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Cytokeratin19 mRNA as a Universal Marker for Detecting Metastatic Lymph Nodes, Irrespective of Racial Groups or Cancer Types

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The mRNA of cytokeratin (CK) 19 in lymph nodes (LNs) has been used as a clinical marker for predicting disease progression and as an aid for deciding therapeutic strategy. Although it has been reported that CK19 mRNA is a useful marker to detect metastasis positive or negative through races and cancers, racial or cancer-related differences in CK19 mRNA which may affect cut-off value have not been evaluated by comparing cohorts directly. The objective of this study was to validate the applicability of CK19 mRNA as a clinical marker and its cut-off value by comparing the distribution of CK19 mRNA expression level per unit of metastasis volume in LNs from Japanese breast cancer patients and Caucasian breast, colon, and gastric cancer patients. Frozen serial sections of lymph nodes were tested both in the OSNA assay and by histopathological examination, and CK19 mRNA expression level per unit of metastasis volume was calculated for each LN. The average (±standard deviation) level (expressed as log number of copies/μL) in Japanese breast (30LNs), Caucasian breast (29LNs), colon (21LNs), and gastric cancer (30LNs) lymph nodes was 3.714 (0.599), 3.433 (0.610), 3.457 (0.574), and 3.599 (0.644), respectively, indicating no significant difference between races or cancer types.

This result confirmed the applicability of CK19 mRNA expression level for Japanese/Caucasian patients as a clinical marker for breast, colon, and gastric cancer. It is expected that further clinical research to assess disease progression using CK19 mRNA will be investigated and that CK19 mRNA will be proved to be a more valuable clinical marker than the standard practice when defining the optimal treatment method for patients.

Key Words Cytokeratin, CK19 mRNA Expression, the OSNA Assay, Lymph Node, Metastasis, Race, Breast Cancer, Colon Cancer, Gastric Cancer

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INTRODUCTION

Sentinel lymph node (SLN) biopsy is accepted as the standard practice for axillary staging in patients with primary breast cancer[1)], and plays an important role in patients with colon or gastric cancer to minimize the extent of surgical resection or to evaluate the precise stage.[2-5)] The One-step Nucleic Acid Amplification (OSNA) assay (Sysmex Corporation, Kobe, Japan) is one of the methods to detect metastasis in LNs by measuring CK19 mRNA expression level automatically within 30-40 minutes for these cancer types.[6-8)] Numerous reports have indicated that the performance of the OSNA assay for detecting lymph node (LN) metastasis is qualitatively equivalent to that of histopathological examination in breast, colon, and gastric cancer.[9-21)] On the basis of these reports, the OSNA assay is now used globally and recommended by guidelines for breast cancer in some countries.[22,23)]

In breast cancer, sentinel lymph node biopsy (SLNB) also helps to judge intraoperatively whether axillary lymph node dissection (ALND) is necessary or not.[1)] Although ALND is carried out to avoid further metastasis to another organ, it also has major long-term side effects such as lymphedema, motor nervous system disorders, and impaired arm mobility. Therefore, it is important to assess the need for ALND with high accuracy and to select only patients who should have ALND.

Previously the prediction of non-SLN metastasis status was based on whether SLNs were metastasis positive or negative. However, this SLN qualitative diagnosis is insufficient

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since metastasis positive SLNs predict additional non-SLN metastasis in only 40-60% of patients.[24,25] Some reports have suggested other more accurate diagnosis to detect non-SLN metastasis by using metastasis size in the SLNs or nomograms that combine several clinicopathological variables related to SLN metastasis.[26,27] Total tumor load (TTL) is total CK19 mRNA expression level in SLNs determined by the OSNA assay. It has been proposed to be a new predictor of metastasis in non-SLNs. Several reports showed that TTL or a nomogram containing TTL is a more useful predictor of metastasis-positive non-SLNs than the number of metastasis-positive SLNs, and CK19 mRNA expression level is a remarkably sensitive marker for lymph node metastasis diagnosis.[28-30] Other reports tried to use CK19 mRNA expression level for predicting non-SLN metastasis and prognosis.[31,32]

The same cut-off value for CK19 mRNA is applied to the OSNA assay for Japanese breast cancer patients as well as for Caucasian breast, colon, and gastric cancer patients, and its clinical performance has been accepted by the scientific community.[9-16,18-20,33] Although there is no expectation of a racial difference between Japanese and Caucasian breast cancer patients nor of differences by cancer type among Caucasian breast, colon, and gastric cancer patients, prior to this study no report has directly compared the CK19 mRNA expression levels of these cohorts. Proof of the absence of racial and cancer-type differences would validate the clinical value of CK19 mRNA in a LN as a clinical marker and its cut-off value, and suggest further investigations and new therapeutic strategies. Therefore, this study aimed to evaluate racial and cancer-type differences in CK19 mRNA expression level per metastasis volume in LNs by comparing a distribution of cohorts.

MATERIALS AND METHODS

Sample collection and diagnosis

Only patients who had one metastatic organ and had not received neoadjuvant chemotherapy were enrolled. LNs collected from Japanese breast cancer patients were obtained from Sagara Hospital (Kagoshima, Japan) and LNs collected from European (Caucasian) breast, colon, and gastric cancer patients were purchased from tissue banks (ProteoGenex, Inc., Inglewood, CA; Asterand Bioscience, Detroit, MI; Geneticist Inc., Glendale CA; EastWestBiopharma, Kyiv, Ukraine; and Trans-HIT Bio, Laval Qc Canada). The LNs were surgically resected and divided into two parts, with one part stored at −80°C and the other stored in formalin. Hematoxylin and eosin (HE) stained formalin-fixed paraffin-embedded (FFPE) specimens were used by pathologists to diagnose LN metastasis status following the criteria of the Union for International Cancer Control (UICC) 7th edition. Only one or two metastasis-positive LNs per patient were selected for this study.

Sample transportation

The part of each LN stored at −80°C was transported to Sysmex Corporation under −20°C on dry ice, and, once there, stored at −80°C until testing.

Preparing frozen serial sections

Excess adipose tissue was removed from the LN sample on dry ice. The LN and OCT compound (Tissue-Tek) were put into a Cryomold (Tissue-Tek) and frozen in dry ice/ethanol. Three sets of serial sections (set No. 1, 2, and 3), each consisting of seven serial sections 10-μm thick (a, b, c, A, B, C, and D) and additional sections between each set were prepared; in all, 25 sections were cut from the center of each frozen LN with a cryostat (Leica Biosystems) (***Fig. 1***).

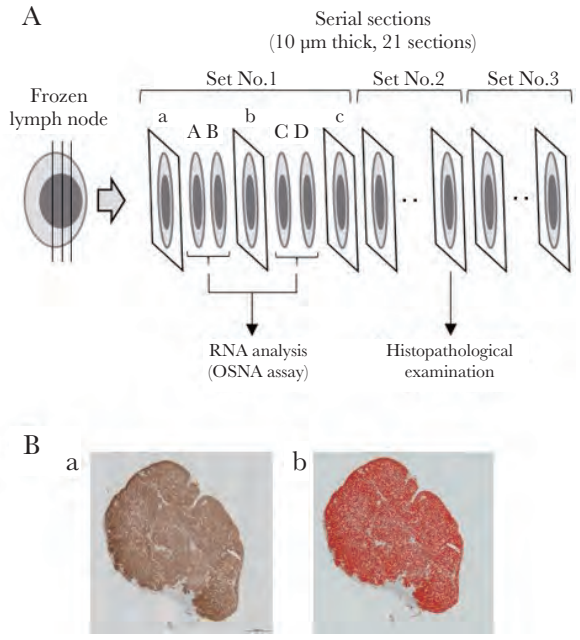

Fig. 1 *Protocols A, preparing serial sections B, image of examination under a microscope (a) and calculation of the metastasis positive area (b).*

In each serial section set, the 3 sections (labeled a, b and c) were put on glass slides and processed for histopathological examination, and the 4 sections (labeled A, B, C, and D) were put into a single micro tube, homogenized, and processed for RNA analysis by the OSNA assay and for calculation of RNA integrity (RIN). If histopathological samples were not stained and RNA analysis samples were not homogenized on the day of LNs sectioning, they were stored in a freezer at −80°C until the day of next examination or analysis.

Homogenization

Four sections (A, B, C, D) in each set were homogenized together in 400μL of LYNORHAG (Sysmex Corporation) using a Handy Pestle (TOYOBO Inc., Osaka, Japan), and the homogenate was centrifuged (10,000×g, 1 min, 4°C) to obtain its supernatant as lysate. If the OSNA assay or RIN analysis was not performed on the same day, the lysate was stored on −80°C.

Calculation of the RNA Integrity Number (RIN)

Total RNA was purified from 150μL of lysate using the RNeasy Plus Mini Kit (QIAGEN, Hilden, Germany) and QIAcube (QIAGEN) and eluted with 50μL of nuclease-free water. The RIN value of the purified total RNA was calculated using software accompanying the Agilent RNA 6000 Pico Kit (Agilent Technologies, Palo Alto, CA), and the Agilent 2100 bioanalyzer (Agilent Technologies) was used for total RNA assessment.

The OSNA assay

Each sample consisting of 10μL of lysate diluted with 90μL of LYNORHAG was analyzed for CK19 mRNA expression twice using a Gene Amplification Detector System RD-100*i* (Sysmex Corporation) and LYNOAMP BC kit (Sysmex Corporation), which are used specifically in the OSNA system. Total CK19 mRNA expression level (log number of copies/μL) in four sections (A+B+C+D) was the average of two measured values (log number of copies/μL) obtained using a calibration curve.

Immunohistochemistry (IHC)

Lymph node sections on slides were subjected to IHC using the Envision FLEX visualization system for Cytokeratin (AE1/AE3) (Dako, Glostrup, Denmark) and an automated immunostainer (Autostainer Link 48; DAKO). The antibody was diluted to 10-50% of its original concentration with antibody dilution buffer (DAKO), and the EnVision FLEX Hematoxylin (RTU) in the EnVision FLEX kit was diluted to 10% of its original concentration with nuclease-free water. The incubation time was optimized for each LN and varied from 30 seconds to 10 minutes. Other solutions were used per the manufacturer's instructions.

Calculation of metastasis volume

Histopathological slides were examined under BZ-X710 microscope (KEYENCE CORPORATION, Osaka, Japan) and the area of staining was calculated digitally using Hybrid Cell Count BZ-H3C software (KEYENCE CORPORATION). The Hybrid Cell Count BZ-H3C software marked the stained area in red and calculated it automatically (***Fig. 2***). When the metastasis positive area was not detected correctly, e.g., because of weak staining of the area, the setting was changed and the stained area was calculated again. Our histopathologist confirmed that the accurate area of metastasis was calculated for each LN. The metastasis volume was calculated as described below based on the thickness of a section:

Metastasis volume [mm^3] = Metastasis area [mm^2] × 0.01 mm

Selection of the final data set

In each LN, we selected one of the three serial section sets

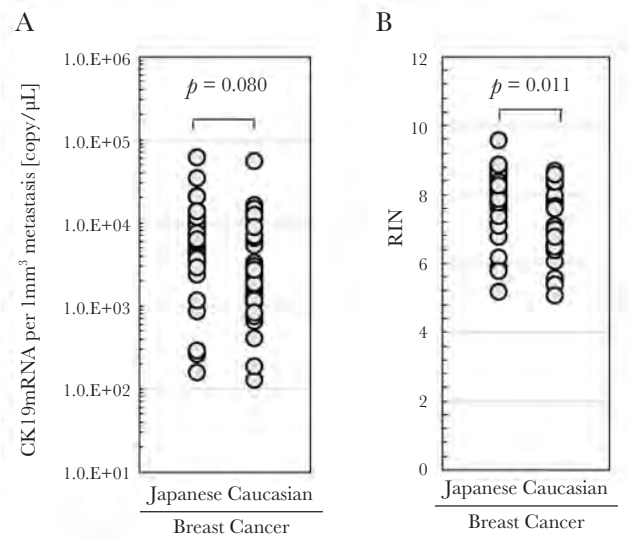

Fig. 2 *LNs of Japanese and Caucasian breast cancer patients A, The distribution of CK19 mRNA expression level per unit of metastasis (p value of the Student t-test) B, The distribution of RIN.*

fulfilling the following two criteria: 1) a RIN value equal to or more than 5.0, and 2) no section peeling, folding, low staining homogeneity, or staining spots on all three slides. When a few serial section sets met both criteria, the minimum set number (set No.1, 2 or 3) was selected as final data set. When the RNA quality in a lymph node was low (i.e., the RIN value of all serial section sets from the same LN was under 5.0), the LN was changed to another LN from that patient. When no serial section set was appropriate for this study, an additional serial section set (No. 4) was prepared and used to obtain the final data set.

Method for determination of CK19 mRNA expression level per unit of metastasis volume

Since metastasis volume varied between sections, it was assumed that the metastasis volume in the center section was an average of the metastasis volumes of sections on either side, that is, A+B=a+b and C+D=b+c, where a, A, B, b, C, D, c, and d represent their metastasis volumes. In addition, after homogenizing each lymph node sections A, B, C, and D with 4,000μL of LYNORHAG, the OSNA assay tested samples consisting of 1 volume of lymph node lysate and 9 volumes of LYNORHAG. The CK19 mRNA expression level per metastasis volume was calculated as described below:

- Total metastasis volume: a+b×2+c [mm^3/set]
- Total CK19 mRNA expression level in four sections: A+B+C+D [number of copies/set]
- CK19 mRNA expression level per unit of metastasis volume: (A+B+C+D)/(a+ b×2+c) [copies/mm^3]
- CK19 mRNA expression level per unit of metastasis volume: (A+B+C+D)/{(a+ b×2+c)×4000×10}[copies/μL·mm^3]

Statistical analysis

Since OSNA is a logarithmic growth reaction of mRNA, the CK19 mRNA expression level per unit of metastasis volume was analyzed in log copy/μL.

We evaluated between-cohort differences in the average and dispersion of data obtained from two cohorts. We used the F-test with a significance level of α=0.05 to assess the difference in dispersion. After confirming the absence of a between-cohort dispersion difference, we used a two-sided Student t-test with a significance level of α = 0.05 to assess the difference in distribution average. Differences were considered significant if $p<0.05$.

RESULTS

Distribution of CK19 mRNA level measured by the OSNA assay was compared among cohorts to assess whether expression level can be utilized as a clinical quantitative index broadly among various races and cancer types. Since the OSNA assay is diagnostically equivalent to histopathological examination, which assesses cancer progression by measuring metastasis size under a microscope, we compared the distribution of CK19 mRNA expression level per mm^3 of metastasis to the metastasis volume measured histopathologically.

A total of 30, 29, 21, and 30 LNs were collected from Japanese breast cancer patients, Caucasian breast, colon, and gastric cancer patients, respectively. The demographic and clinical characteristics of the patients who donated these tumor tissues are summarized in ***Table 1***, ***2***, and ***3***. LNs were harvested from 25 Japanese breast cancer patients, 20 of whom donated only a single LN and five of whom donated two LNs. All the LNs of Caucasian breast, colon, and gastric cancer patients were harvested from different patients. The disease was stage II (66.7%) and III (33.3%) in the Japanese breast cancer patients, stage II (27.6%) and III (65.5%) in the Caucasian breast patients, stage III (61.9%) and IV (28.6%) in the Caucasian colon patients, and stage II (6.7%), III (83.3%), and IV (10.0%) in the Caucasian gastric cancer patients (***Table 1-3***).

Table 1 *Demographic and clinical characteristics of breast cancer patients*

Ethnicity	Japanese	Caucasian
Total	30	29
Age		
Mean	58.5	63.7
SD	11.74	8.94
Median	58	61
Range	35 - 92	41 - 81
Stage		
IIA	2	2
IIB	18	5
IIC	0	1
IIIA	6	10
IIIB	2	2
IIIC	2	7
N/A	–	2
Histopathologic type		
Invasive apocrine carcinoma	–	1
Invasive ductal carcinoma	28	26
Invasive ductal and lobular carcinoma	1	–
Invasive lobular carcinoma	–	1
Invasive micropapillary carcinoma	1	–
Mucinous carcinoma	–	1

Table 2 *Demographic and clinical characteristics of Caucasian colon cancer patients*

Ethnicity	Caucasian
Total	21
Age	
Mean	61.3
SD	8.18
Median	62
Range	45 - 75
Stage	
III	13
IIIA	1
IIIB	6
IIIC	5
n/a	1
IV	6
IVA	4
IVB	1
n/a	1
n/a	2
Cancer type and histological diagnosis	
Adenocarcinoma	20
Metastatic neoplasm	1

Table 3 *Demographic and clinical characteristics of Caucasian gastric cancer patients*

Ethnicity	Caucasian
Total	30
Age	
Mean	61.6
SD	9.15
Median	63
Range	33 - 86
Stage	
IIB	2
IIIA	5
IIIB	14
IIIC	6
IV	3
Cancer type and histological diagnosis	
Adenocarcinoma	27
Signet ring cell carcinoma	1
Adenocarcinoma and signet ring cell carcinoma	1
Mucinous adenocarcinoma and signet ring cell carcinoma	1

Table 4 summarizes the results of the study. CK19 mRNA expression level per mm^3 of metastasis in LNs from Japanese breast cancer patients, Caucasian breast, colon, and gastric cancer patients were (in log number of copies/μL) 3.714 ± 0.599, 3.433 ± 0.610, 3.457 ± 0.574, and 3.599 ± 0.644, respectively.

The Japanese and Caucasian breast cancer patient results almost overlapped (***Fig. 2A***). The p values in the F-test and Student t-test were 0.459 and 0.080, respectively, which indicated the absence of a significant difference both in the dispersion and average of CK19 mRNA expression level per mm^3 of metastasis volume between LNs of Japanese and Caucasian breast cancer patients. This suggests that there is no racial difference in CK19 mRNA expression level in the LNs of breast cancer patients.

The Student t-test about RIN value found some difference in RNA quality between the LNs of Japanese and Caucasian breast cancer patients (***Fig. 2B***). This difference might have some effect on measured CK19 mRNA level in this study.

The p values in the F-test and Student t-test were above 0.05 for all cohort comparisons, which indicated no significant difference both in the dispersion and average of the CK19 mRNA expression level per unit of metastasis volume in LNs of Caucasian breast, colon, and gastric cancer patients (***Fig. 3***, ***Table 5***). In addition, these cohorts overlapped. This proves that there is no difference in LN CK19 mRNA expression level among these three cancer types.

Table 4 *Summary of CK19 mRNA expression level per unit of metastasis volume*

Cancer type Race	Breast Japanese	Breast Caucasian	Colon Caucasian	Gastric Caucasian
Average (log copies/μl)	3.714	3.433	3.457	3.599
SD	0.599	0.610	0.574	0.644
Sample number	30	29	21	30

Table 5 *Statistical analyses of CK19 mRNA expression level per unit of metastasis volume between various cancers in Caucasian patients*

	p value	
	F-test	Student t-test
Breast and colon cancer	0.394	0.890
Breast and gastric cancer	0.389	0.316
Colon and gastric cancer	0.300	0.423

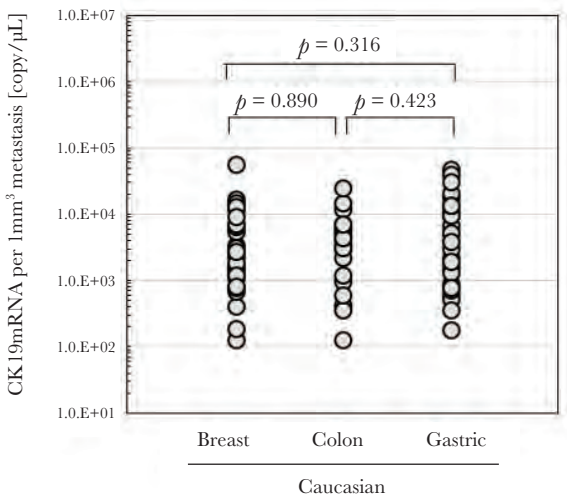

Fig. 3 *The distribution of CK19 mRNA expression level per unit of metastasis calculated with Caucasian breast, colon and gastric cancer patients (p value of the Student t-test).*

DISCUSSION

Our study comparing Japanese with Caucasian breast cancer patients showed no significant difference in CK19 mRNA expression level per unit of lymph node metastasis volume measured by the OSNA reagent kit LYNOAMP BC. Our result implied that CK19 mRNA can be used as a general clinical marker of breast cancer in patients regardless of race. It is certainly predictable that there is no racial difference in CK19 mRNA from the previously published literature. For example, several previous studies validated the use of the same cut-off value through races for qualitative assay to distinguish metastasis positive LNs from metastasis negative LNs in each cohort. Some studies have reported equivalent cut-off values for the CK19 mRNA analysis to detect metastasis to non-sentinel lymph nodes (non-SLN), such as TTL and Nomograms including TTL as an indicator. In regards to predicting one or more metastasis positive non-SLNs, V. Peg et al. suggested 15,000 copies/μL (4.176 log copies/μL) whereas M. Espinosa-Bravo et al. suggested 12,000 copies/μL, (4.079 log copies/μL) and others suggested different but similar cut-off value.[28,29,34,35] In regards to predicting four or more metastasis-positive non-SLNs, which would necessitate additional chemotherapy with irradiation of the supra- and subclavian regions, Y. Ohi et al. suggested 100,000 copies/μL (5.000 log copies/μL) whereas M. Kubota et al. suggested 54,000 copies/μL (4.732 log copies/μL).[36,37] The cut-off values proposed for each study are somewhat different as the cohorts were composed of limited patients within one facility or country, but the values are still close. In this way, it is predictable that there is no racial difference in CK19 mRNA expression level as a clinical marker since the same cut-off value has been shown regardless of race. This is the first report to compare CK19 mRNA expression levels between the races directly and to validate that there is no significant racial difference. As a result of the present study, it is expected that the global scientific community will soon discover a common cut-off value for defining therapeutic strategy in Japanese and Caucasian patients, which will further expand the clinical value of CK19 mRNA expression level in LNs.

In the present study, RNA extracted from Caucasian breast cancer patients was poorer quality than from Japanese patients. It was suggested that the result of RNA quality affected the low p value ($p=0.080$) in Student t-test between the two cohorts (***Fig. 2***). The LNs from Japanese patients were frozen quickly after surgery for this RNA study, while the LNs from European patients were not obtained specifically for this study and not all of them were frozen quickly. It is known that low RNA quality results in low mRNA yield in RT-PCR when using a method principle similar to the OSNA assay.[38,39] It is therefore expected that CK19 mRNA level measured by the OSNA assay will be more comparable between Japanese and Caucasian breast cancer patients if RNA quality is improved.

The present study shows the ability of CK19 mRNA as a clinical marker for the three cancer types by showing no significant difference in dispersion of CK19 mRNA expression level per unit of LN metastasis volume among Caucasian breast, colon, and gastric cancer patients. Numerous reports about breast cancer applied the same cut-off value for CK19 mRNA measured by the OSNA assay to each tested cohort, and indicated that the OSNA assay is the same performance level as histopathological examination for deciding whether the LN is metastasis negative or positive. This suggests that the same cut-off value can be used for various patient populations and the dispersion of CK19 mRNA among breast cancer patients is within a certain range. F-test in this present study showed that the dispersion of Caucasian colon and gastric cancer patients are the same as that of Caucasian breast cancer patients. Therefore, similar to clinical utility in breast cancer, it is indicated that CK19 mRNA could also be used as a clinical marker for colon and gastric cancer to predict metastasis in non-SLNs or disease progression.

The dispersion of each cohort was overestimated due to experimental conditions and sampling method, which were unique to this study protocol and would not occur in actual diagnosis. Some factors such as thickness of serial sections, specificity of IHC staining, operation temperature, calculation of the metastasis area, and homogenization and inter-operator variability are thought to have some effect on the accuracy of data. These effects were minimized by using an automated IHC device and increasing the number of samples. In addition, using different LN tissue parts for the OSNA assay and histopathological examination is also thought to have some effect on the accuracy of data, which was minimized by using sections alternately for these two methods. In spite of these improvements, some differences likely remain and prevent a true comparison of CK19 mRNA level to metastasis volume in each sample. Although there are experimental errors, they have no influence on the cohort comparison since the effect of experimental errors on each cohort is considered to be the same. Therefore, the results obtained from this study are reliable.

In actual diagnosis, the dispersion of the cohort is considered to be narrower than this study. The OSNA assay measures CK19 mRNA level directly without examination variability and converts the result to metastasis volume within the same LN part. In fact, some studies showed good concordance between the results of histopathological examination and that of the qualitative OSNA assay with a given cut-off value common to all the patients.[9-16,18-20,33] These reports indicate that actual data dispersion has little effect on deciding whether an LN is metastasis positive or negative and that the same cut-off value can be applied to all the breast cancer patients.

In conclusion, this present study revealed the applicability of CK19 mRNA as a clinical marker by comparing cohorts directly. We showed no difference in CK19 mRNA expression level per unit metastasis volume in race (among Japanese and Caucasian breast cancer patients) nor cancer type (among Caucasian breast, colon, and gastric cancer patients). For breast cancer, further investigation on a global scale will show the applicability of CK19 mRNA, and it is expected to become a more valuable clinical marker for diagnosis of metastatic stage, prognosis, and postoperative therapy. CK19 mRNA may also be used clinically as a valuable marker for colon and gastric cancer to suggest

treatment strategy by defining new method or cut-off value, again following the relationship between lymph node metastasis and patients' progression of each cancer entity.

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