Table 1. List of antibodies used and working conditions

Antibody	Clone	Dilution	AR	City/location	Source
β-Catenin	14	1:5,000	MW	Lexington/Ky./USA	Transduction
COX-2	160112	1:200	MW	Ann Arbor/Mich./USA	Cayman
Dysadherin	M53	1:4,000	MW	Tokyo/Japan	original
E-cadherin	HECD-1	1:4,000	MW	Tokyo/Japan	original
Ki-67	MIB-1	1:500	MW	Glostrup/Denmark	DAKO
Laminin5γ2	1-97	1:4,000	MW	Tokyo/Japan	original
Matrilysin	141B-2	1:800	MW	Tokyo/Japan	Fine Chemical
MUC-1	Ma695	1:200	MW	Newcastle/UK	Novocastra
p53	DO-7	1:500	MW	Newcastle/UK	Novocastra

AR = Antigen retrieval; MW = microwave.

1; 1:2,000 dilution, established in our laboratory [43]), anti-β-catenin antibody (clone 14; 1:5,000 dilution, Transduction Laboratories, Lexington, Ky., USA), anti-COX-2 antibody (160112; 1:200 dilution, Cayman, Ann Arbor, Mich., USA), anti-laminin5γ2 antibody (1–97; 1:4,000 dilution, established in our laboratory [40]), anti-Ki-67 antibody (MIB-1; 1:500 dilution, Dako, Glostrup, Denmark), anti-matrilysin antibody (141B-2; 1:800 dilution, DFC, Toyama, Japan), anti-MUC-1 antibody (Ma695; 1:200 dilution, Novocastra, Newcastle-upon-Tyne, UK) and anti-p53 antibody (DO7; 1:500 dilution, Novocastra) at 4°C. The sections were washed with phosphate-buffered saline, incubated with biotin-labeled anti-mouse IgG antibody and avidin-biotin complex (ABC kit, Vector Laboratories, Peterborough, UK) and visualized using diaminobenzidine tetrahydrochloride. The sections were counterstained with hematoxylin. As internal positive controls for dysadherin and laminin5γ2 staining, positive staining of endothelial cells present in the primary tumor tissue was used. As an internal positive control for E-cadherin staining, membranous staining of normal epithelial cells adjacent to the tumor specimens was used. As internal positive controls for COX-2, MUC-1, β-catenin, matrilysin, p53 and Ki-67 staining, colon cancer samples known to stain positively for each antibody were used. As a negative control, normal mouse IgG (Vector Laboratories, Burlingame, Calif., USA) was used instead of the primary antibody.

Evaluation of Immunohistochemistry

All the slides were first reviewed by two observers (H.O. and Y.N.) independently without knowledge of the clinical data. All discrepancies were resolved by joint review of the slides in question. After selecting three markers – dysadherin, E-cadherin and matrilysin – from the training cohort, group I, immunohistochemical stainings were scored by a third independent pathologist (Y.F.) to allow validation of the evaluation of the immunohistochemical results.

The percentages of tumor cells positive for p53, Ki-67, β-catenin, COX-2, laminin5γ2, dysadherin, E-cadherin and MUC-1 were evaluated semiquantitatively as the ratio of the number of positive tumor cells relative to the total number of tumor cells. Cutoff indices were fixed according to previous reports as follows. Expression of E-cadherin was defined as preserved when membrane staining of >80% of the tumor cells was observed and reduced when membrane staining ≤80% of the tumor cells was observed [18]. Expression of dysadherin and β-catenin was defined as high when membrane staining >50% of the tumor cells was observed, and as low when membrane staining ≤50% of the cells was observed [18]. Expression of laminin5γ2 was categorized into three groups as: few, <10% of tumor cells positive; moderate, 10–50% of tumor cells positive, and high, >50% of tumor cells positive [17]. Expression of matrilysin was defined as high when >30% of tumor cells were stained at the invasive front, and as low when ≤30% of cells were stained at the invasive front [15, 38]. Expression of COX-2 was defined as positive when cytoplasmic staining of >10% of tumor cells was observed [16]. Expression of MUC-1 [19] and p53 and Ki-67 [34] was defined as positive when >10% of tumor cells were stained.

Statistical Analysis

All the data were tabulated, and statistical tests were performed with SAS version 9.1 (SAS Institute, Cary, N.C., USA). The relationship between clinicopathological findings and the scores of immunohistochemical markers were analyzed by Fisher's exact test for a two-by-two contingency table or by the χ^2 test for other contingency tables.

Selection of the best combination of markers was performed in group I by a stepwise selection procedure in a multivariate logistic regression model. The stepwise procedure was set to a threshold of 0.05 for inclusion and 0.15 for exclusion. Each selected independent liver metastasis factor was given a coefficient suggested by the multivariate logistic regression model, as a parameter estimate. In order to evaluate the goodness of fit for the final model, we applied the Hosmer-Lemeshow test [44] on eight distinct groups, and the Akaike Information Criterion (AIC) test [45] to the combination set of markers. AIC is widely used as a criterion for model selection. The model with the minimum AIC is chosen as the best one, and the AIC is therefore formally biased against overly complex models. The immunohistochemical metastatic score (IMS) was calculated according to the formula composed of selected factors. The scoring formula was applied to patients in groups II and III as well as those in group I. The thresh-

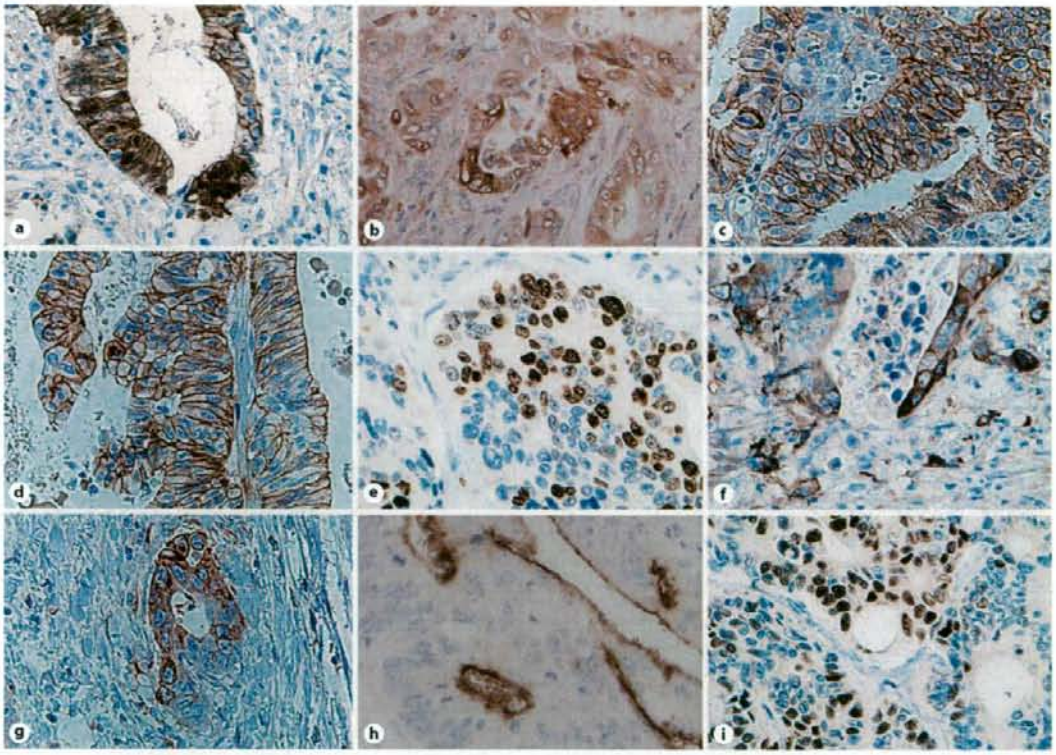

Fig. 1. Immunohistochemical staining pattern of each molecular marker (×400). β-Catenin expression was localized at the cell-cell borders, in the cytoplasm and in the nuclei of cancer cells (**a**). COX-2 expression was observed in the cytoplasm of cancer cells (**b**). Membranous dysadherin (**c**) and E-cadherin (**d**) expression was observed at the cell-cell borders of cancer cells. Ki-67 (**e**) and p53 expression (**i**) was observed in the nuclei of cancer cells. Laminin5γ2 (**f**) and matrilysin expression (**g**) was predominately intracytoplasmic, and preferentially located at the invasive front. MUC-1 (**h**) expression was located at the surface of glandular structures of cancer cells.

old was set at five points. Two theoretical potential groups at risk for liver metastasis were defined as follows: group A, low risk of liver metastasis, total score $0 \leq IMS \leq 4$; group B, high risk of liver metastasis, total score $5 \leq IMS$.

Results

Biomarkers in Primary Colon Cancers with Respect to the Occurrence of Liver Metastasis

The associations between clinicopathological factors and liver metastasis in all samples are shown in table 2. The representative staining pattern of each molecular marker is shown in figure 1. The associations between liver metastasis and immunohistochemical molecular markers in group I are shown in table 3. There was a significant association between liver metastasis and E-cadherin ($p = 0.001$), laminin5γ2 ($p = 0.005$), dysadherin ($p = 0.004$) and matrilysin expression ($p = 0.017$; table 3).

Identification of Candidate Markers in the Training Cohort, Group I, by Stepwise Analysis of the Logistic Regression Model

Although two markers – dysadherin and E-cadherin – were significantly associated with liver metastasis ($p = 0.013$ and 0.004, respectively) by the multivariate re-

Table 2. Association between liver metastasis and clinicopathological factors in all samples

Characteristics	Liver metastasis		p value
	positive (n = 49)	negative (n = 390)	
Age			
<65 years	27	226	
≥65 years	22	164	0.759
Gender			
Female	18	154	
Male	31	236	0.758
Tumor location			
Colon	35	238	
Rectum	14	152	0.210
Maximum tumor diameter			
<4.5 cm	30	191	
≥4.5 cm	19	199	0.129
Pathological tumor status			
T_2	1	31	
T_3	46	345	
T_4	2	14	0.351
Lymph node metastasis			
Absent	11	183	
Present	38	207	0.001
Histological grade			
G_1	18	170	
G_2	30	201	
G_3	1	18	0.474
Lymphatic invasion			
Absent	8	93	
Present	41	297	0.282
Venous invasion			
Absent	11	149	
Present	38	241	0.039

T_2 = Tumor invades the muscularis propia; T_3 = tumor invades through the muscularis propia into the subserosa or peritoneal tissues; T_4 = tumor directly invades other organs or structures and/or perforates the visceral peritoneum; G_1 = well-differentiated adenocarcinoma; G_2 = moderately differentiated adenocarcinoma; G_3 = poorly differentiated adenocarcinoma including signet-ring cell adenocarcinoma and mucinous adenocarcinoma.

Table 3. Association between liver metastasis and immunohistochemical molecular markers

Characteristics	Liver metastasis		p value
	positive (n = 21)	negative (n = 129)	
β-Catenin: membranous			
<70%	3	21	
≥70%	18	108	1.000
β-Catenin: cytoplasmic			
<50%	9	54	
≥50%	12	75	1.000
β-Catenin: nuclear			
<50%	12	88	
≥50%	9	41	0.328
COX-2			
<10%	12	76	
≥10%	9	53	1.000
Dysadherin			
<50%	5	75	
≥50%	16	54	0.004
E-cadherin			
Reduced	4	77	
Preserved	17	52	0.0006
Ki-67			
<30%	11	55	
≥30%	10	74	0.480
Laminin5γ2			
<10%	2	40	
≥10% and <50%	12	67	
≥50%	7	22	0.005
Matrilysin			
<30%	5	69	
≥30%	16	60	0.017
MUC-1			
<10%	12	72	
≥10%	9	57	1.000
p53			
<10%	9	48	
≥10%	12	81	0.801

β-Catenin: membranous/cytoplasmic/nuclear = Membranous/cytoplasmic/nuclear staining of β-catenin.

gression model, three markers – dysadherin, E-cadherin and matrilysin – were selected as candidate markers to establish a formula using a stepwise selection procedure in the multivariate logistic regression model (table 4). This combination set of markers showed an AIC value of 104.9. The receiver-operating characteristic curve of this combination set in the 150 individuals of group I is shown in figure 2. The area under the curve value was 0.807.

We carried out a stepwise method using the minimum value of the AIC as the selecting criterion. In cases where the model included dysadherin and E-cadherin, the AIC was 106.9. On the other hand, when the model included dysadherin, E-cadherin and matrilysin, the AIC was 104.9. As a result, although matrilysin was not significant in the multivariate regression analysis, it was included in the formula. Additionally, we obtained the Hosmer-

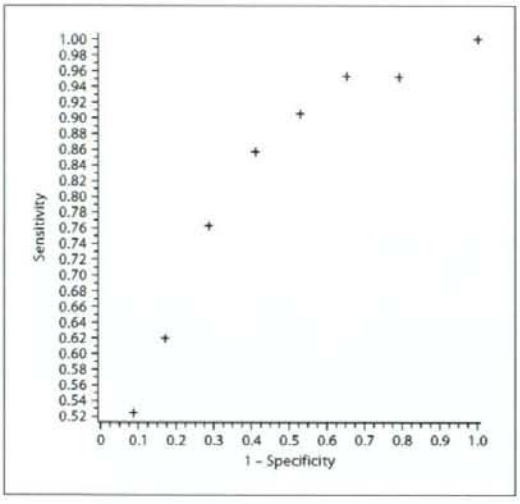

Fig. 2. Receiver-operating characteristic curve of the immunohistochemical metastatic scores for 150 independent patients (group I).

Table 4. Summary of the stepwise selection of the logistic regression model

Variable	Estimate	Standard error	Odds ratio (95% confidence interval)	p value
Intercept	–4.4458	0.787		<0.0001
Dysadherin	1.4216	0.569	4.144 (1.357, 12.66)	0.013
E-cadherin	1.7611	0.603	5.819 (1.782, 19.01)	0.004
Matrilysin	1.0931	0.573	2.984 (0.969, 9.186)	0.056

Table 5. Scoring formula for predicting liver metastasis in CRC patients: IMS

IMS = 3× dysadherin score + 4× E-cadherin score + 2× matrilysin score

Dysadherin score
- 0 for low expression (≤50% of tumor cells positive)
- 1 for high expression (>50% of tumor cells positive)

E-cadherin score
- 0 for preserved (>80% of tumor cells positive)
- 1 for reduced (≤80% of tumor cells positive)

Matrilysin score
- 0 for low expression (≤30% of tumor cells positive)
- 1 for high expression (>30% of tumor cells positive)

IMS = Immunohistochemical metastatic score.

Lemeshow χ^2 with 6 degrees of freedom equal to 2.647 and p = 0.852. It appeared, therefore, that our model fit was acceptable.

Predictive Formula for Liver Metastasis

A formula for predicting liver metastasis was established using the above three markers. The predictive formula: 3× dysadherin score [0 for low expression (≤50% of tumor cells positive) or 1 for high expression (>50% of tumor cells positive)] + 4× E-cadherin score [0 for preserved (>80% of tumor cells positive) or 1 for reduced (≤80% of tumor cells positive)] + 2× matrilysin score [0 for low expression (≤30% of tumor cells positive) or 1 for high expression (>30% of tumor cells positive)] was established (table 5). Total scores calculated using this formula predicted liver metastasis with a sensitivity of 85.7% (18 of 21) and a specificity of 58.9% (76 of 129) in the training cohort (group I).

Confirmation of the Evaluation of Immunohistochemistry by the Third Independent Pathologist

Slides immunostained for dysadherin, E-cadherin and matrilysin were also evaluated by the third independent pathologist, and the expression of these markers was significantly correlated with liver metastasis in the training cohort (group I), confirming the evaluation done by the other two pathologists. Each concordance rate for the dysadherin, E-cadherin and matrilysin expression scores between a third pathologist and the other two pathologists was 72, 70 and 78%, respectively. The concordance rate for the risk of liver metastasis calculated by our new formula between a third pathologist and the other two pathologists was 69%.

Confirmation of the Prediction Formula in the Validation Cohort (Group II)

The discriminating performance of the prediction formula was validated in a blinded manner using an independent validation cohort (group II), consisting of 190 patients. The same calculation showed a predictive accuracy with a sensitivity of 87.0% (20 of 23) and a specificity of 66.5% (111 of 167).

Confirmation of the Prediction Formula in the Second Validation Cohort (Group III) from the Kitasato University

The discriminating performance of the prediction formula was validated in a blinded manner using the second independent validation cohort, group III, consisting

of 99 patients from the Kitasato University Hospital. The same calculation showed a predictive accuracy with a sensitivity of 80% (4 of 5) and a specificity of 60.0% (56 of 94).

Discussion

We used a supervised learning method which requires the use of a training data set of known markers to identify the best combination of immunohistochemical markers for predicting liver metastasis in patients with CRC after curative surgery, and dysadherin, E-cadherin and matrilysin expression was found to be the best combination for this purpose. Patients were divided into two categories – a high-risk group for liver metastasis and a low-risk group for liver metastasis – based on the scores obtained using the formula. The choice of a threshold should primarily depend on the purpose of the overall clinical scheme; some investigators may require a higher sensitivity for clinical applications while sacrificing specificity, whereas others may choose the opposite. In this study, we determined 5 as the threshold, for which the sensitivity was >80%, and can be regarded as sufficient for use as a screening test. Liver metastasis was predicted with an accuracy of 85.7% in terms of sensitivity and 58.9% in terms of specificity using our formula. Pathological risk factors for liver metastasis have been reported to be venous, lymphatic and serosal invasion, tumor dedifferentiation, lymph node metastasis and white streak sign, observed macroscopically at the invasive front of the cut surface of a tumor [1, 8–14]. We used stepwise multivariate analysis to look for the best combination set of markers for predicting liver metastasis, including conventional clinicopathological factors. However, no conventional clinicopathological factors were selected as candidate markers useful for constructing a predictive formula for liver metastasis, indicating that our formula is able to predict liver metastasis more precisely than conventional clinicopathological factors. Additionally, we applied survival analysis to liver metastasis event data. The results obtained were similar to those of logistic regression analysis, and the selected markers were the same as those selected by the Cox regression models (data not shown). We also performed multivariate analysis using the logistic regression model between liver metastasis and immunohistochemical molecular markers for patients with 219 colon cancers and patients with 140 rectal cancers separately. In both cancer groups, all three selected markers – dysadherin, E-cadherin and matrylsin – showed a similar tendency in the stepwise logistic regression model (data not shown). Our formula was validated using independent sets of patients, including 190 from our institution and 99 from another institution. Furthermore, our new predictive formula was validated not only in cases from an outside hospital but also by a third independent pathologist who was instructed to evaluate immunostained slides without prior knowledge of the cases. This predictive formula might be helpful for selecting patients who should undergo adjuvant chemotherapy after curative surgery, or who require close follow-up to detect liver metastasis at a sufficiently early stage for curative resection, and ultimately for avoiding unnecessary adjuvant chemotherapy in patients who are unlikely to develop liver metastasis. In order for our formula to be applied for practical clinical care, however, it must be validated in a large-scale prospective clinical trial.

We examined the differences in immunohistochemical positivity for the three molecular markers between older samples (resected between 1995 and 1996) and relatively new samples (resected between 1997 and 2001) in order to evaluate the suitability of older samples for immunohistochemical study. There were no differences in immunohistochemical positivity for the three molecular markers between the two sample groups (data not shown). Therefore, we consider that even older samples, such as specimens resected over 10 years ago, are reliably applicable for immunohistochemical study for prediction of liver metastasis.

Our study showed that E-cadherin, dysadherin and matrilysin expression was significantly correlated with liver metastasis, confirming the results of previous studies [15, 18–38]. Although multivariate logistic analysis failed to reveal a significant association between laminin5γ2 expression and liver metastasis, the χ^2 test showed that laminin5γ2 was significantly associated with liver metastasis, confirming the results of previous studies [17, 39]. The expression of p53, Ki-67, COX-2, β-catenin or MUC-1 failed to demonstrate any significant association with liver metastasis, even though these markers were selected on the basis of the fact that their prognostic significance had been reported in several previous papers [3, 16, 19, 26, 28, 34]. These discrepancies could be explained on the basis of differences in treatment modalities, scoring system, sample size analyzed, tumor heterogeneity and interobserver variations in evaluating immunostained slides.

A number of previous studies have investigated the usefulness of combining several molecular markers for predicting liver metastasis in CRC patients [46–48]. Na-

gai et al. [11] analyzed 100 patients, comprising 48 with liver metastasis and 52 without evidence of liver metastasis, and established a predictive formula for liver metastasis using a combination of factors such as tumor location, host inflammatory cell reaction, p53 staining, and extent of tumor and venous invasion using multivariate analysis. The predictive value for liver metastasis was 81.3% in terms of sensitivity and 92.3% in terms of specificity [11]. Barozzi et al. [49] investigated five clinicopathological factors and seven molecular markers – TGF-α, IGF-II, MMP-2, VEGF, CD34, c-erb B2 and EGFR – in 101 patients, comprising 49 patients without evidence of metastasis, 27 with synchronous liver metastasis and 25 with metachronous liver metastasis. Using multivariate analysis, they found that TGF-α, IGF-II and MMP-2 were independent predictors of liver metastasis. They reported that if the expression levels of all three of these molecular markers were high, then the probability of liver metastasis was 99.5%, whereas if the expression levels of all three were low, then the probability of liver metastasis was only 0.3% [49]. Although the sensitivity and specificity in these previous reports were high, their sample sizes were rather small in comparison with our present study. Also, before drawing any conclusions about their usefulness, these previous reports need to be validated in patients from an outside hospital and by another independent pathologist to confirm the accuracy of the immunostaining evaluation.

Several recent studies have demonstrated the potential clinical utility of gene expression profiles, including the identification of prognostic subclasses. Eschrich et al. [50] reported that in 78 patients with Dukes B and C stage disease, a 43-gene signature was demonstrated to identify 3-year survival significantly better with a sensitivity of 73% and a specificity of 84%. Wang et al. [51] identified a 23-gene signature that predicted prognosis in 74 patients with Dukes B stage disease with a sensitivity of 72% and a specificity of 83%. Bertucci et al. [52] found a 244-gene signature that separated 22 patients from among a group with all stages of CRC with a significant difference in 5-year survival of 100 vs. 30% ($p = 0.001$). These previous reports suggest that microarray gene expression profiling could be a valuable tool for highly accurate prognostication in CRC patients. At present, however, the cost of cDNA analysis, the complexity of the method and accuracy in the interpretation of DNA microarrays are problems that remain to be solved before this approach can be applied routinely in a standard clinical setting. On the other hand, immunohistochemistry is an already standardized method that can easily be performed in every laboratory. Although application of the specific scoring calculation is less feasible for timely routine diagnostics, our formula based on immunohistochemical results has the advantage of feasibility compared with methods using DNA extracted from tumor tissue.

In conclusion, we have established a formula for predicting liver metastasis in CRC patients and confirmed its high sensitivity potentially for clinical application.

Acknowledgments

The authors are grateful to Dr. Y. Ino, Ms. A. Miura and Ms. F. Kaiya for their expert technical assistance.

This study was supported by a Grant-in-Aid for the 3rd-Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health, Labor and Welfare of Japan. H. Ochiai is a recipient of a Research Resident Fellowship from the Foundation for Promotion of Cancer Research in Japan.

References

1 Weitz J, Koch M, Debus J, Hohler T, Galle PR, Buchler MW: Colorectal cancer. Lancet 2005;365:153–165.

2 Cascinu S, Georgoulias V, Kerr D, Maughan T, Labianca R, Ychou M: Colorectal cancer in the adjuvant setting: perspectives on treatment and the role of prognostic factors. Ann Oncol 2003;14(suppl 2):ii25–ii29.

3 Watanabe T, Wu TT, Catalano PJ, Ueki T, Satriano R, Haller DG, Benson AB 3rd, Hamilton SR: Molecular predictors of survival after adjuvant chemotherapy for colon cancer. N Engl J Med 2001;344:1196–1206.

4 O'Connell MJ, Laurie JA, Kahn M, Fitzgibbons RJ Jr, Erlichman C, Shepherd L, Moertel CG, Kocha WI, Pazdur R, Wieand HS, Rubin J, Vukov AM, Donohue JH, Krook JE, Figueredo A: Prospectively randomized trial of postoperative adjuvant chemotherapy in patients with high-risk colon cancer. J Clin Oncol 1998;16:295–300.

5 Doci R, Gennari L, Bignami P, Montalto F, Morabito A, Bozzetti F: One hundred patients with hepatic metastases from colorectal cancer treated by resection: analysis of prognostic determinants. Br J Surg 1991;78:797–801.

6 Fortner JG: Recurrence of colorectal cancer after hepatic resection. Am J Surg 1988;155: 378–382.
7 Chang GJ, Rodriguez-Bigas MA, Skibber JM, Moyer VA: Lymph node evaluation and survival after curative resection of colon cancer: systematic review. J Natl Cancer Inst 2007; 99:433–441.
8 Galandiuk S, Wieand HS, Moertel CG, Cha SS, Fitzgibbons RJ Jr, Pemberton JH, Wolff BG: Patterns of recurrence after curative resection of carcinoma of the colon and rectum. Surg Gynecol Obstet 1992;174:27–32.
9 Krasna MJ, Flancbaum L, Cody RP, Shneibaum S, Ben Ari G: Vascular and neural invasion in colorectal carcinoma. Incidence and prognostic significance. Cancer 1988;61:1018–1023.
10 Adachi Y, Inomata M, Kakisako K, Sato K, Shiraishi N, Kitano S: Histopathologic characteristics of colorectal cancer with liver metastasis. Dis Colon Rectum 1999;42:1053–1056.
11 Nagai E, Yao T, Sakamoto M, Akazawa K, Utsunomiya T, Tsuneyoshi M: Risk factors related to liver metastasis in colorectal carcinoma: a multivariate analysis of clinicopathologic and immunohistochemical variables. Jpn J Cancer Res 1994;85:1280–1287.
12 Ouchi K, Sugawara T, Ono H, Fujiya T, Kamiyama Y, Kakugawa Y, Mikuni J, Tateno H: Histologic features and clinical significance of venous invasion in colorectal carcinoma with hepatic metastasis. Cancer 1996;78: 2313–2317.
13 Ono M, Sakamoto M, Ino Y, Moriya Y, Sugihara K, Muto T, Hirohashi S: Cancer cell morphology at the invasive front and expression of cell adhesion-related carbohydrate in the primary lesion of patients with colorectal carcinoma with liver metastasis. Cancer 1996;78:1179–1186.
14 Inomata M, Ochiai A, Sugihara K, Moriya Y, Yamaguchi N, Adachi Y, Kitano S, Hirohashi S: Macroscopic features at the deepest site of tumor penetration predicting liver metastases of colorectal cancer. Jpn J Clin Oncol 1998;28:123–128.
15 Adachi Y, Yamamoto H, Itoh F, Arimura Y, Nishi M, Endo T, Imai K: Clinicopathologic and prognostic significance of matrilysin expression at the invasive front in human colorectal cancers. Int J Cancer 2001;95:290–294.
16 Yamauchi T, Watanabe M, Kubota T, Hasegawa H, Ishii Y, Endo T, Kabeshima Y, Yorozuya K, Yamamoto K, Mukai M, Kitajima M: Cyclooxygenase-2 expression as a new marker for patients with colorectal cancer. Dis Colon Rectum 2002;45:98–103.
17 Aoki S, Nakanishi Y, Akimoto S, Moriya Y, Yoshimura K, Kitajima M, Sakamoto M, Hirohashi S: Prognostic significance of laminin-5 gamma2 chain expression in colorectal carcinoma: immunohistochemical analysis of 103 cases. Dis Colon Rectum 2002;45: 1520–1527.
18 Aoki S, Shimamura T, Shibata T, Nakanishi Y, Moriya Y, Sato Y, Kitajima M, Sakamoto M, Hirohashi S: Prognostic significance of dysadherin expression in advanced colorectal carcinoma. Br J Cancer 2003;88:726–732.
19 Hiraga Y, Tanaka S, Haruma K, Yoshihara M, Sumii K, Kajiyama G, Shimamoto F, Kohno N: Immunoreactive MUC1 expression at the deepest invasive portion correlates with prognosis of colorectal cancer. Oncology 1998;55:307–319.
20 Sobin LH, Fleming ID: TNM Classification of Malignant Tumors, fifth edition (1997). Union Internationale Contre le Cancer and the American Joint Committee on Cancer. Cancer 1997;80:1803–1804.
21 World Health Organization: Classification of Tumors. IARC Press, Lyon, 2000.
22 Hugh TJ, Dillon SA, Taylor BA, Pignatelli M, Poston GJ, Kinsella AR: Cadherin-catenin expression in primary colorectal cancer: a survival analysis. Br J Cancer 1999;80:1046–1051.
23 Nakanishi Y, Ochiai A, Akimoto S, Kato H, Watanabe H, Tachimori Y, Yamamoto S, Hirohashi S: Expression of E-cadherin, alpha-catenin, beta-catenin and plakoglobin in esophageal carcinomas and its prognostic significance: immunohistochemical analysis of 96 lesions. Oncology 1997;54:158–165.
24 Gunther K, Brabletz T, Kraus C, Dworak O, Reymond MA, Jung A, Hohenberger W, Kirchner T, Kockerling F, Ballhausen WG: Predictive value of nuclear beta-catenin expression for the occurrence of distant metastases in rectal cancer. Dis Colon Rectum 1998;41:1256–1261.
25 Maruyama K, Ochiai A, Akimoto S, Nakamura S, Baba S, Moriya Y, Hirohashi S: Cytoplasmic beta-catenin accumulation as a predictor of hematogenous metastasis in human colorectal cancer. Oncology 2000;59: 302–309.
26 Wong SC, Lo ES, Lee KC, Chan JK, Hsiao WL: Prognostic and diagnostic significance of beta-catenin nuclear immunostaining in colorectal cancer. Clin Cancer Res 2004;10: 1401–1408.
27 Fux R, Schwab M, Thon KP, Gleiter CH, Fritz P: Cyclooxygenase-2 expression in human colorectal cancer is unrelated to overall patient survival. Clin Cancer Res 2005;11: 4754–4760.
28 Soumaoro LT, Uetake H, Higuchi T, Takagi Y, Enomoto M, Sugihara K: Cyclooxygenase-2 expression: a significant prognostic indicator for patients with colorectal cancer. Clin Cancer Res 2004;10:8465–8471.
29 Ino Y, Gotoh M, Sakamoto M, Tsukagoshi K, Hirohashi S: Dysadherin, a cancer-associated cell membrane glycoprotein, down-regulates E-cadherin and promotes metastasis. Proc Natl Acad Sci USA 2002;99:365–370.
30 Nakanishi Y, Akimoto S, Sato Y, Kanai Y, Sakamoto M, Hirohashi S: Prognostic significance of dysadherin expression in tongue cancer: immunohistochemical analysis of 91 cases. Appl Immunohistochem Mol Morphol 2004;12:323–328.
31 Shimamura T, Sakamoto M, Ino Y, Sato Y, Shimada K, Kosuge T, Sekihara H, Hirohashi S: Dysadherin overexpression in pancreatic ductal adenocarcinoma reflects tumor aggressiveness: relationship to E-cadherin expression. J Clin Oncol 2003;21:659–667.
32 Hirohashi S: Inactivation of the E-cadherin-mediated cell adhesion system in human cancers. Am J Pathol 1998;153:333–339.
33 Aoki R, Tanaka S, Haruma K, Yoshihara M, Sumii K, Kajiyama G, Shimamoto F, Kohno N: MUC-1 expression as a predictor of the curative endoscopic treatment of submucosally invasive colorectal carcinoma. Dis Colon Rectum 1998;41:1262–1272.
34 Allegra CJ, Paik S, Colangelo LH, Parr AL, Kirsch I, Kim G, Klein P, Johnston PG, Wolmark N, Wieand HS: Prognostic value of thymidylate synthase, Ki-67, and p53 in patients with Dukes' B and C colon cancer: a National Cancer Institute-National Surgical Adjuvant Breast and Bowel Project collaborative study. J Clin Oncol 2003;21:241–250.
35 Crowe PJ, Yang JL, Berney CR, Erskine C, Ham JM, Fisher R, Russell PJ, Genetic markers of survival and liver recurrence after resection of liver metastases from colorectal cancer. World J Surg 2001;25:996–1001.
36 Soong R, Grieu F, Robbins P, Dix B, Chen D, Parsons R, House A, Iacopetta B: p53 alterations are associated with improved prognosis in distal colonic carcinomas. Clin Cancer Res 1997;3:1405–1411.
37 Adachi Y, Yamamoto H, Itoh F, Hinoda Y, Okada Y, Imai K: Contribution of matrilysin (MMP-7) to the metastatic pathway of human colorectal cancers. Gut 1999;45:252–258.
38 Masaki T, Matsuoka H, Sugiyama M, Abe N, Goto A, Sakamoto A, Atomi Y: Matrilysin (MMP-7) as a significant determinant of malignant potential of early invasive colorectal carcinomas. Br J Cancer 2001;84:1317–1321.
39 Lenander C, Habermann JK, Ost A, Nilsson B, Schimmelpenning H, Tryggvason K, Auer G: Laminin-5 gamma 2 chain expression correlates with unfavorable prognosis in colon carcinomas. Anal Cell Pathol 2001;22: 201–209.
40 Ono Y, Nakanishi Y, Ino Y, Niki T, Yamada T, Yoshimura K, Saikawa M, Nakajima T, Hirohashi S: Clinicopathologic significance of laminin-5γ2 chain expression in squamous cell carcinoma of the tongue: immunohistochemical analysis of 67 lesions. Cancer 1999; 85:2315–2321.
41 Japanese Society for Cancer of the Colon and Rectum: General Rules for Clinical and Pathological Studies on Cancer of the Colon, Rectum and Anus, ed 7. Tokyo, Kanehara, 2006.

42 Nakanishi Y, Noguchi M, Matsuno Y, Saikawa M, Mukai K, Shimosato Y, Hirohashi S: p53 expression in multicentric squamous cell carcinoma and surrounding squamous epithelium of the upper aerodigestive tract. immunohistochemical analysis of 95 lesions. Cancer 1995;75:1657–1662.
43 Shimoyama Y, Hirohashi S, Hirano S, Noguchi M, Shimosato Y, Takeichi M, Abe O: Cadherin cell-adhesion molecules in human epithelial tissues and carcinomas. Cancer Res 1989;49:2128–2133.
44 Hosmer DW, Hosmer T, Le Cessie S, Lemeshow S: A comparison of goodness-of-fit tests for the logistic regression model. Stat Med 1997;16:965–980.
45 Akaike H: A new look at the statistical model identification. IEEE Trans Autom Control 1974;19:716–723.
46 Mitomi H, Mori A, Kanazawa H, Nishiyama Y, Ihara A, Otani Y, Sada M, Kobayashi K, Igarashi M: Venous invasion and down-regulation of p21(WAF1/CIP1) are associated with metastasis in colorectal carcinomas. Hepatogastroenterology 2005; 52: 1421–1426.
47 Gunther K, Dworak O, Remke S, Pfluger R, Merkel S, Hohenberger W, Reymond MA: Prediction of distant metastases after curative surgery for rectal cancer. J Surg Res 2002;103:68–78.
48 Maeda K, Kang SM, Ogawa M, Onoda N, Sawada T, Nakata B, Kato Y, Chung YS, Sowa M: Combined analysis of vascular endothelial growth factor and platelet-derived endothelial cell growth factor expression in gastric carcinoma. Int J Cancer 1997;74:545–550.
49 Barozzi C, Ravaioli M, D'Errico A, Grazi GL, Poggioli G, Cavrini G, Mazziotti A, Grigioni WF: Relevance of biologic markers in colorectal carcinoma: a comparative study of a broad panel. Cancer 2002;94:647–657.
50 Eschrich S, Yang I, Bloom G, Kwong KY, Boulware D, Cantor A, Coppola D, Kruhoffer M, Aaltonen L, Orntoft TF, Quackenbush J, Yeatman TJ: Molecular staging for survival prediction of colorectal cancer patients. J Clin Oncol 2005;23:3526–3535.
51 Wang Y, Jatkoe T, Zhang Y, Mutch MG, Talantov D, Jiang J, McLeod HL, Atkins D: Gene expression profiles and molecular markers to predict recurrence of Dukes' B colon cancer. J Clin Oncol 2004;22:1564–1571.
52 Bertucci F, Salas S, Eysteries S, Nasser V, Finetti P, Ginestier C, Charafe-Jauffret E, Loriod B, Bachelart L, Montfort J, Victorero G, Viret F, Ollendorff V, Fert V, Giovaninni M, Delpero JR, Nguyen C, Viens P, Monges G, Birnbaum D, Houlgatte R: Gene expression profiling of colon cancer by DNA microarrays and correlation with histoclinical parameters. Oncogene 2004;23:1377–1391.