the risk of lymph node metastasis. The precise depth or extent of submucosal invasion of carcinoma that correlates with a risk of lymph node metastasis has not yet been established.

On the other hand, immunohistochemical[19,20] and molecular biological[21,22] techniques have enabled us to identify the presence of a single or several small clusters of metastatic carcinoma cells that are undetectable by routine H&E histopathologic examination (so-called micrometastasis). Analysis using cytokeratin immunohistochemistry indicates that 25-60% of colorectal carcinomas show micrometastasis[20,23-25]. Micrometastasis is thought to be a more sensitive marker than overt lymph node metastasis (as detected by routine histopathologic examination) for the prediction of cancer recurrence and the prognosis of patients[21,26].

However, earlier studies have examined mainly advanced CRCs (carcinomas invading below the proper muscle coat), and no systematic study has been made of micrometastasis in sm-CRC. The histopathologic features of sm-CRCs that correlate with micrometastasis would be useful prognostic markers for sm-CRCs treated by EMR. If the EMR-treated sm-CRC is regarded as not even presenting any risk of micrometastasis, the patient could be cured by EMR alone without subsequent surgery.

In this study, we performed immunohistochemical examinations using an anti-human cytokeratin antibody (CAM5.2), in order to detect the micrometastasis of sm-CRCs, and investigated the correlation between histopathologic characteristics of the primary tumor, particularly the extent of cancer invasion, and lymph node metastasis (including both overt metastasis and micrometastasis) in order to assess the curative potential of EMR for sm-CRC.

MATERIALS AND METHODS

Patients

One hundred seventeen sm-CRC and their regional lymph nodes were examined after surgical resection. The following were excluded from the study: 1)cases accompanied by other sm-CRC or advanced CRC; 2) cases of familial polyposis or inflammatory bowel disease; and 3)cases in which EMR was followed by bowel resection.

Histopathologic examination of the primary tumor

The resected bowels were opened and fixed in 10% formalin, and the entire tumor was cut into step-wise sections and embedded in paraffin. All cases were examined after H&E staining by experienced histopathologists according to the General Rules for Clinical and Pathological Studies on Cancer of the Colon, Rectum and Anus (Japanese Society for Cancer of the Colon and Rectum)[18]. Gross type, site of tumor (colon or rectum), grade of differentiation (well, moderate, or poor), lymphatic and venous invasion, and the extent of cancer invasion to the submucosa were selected as histopathologic factors.

The extent of cancer invasion was analyzed according to the depth (extent of vertical invasion: Vsm) and width (extent of horizontal invasion: Hsm) on an ocular lens scale (Fig. 1)[27]. Vsm was defined as the distance between the deepest point of the cancer invasion and the lower edge of the muscularis mucosae[28]. For cases in which the muscularis mucosae had been destroyed and had disappeared, Vsm was measured as the distance between the deepest point and the surface of the tumor. Hsm was defined as the maximum horizontal extent of the cancer in the submucosa. In order to confirm the deepest and widest point of the cancer invasion, serial sections (at least 27 finally) were cut for each paraffin block.

Immunohistochemical stainings using monoclonal antibodies against Desmin (clone D33; diluted 1 : 100; Dako) and α-smooth muscle actin (ASMA) (clone 1A4; diluted 1 : 2000; Sigma) were performed to confirm the presence of the muscularis mucosae. For the

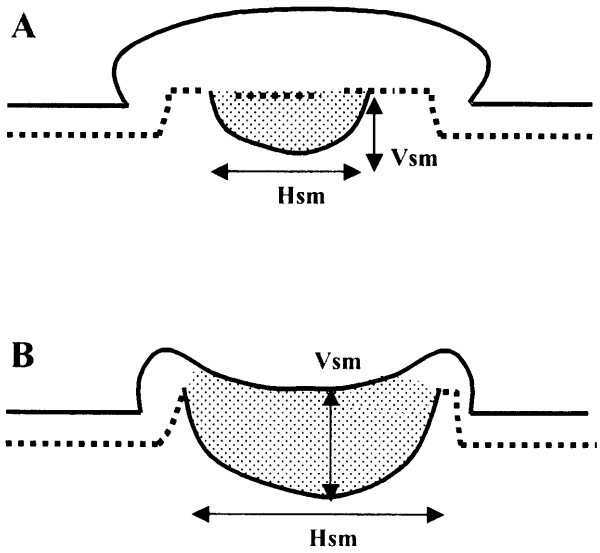

Fig. 1. Analysis of the extent of submucosal cancer invasion. Vsm, maximum vertical distance of submucosal invasion; Hsm, maximum horizontal distance of submucosal invasion.

Table 1. Relationship between clinicopathologic factors of primary tumors and lymph node metastasis

	Lymph nodal metastasis					
	N(+)		MM(+)		N(+) or MM(+)	
Gross Type						
Ip, Isp	1/21(4.8%)		2/20(10.0%)		3/21(14.3%)	
Is	14/59(23.7%)		8/45(17.8%)		22/59(37.3%)	
IIa	2/17(11.8%)		4/15(26.7%)		6.17(35.3%)	
IIa+IIc, IIc, IIc+IIa	1/20(5.0%)		3/19(15.8%)		4/20(20.0%)	
Site of tumor						
Colon	9/62(14.5%)		6/53(11.3%)		15/62(24.2%)	
Rectum	9/55(14.5%)		11/46(23.9%)		20/55(36.4%)	
Histologic type						
well	12/89(13.5%)		11/77(14.3%)		23/89(25.8%)	
moderate	6/28(21.4%)		6/22(27.3%)		12/28(42.9%)	
poor	-		-		-	
Lymphatic invasion						
Negative	7/91(7.6%)	$p<0.0001$	11/84(13.1%)	$p<0.030$	18/91(19.8%)	$p<0.0001$
Positive	11/26(42.3%)		6/15(40.0%)		17/26(65.4%)	
Venous invasion						
Negative	11/89(12.4%)		12/78(15.4%)		23/89(25.8%)	
Positive	7/28(25.0%)		5/21(23.8%)		12/28(42.9%)	
Total	18/117(15.4%)		17/99(17.2%)		35/117(29.9%)	

N(+), overt metastasis; MM(+), micrometastasis; Histologic type represents the predominant type.

examination of venous and lymphatic invasion, Victoria blue elastic fiber staining was performed to visualize the elastic fibers of the venous wall, and immunostaining with anti-human endothelial cell antibody CD31 (JC/70A; diluted 1 : 100; Dako) and CD34 (QBEnd 10; diluted 1 : 200; Dako) were performed to visualize the endothelial cells in the lymphatic channels.

Investigations of lymph node metastasis

The lymph nodes obtained from each case were first examined using routine H&E sections. Following this, all lymph nodes diagnosed as negative by H&E staining were subjected to an immunohistochemical assay for micrometastasis. To detect micrometastasis, two 3 μm-thick sections and three consecutive 10 μm-thick sections were prepared from all lymph nodes as described in our previous study[26,27]. The 3 μm-thick sections were stained with H&E, and the 10 μm-thick sections were immunostained with anti-human cytokeratin 8 and 18 antibody (CAM5.2; diluted 1 : 3; Becton Dickinson) to visualize the epithelial cells in the lymph nodes.

Immunohistochemistry

Immunohistochemical assays for Desmin, ASMA, CD31, CD34 and CAM5.2 were performed by the conventional streptavidin-biotin (SAB) immunoperoxidase method, using a commercially available SAB complex (Nichirei, Tokyo, Japan). Diaminobenzidine was used as the chromogen, and the counterstaining was done with hematoxylin for Desmin, ASMA, CD31 and CD34, and with methyl green for CAM5.2.

Statistical analysis

A chi-square test was used for the statistical analysis, and values of $p<0.05$ were considered to indicate statistical significance. Fisher's exact test was applied where appropriate.

RESULTS

One thousand thirty-one lymph nodes from 117 sm-CRC (11.3 lymph nodes per case) were examined, and 18 cases (15.4%) were positive for cancer metastasis by H&E examination (overt metastasis). In the

Table 2. Clinicopathologic findings of the patients with lymph node metastasis

1) Patients with overt metastasis

No.	Gross type	Tumor size (mm)	Site	Histologic type	Lymphatic invasion	Venous invasion	Extent of sm invasion	
							Vsm(μm)	Hsm(μm)
1	Is	37	A	well	-	-	1000	4800
2	Is	22	A	well	+	-	1500	3300
3	Is	8	Ra	well	+	-	1600	3000
4	Is	48	A	well	-	-	2000	3000
5	IIa	7	S	well	+	-	2100	3300
6	IIa+IIc	14	Rb	well	+	+	2300	5500
7	IIa	15	Rb	well	-	-	2300	10000
8	Is	30	S	moderate	+	+	3000	5000
9	Is	19	T	well	-	-	3500	8000
10	Is	23	Rb	well	-	-	3500	16000
11	Is	19	Rb	well	+	+	3800	14000
12	Is	15	A	well	+	+	4400	12500
13	Is	13	T	well	+	+	4500	5000
14	Isp	20	S	moderate	-	-	4500	11500
15	Is	21	Rs	well	-	-	4500	13000
16	Is	21	Rs	moderate	+	+	5100	15000
17	Is	25	Rs	moderate	+	+	5500	12000
18	Is	28	Rb	moderate>poor	+	-	6000	22000

2) Patients with micrometastasis

No.	Gross type	Tumor size (mm)	Site	Histologic type	Lymphatic invasion	Venous invasion	Extent of sm invasion		Number of metastatic cells
							Vsm(μm)	Hsm(μm)	
1	IIa	48	A	well	-	-	850	2500	Single cell
2	Is	30	Rb	well	-	-	1000	2500	2 clusters
3	IIc+IIa	22	Rs	well	+	-	1500	3300	Single cell
4	IIa+IIc	10	A	well	+	+	1800	5500	Cluster
5	Is	52	Rs	well	-	-	2000	3800	Single cell
6	IIc+IIa	17	Ra	well	-	-	2000	4200	3 single cells
7	IIa	18	T	well	-	+	2300	5800	Cluster
8	Isp	15	S	well>moderate	-	-	2400	10000	Single cell
9	Is	20	Ra	moderate	+	-	2700	11000	3 single cells+cluster
10	IIa	11	Ra	moderate	-	-	3000	5600	Cluster
11	Is	20	Ra	moderate>poor	-	+	3300	8300	Single cell+cluster
12	IIa	18	Rb	well	+	+	3500	6200	Single cell+cluster
13	Is	16	Ra	well	-	+	4200	7500	Single cell
14	Isp	22	Ra	moderate	-	-	4500	6000	Single cell
15	Is	25	S	moderate	+	-	4500	16000	Single cell
16	Is	42	Rb	well>moderate	+	-	7800	15000	Single cell
17	Is	15	S	moderate	-	-	8000	11000	Cluster

F, female; M, male; Rb, rectum below the peritoneal reflection; Ra, rectum above the peritoneal reflection; Rs, rectosigmoid; S, sigmoid colon; T, transverse colon; A, ascending colon; sm, submucosal; Vsm, vertical extent; Hsm, horizontal extent.

Fig. 2. Immunostaining of lymph node micrometastasis consisting of a single cancer cell (×100, CAM5.2).

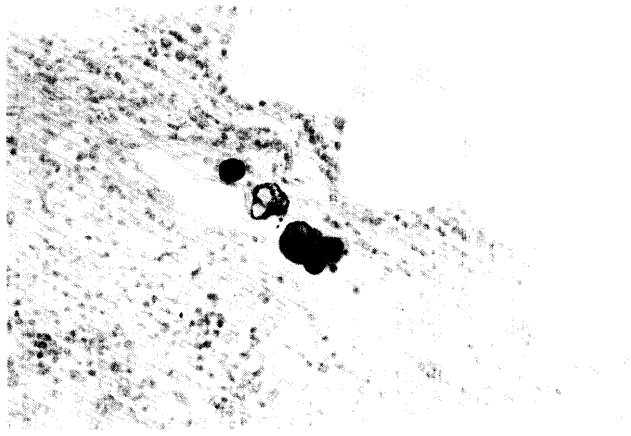

Fig. 3. In some foci, micrometastasis consist of a cluster of several cancer cells (×100, CAM5.2).

remaining 99 cases that were negative for overt metastsis, a total of 802 lymph nodes (8.1 per case) were examined, and micrometastasis was detected in 17 cases (17.2%) by CAM5.2 immunostaining (Fig. 2 and 3). The incidence of micrometastasis in cancer-free nodes by H&E examination was 23/802 (2.9%). The overall incidence of lymph node metastasis (overt or micrometastasis) in sm-CRC was 29.9% (35/117).

The relationships between lymph node metastasis and the clinicopathologic factors of the primary tumor are summarized in Table 1. Lymphatic invasion was the only factor correlated with overt metastasis, micrometastasis and overall lymph node metastasis ($p<0.0001$, $p=0.03$, $p<0.0001$, respectively).

The clinicopathologic findings of the 18 patients with overt metastasis and 17 patients with micrometastasis are shown in Table 2. The minimal depth (Vsm) and width (Hsm) of cancer invasion in patients with overt metastasis were 1000 μm and 3000 μm, respectively, and in patients with micrometastasis, they were 850 μm and 2500 μm. There was no significant difference in the average Vsm or Hsm between cases with overt metastasis and those with micrometastasis. Micrometastasis was detected as a single cancer cell (Fig. 2) and/or cluster(s) consisting of up to 8 cancer cells (Fig. 3). Nine out of the 17 cases showed a micrometastasis focus (or foci) consisting of a single cancer cell in the marginal or medullary sinus of the lymph node. No correlation was observed between the number of micrometastatic cells and the extent of cancer invasion in the submucosa.

The relationship between the extent of submucosal invasion of the cancer and the status of lymph node metastasis is depicted for all 117 patients in the scattergraph in Fig. 4. Fifteen of the 117 (12.8%) showed a submucosal invasion of less than 850 μm in depth (Vsm) and 2500μm in width (Hsm). These 15 tumors were not only negative for lymph node metastasis, but also lacked both lymphatic and venous invasion.

DISCUSSION

In this study, micrometastasis was detected in 17 of 99 (17.2%) sm-CRC by immunohistochemical analysis using an anti-human cytokeratin antibody (CAM5.2). Although this incidence was less than that of micrometastasis in advanced CRC (25-60%)[20,23-25], it is noteworthy that nearly 20% of sm-CRC had lymph node metastasis that was not discernible by routine H&E examination. The incidence of overall lymph nodal metastasis of sm-CRC, including both overt and micrometastasis, was 29.9% (35/117).

The clinical significance of micrometastasis in sm-CRC remains to be conclusively established. A long-time follow-up study will be needed to clarify whether or not such micrometastasis is related to cancer recurrence and the prognosis of patients. However, the presence of micrometastasis indicates that cancer cells are circulating in the lymphatic system apart from the bowels, and thus it must be considered that the cancer with micrometastasis may already be a systemic disease. Micrometastatic cells may be a precursor of overt metastasis, as indicated by experimental studies showing the tumorigenecity and metastatic potential of these cells[28]. Inversely, sm-CRCs lacking even micrometastasis might be

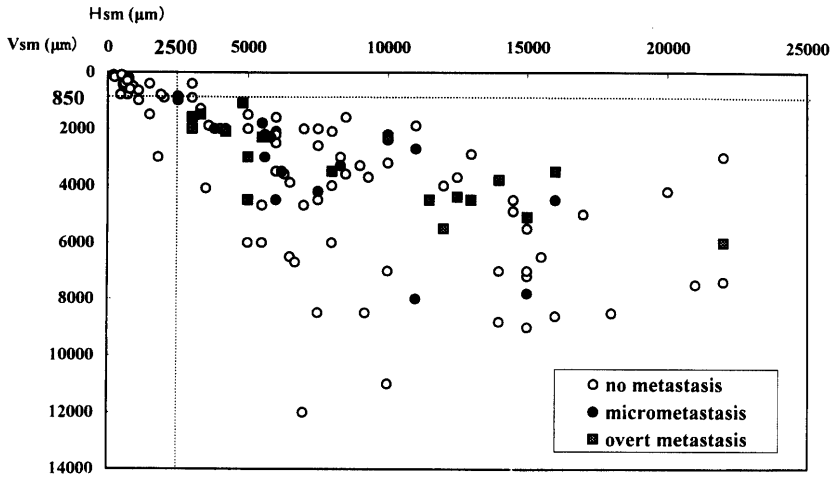

Fig. 4. Relationship between lymph node metastasis (overt and micrometastasis) and the extent of submucosal cancer invasion. Vsm, maximum vertical distance of submucosal invasion; Hsm, maximum horizontal distance of submucosal invasion.

regarded as cases of local disease in which cancer cells are confined within the primary tumor.

Since lymph node metastasis is the major prognostic factor in patients with carcinomas, the complete curative potential of EMR for sm-CRCs should be evaluated in terms of whether the histopathologic features of the primary tumor are those expected to have considerably low potential for lymph node metastasis. Several studies have stressed the importance of the extent of cancer invasion to the submucosa as the indicative factor for the curative potential of EMR-treated sm-CRC. Tanaka et al.[15] reported that sm-CRCs showing even slight submucosal invasion (less than 400 μm in depth) are indicated for EMR provided that the tumor is well or moderately well differentiated and shows no lymphatic invasion. Okabe et al[13,16]. demonstrated that cases of submucosal invasion of less than 500 μm in depth and 2000 μm in width showed neither lymph node metastasis nor vascular invasion. In our previous study[14], neither lymph node metastasis nor vascular invasion were detected in sm-CRCs less than 1000 μm in depth and 3000 μm in width. The extent of submucosal invasion of a carcinoma is expected to be a useful histopathologic marker for the curative potential EMR treatment of sm-CRCs in practical routine diagnosis. However, the precise degree of submucosal invasion that constitutes no risk of lymph node metastasis has yet to be agreed upon. Further, many earlier studies, including ours, were based on the correlation between the histopathologic features of the primary tumor and overt metastasis, and it seems necessary to re-evaluate such results in the light of micrometastasis.

In this study, lymphatic invasion was the only factor correlated with both overt and micrometastasis ($p<0.0001$ and $p=0.030$, respectively). This finding was consistent with earlier studies[4,8–10] that emphasized lymphatic invasion as the most significant risk factor for lymph node metastasis.There were no cases of poorly differentiated or undifferentiated-type adenocarcinoma in the present cohort, and thus we were unable to evaluate the correlation between such tumors and lymph node metastasis.

The minimum extent of cancer invasion in patients with lymph node metastasis was 850 μm in depth and 2500 μm in width (Fig. 4), and the micrometastasis in this case consisted of a single cancer cell(Table 2). Tumors in which the extent of invasion was smaller than these values showed no lymphatic or venous invasion. These cutoff values are greater than the cutoff value of the 400–500 μm depth that was estimated earlier for patients without overt lymph node metastasis or other risk factors for lymph node metastasis[13,15,16]. We speculate that the earlier studies may have underestimated the minimum extent of cancer invasion with overt lymph node metastasis. The deepest or widest points of submucosal invasion do not always appear in the representative or first-prepared H&E section. It is also not clear in earlier studies whether the entire lesions were examined by stepwise sectioning[13,16] or by producing serial sections[13,15,16] to confirm the deepest

and/or widest point of submucosal invasion. In this study, we examined the entire lesion using stepwise sections and confirmed the deepest and widest point of submucosal invasion by producing over 27 serial sections for each paraffin block.

The present results indicate that sm-CRCs with a submucosal invasion of less than 850 μm in depth and 2500 μm in width may present no risk of even micrometastasis unless histological analysis indicates a carcinoma of a poorly or undifferentiated type, which we were unable to investigate in this study. In these sm-CRCs, EMR alone would be expected to have complete curative potential without additional surgery.

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REFERENCES

1) Yokota T, Sugihara K, Yoshida S: Endoscopic mucosal resection for colorectal neoplastic lesions. *Dis Colon Rectum* **38:** 959-963, 1994.
2) Kudo S, Kashida H, Nakajima T, Tamura S, Makajo K: Endoscopic diagnosis and treatment of early colorectal cancer. *World J Surg* **21:** 694-701, 1997.
3) Mainprize KS, Mortensen NJ, Warren BF: Early colorectal cancer: recognition, classification and treatment. *Br J Surg* **85:** 469-476, 1998.
4) Coverlizza S, Risio M, Ferrari A, Fenoglio-Preiser CM, Rossini FP: Colorectal adenomas containing invasive carcinoma. Pathologic assessment of lymph node metastatic potential. *Cancer* **64:** 1937-1947, 1989.
5) Nivatvongs S, Rojanasakul A, Reiman HM, Dozois RR, Wolff BG, Pemberton JH, Beart RW, Jacques LF: The risk of lymph node metastasis in colorectal polyps with invasive adenocarcinoma. *Dis Colon Rectum* **34:** 323-328, 1991.
6) Tanaka S, Yokota T, Saito D, Okamoto S, Oguro Y, Yoshida S: Clinicopathologic features of early rectal carcinoma and indications for endoscopic treatment. *Dis Colon Rectum* **38:** 959-963, 1995.
7) Hase K, Shatney CH, Mochizuki H, Johnson DL, Tamakuma S, Vierra M, Trollope M: Long-term results of curative resection of "minimally invasive" colorectal cancer. *Dis Colon Rectum* **38:** 19-26, 1995.
8) Shatney CH, Lober PH, Gilbertsen VA, Sosin H: The treatment of pedunculated adenomatous colorectal polyps with focal cancer. *Surg Gynecol Obstet* **139:** 845-850, 1974.
9) Wolff WI, Shinya H: Definitive treatment of "malignant" polyps of the colon. *Ann surg* **182:** 516-524, 1975.
10) Christie JP: Malignant colon polyps-cure by colonoscopy or colectomy? *Am J Gastroenterol* **79:** 543-547, 1984.
11) Kudo S, Soga J, Yamamoto S, Yamamoto M, Koyama M, Muto T: Treatment of colorectal sm-carcinomas. *Stomach and Intestine (I to Cho)* **19:** 1349-1355, 1984. (in Japanese with English abstract)
12) Haggitt RC, Glotzbach RE, Soffer EE, Wruble LD: Prognostic factors in colorectal carcinomas arising in adenomas: Implications for lesions removed by endoscopic polypectomy. *Gastroenterology* **89:** 328-336, 1985.
13) Okabe S: Histopathological investigation of risk factors of metastasis in submucosal invasive carcinomas of the colon and rectum. *J Jpn Soc Coloproctol* **47:** 564-575, 1994 (in Japanese with English abstract)
14) Kobayashi M, Watanabe H, Naeo S, Ajioka Y, Yoshida M, Saito H, Hitomi J, Suda T: Correlation of histological atypia and cancer sprouting with vascular permeation and lymph node metastasis by our new histological classification of submucosal invasion by colorectal carcinoma. *Stomach and Intestine (I to Cho)* **29:** 1151-1160, 1994. (in Japanese with English abstract)
15) Tanaka S, Haruma K, Teixeira CR, Tatsuta S, Ohtsu N, Hiraga Y, Yoshihara M, Sumii K, Kajiyama G, Shimamoto F: Endoscopic treatment of submucosal invasive colorectal carcinoma with special reference to risk factors for lymph node metastasis. *J Gastroenterol* **30:** 710-717, 1995.
16) Okabe S, Arai T, Maruyama S, Murase N, Tsubaki M, Endo M: A clinicopathological investigation on superficial early invasive carcinomas of the colon and rectum. *Surg Today* **28:** 687-695, 1998.
17) Minamoto T, Mai M, Ogino T, Sawaguchi K, Ohta T, Fumimoto T, Takahashi Y: Early invasive colorectal carcinomas metastatic to the lymph node with attention to their nonpolypoid development. *Am J Gastroenterol* **88:** 1035-1039, 1993.
18) Japanese Society for Cancer of the Colon and Rectum. General Rules for Clinical and Pathological Studies on Cancer of the Colon, Rectum and Anus, 6 th ed., Tokyo, Kanehara, 1998, pp. 34-35.
19) Moll R, Franke WW, Schiller DL, Geiger B, Krepler R: The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. *Cell* **31:** 11-24, 1982.
20) Greenson JK, Isenhart CE, Rice R, Mojzisik C, Houchens D, Martin EW: Identification of occult micrometastases in pericolic lymph nodes of Dukes' B colorectal cancer patients using monoclonal antibodies against cytokeratin and CC49. *Cancer* **73:** 563-569, 1994.
21) Hayashi N, Arakawa H, Nagase H, Yanagisawa A, Kato Y, Ohta H, Takano S, Ogawa M, Nakamura Y: Genetic diagnosis identifies occult lymph node

metastases undetectable by the histopathological method. *Cancer Res* **54:** 3853-3856, 1994.

22) Mori M, Mimori K, Inoute H, Barnard GF, Tsuji K, Nanbara S, Ueno H, Akiyoshi T: Detection of cancer micrometastases in lymph nodes by reverse transcriptase-polymerase chain reaction. *Cancer Res* **55:** 3417-3420, 1995.

23) Jeffers MD, O'Dowd GM, Mulcahy H, Stagg M, O'Donoghue DP, Toner M: The prognostic significance of immunohistochemically detected lymph node micrometastases in colorectal carcinoma. *J Pathol* **172:** 183-187, 1994.

24) Adell G, Boeryd B, Franlund B, Sjodahl R, Hakansson L: Occurrence and prognostic importance of micrometastases in regional lymph nodes in Dukes' B colorectal carcinoma- an immunohistochemical study. *Eur J Surg* **162:** 637-642, 1996.

25) Haboubi NY, Abdall SA, Amini S, Clark P, Dougal M, Dube A, Schofield P: The novel combination of fat clearance and immunohistochemistry improves prediction of the outcome of patients with colorectal carcinomas: a preliminary study. *Int J Colorectal Dis* **13:** 99-102, 1998.

26) Sasaki M, Watanabe H, Jass JR, Ajioka Y, Kobayashi M, Matsuda K, Hatakeyama K: Occult lymph node metastases detected by cytokeratin immunohistochemistry predict recurrence in "node-negative" colorectal cancer. *J Gastroenterol* **32:** 758-764, 1997.

27) Kashimura H, Ajioka Y, Watanabe H, Nishikura K, Iiri T, Asakura H: Risk factors for nodal micrometastasis of submucosal gastric carcinoma: assessment of indications for endoscopic treatment. *Gastric Cancer* **2:** 33-39, 1999.

28) Hosch S, Kraus J, Scheunemann P, Izbicki JR, Schneider C, Schumacher U, Witter K, Speicher MR, Pantel K: Malignant potential and cytogenetic characteristics of occult disseminated tumor cells in esophageal cancer. *Cancer Res* **60:** 6836-6840, 2000.