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Multiple-sectioning Study to Establish a Standardized Immunohistochemical Method for Detecting Isolated Tumor Cells in Lymph Nodes of Patients with Colorectal Cancer

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Summary. To establish a standardized immunohistochemical method for detecting isolated tumor cells (ITC) in lymph nodes of patients with colorectal cancer (CRC), we examined the detection rates of ITC using sections of various thicknesses and two different kinds of cytokeratin monoclonal antibodies, CAM5.2 and AE1/AE3. According to the sixth edition of the TNM classification, ITC was defined as a single tumor cell or small cell clusters not greater than 0.2 mm. From 29 patients with stage III colorectal cancer, 149 lymph nodes diagnosed as negative for cancer metastasis by routine hematoxylin and eosin (H&E) staining were randomly selected. Twelve serial sections were cut for each lymph node. Sections one to eight were cut at a 4 μm thickness, and sections nine to 12 were cut at a 10 μm thickness. Sections one and eight, two to six and nine to 11, and seven and 12, were stained with H&E, the CAM5.2 antibody, and the AE1/AE3 antibody, respectively. Comparisons of cumulative ITC positive rates in sections of varying total thicknesses stained with the CAM5.2 antibody revealed that no significant differences were found when the total thickness of the sections examined exceeded 8 μm. There was no significant difference in ITC positive rates between CAM5.2 and AE1/AE3 staining. Our data indicate that, when monoclonal antibodies CAM5.2 or AE1/AE3 are used, ITC can be optimally detected immunohistochemically in sections with a total thickness of more than 8 μm. This practical suitable procedure can be used for standardizing the detection method for ITC.

Key words— isolated tumor cell; occult node metastasis; immunohistochemistry; CAM5.2; AE1/AE3; colorectal cancer.

INTRODUCTION

The presence of lymph node metastasis, detected by routine histologic examination using hematoxylin and eosin (H&E) after surgical resection, is one of the most important prognostic factors in patients with colorectal cancer (CRC).[1,2] However, it remains unclear whether or not occult node metastasis (also termed as micrometastasis) detected by immunohistochemistry (IHC) can also be used as a prognostic factor in colorectal cancer patients who are diagnosed as node-negative by routine H&E histologic examination. In earlier studies, the prevalences of occult metastasis in patients and lymph nodes revealed a wide variation, from 15.8% to 100%, and from 1.8% to 25.1%, respectively (Table

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Abbreviations – CRC, colorectal cancer; H&E, hematoxylin and eosin; IHC, immunohistochmistry; ITC, isolated tumor cell.

1); some have suggested a correlation between occult metastasis and a worse prognosis and recurrence[3,4,5,6] while others argued against this.[7-16] These discrepancies are likely to be ascribed to several factors, namely, the vagueness of the definition of occult node metastasis, the absence of a standardized detection method by IHC, the number of lymph nodes examined, and the number of patients included in the study. Among these factors, problems with definition and the detection method may be most critical since they constitute the bases of any study.

Occult lymph node metastasis has generally been defined as minimal cancer cells that cannot be detected by routine histologic examination.[17] On IHC using a monocolonal antibody against cytokeratin, which is a specific marker of epithelial cells, occult metastasis is represented by single or small clusters of tumor cells that can not be recognized on routine H&E staining of lymph nodes. However, the used definition of occult metastasis is conventionally subjective in its failure to specify which size of small clusters is considered minimal, as such clusters cannot be recognized on H&E staining but only on IHC. A large cluster of cancer cells identified by IHC staining can be diagnosed as cancer metastasis if the IHC section has been prepared for routine H&E staining. Consequently, earlier studies can label various sizes of cancer clusters as occult metastasis, some of which can be overlooked by H&E examination. On the other hand, IHC as a detection method for occult lymph node metastasis has been shown to have great variability among researchers, including differences in the IHC markers used, the thickness of cut sections, and the total thickness of the lymph nodes examined (Table 1).

Recently, the sixth edition of the TNM classification[18,19] defined lymph node metastasis as comprising three categories according to the size of the tumor tissue: 1) those 2 mm or larger; 2) those larger than 0.2 mm but not more than 2 mm; and 3) a single or small clusters of cells not larger than 0.2 mm. Tumor statuses 2) and 3) are termed as micrometastasis (MM) and isolated tumor cells (ITC), respectively. This definition of ITC according to the size of the cancer focus has resolved the problem concerning a definitive description of occult metastasis. Thus, the frequency and prognostic significance of ITC, and not of occult metastasis, will be investigated. However, to do this, the problem of the establishment of a standardized detection method for ITC must be addressed. In this study, we examined the detection rates of ITC in lymph nodes of CRC patients using sections of various thicknesses and two different kinds of cytokeratin monoclonal antibodies in order to achieve an optimal detection method for ITC by IHC.

MATERIALS AND METHODS

Definition of ITC

ITC was defined as a single tumor cell or small cell clusters not greater than 0.2 mm according to the sixth edition of the TNM classification.[18,19]

Lymph nodes samples

One hundred forty-nine lymph nodes diagnosed as negative for cancer metastasis by routine H&E staining were randomly selected from 29 patients with stage III (pT any, pN1 or 2, M0) CRC who underwent a radical resection at Niigata University Hospital between January 1991 and December 2001. We selected lymph nodes from stage III cases because the ITC positivity of stage III lymph nodes is expected to be greater than those of stage I or II lymph nodes, thus allowing us to perform studies with greater efficiency.

Immunohistochemistry

Monoclonal antibodies CAM5.2 (Becton Dickinson, San Jose, CA, USA) and AE1/AE3 (Dako, Carpinteria, CA, USA) were used. They are reported to recognize cytokeratins in colorectal epithelial cells.[20,21,22] The streptavidin-biotin immunoperoxidase (SAB) method was applied. The sections were pre-treated with 0.1% trypsin (Sigma Chemical, St. Louis, MO, USA) in 0.1% calcium chloride (pH 7.8) at 37°C for 20 min prior to immunostaining. Reagents for the subsequent step, biotinylated rabbit anti-mouse immunoglobulin, and the SAB complex were supplied commercially (Nichirei, Tokyo). Diaminobenzidine was used as the chromogen, and the sections were counterstained with hematoxylin.

Multisection preparation

Twelve serial sections were cut for each paraffin block of 149 lymph nodes, and a total of 1490 sections were examined. Sections one to eight were cut at a 4 μm thickness, and sections nine to 12 were cut at a 10 μm thickness. To detect ITC, sections two to six (4 μm thickness) and nine to 11 (10 μm thickness) were stained with the CAM5.2 antibody, while sections seven (4 μm thickness) and 12 (10 μm thickness) were stained with the AE1/AE3 antibody (Fig. 2).

Statistical analysis

Statistical analyses were performed using the chi-squared test. P values < 0.05 were considered statistically significant. SPSS software (version 11.5J, SPSS Japan

Table 1. Frequencies of occult node metastasis and their immunohistochemical detection method

Author	TNM stage	Patients		Lymph nodes		Immunohistochemistry		
		No.	Occult metastasis positive	No.	Occult metastasis positive	Monocolonal antibody	Thickness and number of sections	Total thickness of sections
Palma[7]	II	38	15.8%	383	1.8%	AE1/AE3	4 μm × 1	4 μm
Isaka[3]	II	42	21.4%	644	3.0%	CAM5.2	3 μm × 1	3 μm
Jeffers[8]	II	77	24.7%	(Not stated)		AE1/AE3	(Not stated)	
Cutait[9]	I / II	46	26.1%	603	3.6%	AE1/AE3	(Not stated)	
Broll[10]	I / II / III	49	26.5%	(Not stated)		AE1/AE3	3 μm × 1	3 μm
Greenson[4]	II	50	28.0%	568	5.8%	AE1/AE3	(Not stated)	
Zhou[11]	I / II	114	28.9%	2481	2.1%	AE1/AE3	3 μm × 1	3 μm
Kronberg[12]	I / II	90	28.9%	(Not stated)		AE1/AE3, PCK2	5 μm × 2	10 μm
Choi[13]	II	93	31.2%	1808	3.0%	MNF116	4 μm × 1	4 μm
Adell[14]	II	100	39.0%	467	17.3%	anti-CK	4 μm × 4	16 μm
Noura[15]	I / II / III	98	45.9%	878	11.8%	AE1/AE3	4 μm × 5	20 μm
Tschmelitsch[16]	II	50	76.0%	814	19.4%	AE1/AE3	4 μm × 1	4 μm
Yasuda[5]	II	42	76.2%	373	15.8%	CAM5.2	6 μm × 5	30 μm
Sasaki[6]	I / II	19	100%	358	25.1%	CAM5.2	10 μm × 3	30 μm

TNM, tumor node metastasis factor [classification].

Table 2. Number of isolated tumor cell (ITC) positive nodes and cumulative number of ITC positive nodes in multiple-sectionings

Section number	Monoclonal antibody	Thickness of a section	Number of ITC positive nodes per single section	Total thickness of sections (section number)	Cumulative number of ITC positive nodes
1	H&E	4 μm	–	–	–
2	CAM5.2	4 μm	50/149 (33.6%)	4 μm (2)	50/149 (33.6%) *
3	CAM5.2	4 μm	52/149 (34.9%)	8 μm (2-3)	56/149 (37.6%)
4	CAM5.2	4 μm	59/149 (39.6%)	12 μm (2-4)	64/149 (43.0%)
5	CAM5.2	4 μm	62/149 (41.6%)	16 μm (2-5)	72/149 (48.3%) **
6	CAM5.2	4 μm	55/149 (36.9%)	20 μm (2-6)	72/149 (48.3%) **
7	AE1/AE3	4 μm	56/149 (37.6%)	–	–
8	H&E	4 μm	–	–	–
9	CAM5.2	10 μm	59/149 (39.6%)	10 μm (9)	59/149 (39.6%)
10	CAM5.2	10 μm	60/149 (40.3%)	20 μm (9-10)	70/149 (47.0%) #
11	CAM5.2	10 μm	56/149 (37.6%)	30 μm (9-11)	71/149 (47.7%) ##
12	AE1/AE3	10 μm	50/149 (33.6%)	–	–

* vs **, P = 0.010; * vs #, P = 0.018; * vs ##, P = 0.013.

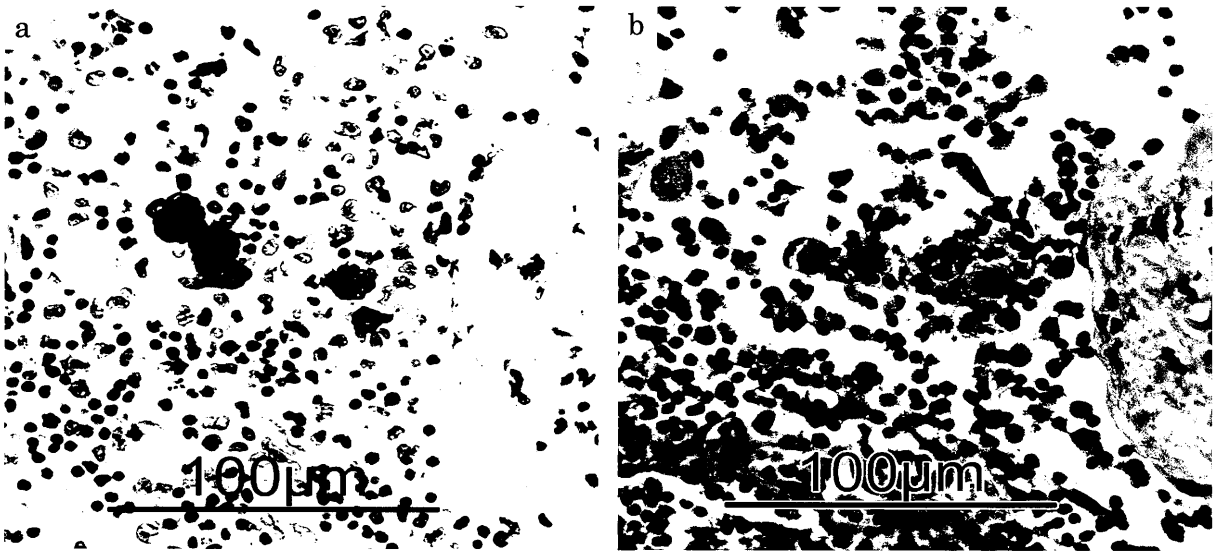

Fig. 1. ITC. **a.** Immunostaining with the CAM5.2 monoclonal antibody detects three foci of ITC (two single cancer cells and a small cluster consisting of three cancer cells, respectively, right and left) in the peripheral nodal sinus. **b.** In the consecutive H&E section however, it is difficult to make a definitive diagnosis of cancer cells. Original magnification.

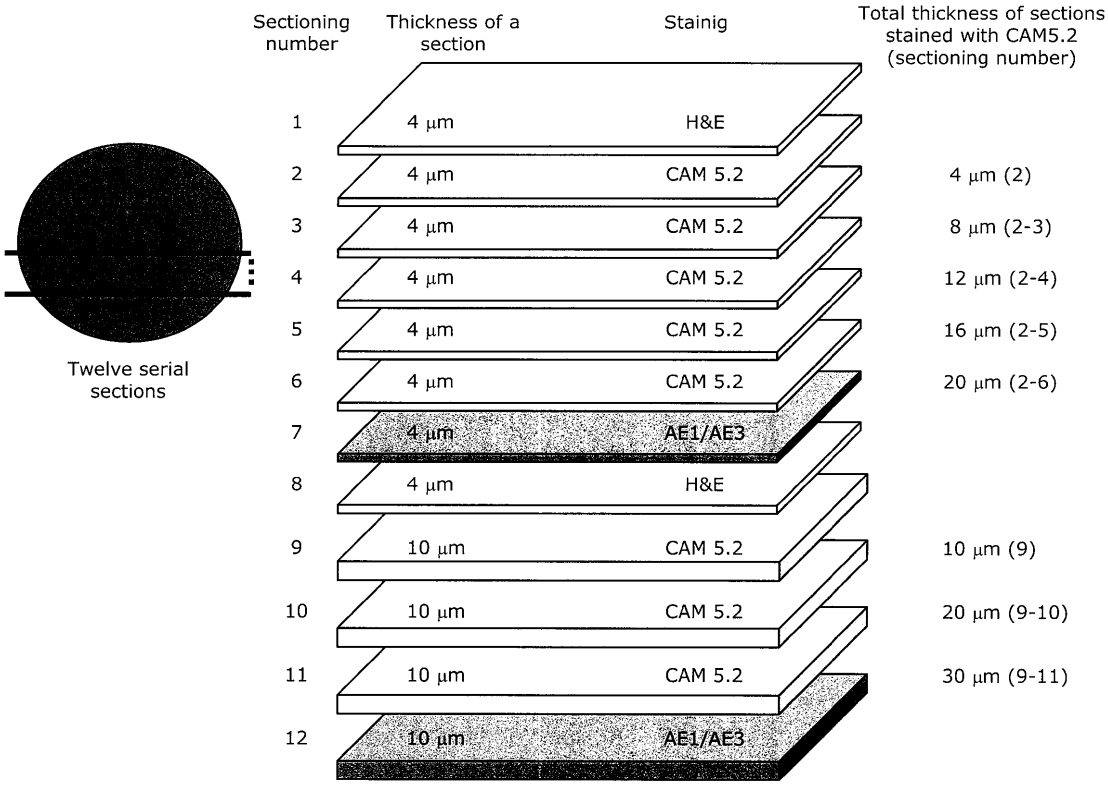

Fig. 2. Multipe-sectioning preparation for detecting ITC. Twelve serial sections were cut for each lymph nodes. Sections one to eight were cut at a 4 μm thickness, and sections nine to 12 were cut at a 10 μm thickness, and the sections were stained with monoclonal antibodies CAM5.2 and AE/AE3 and H&E, respectively.

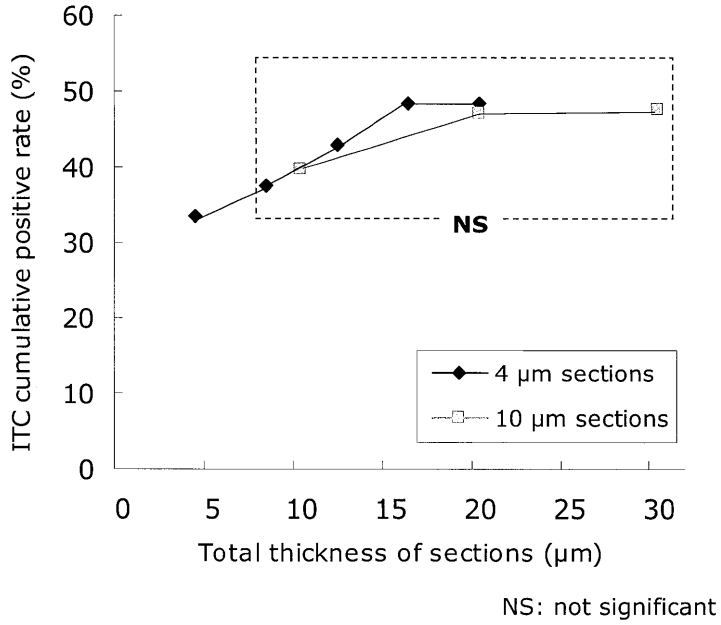

Fig. 3. Correlation between the number of sections (The total thickness of sections) and cumulative positive rates of ITC among 149 lymph nodes. Five consecutive 4-μ-thick sections and three consecutive 10-μm-thick sections were used for immunohistochemical staining. The ITC positive rate tended to increase contingent upon the number of sections in both the 4 μm-thick and 10-μm-thick sections. However, when the total thickness of the sections exceeded 8 μm, there were no significant differences in the ITC cumulative positive rate.

Inc., Tokyo) was used for statistical analyses.

RESULTS

Isolated tumor cells (ITC)

ITCs were usually located in the marginal and medullary sinuses of lymph nodes. They showed morphological features of malignant cells, such as large nuclei, or hyperchromatic and dotted small nuclear bodies. A strong immunoreactivity of cytokeratins was found in the cytoplasm, particularly peripherally (Fig. 1).Weak immunoreactive signals were occasionally found in reticulum and plasma cells, and strong spotty immunoreactivity was observed in macrophages. These cells were easily differentiated from cancer cells by their morphological features.[23)]

ITC positive rates in single 4-μm-thick and 10-μm-thick sections stained with CAM5.2 and AE1/AE3 IHC antibodies, and cumulative positive rates in IHC sections two to six (4, 8, 12, 16, and 20 μm total thickness), and sections nine to 11 (10, 20, and 30 μm total thickness) stained with CAM5.2 antibody are shown in Table 2.

Comparison of cumulative ITC positive rates by total thickness of sections stained with CAM5.2 antibody

In 4-μm-thick sections, the cumulative ITC positive rates of sections two to six (4, 8, 12, 16, and 20 μm total thickness) were 33.6%, 37.6%, 43.0%, 48.3%, and 48.3%, respectively. In the 10-μm-thick sections, the cumulative ITC positive rates of sections nine to 11 (10, 20, and 30 μm total thickness) were 39.6%, 47.0%, and 47.7%, respectively. Although the cumulative positive rate increased with the total thickness of the IHC sections examined, a significant difference was revealed only between 4-μm-thick to 16-μm-thick sections (four 4-μm-thick sections), 20-μm-thick sections (five 4-μm-thick sections and two 10-μm-thick sections), and 30-μm-thick sections (three 10-μm-thick sections). When the total thickness of the sections examined was 8 μm (two 4-μm-thick sections or one 10-μm-thick section) or more, there was no significant difference in cumulative positive rates (Table 2 and Fig. 3).

Comparison of ITC positive rates by CAM5.2 and AE1/AE3 staining

In 4-μm-thick sections, the positive rates of any single CAM5.2-stained IHC section (33.6% to 41.6%) showed no significant differences when compared with those of the AE1/AE3-stained IHC section (37.6%). In 10-μm-thick sections, the positive rates of any single CAM5.2-stained IHC section (37.6% to 40.3%) showed no significant difference when compared with those of the AE1/AE3-stained IHC section (33.6%) (Table 2).

DISCUSSION

This study found that the detection rate of cancer cells in lymph nodes on IHC increases with the number of sections (or total thickness of sections) examined. By investigating occult node metastasis, Sasaki et al.[23] reported that the cumulative positive rate detected in 3-μm-thick sections stained with the CAM5.2 monoclonal antibody increased until the ninth serial section (total thickness of 27 μm). Noura et al.[15] used five serial 4-μm-thick sections stained with AE1/AE3 monoclonal antibodies and demonstrated that a higher cumulative positive rate was obtained as the slice number was increased from one to two to five. The wide variation in frequency of occult node metastasis in earlier studies can partly be ascribed to variability among the numbers or thicknesses of sections for IHC examination among researchers (Table 1). A similar discrepancy will occur in the investigation of newly defined ITC unless a standardized method is established.

Prior to our study, there was no previous investigation to determine the statistically sufficient thickness of sections for IHC to detect cancer cells. The production of serial sections of multiple lymph nodes which was demonstrated in the studies of Sasaki et al.[23] and Noura et al.[15] requires a considerable costs and efforts and is not practically applicable. In the present study, we compared the cumulative ITC positive rates detected in IHC sections of various thicknesses. The maximum total thickness of the sections was set at 30 μm, which is based on our previous study,[23] demonstrating that the rate of detection of cancer cells with CAM5.2 staining reached a plateau once nine 3-μm-thick sections (27 μm total thickness) had been examined. The results of our multiple-sectioning study revealed that, while the ITC cumulative positive rate increased with the total thickness of sections for IHC, the positive rates showed no significant difference when the total thickness of sections exceeded 8 μm.

Differences in the monoclonal antibody used for IHC may be an important factor to explain the discrepancies in the frequency of occult metastasis. Earlier studies generally used either CAM5.2 or AE1/AE3 monoclonal antibodies. Although the two antibodies are able to detect colorectal epithelial cells,[20,21,22] their specificity for cytokeratin is not identical. The CAM5.2 antibody is specific for cytokeratins 8 and 18,[20] while the AE1/AE3 antibody is specific for cytokeratins 1-5, 8, 10, 11, 13-15, and 19.[21] However, our present study demonstrated that there was no significant difference in the positive rate between these two antibodies. It is thus suggested that the wide variation in frequency of occult node metastasis in earlier studies may not be ascribed to the difference in immunohistochemical markers used.

Our study indicated that, when monoclonal antibodies CAM5.2 or AE1/AE3 are used, ITC can be optimally examined in either two serial 4-μm-thick sections or one 10-μm-thick section (in which the total thickness is more than 8 μm), without producing serial multiple sections up to a total of 30 μm thickness, as demonstrated by Sasaki et al.,[23] or 20 μm thickness, as shown by Noura et al.[15]

Categorization of the status of lymph node metastasis as proposed by the sixth edition of the TNM classification[18,19] seems to be objective since the definition is based on the size of cancer focus in lymph nodes. This can resolve the vagueness of the definition of occult metastasis, and investigation of the prognostic significance of ITC can become the focus instead.[24,25] To identify the prognostic significance of ITC, it will be necessary to perform a multicentric large-scale study based on identical detection methods. We believe that the results of our present study can be used for standardization of the detection method for ITC, which usually incurs a considerable costs and efforts for pathologists, with the sectioning method used in the present study being practically more applicable.

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