 mm		(+)	5000 copies/μL > [*CK19* mRNA] ≥ 250 copies/μL	5000 cCP/μL > [*CK19* mRNA] ≥ 250 cCP/μL
			+I	The result is positive but (++) and (+) were indistinguishable	Not applicable
Negative	Isolated tumor cells (ITCs): size of metastatic lesions in the greatest dimension ≤ 0.2 mm or < 200 metastasis-positive cells	Negative	(−)	[*CK19* mRNA] < 250 copies/μL	[*CK19* mRNA] < 250 cCP/μL
	No metastasis: No test-positive cells				

[a]cCP is the unit that means conversion from the new system to the conventional one

instructions. A calibration curve was drawn based on the rise time of calibrators, and *CK19* mRNA concentrations in the samples were calculated by applying rise time to the calibration curve. The conventional system and new system automatically calculated *CK19* mRNA concentrations in analysis samples via comparison with respective valid standard curves and a predetermined cut-off value of 250 cCP/μL (The improved reaction efficiency of the new system results in higher copy number than that obtained by conventional system. Using a conversion formula, measurements from the new system are converted into values equivalent to measurements from the conventional system and displayed in the unit of "cCP."). The conventional system and new system identified each sample automatically as (−), (+), (++), or +I following the criteria listed in Table 1.

Investigation of discordant cases

To evaluate the cases discordant between the new system and histopathological analysis, β-actin expression levels were evaluated via the new OSNA system to determine whether RNA quality was sufficient. β-actin mRNA was amplified following RT-LAMP method using six primers (βact-FA, βact-RA, βact-F3, βact-R3, βact-LPF, βact-LPR). To evaluate a case discordant between the new system and conventional system, the OSNA assay was redone three times.

Statistical analysis

The data on recruited patients are presented as percentages, ranges, or counts. To compare clinical performance between

the new system and histopathological analysis or the conventional system, concordance, sensitivity, and specificity were calculated.

Results

Study subjects

Sixty-three patients who met the inclusion criteria were enrolled from the three institutions. All of them underwent the tests for comparison of the new system with histopathological analysis and the conventional system. A total of 150 LNs (SLNs or non-SLNs) resected from the 63 patients were examined. Age and clinical characteristics of the enrolled patients are described in Table 2.

Diagnostic performance of new system, of histopathological analysis, and of the conventional system

Table 3 shows the comparison of diagnostic performance between the new system and histopathological analysis, or the conventional system on the LN specimens. When compared with histopathological analysis, the concordance rate, sensitivity, and specificity of the new system were 96.7% (95% confidence interval [CI] 92.3–99.0%), 93.9% (95% CI 88.8–97.2%), and 98.8% (95% CI 95.3–99.9%), respectively. The concordance rate, sensitivity, and specificity of the new system relative to histopathological analysis were equivalent to those relative to the conventional system [6–9]. When compared with the conventional system, the concordance rate, sensitivity, and specificity of the new system were 98.0% (95% CI 94.3–99.6%), 96.9% (95% CI 92.7–99.1%), and 98.8% (95% CI 95.3–99.9%), respectively. In addition, when compared with histopathological analysis, the concordance rate, sensitivity, and specificity of the conventional system were 97.3% (95% CI 93.3–99.3%), 95.5% (95% CI 90.8–98.3%), and 98.8% (95% CI 95.3–99.9%), respectively.

Investigation of the results discordant between the new system and histopathological analysis

The results on five LN specimens were discordant between histopathological analysis and the new system (Table 4A). On histopathological slides, discordant LNs No. 1–3 contained relatively small numbers of cancer cells, and some of their sections had no metastatic cells, suggesting that metastatic cells were detected only in a histopathological sample. Although only relatively small numbers of cancer cells were observed on all the surfaces of LN No. 4, it was identified as metastasis-negative by the new system despite detection of some *CK19* mRNA. This finding suggested that the number of cancer cells present in slices of the OSNA sample was smaller than that on slices for histopathological analysis. In LN No. 5, even though metastasis was not visible in all histopathological sections, the result of the new system was positive. Because *CK19* mRNA concentration was near the cut-off for the new system, it was assumed that a small

Table 2 Characteristics of the patients

Characteristic	($n=63$)
Age	
Median	64 (38–83)
T	
Tis	2 (3.2%)
T1	17 (27.0%)
T2	23 (36.5%)
T3	10 (15.9%)
T4	4 (6.4%)
Unknown	7 (11.1%)
Tumor histology	
IDC	52 (82.5%)
ILC	4 (6.3%)
DCIS	1 (1.6%)
Unknown	5 (7.9%)
Histological grade	
Grade1	12 (19.0%)
Grade2	18 (28.6%)
Grade3	28 (44.4%)
Unknown	5 (7.9%)
ER	
Negative	15 (23.8%)
Positive	47 (74.6%)
Unknown	1 (1.6%)
PR	
Negative	22 (34.9%)
Positive	40 (63.5%)
Unknown	1 (1.6%)
Her-2/neu	
Negative	50 (79.3%)
Positive	12 (19.0%)
Unknown	1 (1.6%)
LVI	
Positive	19 (30.2%)
Negative	39 (61.9%)
Unknown	5 (7.9%)
Neoadjuvant chemotherapy	
Performed	20 (31.8%)
Not performed	43 (68.3%)

IDC Invasive ductal carcinoma, *ILC* invasive lobular carcinoma, *DCIS* ductal carcinoma in situ, *LVI* lymph vascular invasion, *ER* Estrogen receptor, *PR* Progesterone receptor, *Her-2/neu* human epidermal growth factor receptor 2

Table 3 Comparison of diagnostic performance between the new system and histopathological analysis or the conventional system

	Histopathological diagnosis				
	Positive		Negative		Total
	Macro-metastasis	Micro-metastasis	ITC	No metastasis	
New system					
Positive					
++	42	3	0	0	45
+	13	4	0	1[a]	18
Negative					
−	0	4[a]	2	81	87
Total	55	11	2	82	150
	Conventional system				
	Positive			Negative	Total
	++	+	+I	−	
New system					
Positive					
++	39	1	5	0	45
+	2	14	1	1[b]	18
Negative					
−	0	2[b]	0	85	87
Total	41	17	6	86	150
	Histopathological diagnosis				
	Positive		Negative		Total
	Macro-metastasis	Micro-metastasis	ITC	No metastasis	
Conventional system					
Positive					
++	38	3	0	0	41
+	11	5	0	1[c]	17
+I	6	0	0	0	6
Negative					
−	0	3[c]	2	81	86
Total	55	11	2	82	150

[a]Discordant results between the new system and histopathological analysis

[b]Discordant results between the new system and the conventional system

[c]Discordant results between the conventional system and histopathological analysis

number of cancer cells were present only in the OSNA sample. These data suggested that metastatic lesions in blocks used for the assay were small thus causing allocation bias.

Analysis of the results discordant between the new system and conventional system

Three LN specimens (No. 6, 7, and 8), for which the test results were discordant between the conventional system and new system, were re-assayed three additional times to examine the cause of discordance (Table 4B). These three discordant cases yielded both positive and negative results in four measurements using at least one of these systems. *CK19* mRNA concentrations in these discordant cases were likely near the cut-off value and yielded both positive and negative results frequently within the margin of error of these systems. It is likely that the reason for these discordant cases is reproducibility of quantitative assays around the cut-off value in these systems.

Table 4 Samples discordant between the new system and histopathological analysis or the conventional system

(A)

LN No.	New system			Histopathological diagnosis			
	CK19 mRNA		β-actin mRNA		Metastasis size (mm)		
	Analysis samples, cCP/μL[a]	Finding	Decision		Surface No. 1	Surface No. 2	Surface No. 3
1	ND	−	> Cut-off	Micro	0.4	0	0
2	1.7×10^{0}	−	> Cut-off	Micro	1	0	0
3	1.8×10^{2}	−	> Cut-off	Micro	0.3	0	0.2
4	1.4×10^{2}	−	> Cut-off	Micro	0.1	0.1	0.5
5	4.1×10^{2}	+	> Cut-off	Negative	0	0	0

(B)

LN No.	New system			Conventional system			
	Analysis samples, CK19 mRNA cCP/μL[a]	Finding	Findings of additional measurements (three)	Analysis samples, CK19 mRNA copy/μL	Diluted samples, CK19 mRNA copy/μL	Finding	Findings of additional measurements (three)
6	1.8×10^{2}	−	−, −, −	2.5×10^{3}	ND	+	−, −, −
7	2.9×10	−	−, −, −	2.6×10^{2}	ND	+	−, −, −
8	4.1×10^{2}	+	+, −, −	4.5×10	ND	−	+, +, +I

[a]cCP is the unit that means conversion from the new system to the conventional one. ND: note determined

Table 5 Further analysis of samples identified as +I by the conventional system

LN No.	New System		Histopathological analysis	Conventional system		
	Analysis samples, CK19 mRNA cCP/μL[a]	Finding	Finding	Analysis Samples, CK19 mRNA copy/μL	Diluted samples, CK19 mRNA copy/μL	Finding
9	2.2×10^{4}	++	Macro	4.0×10^{3}	6.3×10^{3}	+I
10	2.2×10^{4}	++	Macro	9.6×10^{2}	3.2×10^{4}	+I
11	2.4×10^{4}	++	Macro	1.6×10^{3}	1.0×10^{4}	+I
12	2.9×10^{4}	++	Macro	4.0×10	1.6×10^{4}	+I
13	6.9×10^{3}	++	Macro	2.2×10^{3}	1.8×10^{4}	+I
14	2.5×10^{2}	+	Macro	1.9×10^{2}	4.1×10^{2}	+I

[a]cCP is the unit that means conversion from the new system to the conventional one

Analysis of +I results produced by the conventional system

For analysis of PCR inhibition in the new system, we assessed the results on the samples identified as +I by the conventional system because of strong gene amplification inhibition (Table 5). The results on five samples identified as ++ by the new system out of the six were consistent with histopathological findings. These five samples belonged to the different patients. The discordance between the new system and histopathological analysis regarding LN specimen No. 14 was probably due to the borderline diagnosis of micrometastases and/or allocation bias because measurement results from both the new system and the conventional system were near the cut-off. Thus, although the new system was not applied to the samples that were highly diluted to reduce inhibition of gene amplification, it was confirmed that clinical performance of the new system was equivalent to that of histopathological analysis.

Discussion

This study evaluated clinical performance of a new OSNA assay. First, the results confirmed good concordance in diagnostic performance between the new system and

histopathological analysis. Allocation bias was suspected as the reason for discordance regarding several samples because these two methods were applied to different LN slices. In clinical practice, if the new system is employed as a complete substitute of histopathological analysis for the same LNs, allocation bias of metastasis samples is less likely because the whole LN can be subjected to OSNA. Therefore, the concordance rate is expected to be better than that seen in the current results. Second, a comparison between the new OSNA system and conventional system also revealed high concordance in positive and negative diagnostic results. Because the decisions on three discordant samples were changed after the additional measurements (Table 4B), analytical reproducibility of calibrators and samples around a cut-off value was suspected as the cause of the observed discordance. These results suggest that the new OSNA system shows good clinical performance equal to that of histopathological analysis, the gold standard of diagnosing LNM, and to performance of the conventional system, which is recommended for diagnosing LNM in some guidelines. When the conventional OSNA system were first introduced, many studies have validated the performance with histopathological examination used as the gold standard for comparison (lymph nodes were divided and half were subjected to OSNA and the other half to histopathological examination). Being consistent with our finding, the results of these studies showed a pooled 96% concordance rate with histopathological examination [16]. Most discordant results were explained by allocation bias and low volume metastases around cut-off value as described in this study.

Our results indicate that the new system can replace the conventional system in clinical practice. Replacing the analytical system should improve operator productivity because the new system can make diagnoses faster and without high dilution of samples. Furthermore, the new system is useful for quality control because it allows for easy and automatic management of reagent lots with barcodes. With an increased number of samples measured, the system may be next developed into a pre- or postoperative diagnostic tool for other cancer types and LNs, providing additional clinical value [17, 18]. For example, AJCC-2013 states that accurate staging of colorectal cancer requires testing of 12 or more LNs [19] Thus, the utility of the new system, which enables analysis of 14 samples in a single run, is promising.

In addition, this study evaluated inhibition of DNA amplification. In general, substances present in biological samples, e.g., nucleases, proteases, polysaccharides, proteins, and metal ions, inhibit an amplification reaction. Concentrations of these components vary among samples, and hence the magnitude of inhibition of PCR amplification depends on individual differences. If this inhibition is strong enough to affect clinical findings, then the conventional system yields the +I result; the same is true for high-dilution samples. In the new system, the effect of the gene amplification reaction inhibition was reduced by the reagent improvement, and it has been confirmed that the determination equivalent to the conventional system can be made only with the analysis sample by another study (data not shown). Although the new system is not designed to perform measurements on highly diluted samples, it showed high concordance with histopathological analysis regarding the six samples that were identified as +I by the conventional system. This result proved that the new system has high PCR efficiency without inhibition by components of LNs. It is expected that the new system will be resistant to individual differences and other sources of systematic error because the samples with strong PCR inhibition are analyzed more accurately with high quantitative performance. The +I result is one of disadvantages of the conventional system because this result is not quantitative, and a nomogram (which requires a copy number of *CK19* mRNA) cannot be constructed. The new system overcomes this problem and determines a copy number of *CK19* mRNA more accurately in such clinical samples.

The improved reaction efficiency of the new system results in higher copy number than that obtained by conventional system. However, the copy number by the conventional system has already been used for decision making regarding the treatment of breast cancer in many countries, because it has been reported that the amount of CK19 mRNA in SLN was significantly correlated with non-SLN metastasis and prognosis [20, 21]. Thus, to avoid confusion due to the change in the measurement value by the new system, a conversion formula that makes the measured value of the new system the same value as the conventional system. Further studies are required to evaluate whether amount of cCP obtained by the new system had a significant impact on non-SLN metastasis and prognosis.

In this study, the performance of the new system was proved to be equal to that of histopathological analysis and of the conventional system. This study is one of the first reports presenting a new easy-to-use system as a robust diagnostic tool that is a possible substitute for the conventional system in clinical practice. Nevertheless, our results on the new OSNA system need to be validated in a future study involving a larger number of patients.

Funding All the funding was provided by the Sysmex Corporation.

Compliance with ethical standards

Conflict of interest KS. has received honoraria from AstraZeneca, Novartis and Sysmex. Y.T. has received honoraria from AstraZeneca, Eisai, Pfizer, Allergan, Johnson & Johnson. T.S and A.O. have received research funding AstraZeneca, Eisai, Ono, Kyowa Hakko, Daiichi-Sankyo, Taiho, Chugai, Nippon Kayaku, Bayer, MSD, Nihon-

Mediphysics, Hamamatsu-photonics and Parexel on the other studies. S.N. has been an advisor for AstraZeneca, Novartis and Taiho, has received honoraria from Sysmex, AstraZeneca, Chugai, Nippon Kayaku, Novartis and Takeda, and has received research funding from Sysmex, AstraZeneca, Chugai, Daiichi-Sankyo, Eisai, Nippon Kayaku, Novartis, Ono, Pfizer, Taiho and Takeda on the other studies. S.N. hold the joint patents with Sysmex on subjects not related to this study. M.D. and M.K. are employees of Sysmex Corporation. The other Authors do not declare any conflict of interest related to this study.

Ethics approval The study protocol was approved by the institutional review boards of all participating institutions.

Informed consent Informed consent was obtained from all patients included in the study.

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