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Diagnosis of Lymph Node Metastasis in Colorectal Cancer by a Semi-dry Dot-blot Method

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Abstract. *Background/Aim: This study aimed to examine whether the semi-dry dot-blot (SDB) method can correctly identify metastasis to lymph nodes in colorectal cancer. Materials and Methods: A total of 200 dissected lymph nodes from 83 patients with colorectal cancer who underwent surgery between November 2013 and May 2016 were examined. Each lymph node was first examined by SDB using anti-pancytokeratin antibody (AE1/AE3). Pathological Stage I/II patients with a negative reaction were further analyzed by SDB using anti-cytokeratin 20 antibody (CK-20) to detect micrometastasis or isolated tumor cells. Results: The sensitivity, specificity, and accuracy of SDB using AE1/AE3 were 91.3%, 100%, and 98.0%, respectively. Five of 99 lymph nodes of pathological Stage I/II patients had a negative reaction to AE1/AE3, but were positive to CK-20, while 3 showed isolated tumor cells. Conclusion: The SDB is a useful diagnostic tool to detect lymph node metastases in colorectal cancer.*

Colorectal cancer (CRC) is the third most frequently diagnosed cancer in men and the second in women worldwide (1). The number of CRC cases in elderly patients is expected to rise in the next decades in concert with the general aging of the population (2, 3). CRC resection with regional lymphadenectomy is a standard procedure in CRC. However, lymph node (LN) metastasis is an important prognostic indicator in CRC and essential in the decision-making process of administering adjuvant chemotherapy (4-7). So far, definitive LN analysis is usually performed by fixed-tissue sections. The midline division of a LN is subjected to pathological examination with hematoxylin and eosin (H&E) staining. Regardless of the decision to perform complete resection in pathological N0 (pN0) CRC, tumor recurrence in pathological Stage II (pStage II) accounts for 20-30% of cases (8, 9). Such a high recurrence rate might be due to understaged pStage I/II CRC patients who are not detected with micrometastasis (MM) or isolated tumor cells (ITCs) by conventional pathological examination. There are ongoing discussions on whether pN0 CRC patients have a high-risk of recurrence and can benefit from adjuvant chemotherapy. It has been suggested that MM/ITC might be a high-risk factor for disease progression. Approximately 20-40% of colon cancer patients who are classified as node-negative by H&E staining are found to have MM/ITC on intensive histopathologic analysis of stepwise sections and immunohistochemistry (10-12). The present pathological examinations do not have the sensitivity to detect MM/ITC in LNs in CRC; therefore, the development of supportive analysis techniques for the detection of LN metastases is required.

The semi-dry dot-blot (SDB) method is a simple detection system for LN metastases, which is based on the antigen-antibody reaction using anti-cytokeratin antibody and a chromogen on a dot-blot membrane (13-15). According to this principle, it is possible to visualize the presence of cancer cells on the membrane. The SDB is an easy, rapid, and cost-effective method, taking approximately 40 min (14). Furthermore, it requires only a lavage liquid to wash the sectioned LN and suspend the cells in the liquid for processing with the SDB method, while the tissue can be later used for pathological examination.

The SDB method usually uses the AE1/AE3 monoclonal antibody, which recognizes a broad spectrum of different cytokeratins. Hence, AE1/AE3 demonstrates poor specificity for detecting MM or ITC in pN0 CRC (16) and lacks cytokeratin 20 (CK-20) immunoreactivity, which is a tumor-specific antigen

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with a stable expression in LN metastases of CRC (17-19). Several studies have shown high detection of MM/ITC by immunohistochemistry and reverse transcriptase-polymerase chain reaction for CK-20 (20, 21). In this study, if the use of the AE1/AE3 antibody gave negative results, the SDB method was repeated using an anti-CK-20 antibody to detect MM/ITC; this method was limited to pStage I or II patients.

The effectiveness of this method for the detection of cancer cells in sectioned LNs, has been already demonstrated experimentally in breast and lung cancer (14, 15). However, in CRC, the effectiveness of the SDB method in detecting LN metastasis has not yet been evaluated. The aims of this study were to assess the effectiveness of the SDB method in comparison with conventional pathological examination by H&E staining in CRC and evaluate whether the SDB method could detect MM/ITC.

Patients and Methods

Patients. This study was approved by the Genetic and Medical Ethics Commission at Nagasaki University (Approval No. 13072240), and all patients included in this study provided informed consent. Overall, 200 LN samples were obtained from 83 patients with CRC who underwent CRC resection with regional lymphadenectomy by a single surgeon at our institution between November 2013 and May 2016. Patient characteristics are shown in Table I. Nine patients received neoadjuvant chemotherapy. Patients with synchronous second CRC were excluded from this study.

Semi-dry dot-blot method. LNs were harvested by surgery. The median number of resected LNs per patient was 25 (range=8–58 LNs), of which 2 or 3 of the larger LNs were selected. LNs were separated from the mesentery using a clean and sterilized Cooper. Sampled LNs were separated from fat tissue and cut in the middle for maximum diameter. Divided LNs were washed with 2 ml of phosphate-buffed saline (PBS) in a 15 ml silicon tube. The lavage fluid containing the suspended cells was centrifuged for 1 min. The collected cell pellets were lysed with 20 μl of lysis buffer (50 mmol/l Tris-Cl [pH 7.6], 150 mmol/l NaCl, 0.1% Nonidet P-40) for 3 min. The lysate was filtered through a spin column filter to remove surplus tissues prior to protein (cytokeratin) detection. Samples were stored at –80˚C if dot-blot analysis was not performed immediately after the filtration step. Two circles, 10 mm in diameter, were placed on a polyethersulfone membrane (Sartrius Stedim Biotech GmbH, Goettingen, Germany), which had been indicated with a hydrophobic pen. Then, 2 μl of the lysate was spotted on each circle. The MCF-7 human breast cancer cell line was used as positive control, and lysis buffer as negative control. The membrane was soaked using an absorption paper to generate semi-dry conditions. In order to avoid nonspecific reactions, the membrane was incubated with 20 μl of blocking solution (0.05% skim milk in PBS with 0.05% Tween 20 (PBS-T)) for 30 sec. The membrane was incubated with the anti-pancytokeratin mouse monoclonal antibody (AE1/AE3; Nichirei, Tokyo, Japan) at room temperature for 3 min, followed by peroxidase-coupled anti-mouse immunoglobulin G (ImmPRESS anti-mouse IgG; Vector Laboratories, Burlingame, CA, USA). For the test sample (one circle), both the primary and secondary antibodies were spotted on the circle, while for the reference sample (the second circle), only the primary antibody was spotted. The membrane was washed with PBS-T between incubations with each antibody. The presence of cytokeratin was visualized with the Vector® VIP substrate kit (Vector Laboratories, Burlingame, CA, USA). A positive reaction was illustrated by a denser purple color in the center of the test sample relative to the reference sample. In contrast, a negative reaction produced the same dense color in both circles. The procedure of the SDB method is schematically illustrated in Figure 1. Potential false-negative results of the SDB method using AE1/AE3 were subjected to immunohistochemistry with AE1/AE3.

Table I. *Clinicopathological characteristics of the enrolled 83 patients*.

Characteristic	Values
Male/Female	45/38
Age	69 (40-90)
Location (C/A/T/D/S/R)	13/15/6/3/19/27
Tumor size (mm)	49 (12-90)
Depth (M/SM/MP/SS•A/SE/SI•AI)	2/ 13/15/40/8/5
Lymph node metastasis (N0/1a/1b/1c/2a/2b)	48/7/12/0/10/6
pStage (0/I/II A/II B/II C/III A/III B/III C/IV A/IV B)	2/22/20/2/ 1/3/20/7/6/0
Lymphatic invasion (ly0/1/2/3)	24/30/23/6
Vessel invasion (v0/1/2/3)	29/29/21/4
Pathology (well/mod/por)	26/51/6
Neoadjuvant chemotherapy (yes/no)	9/74
Number of dissected LN per patients	26 (2-58)
Size of lymph node for SDB (mm)	8 (3-18)

C: Cecum; A: ascending colon; T: transverse colon; D: descending colon; S: sigmoid colon; R: rectum; M: mucosa; SM: submucosa; MP: muscularis propria, SS: subserosa, SE: serosa exposed; SI: serosa infiltrating; AI: adventitia infiltrating; well: well differentiated; mod: moderately differentiated; por: poorly differentiated; SDB: Semi-dry dot-blot.

Further analysis by the SDB method using CK-20. In the case of a negative reaction on the SDB method using AE1/AE3, and a pStage I/II CRC, the SDB method using CK-20 was also performed following the same procedure as mentioned above to detect whether CK-20 can effectively detect cytokeratin in pStage I/II samples. The positive cases by the SDB method using CK-20 were subjected to immunohistochemistry with CK-20 (Figure 2). In addition, the positive cases by immunohistochemistry with CK-20 were subjected to immunohistochemistry with AE1/AE3.

Immunohistochemistry. Immunohistochemistry was performed on formalin-fixed paraffin-embedded LNs. Deparaffinized and rehydrated sections were washed with PBS. Slides were boiled in a sodium citrate solution (0.01 mol/l; pH=6) at 105˚C for 10 min. To inhibit endogenous peroxidase activity, the slides were incubated with 3% hydrogen peroxide at room temperature for 10 min, followed by serum blocking at room temperature for 1 h. In immunohistochemistry with AE1/AE3, primary AE1/AE3 antibody (DAKO, Tokyo, Japan) was added and incubated with the slide at room temperature for 20 min. In immunohistochemistry with CK-20, the sections were incubated overnight at 4˚C with CK-20 (1:50). Mouse MAX-PO (Nichirei, Tokyo, Japan) was added as secondary antibody, and the sections were incubated at room temperature for 20 min. Finally, the slides were incubated with liquid DAB

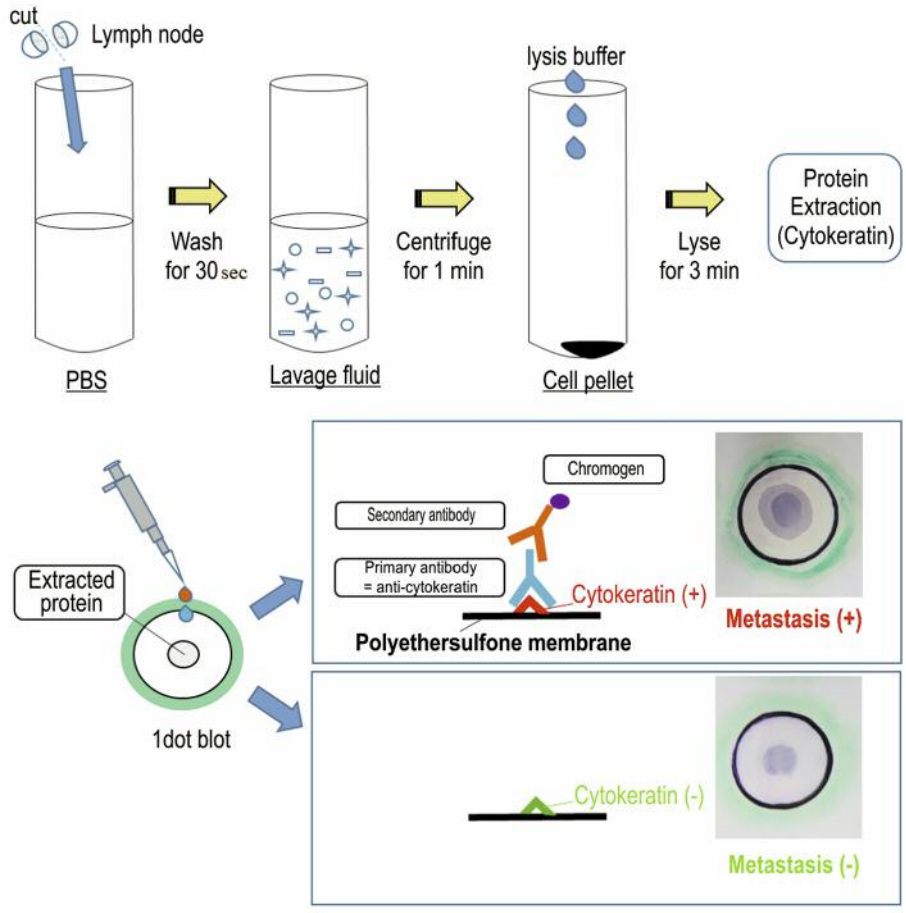

Figure 1. *Diagram of the SDB method. The process starts with protein extraction from harvested lymph nodes (LNs). Each LN is cut and washed with phosphate-buffered saline. The lavage fluid is centrifuged to collect the cell pellet, which is then lysed with lysis buffer. The lysate is spotted onto a dot-blot membrane under semi-dry conditions. The membrane is then incubated with the primary anti-cytokeratin and species-specific secondary antibodies. The immunoreaction is detected using a chromogen; the membrane turns purple in response to the antigen-antibody reaction, indicating LN metastasis.*

substrate-chromogen at room temperature for 10 min to visualize the antibody-antigen reaction, and counterstained with hematoxylin.

Pathological examination and Staging. All LNs examined by the SDB method were subjected to pathological examination. Sections were formalin-fixed and paraffin-embedded with H&E staining for final diagnosis. Pathological Staging was based on the 7th edition of the tumor-node-metastasis classification of the Union for International Cancer Control (22). Metastasis was defined as a malignant cell cluster larger than 2 mm, MM as deposits of tumor cells between 0.2 mm and 2 mm in diameter, ITC as either single tumor cells or clusters of tumor cells of 0.2 mm or less on H&E staining or immunohistochemistry; as previously reported, LNs with a positive reaction by the SDB method using CK-20 were subjected to immunohistochemistry. Patients with MM-positive status were staged as pN1 (mi), and those with ITC-positive status as pN0 (i+) (23).

Statistical analysis. The sensitivity, specificity, and accuracy of the SDB method were determined and compared to pathological examination with H&E staining. The statistical program JMP 11 for binominal distribution analysis with 95% confidence intervals (95% CI) was used for all statistical analyses.

Results

Analysis of the SDB method with anti-pancytokeratin antibody (AE1/AE3). In this prospective study, 200 LNs from 83 patients with CRC were analyzed. Of the 200 LNs, 46 (23%) were identified as positive and 154 (77%) as negative by the conventional pathological examination with H&E staining. A positive H&E result did not include MM or ITC. By the SDB method using AE1/AE3, only 42 of the 46 pathologically-positive LNs (91.3%) were identified as positive. Therefore, the sensitivity, specificity, and accuracy of the SDB method relative to pathological examination were 91.3% (95% CI=79.7-96.6%), 100% (95% CI=97.6-100%), and 98.0% (95% CI=88.5-99.8%), respectively (Table II). Four LNs showed discordant results between pathological examination

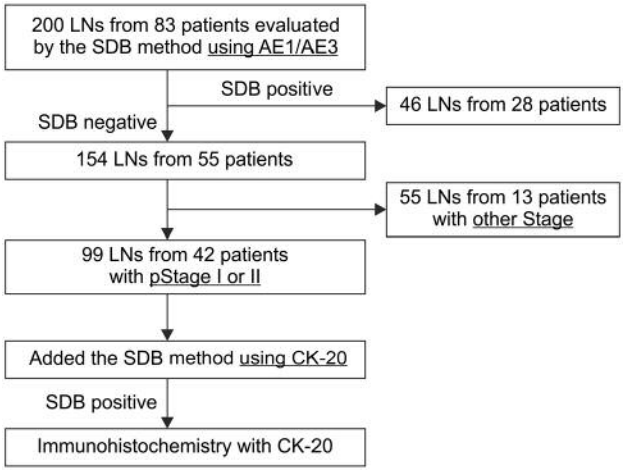

Figure 2. *Flow chart of the analysis of LNs by the SDB method using sequentially AE1/AE3 and CK-20 antibodies. This process was applied in order to determine whether occult cancer in histologic pStage I or II colorectal cancer patients could be detected following a negative reaction in SDB using AE1/AE3.*

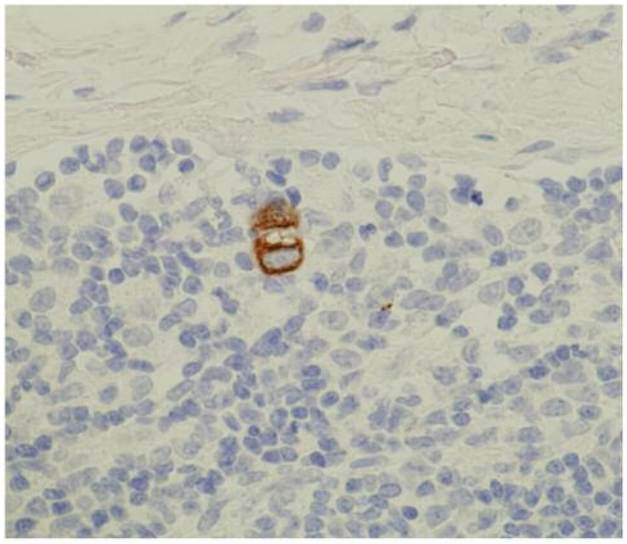

Figure 3. *Representative image of immunohistochemistry staining for CK-20 of isolated tumor cells in a regional node metastasis of colorectal cancer (×400).*

Table II. *Comparison of data between the SDB method using AE1/AE3 and permanent pathological examination to diagnose LN metastasis.*

		Pathology (permanent)		Total
		Positive	Negative	
SDB	Positive	42	0	42
	Negative	4	154	158
	Total	46	154	

Sensitivity=42/46 (91.3%)
Specificity=154/154 (100%)
Accuracy=196/200 (98.0%)

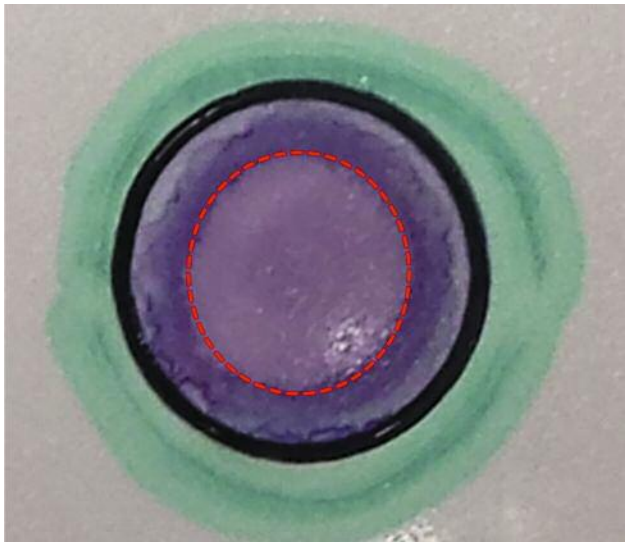

Figure 4. *False-negative result of the SDB method using immunohistochemistry staining for AE1/AE3. The area delineated by the red dotted line shows fat tissue contamination, which blocks antigen-antibody reaction.*

and the SDB method using AE1/AE3. For this reason, immunohistochemistry with AE1/AE3 was performed. All 4 false-negative cases by the SBD method using AE1/AE3 were positive by immunohistochemistry with AE1/AE3, indicating cytokeratin-positive CRC.

Further analysis by the SDB method and immunohisto-chemistry using CK-20. Of the 200 LNs, 99 (49.5%) from 42 histologically pStage I or II patients, were negative by the SDB method using AE1/AE3. Those LNs were subjected to further analysis by the SDB method using CK-20, and 5 (5.1%) were identified as positive. These positive samples were further subjected to immunohistochemistry with CK-20, of which ITC was observed in 3 LNs (Figure 3). ITC in the 3 LNs was not observed on staining with AE1/AE3.

Discussion

The SDB method is a novel procedure for detecting LN metastasis, developed by Hirakawa *et al.* in 2010 (13). There is usually no epithelial component in the LN; however, this method identifies the tumor cells within the LN with epithelial component, containing cytokeratin. Specifically, the cytokeratin

Table III. *Comparison of data between the SDB method and OSNA.*

Diagnostic method	Total number of lymph nodes	Permanent pathological diagnosis		
		Sensitivity (%)	Specificity (%)	Accuracy (%)
SDB (present study)	200	91.3	100	98
OSNA [Yamamoto H *et al*. (24)]	1925	86.2	96.5	95.7
OSNA [Yamamoto N *et al*. (25)]	66	96.4	100	NA
OSNA [Güller U *et al*. (26)]	313	94.5	97.6	97.1
OSNA [Croner RS *et al*. (27)]	184	92.5	96.5	95.7

NA: Not available; SDB: semi-dry dot-blot; OSNA: one-step nucleic amplification.

is detected by an antigen-antibody reaction and chromogen on a dot-blot membrane. It has been proven that this protocol has the ability of identifying the protein of interest at the concentration of 0.01 mg/ml extracted protein from cancer tissue or 20 cells from a tissue suspension (13, 15). Otsubo *et al*. analyzed 174 sentinel LNs from 100 patients with breast cancer using the SDB method. Results showed that sensitivity, specificity, and accuracy compared with pathological examination were 93.3%, 96.9%, and 96.6%, respectively (14). Tomoshige *et al*. reported that the sensitivity, specificity, and accuracy of the SDB method compared with pathological examination were 94.7, 97.7, and 97.3%, respectively, after analysis of 147 LNs from 50 patients with lung cancer (15). Our data are in line with those studies, showing sensitivity, specificity, and accuracy of 91.3%, 100%, and 98.0%, respectively. These results suggest that the SDB method has high concordance with conventional pathological examination of LNs in CRC. Therefore, the SDB method could be considered as a diagnostic method for LN metastasis in CRC.

The diagnostic accuracy of the SDB method is also comparable to that of the one-step nucleic amplification (OSNA), which is a molecular technique for detecting LN metastasis. This assay identifies cytokeratin-19 mRNA, a molecular marker of metastasis, using the reverse-transcription loop-mediated isothermal amplification method for the diagnosis of LN metastasis (24-28). Importantly, it has demonstrated high sensitivity, specificity, and accuracy in various diseases. However, the disadvantage of the OSNA assay is the need of a specific equipment for the quantitative reverse-transcription polymerase chain reaction and the homogenization of the LN tissue. Therefore, this assay involves high cost and loss of LN tissue. In contrast to the OSNA assay, the SDB method has the strengths of a simple, fast, and cost-effective diagnosis of LN metastases (13, 15). Besides, in the SDB method, the same sample could be used for pathological examination due to no loss of LN tissue. In the field of CRC, there were no significant differences between the data on LN metastasis from the SDB method and the OSNA assay (Table III).

The high recurrence rate in Stage I/II CRC remains unclear. It can be hypothesized that Stage I/II patients were understaged by conventional pathological examination with H&E staining, since this method demonstrates poor detection of MM/ITC in LNs (8-10). The prognostic value of these findings remains controversial, but several studies revealed that ITC could be associated with worse prognosis (12, 29-35). Based on these data, ITC-positive status predicts poor survival. Specifically, ITC in Stage I/II colon cancer is associated with worse disease-free and overall survival, indicating patients of high-risk who may benefit from adjuvant chemotherapy.

A systematic review and meta-analysis showed that molecular detection of tumor cells in regional LNs is associated with disease recurrence and poor survival in node-negative CRC (31). Conventional pathological examination with H&E staining is limited to detection of ITC. For detecting MM/ITC, molecular analysis, such as immunohistochemistry, reverse transcriptase-polymerase chain reaction (RT-PCR), and OSNA should be performed (10-12, 26-28). Although the detection rate of MM/ITC can be improved by using these techniques, the downside is that they tend to be rather labor intensive, time-consuming, and extremely expensive. This study demonstrated that the SDB method using CK-20, can effectively detect ITC, despite the small sample size. Three of the five LNs (60%) with positive reaction on the SDB method using CK-20, were diagnosed with ITC. It is suggested that the use of a broad-spectrum antibody, such as AE1/AE3, might not be as sensitive as other subset-specific antibodies, such as CK-20, to detect MM/ITC. In the two false positive cases, it is possible that the LN cut for H&E did not contain evidence of ITC on the surface. Another plausible reason for the two false positive results is the high sensitivity of the SDB method, although the possible contamination of detached tumor cells from the primary site cannot be excluded, in spite of careful treatment. Therefore, the SDB method using CK-20 could be considered as an option for improving diagnostics for Stage I/II CRC.

The limitations of this study should be addressed. Firstly, technical issues arose with the SDB methods. We noted metastasis with H&E staining in four LNs that were identified as false-negative by the SDB method using AE1/AE3. These results might be caused by the presence of fat tissue in the process of protein extraction. The fat tissue, when adhered onto the membrane, can block the antigen-antibody reaction and disturb the solid phase of the antibodies on the center of the dot (Figure 4). Therefore, careful removal of the fat tissue around sampled LNs is crucial for this method to improve sensitivity. Furthermore, poor procedure and small lysate sample (2 μl) may have led to inadequate loading. The SDB method depends on the cytokeratin concentration. Therefore, use of another lysis buffer may improve protein retrieval and prevent negative results. Secondly, since the sample size was rather small, further population samples are needed to confirm the effectiveness of the SDB method for detecting MM/ITC.

In general, the detection of MM/ITC using the SDB method may be a new diagnostic method to improve the prognosis for high recurrence of pStage I/II of CRC. In addition, the SDB method is expected to be applied for the diagnosis of lateral sentinel LN (SLN) in low rectal cancer. SLN is the first LN to receive lymphatic drainage from the primary tumor. Several studies have shown the usefulness of lateral SLN using SLN navigation surgery in low rectal cancer (36-39). The usefulness of the diagnosis of lateral SLN using the SDB method should be further investigated, since the combination of the SDB method and SLN navigation surgery may lead to accurate diagnosis.

Conclusion

In conclusion, the SDB method is a promising technique to detect LN metastasis in CRC that provides a simple and accurate diagnostic tool without losing any LN tissue. Specifically, the SDB method using CK-20 has been identified as a new effective way for detecting ITC.

Conflicts of Interest

The Authors have no conflicts of interest to declare regarding this study.

Authors' Contributions

Data curation: Kiyoaki Hamada, Ryota Otsubo, Tetsuro Tominaga; Formal analysis: Kiyoaki Hamada, Ryota Otsubo, Tetsuro Tominaga; Funding acquisition: Takeshi Nagayasu, Ryota Otsubo; Investigation: Kiyoaki Hamada, Ryota Otsubo, Tetsuro Tominaga, Hiroaki Takeshita, Takashi Nonaka, Kuniko Abe; Methodology: Takeshi Nagayasu, Terumitsu Sawai, Yorihisa Sumida, Shigekazu Hidaka, Ryota Otsubo; Project administration: Takeshi Nagayasu, Terumitsu Sawai, Yorihisa Sumida, Shigekazu Hidaka, Ryota Otsubo; Resources: Takeshi Nagayasu, Ryota Otsubo; Software: Kiyoaki Hamada; Supervision: Takeshi Nagayasu, Terumitsu Sawai, Yorihisa Sumida, Shigekazu Hidaka, Ryota Otsubo; Validation: Kiyoaki Hamada, Ryota Otsubo, Tetsuro Tominaga; Visualization: Kiyoaki Hamada, Ryota Otsubo, Tetsuro Tominaga; Writing – original draft: Kiyoaki Hamada, Ryota Otsubo, Tetsuro Tominaga; Writing – review & editing: Kiyoaki Hamada, Ryota Otsubo, Tetsuro Tominaga.

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