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Microenvironmental markers are correlated with lymph node metastasis in invasive submucosal colorectal cancer

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Aims: Recent studies have shown that the microenvironment can include cancer cells and cancer-associated fibroblasts (CAFs), and that both play important roles in the progression and metastasis of CRC. Here, we aimed to analyse the expression patterns of cancer cell- and CAF-related proteins in submucosal invasive colorectal cancer (SiCRC) and whether such markers are correlated with lymph node metastasis (LNM).

Methods and results: Quantitative analysis was conducted for Ki-67, p53, β-catenin and matrix metalloproteinase-7 (MMP7) to assess cancer cell markers. In addition, we examined CAF markers, including smooth muscle alpha-actin (α-SMA), CD10, podoplanin, fibroblast-specific protein 1 (FSP-1), platelet-derived growth factor receptor (PDGFR)-α, PDGFR-β, adipocyte enhancer-binding protein 1 (AEBP1), fibroblast-associated protein 1 (FAP-1), zinc finger E-box binding homeobox 1 (ZEB1) and TWIST-related protein 1 (TWIST1). In both cases, we conducted digital pathology with Aperio software. We also examined the expression patterns of biomarkers using hierarchical cluster analysis. Two subgroups were established based on the expression patterns of cancer cell- and CAF- related markers, and the associations of these subgroups with clinicopathological variables. In multivariate analysis, subgroup 2, which was characterised by high expression of Ki-67, p53, FAP-1, platelet-derived growth factor receptor (PDGFR)-α, PDGFR-β and TWIST1, was correlated with LNM ($P < 0.01$). Next, we examined the associations of individual biomarkers with LNM. Multivariate analysis showed that high expression levels of Ki-67 and FAP-1 were significantly associated with LNM ($P < 0.05$).

Conclusions: Our findings showed that expression patterns of cancer cell- and CAF-related proteins may allow for stratification of patients into risk categories for LNM in SiCRC. In addition, Ki-67- and FAP-1-expressing microenvironmental cells might be helpful for identification of correlations with LNM in SiCRC.

Keywords: cancer-associated fibroblast, cancer cell, epithelial-mesenchymal transition, hierarchical cluster analysis, submucosal colorectal cancer

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Introduction

Colorectal cancer (CRC) is one of the most common cancers diagnosed worldwide.[1] The death rate from colorectal cancer has been increasing in both men and women for several decades.[1] According to a recent study, most CRC is discovered at an advanced stage. There are limitations to the efficacy of additional treatment for advanced CRC. To resolve this dilemma, it would be advantageous to detect submucosal invasive colorectal cancer (SiCRC) early in the course of disease. SiCRC is an intermediate lesion between intramucosal cancer and advanced invasive CRC and is probably curable.[2] Previous study has shown that although patient prognosis of SiCRC is favourable, the frequency of lymph node metastasis (LNM) in SiCRC is estimated to be approximately 10%.[3] Many investigations have developed predictive risk factors for LNM in SiCRC. For example, a submucosal invasion depth (SID) >1000 μm, lymphovascular invasion, poorly differentiated components and tumour budding have been postulated by many studies as suggestive predictive factors of LNM.[4–8] However, these parameters have not proved reliable or reproducible in predicting LNM. Instead of these pathological factors, biomarkers that might predict LNM have also been explored recently, and a combination of pathological factors and biomarkers may be the ideal method.[2]

Molecular alterations related to the progression or metastatic potential of CRC have been investigated, with a focus on cancer cells. However, recent studies have shown that the microenvironment, which is composed of both cancer cells and surrounding stromal cells at the invasive front, is closely associated with tumour invasion and metastasis.[9–11] In this model, stromal cells are thought to play particularly important roles in invasion and/or metastasis.[9,12] Thus, both cancer cells and stromal cells [cancer-associated fibroblasts (CAFs)] contribute to neoplastic progression.[2,12] Furthermore, recent study has shown that cancer metastasis might begin with the process of epithelial–mesenchymal transition (EMT), in which well-polarised epithelial cells are converted into non-polarised mesenchymal cells that acquire invasive and metastatic properties.[13–15] EMT occurs in the microenvironment and facilitates tumour invasion and metastasis[13–15] Therefore, EMT-related proteins, including TWIST-related protein 1 (TWIST1) and zinc finger E-box binding homeobox 1 (ZEB1), could be CAF-related markers.[15]

We previously reported that the immunohistochemical expression pattern in CAF helped to predict LNM in SiCRC.[2] However, cancer cells and the surrounding stromal cells presented together at the invasive front were not assessed in detail. In the present study, we evaluated the expression patterns in both cancer cells and CAF-related markers, including EMT-related proteins as well as clinicopathological variables to identify correlations with LNM in submucosal invasive CRC.

Materials and methods

PATIENTS

Sections of invasive submucosal colorectal cancer (SiCRC) were reviewed and examined by a senior gastrointestinal pathologist blinded to patient outcomes. One hundred and fifty-five patients who underwent curative surgery for submucosal CRC (SiCRC) at Iwate Medical University were enrolled into the present study. SiCRC samples were divided into two categories according to the absence or presence of LNM. Depth of submucosal invasion was also subclassified into pSM1 and pSM2 based on the criteria used in the Classification of the Japanese Society for Cancer of the Colon and Rectum.[16] The pathological factors, including tumour location and macroscopic classification and histological diagnosis, were also described according to this classification. Tumour budding was defined as an isolated single cancer cell or a cluster composed of fewer than five cancer cells.[17,18] Tumour budding was scored into two subgroups: low/negative and high, based on the criteria used in the Classification of the Japanese Society for Cancer of the Colon and Rectum.[18] Lymphatic and vascular invasion of the examined cases was assessed by D2-40 immunostaining and elastin staining, respectively. In addition, desmoplasia was defined as proliferation of active fibroblasts with expression of α-smooth muscle actin (α-SMA) and loss of desmin expression. In the present study, we examined perineural invasion (PNI),[19] tumour border configuration[20] and peritumoral inflammation (using a 'Jass-Klintrup score with a modification': 0, 1, mild; 2, moderate; 3, strong).[21] These characteristics were reported to be helpful in the prediction of LNM.[19–21] Notably, PNI was not observed in the present study. Tumour border configuration was classified into three classes, including expansive, intermediate and invasive types, in accordance with a previous study, with modification.[20]

Patients who showed evidence of hereditary non-polyposis CRC or familial adenomatous polyposis were excluded from the study. The detailed clinicopathological findings are listed in Table 1.

Table 1. Demographic and pathological characteristics of patients with submucosal invasive colorectal cancer

Variables	Frequency	(%)
Total number of patients	115	
Sex		
Male/female	57/58	
Age (years; median) (range)	67	(39–93)
Median size (mm) (range)	20	(8–85)
Locus		
Right	37	(32.2)
Left (rectum)	78 (47)	(67.8) (40.9)
Macroscopic type		
I	43	(37.4)
IIa	27	(23.5)
IIc	15	(13.0)
IIa + IIc	25	(21.7)
LST	5	(4.3)
Differentiation		
Well-differentiated	33	(28.7)
Moderately differentiated	80	(69.6)
Poorly differentiated	2	(1.7)
Depth of submucosal invasion		
<1000 μm	9	(7.8)
≥1000 μm	106	(92.2)
Lymphatic invasion		
Low	109	(94.8)
High	6	(5.2)
Venous invasion		
Low	107	(93.0)
High	8	(7.0)
Perineural invasion		
Negative	115	(100)
Positive	0	(0)
Tumour budding		
Low	104	(90.4)
High	11	(9.6)
Tumour border configuration		
Expansive type	3	(2.6)
Intermediate type	112	(97.4)
Infiltrative type	0	0 (0)
Peritumoral inflammation		
Mild (0, 1)	81	(70.4)
Moderate (2)	17	(14.8)
Marked (3)	12	(14.8)
Positive for lymph node metastasis	37	(32.2)

LST, Laterally spreading tumour.

This study was approved by the Ethical Research Committee of Iwate Medical University (reference number: H28-185).

CONSTRUCTION OF TISSUE MICROARRAYS (TMAS)

The construction of tissue microarrays (TMAs) was described previously.[2] Briefly, formalin-fixed, paraffin-embedded tissue blocks containing SiCRC samples were retrieved from the archives of the Iwate Medical University Department of Diagnostic Molecular Pathology. Areas of submucosal invasive cancer that included the deepest areas were identified on corresponding haematoxylin and eosin (H&E)-stained slides, and the tissue blocks were scored and transferred to a recipient 'master' block for use in TMAs.

IMMUNOHISTOCHEMISTRY

Tissue sections were deparaffinised and rehydrated using xylene and alcohols, and incubated in 3% hydrogen peroxide to block endogenous peroxidase. Antigen retrieval was performed using an autoclave-based method, followed by incubation with the primary antibody overnight at 4°C in a high-humidity cabinet. Slides were processed using the Dako Autostainer Universal Staining System (Dako, Glostrup, Denmark).

Antibodies used in this study were classified into two subgroups: epithelial (cancer cells) and interstitial (CAF) markers. Antibodies targeting interstitial markers (CAFs) included the following: (α-SMA; Dako 1A4), CD10 (Dako, 56C6), podoplanin (Dako,

D2-40), fibroblast-specific protein 1 (FSP-1; S100A4, Dako, polyclonal), platelet-derived growth factor recepotr (PDGFR-α, CST, polyclonal), PDGFR-β (CST, 28El), fibroblast-associated protein-1 (FAP-1; Abcam, Cambridge, MA, USA, EPR20021) and adipocyte enhancer-binding protein 1 (AEBP1; Abcam, ab54820). For EMT, the following were used: (ZEB1; Sigma-Aldrich, St Louis, MO, USA, polyclonal and TWIST1; Abcam, TWIST2C1A) (Table S1). CAFs were recognised as 'spindle-shaped cells' by experienced pathologists (T.S. and N.U.). Cytoplasmic staining of tumour cells was conducted with antibodies against α-SMA, CD10, podoplanin, FSP-1, PDGFR-α, PDGFR-β and FAP-1. Nuclear staining of fibroblasts was based on positivity for ZEB1 and TWIST1 expression. Next, antibodies targeting cancer cells in this study included Ki-67 (Dako; MIB1) for proliferative activity, p53 (Dako; Do7) for p53 mutation, β-catenin (Dako; β-catenin-1) for activation of the Wnt signal, which is a central signal transducer in CRC, and matrix metalloproteinase-7 (MMP7) (Daiichi Fine Chemical, Toyama, Japan; 141-7B2) for cancer progression.

ASSESSMENT OF IMMUNOHISTOCHEMICAL MARKERS

Quantitative analysis of cancer cell markers (Ki-67, p53, β-catenin and MMP7) and CAF markers (α-SMA, CD10, podoplanin, FSP-1, PDGFR-α, PDGFR-β, FAP-1, AEBP1, ZEB1 and TWIST1) was performed using digital pathology with Aperio software (Leica Biosystems, Tokyo, Japan).[2,12] Tissue sections were scanned on an Aperio AT2 scanner with an average scan time of 120 s (compression quality: 70). Images were analysed using colour deconvolution and colocalisation. The Aperio pixel count version 9 algorithm in Aperio Image Analysis software (for cytoplasmic analysis) was used, and the nuclear version 9 algorithm was applied to detect the nuclear staining of individual tumour cells in the selected regions for nuclear analysis. The intensity of staining was measured on a continuous scale from 0 (black) to 255 (bright white) and was automatically calculated by the software as the ratio of positively stained nuclei to all nuclei (negative, weak, moderate, strong and very strong). Greater than 'moderate intensity' (moderate, strong and very strong) was considered to be positive. Stained areas were colour-separated from H&E-counterstained sections and measured by the software. The score for the area of the positively stained cells (percentage of positive cells) was based on the average score observed in a hot-spot at ×400 (deepest invasive front, which was the high score of tumour budding). Representative images are shown in Figure 1.

HIERARCHICAL ANALYSIS OF THE EXPRESSION OF CAF AND EMT MARKERS

Hierarchical cluster analysis was performed in order to group the samples according to the expression level, thereby achieving maximal homogeneity for each group and the greatest difference between the groups using open-access clustering software (Cluster version 3.0 software; bonsai.hgc.jp/~mdehoon/software/cluster/software.htm). The clustering algorithm was set to centroid linkage clustering, which is the standard hierarchical clustering method used in biological studies.

STATISTICAL ANALYSIS

Data were analysed using JMP Pro version 13.0 software (SAS, Tokyo, Japan). Data obtained for clinicopathological features (sex, macroscopic type, location, histological type, classification of submucosal invasion and LNM) and those in subgroups 1 and 2 were analysed using Fisher's exact test. In addition, the comparison of the age distributions within each subgroup was performed using the Kruskal–Wallis test.

For statistical analysis of the expression of p53, Ki-67, β-catenin, MMP7, α-SMA, CD10, podoplanin, FSP-1, PDGFR-α, PDGFR-β, FAP-1, AEBP1, ZEB1 and TWIST1 in SiCRC and their associations with various clinicopathological factors and LNM, we used χ^2 tests, Fisher's exact tests and Mann–Whitney *U*-tests with a 2×2 table to compare the categorical data. Univariate and multivariate analyses were conducted with logistic regression tests to identify statistical differences in the correlations of examined markers with LNM. The level of significance was $P < 0.05$, and the confidence interval (CI) was determined at the 95% level.

ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

Informed consent was obtained from each patient according to institutional guidelines, and the research protocols were approved by the ethics committee of Iwate Medical University Hospital (reference number: H28-185).

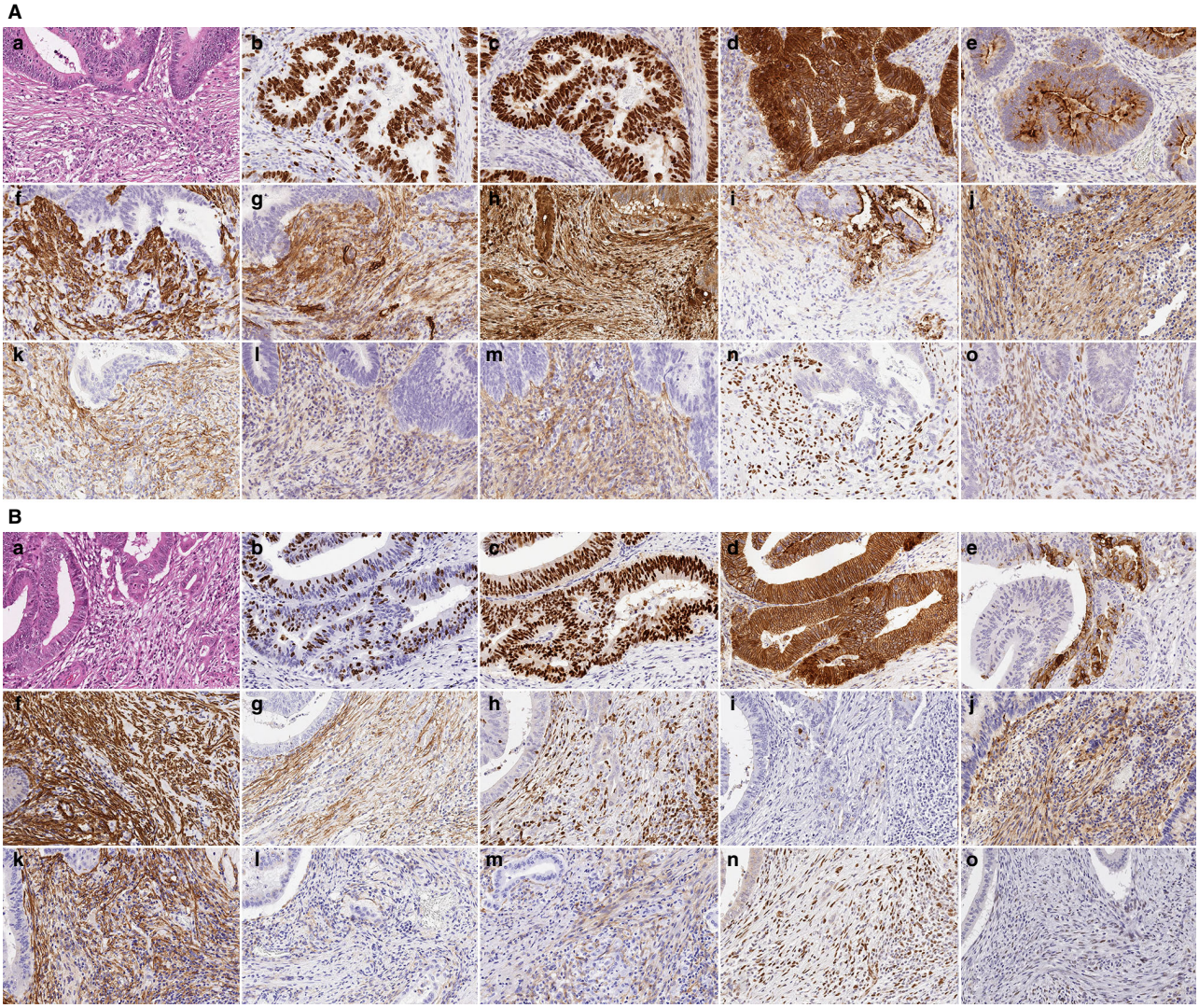

Figure 1. Representative data from submucosal invasive colorectal cancer with/without lymph node metastasis. **A**, Representative illustrations of submucosal invasive colorectal cancer with lymph node metastasis. (a) Haematoxylin and eosin (H&E) section. (b) Ki-67, 88.2% positive. (c) p53, 96.8% positive. (d) β-catenin, 45.4% positive. (e) Matrix metalloproteinase-7 (MMP7), 82.4% positive. (f) Smooth muscle alpha-actin (α-SMA), 81.9% positive. (g) Podoplanin, 65.9% positive. (h) Fibroblast-specific protein 1 (FSP-1), 83.3% positive. (i) CD10, 7.8% positive. (j) Adipocyte enhancer-binding protein (AEBP1), 82.2% positive. (k) Fibroblast-associated protein 1 (FAP-1), 42.7% positive. (l) Platelet-derived growth factor (PDGFR-α), 14.9% positive. (m) PDGFR-β, 55.4% positive. (n) Zinc finger E-box binding homeobox 1 (ZEB1), 58.4% positive, (O) TWIST-related protein 1 (TWIST1), 82.8% positive. **B**, Representative illustrations of submucosal invasive colorectal cancer without lymph node metastasis. (a) H&E section. (b) Ki-67, 38.8% positive. (c) p53, 97.7% positive. (d) β-catenin, 15.8% positive. (e) MMP7, 42.9% positive. (f) α-SMA, 87.1% positive. (g) podoplanin, 39.8% positive. (h) FSP-1, 28.2% positive. (i) CD10, 7.8% positive. (j) AEBP1, 61.8% positive. (k) FAP-1, 28.2% positive. (l) PDGFR-α, 2% positive. (m) PDGFRβ, 18.1% positive. (n) ZEB1, 47.6% positive. (o) TWIST1, 12.8% positive.

HUMAN RIGHTS STATEMENT AND INFORMED CONSENT

All procedures were performed in accordance with the ethical standards of the Iwate Medical University and with the Declaration of Helsinki. Substitute for informed consent (approval by the institutional review board of Iwate Medical University) was obtained from all patients included in the study.

Results

In the present study, heterogeneous levels of expression were observed in all the markers that we

examined. Thus, we selected the deepest invasive region as a target area to measure the expression levels of markers.

HIERARCHICAL CLUSTERING BASED ON MARKER SCORES

First, we performed hierarchical clustering based on marker scores to evaluate differences in expression patterns of cancer cell-, CAFs- and EMT-related markers in patients with SiCRC. Two distinct subgroups were stratified, as shown in Figure 2 (subgroups 1 and 2). The vertical line shows the expression of each marker in cancer cells and fibroblasts and the horizontal lines denote 'relatedness' between samples. There were no statistical differences between subgroups 1 and 2 in the frequencies of clinicopathological findings listed in Table 1 (Table 2).

The positive ratios of Ki-67 ($P = 0.0058$) and p53 ($P < 0.0001$) in cancer cells, as well as FAP-1 ($P = 0.0013$), PDGFR-α ($P = 0.0199$) and PDGFR-β ($P = 0.0099$) in fibroblasts in subgroup 2 were significantly higher than those in subgroup 1 (Figure 3). In addition, the ratio of men to women was significantly higher in subgroup 1 than in subgroup 2 (Table 2). Finally, there was a significant difference in the frequencies of LNM between subgroups 1 and 2 (subgroup 2 > subgroup 1; $P = 0.0032$) (Table 2).

ASSOCIATION OF CLINICOPATHOLOGICAL VARIABLES AND EXPRESSION PATTERNS OF EXAMINED MARKERS WITH LYMPH NODE METASTASIS IN SICRC USING UNIVARIATE AND MULTIVARIATE ANALYSES

To determine whether the clinicopathological variables and expression patterns of examined markers were independent predictors of LNM among patients with SiCRC, we used univariate analysis for preliminary screening of the variables, followed by a stepwise logistic regression of the risk of LNM with the significant univariate correlators (predictors). The univariate analysis (Table 3) identified four factors, including age, tumour location, macroscopic type and subgroups 1 versus 2, as associated with increased LNM in patients with SiCRC. Pathological markers reported as useful markers correlated with LNM were not identified in univariate analysis.

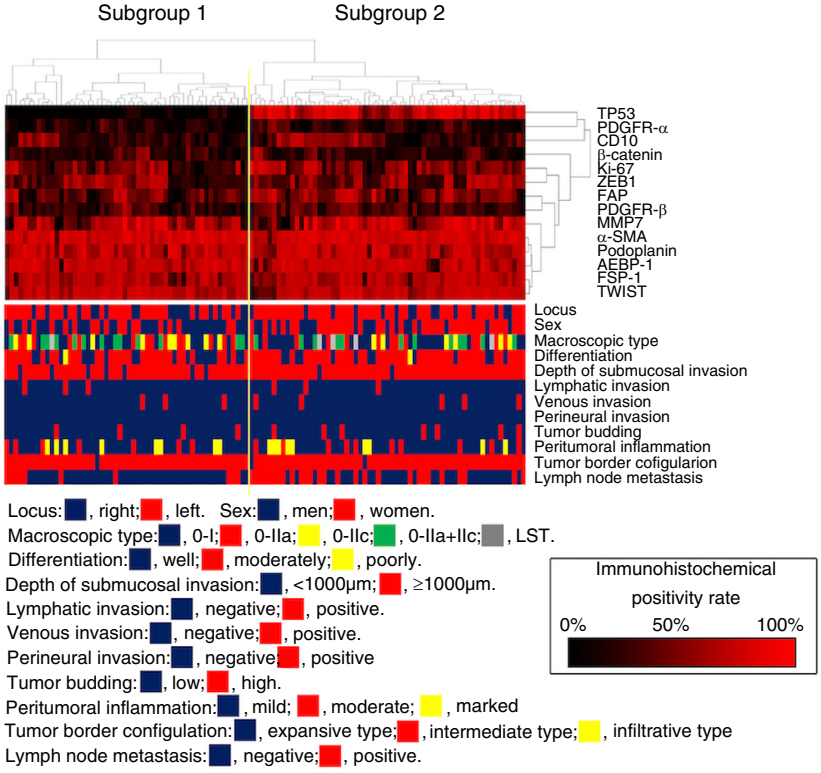

Figure 2. Hierarchical cluster analysis of submucosal invasive colorectal cancer (SiCRC) based on the expression patterns of cancer cells and cancer-associated fibroblast (CAF) proteins. The examined SiCRCs were subclassified into two subgroups.

Table 2. Clinicopathological findings in each subgroup by hierarchical cluster analysis

	Total (%)	Subgroup 1 (%)	Subgroup 2 (%)	*P*-value
Total	115	54 (47.0)	61 (53.0)	
Male/female	57/58	36/18	21/40	0.0006
Age (years; median) (range)	67 (39–93)	67 (40–93)	68 (39–87)	0.9263
Size (mm; median) (range)	20 (8–85)	20 (8–85)	19 (8–68)	0.6820
Locus				
Right	37 (32.2)	20 (37.0)	17 (27.9)	0.2935
Left	78 (67.8)	34 (63.0)	44 (72.1)	
Macroscopic type				
I	43 (37.4)	17 (31.5)	26 (42.6)	0.4278
IIa	27 (23.5)	14 (25.9)	13 (21.3)	
IIc	15 (13.0)	9 (16.7)	6 (9.8)	
IIa + IIc	25 (21.7)	13 (24.1)	12 (19.7)	
LST	5 (4.3)	1 (1.8)	4 (6.6)	
Differentiation				
WDA	33 (28.7)	20 (37.0)	13 (21.3)	0.1069
MDA	80 (69.6)	33 (61.1)	47 (77.0)	
PDA	2 (1.7)	1 (1.9)	1 (1.6)	
Depth of submucosal invasion				
<1000 μm	9 (7.8)	7 (13.0)	2 (3.3)	0.0809
≥1000 μm	106 (92.2)	47 (87.0)	59 (96.7)	
Lymphatic invasion				
Low	109 (94.8)	51 (94.4)	58 (95.1)	1.0000
High	6 (5.2)	3 (5.6)	3 (4.9)	
Venous invasion				
Low	107 (93.0)	51 (94.4)	56 (91.8)	0.7210
High	8 (7.0)	3 (5.6)	5 (8.2)	
Perineural invasion				
Negative	115 (100)	54 (100)	61 (100)	NS
Positive	0 (0)	0 (0)	0 (0)	
Tumour budding				
Low	104 (90.4)	50 (92.6)	54 (88.5)	0.5373
High	11 (9.6)	4 (7.4)	7 (11.5)	

Table 2. (*Continued*)

	Total (%)	Subgroup 1 (%)	Subgroup 2 (%)	*P*-value
Tumour border configuration				
Expansive type	3 (2.6)	1 (1.6)	2 (3.3)	0.8972
Intermediate type	112 (97.4)	53 (98.4)	59 (96.7)	
Infiltrative type	0 (0)	0 (0)	0 (0)	
Peritumoral inflammation				
Mild	81 (70.4)	39 (72.2)	42 (68.9)	0.8719
Moderate	17 (14.8)	7 (13.0)	10 (16.4)	
Marked	17 (14.8)	8 (14.8)	9 (14.8)	
Positive for lymph node metastasis	37 (32.2)	10 (18.5)	27 (44.3)	0.0032

LST, Laterally spreading tumour; WDA, Well–differentiated adenocarcinoma; MDA, Moderately differentiated adenocarcinoma; PDA, Poorly differentiated adenocarcinoma; NS, Not significant.

Table 3 shows the single factor that was retained in the multivariate logistic regression analysis. We found that subgroups 1 versus 2 [odds ratio (OR) = 3.32; 95% confidence interval (CI) = 1.32–8.33; $P = 0.0107$] remained significantly correlated with LNM, even after controlling for the other variables.

COMPARISON OF INDIVIDUAL MARKERS IN THE PRESENCE AND ABSENCE OF LYMPH NODE METASTASIS

We asked whether the expression levels of individual markers we selected were correlated with LNM in SiCRC. A significantly higher expression level of Ki-67 in cancer cells was seen in SiCRC patients positive for LNM than in those negative for LNM ($P \sim 0.0005$). There were significant differences in the expression levels of MMP7 ($P = 0.0394$), FAP-1 ($P = 0.0489$), FSP-1 ($P = 0.0182$) and TWIST1 ($P = 0.0001$) in SiCRC patients positive for LNM than those negative for LNM. These results are shown in Figure 4.

MEASUREMENT OF THE SENSITIVITY AND SPECIFICITY OF SICRC

We determined the cut-off value of Ki-67, MMP7, FAP-1, FSP-1 and TWIST1 to assess whether this marker is correlated with LNM in SiCRCs. The selection of cut-off scores for individual examined markers was based on receiver operating characteristic (ROC) curve analysis (Figure S1). At each expression level, the sensitivity and specificity for the outcome (LNM) under study was plotted, thus generating a ROC curve. If a ROC curve was generated from the pairs of weighted mean sensitivities and mean specificities, then discrimination of the programme for the presence or absence of correlations with LNM was expressed by the area under the curve (Figure S1). We used a statistical package from JMP (SAS Institute, Cary, NC, USA). The data are depicted in Table S2.

ASSOCIATION OF CLINICOPATHOLOGICAL VARIABLES AND EXPRESSIONS OF INDIVIDUALLY EXAMINED MARKERS WITH LYMPH NODE METASTASIS IN SICRC USING UNIVARIATE AND MULTIVARIATE ANALYSES

We analysed the expression levels of individual markers and their associations with LNM in SiCRC. Univariate analysis revealed that several clinicopathological factors, including age, tumour location, macroscopic type and elevated expression levels of Ki-67 ($P < 0.0001$), p53 ($P = 0.0020$), MMP7 ($P = 0.0155$), FAP-1 ($P = 0.0217$), CD10 ($P = 0.0306$), FSP-1 ($P = 0.0110$), ZEB1 ($P = 0.0089$) and TWIST1 ($P = 0.0061$), were significantly associated with LNM (Table 4; $P < 0.05$). In addition, high expression levels of MMP7 (OR = 0.36, 95% CI = 0.16–0.82), FSP-1 (OR = 0.35, 95% CI = 0.15–0.79), AEBP-1 (OR = 0.33, 95% CI = 0.15–0.75), ZEB1 (OR = 0.33, 95% CI = 0.14–0.76) and TWIST1 (OR = 0.32, 95% CI = 0.13–0.73) were inversely correlated with LNM. Importantly, high expression levels of Ki-67 (cut-off value, 61.4%; $P = 0.0037$), FAP-1 (cut-off value, 43.4%; $P = 0.0468$) and ZEB1 (cut-off value, 52.1%;

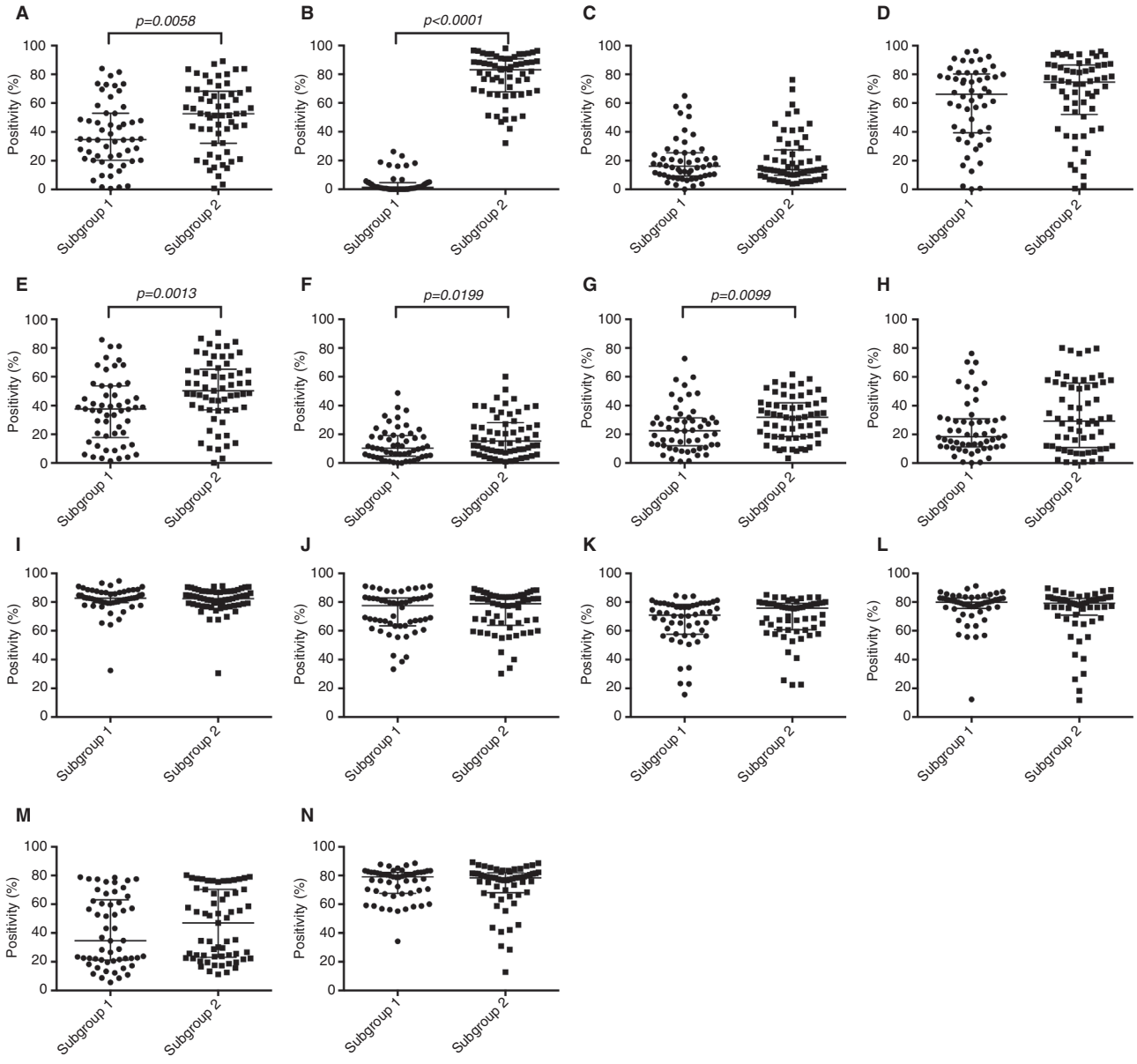

Figure 3. Expression levels in each subgroup of (**A**) Ki-67. (**B**) p53. (**C**) β-catenin. (**D**) Matrix metalloproteinase-7 (MMP7). (**E**) Fibroblast-associated protein 1 (FAP-1), 28.2% positive. (**F**) Platelet-derived growth factor receptor (PDGFR)-α. (**G**) PDGFR-β. (**H**) CD10. (**I**) α-SMA. (**J**) Podoplanin. (**K**) Fibroblast-specific protein 1 (FSP-1). (**L**) Adipocyte enhancer-binding protein (AEBP1). (**M**) Zinc finger E-box binding homeobox 1 (ZEB1). (**N**) TWIST-related protein 1 (TWIST1).

$P = 0.0133$) were retained in multivariate analysis, but a high expression level of ZEB1 was inversely correlated with LNM (Table 4). These relationships are depicted in Table 4.

INTEROBSERVER VARIATION OF TUMOUR BUDDING

We evaluated interobserver variations among four pathologists (T.S., N.U., M.O. and N.Y.) to identify the agreement with regard to tumour budding. Interobserver agreement was determined using kappa statistics. We found that the κ coefficient was excellent, as shown in Table S3 (0.6958–0.9287) in the present study.

Discussion

Tumour budding is an important factor to consider in the evaluation of the metastatic potential in CRC.[17]

Table 3. Univariate and multivariate logistic regression analysis in submucosal invasive colorectal cancer based stratified analysis

	Univariate		Multivariate	
	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
Sex		0.5927		
Age (years)	0.96 (0.93–0.99)	0.0357		0.0851
Size (mm)		0.3421		
Locus				
Right	1 (Reference)			0.1561
Left	3.41 (1.34–9.91)	0.0088		
Macroscopic type		0.0388		0.1813
0–I	1 (Reference)			
0–IIa	0.36 (0.12–1.07)	0.0753		
0–IIc	0.09 (0.01–0.75)	0.0039		
0–IIa + IIc	0.71 (0.26–1.96)	0.5069		
LST	0.84 (0.13–5.56)	0.8578		
Differentiation		0.1021		
Depth of submucosal invasion		0.4925		
Lymphatic invasion		0.0751		
Venous invasion		0.2783		
Tumour budding		0.7567		
Tumour border configuration		0.9346		
Peritumoral inflammation		0.2150		
Subgroup				
Subgroup 1	1 (Reference)		1 (Reference)	
Subgroup 2	3.49 (1.53–8.51)	0.0027	3.32 (1.32–8.33)	0.0107

OR, Odds ratio; CI, Confidence interval; LST, Laterally spreading tumour.

Previous studies have revealed that tumour budding is a strong predictive factor for LNM in SiCRC.[17,22] However, in the present study, the utility of tumour budding as a predictive marker of LNM in SiCRC could not be verified. This finding suggests that observations of tumour budding may differ among individual pathologists and might not constitute an objective factor that could predict LNM in SiCRC. Note, however, that in the present study, the presence of tumour budding was found with a high frequency of agreement among pathologists who worked at the same hospital. Nonetheless, tumour budding did not correlate with LNM in SiCRC. Moreover, in the present study, perineural invasion (not found here), tumour border configuration and peritumoral inflammation, which might be closely associated with metastatic potential, were not correlated with LNM. Therefore, another objective marker, such as a biological factor, will be required to identify correlation with LNM in SiCRC.[2] In the present study we examined the expression patterns in target cells, including cancer cells and the surrounding stromal cells (CAFs) in SiCRC. In addition, we suggested that auto-imaging analysis for the assessment of

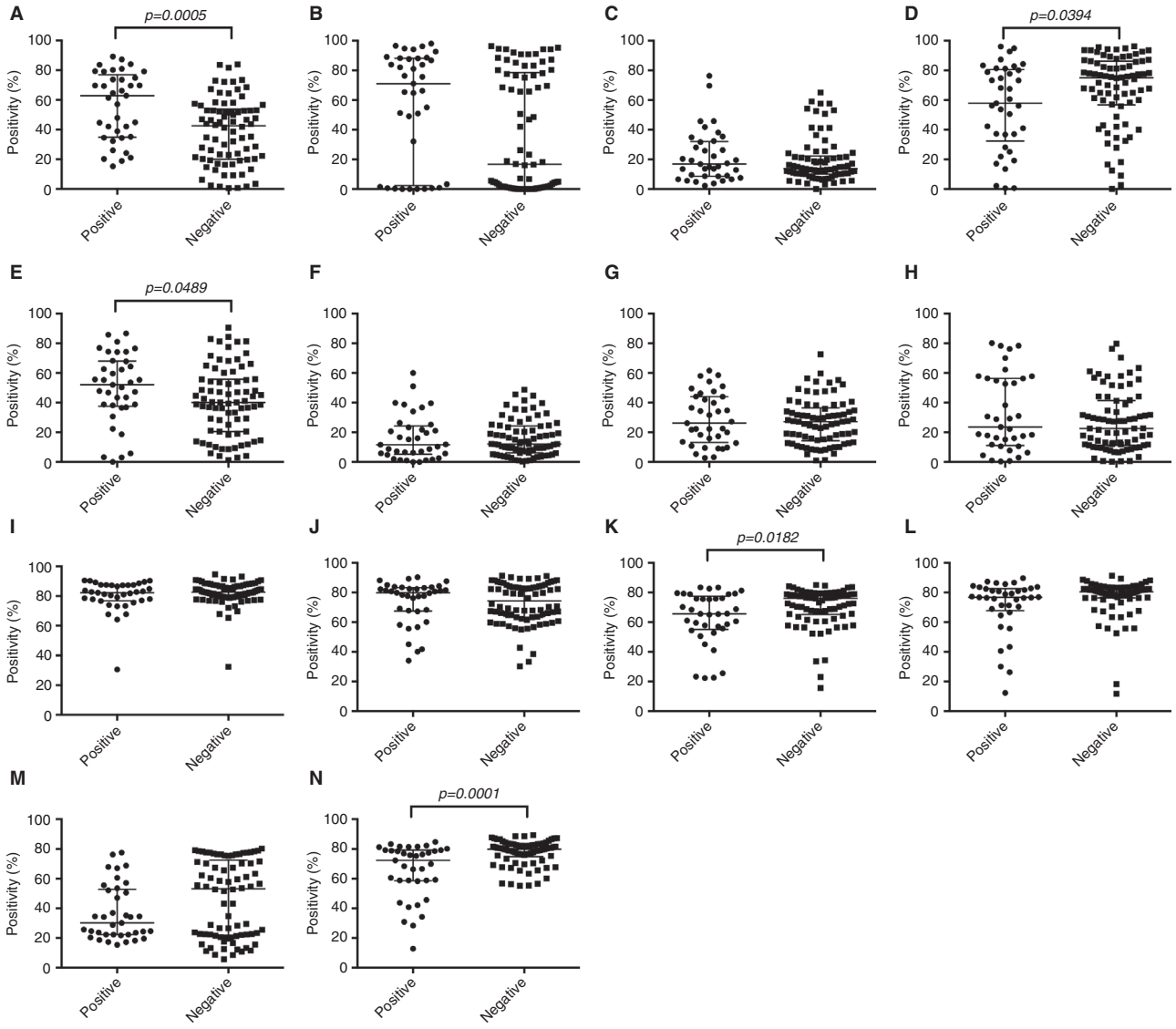

Figure 4. Expression levels in submucosal invasive colorectal cancer (SiCRC) with lymph node metastasis and SiCRC without lymph node metastasis as follows: (**A**) Ki-67. (**B**) p53. (**C**) β-catenin. (**D**) Matrix metalloproteinase-7 (MMP7). (**E**) Fibroblast-associated protein 1 (FAP-1). (**F**) Platelet-derived growth factor receptor(PDGFR)-α. (**G**) PDGFR-β. (**H**) CD10. (**I**) α-SMA. (**J**) Podoplanin. (**K**) Fibroblast-specific protein 1 (FSP-1). (**L**) Adipocyte enhancer-binding protein (AEBP1). (**M**) Zinc finger E-box binding homeobox 1 (ZEB1). (**N**) TWIST-related protein 1 (TWIST1).

immunohistochemical expression compiles evaluation criteria with better objectivity. However, we suggest that the present results do not exclude tumour budding, which is widely used as a predicting marker of LNM in SiCRC.[7,8]

A protein that contributes to tumour invasion and metastasis probably does not act alone.[2,12] Rather, multiple proteins may guide the behaviour of target cells.[2,12] If so, the expression pattern of various proteins present in the target and surrounding cells might provide more useful information necessary for the evaluation of neoplastic progression, including tumour invasion and metastasis. Previous studies have shown that the expression pattern of cancer-related proteins, including CAF- and EMT-related markers, could predict patient prognosis or LNM of CRC in SiCRC.[2,12] In the present study we classified markers into two expression patterns, subgroups 1 and 2. As a result, we found that the group of markers in subgroup 2 was correlated with LNM in a multivariate analysis. Thus, we suggest that specific expression patterns stratified by multiple markers can

Table 4. Univariate and multivariate logistic regression analysis in invasive submucosal colorectal cancer

	Univariate		Multivariate	
	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
Sex		0.5927		
Age (years)	0.96 (0.93–0.99)	0.0357		0.2918
Size (mm)		0.3421		
Locus				
Right	1 (Reference)			0.0895
Left	3.41 (1.34–9.91)	0.0088		
Macroscopic type		0.0388		0.8209
0–I	1 (Reference)			
0–IIa	0.36 (0.12–1.07)	0.0753		
0–IIc	0.09 (0.01–0.75)	0.0039		
0–IIa + IIc	0.71 (0.26–1.96)	0.5069		
LST	0.84 (0.13–5.56)	0.8578		
Differentiation		0.1021		
Depth of submucosal invasion		0.4925		
Lymphatic invasion		0.0751		
Venous invasion		0.2783		
Tumour budding		0.7567		
Tumour border configuration		0.9346		
Peritumoral inflammation		0.2150		
Expression of Ki–67				
Low (<cut-off)	1 (Reference)		1 (Reference)	
High (≥cut-off)	5.88 (2.48–14.54)	<0.0001	10.09 (2.12–47.96)	0.0037
Expression of p53				
Low (<cut off)	1 (Reference)			0.1359
High (≥ cut off)	3.58 (1.58–8.55)	0.0020		
Expression of β-catenin		0.0684		
Expression of MMP7				
Low (<cut-off)	1 (Reference)			0.0911
High (≥cut-off)	0.36 (0.16–0.82)	0.0155		
Expression of FAP-1				
Low (<cut-off)	1 (Reference)		1 (Reference)	
High (≥ cut-off)	2.56 (1.15–5.96)	0.0217	3.77 (1.02–13.96)	0.0468
Expression of PDGFR-α		0.1753		

Table 4. (*Continued*)

	Univariate		Multivariate	
	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
Expression of PDGFR-β		0.1127		
Expression of CD10				
Low (<cut-off)	1 (Reference)			0.1205
High (≥ cut-off)	2.71 (1.10–6.73)	0.0306		
Expression of SMA		0.1252		
Expression of podoplanin		0.0739		
Expression of FSP-1				
Low (<cut-off)	1 (Reference)			0.7832
High (≥cut-off)	0.35 (0.15–0.79)	0.0110		
Expression of AEBP-1				
Low (<cut-off)	1 (Reference)			0.0659
High (≥ cut-off)	0.33 (0.15–0.75)	0.0077		
Expression of ZEB1				
Low (<cut-off)	1 (Reference)		1 (Reference)	
High (≥cut-off)	0.33 (0.14–0.76)	0.0089	0.14 (0.03–0.64)	0.0113
Expression of TWIST				
Low (<cut-off)	1 (Reference)			0.1296
High (≥cut-off)	0.32 (0.13–0.73)	0.0061		

OR, Odds ratio; CI, Confidence interval; LST, Laterally spreading tumour; AEBPO-1, Adipocyte enhancer-binding protein 1; ZEB1, Zinc finger E-box binding homeobox 1; TWIST1, TWIST-related protein 1; FSP-1, Fibroblast-associated protein 1; SMA, Smooth muscle actin; PDGFR, platelet-derived growth factor receptor; FAP-1, Fibroblast-specific protein 1; MMP7, Matrix metalloproteinase-7.

provide novel and significant findings regarding the correlations of LNM in SiCRC.

Using cluster analysis of the expression pattern in target cells, we further examined the association of selected individual markers with LNM in SiCRC. Our data showed that only three markers, Ki-67, FAP-1 and ZEB1, were retained in multivariate analysis (note that high expression of ZEB1 was inversely correlated with LNM). These findings suggest that, whereas high proliferative activity of cancer cells contributes to LNM in SiCRC, high expression of FAP-1 by CAF plays crucial roles in LNM in SiCRC. Thus, cancer cell behaviour and CAF-related proteins cooperate in LNM in SiCRC.

The Ki-67 labelling index (LI) has been widely used as a marker of tumour proliferation, and several studies have compared the Ki-67 index with clinicopathological and follow-up data in CRC.[23] Expression of Ki-67 in a high percentage in cancer cells was correlated with LNM in the present study. Previous studies of CRC showed that the survival of patients with high expression of Ki-67, compared with those with low expression, could be predicted.[24,25] However, contrasting results were obtained in another study.[26] In previous work the hot-spot has been employed for measuring the Ki-67 LI, unlike the present method.[24,26] Here, our findings showed that the prognostic utility of Ki-67 may depend upon both the measurement site for determining the Ki-67 labelling index and the cut-off score. We suggest that Ki-67 LI measured at the deepest invasive front may be a useful marker to examine correlations with LNM.

Expression of FAP-1 is highly restricted to cancer-associated fibroblasts.[27–29] It is not expressed by resting fibroblasts in normal tissue, but can be induced in non-transformed, activated stromal fibroblasts.[28]

Here, we found that high expression of FAP-1 by CAF might be a useful marker to examine correlations with LNM in SiCRC. This is supported by the finding that FAP-1 is thought to promote tumour cell growth and proliferation; moreover, a previous study showed that high expression at the invasive front of CRC could predict patient outcome in CRC.[28,29] In addition, recent studies have shown that FAP-1 could possibly act directly in the degradation of the extracellular matrix (ECM) or indirectly as a regulatory protease involved in the activation/modification of other ECM-proteases/protease inhibitors.[28–30] Lee *et al.* reported that FAP-1 remodels the ECM through modulating protein levels and collagen fibre organisation.[31] A recent study revealed that such a process may be a therapeutic target in cancer research.[32] According to this theory, the present finding suggests that cancer-specific FAP-1 may be a novel marker for the prediction of LNM in SiCRC.

The enhancement of cancer cell activity is a possible driver of neoplastic progression. Cell plasticity also plays an important role in neoplastic invasion and metastasis and is dependent upon ZEB1, which is a key regulator of EMT.[33–35] ZEB1-induced EMT results from reduced expression of E-cadherin which causes the consequent loss of epithelial cell properties.[15] ZEB1 also enhances the motility of cancer cells through reduced intercellular adhesion.[33–35] Thus, this finding suggests that ZEB1 may contribute to tumour invasion and LNM. In the present study, however, high expression of ZEB1 was inversely correlated with LNM of SiCRC. Although the reason remains unknown, it is possible that multiple proteins expressed in cancer tissue at the invasive front interact with one another, and consequently inverse biological effects may occur at that site with some molecules (e.g. ZEB1).

There are some limitations to this study. First, the present study lacks a second cohort to validate the results. However, OR and *P*-values in multivariate analysis are convincing. Thus, our findings are probably reliable and reproducible. Secondly, the results may depend upon the selection of markers used in a study. However, the markers used here have been widely applied in previous studies and are available, reliable and reproducible. This immunohistochemical panel may be helpful to validate our results. We believe that the markers utilised here are appropriate to characterise microenvironmental behavior, including promotion of metastasis. Finally, this study was performed using surgical resection, where there is no need for tumour and stromal markers to 'predict' whether or not nodal metastases are present. The usefulness of such markers may be evident in cases in which tumours but not lymph nodes are available, such as polypectomy or endoscopic mucosal/submucosal resections, in which 'prediction' is useful for assessing risk and guiding disease management. Further studies are required to predict LNM in cases of endoscopic treatment of SiCRC with LNM.

In conclusion, we divided target cells within the microenvironment into cancer cells and CAF. In addition, we examined the expression patterns of various markers in cancer cells and CAF in SiCRC. As a result, expression patterns were classified into subgroups 1 and 2. We examined the association of individual markers that constituted subgroup 2, particularly those expressed at high levels, including Ki-67, p53, FAP-1, PDGFR-α and PDGFR-β. Multivariate analysis showed that high expression of Ki-67 and FAP-1 may be correlated with LNM in SiCRC. Further study for validation will be required.

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Conflicts of interest

We declare that we have no conflicts of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. ROC in individual makers used in this study. (A) Ki-67. (B) p53. (C) β-catenin. (D) MMP7. (E) FAP-1. (F) PDGFR α. (G) PDGFR β. (H) CD10. (I) α-SMA. (J) podoplanin. (K) FSP-1. (L) AEBP1. (M) ZEB1. (N) TWIST1.

Table S1. List of primary antibodies.

Table S2. Lymph node metastasis in patients with invasive submucosal colorectal cancer: sensitivity, specificity, positive predictive value, negative predictive value, positive likelihood ratio, and negative likelihood ratio according to cut-off values of each marker expression.

Table S3. Examination of consistency in inter-observer findings for tumor budding.