Biological predictors of survival in stage II colorectal cancer

YOSHITAKE UEDA1, KAZUHIRO YASUDA1, MASAFUMI INOMATA1, NORIO SHIRAISHI1, SHIGEO YOKOYAMA1 and SEIGO KITANO3

Departments of 1Gastroenterological Surgery and 2Pathology, Faculty of Medicine, Oita University; 3Oita University, Yufu, Oita 879-5593, Japan

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Abstract. The routine use of postoperative adjuvant chemotherapy in patients with stage II colorectal cancer is not recommended. However, the incidence of tumor recurrence or distant metastasis in these patients is reported to be 25-35%. The identification of high-risk patients with stage II colorectal cancer remains difficult. Therefore, the aim of this study was to determine the risk factors that may help identify stage II colorectal cancer patients with unfavorable prognosis. Paraffin-embedded tissue samples from 109 patients with stage II colorectal cancer following curative operation were analyzed. Thirteen clinicopathological variables and 5 biological markers were assessed using immunohistochemistry, including p53 (tumor suppressor gene), CD10 (tumor invasion marker), CD34 (angiogenic marker), Ki-67 (cell proliferation index) and CAM 5.2 (marker of lymph node micrometastasis) and investigated for associations with disease-specific survival. Univariate analysis revealed bowel obstruction, lymph node micrometastasis and lymphatic invasion (P<0.01) to be highly significant factors for determining the 5-year disease-specific survival. By contrast, the multivariate analysis revealed lymph node micrometastasis and lymphatic invasion to be independent prognostic factors. Stage II colorectal cancer patients with lymph node micrometastasis and lymphatic invasion may therefore be suitable candidates for adjuvant chemotherapy to improve prognosis.

Introduction

Lymph node metastasis is the most powerful predictor of recurrence or survival in patients with colorectal cancer. Although the majority of patients with node-negative colorectal cancer are potentially cured with surgery alone, ≤25% are likely to present with recurrence and succumb to the disease (1).

Identifying high-risk patients with stage II colorectal cancer is important for determining which patients may benefit the most from adjuvant chemotherapy. The effect of clinicopathological factors on recurrence and survival following curative resection has been the subject of several studies and numerous clinicopathological factors have been suggested as prognostic indicators for colorectal cancer. It is important to determine which of these factors affect the risk of recurrence in the node-negative colorectal cancer patients and which factors should be prospectively applied in the routine clinicopathological evaluation of colorectal cancer.

With the recent developments in immunohistochemistry and molecular biology, several biological markers have been extensively investigated (2-12). In this study, the expression of several biological markers, including p53, CD10, CD34 and Ki-67, which are strongly suspected of playing a significant role in tumor progression, was evaluated. Additionally, we focused on lymph node micrometastasis, which is easily detected by antibody CAM 5.2.

The aim of this study was to conduct a multivariate analysis of the prognostic impact of a wide range of clinicopathological and biological variables in patients with stage II colorectal cancer.

Materials and methods

Patients. We reviewed all the patients who underwent curative resection for stage II colorectal cancer at the Department of Gastroenterological Surgery, Oita University Hospital, between 1984 and 2002. Patients who had received preoperative chemoradiation for locally advanced lower rectal cancer were excluded from this cohort study. Ultimately, 109 patients (61 males and 48 females; average age, 67 years; range, 31-89 years) were enrolled and the tumors were diagnosed as clinical stage T3, N0 and M0.

Evaluation. The survival analysis was performed for the following clinicopathological factors: age, gender, location of tumor (right vs. left colon vs. rectum), number of resected lymph nodes (0-11 vs. ≥12), bowel obstruction (absent vs. present), tumor size (0-4 vs. >4 cm), depth of tumor invasion (subserosa vs. serosa), tumor differentiation (high vs. moderate vs. mucinous), lymphatic invasion (absent or mild vs. moderate or severe), venous invasion (absent vs.

Correspondence to: Dr Yoshitake Ueda, Department of Gastroenterological Surgery, Faculty of Medicine, Oita University 1-1 Idaigaoka, Hasama-machi, Yufu, Oita 879-5593, Japan
E-mail: yoshimd@med.oita-u.ac.jp

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The study included a total of 109 patients were fixed in 10% formalin solution and embedded in paraffin. Representative tissue sections, each containing the deepest site of cancer invasion, were cut at 4-µm. As regards the lymph node specimens, one 3-µm section was obtained for hematoxylin and eosin staining and five serial 6-µm sections for immunohistochemical staining. The avidin-biotin peroxidase complex method was used for detection of the five monoclonal antibodies in deparaffinized and rehydrated tissue sections. Antigen retrieval was performed by placing the sample in a microwave oven at 95°C for 40 min, followed by cooling for 30 min to room temperature, except for CD34 and CAM 5.2. CAM 5.2 sections were trypsinized with 0.1% calcium chloride solution. p53, Ki-67, CD10 and CD34 were incubated for 2 h at room temperature and CAM 5.2 was incubated overnight at 4°C. The slides were then incubated for 30 min with EnVision™ peroxidase mouse system (DAKO Corporation, Carpinteria, CA, USA). The color reaction was visualized by incubation for 30 min with DAB (Sigma Chemical Co., St. Louis, MO, USA) as the chromogen for 5 min. Using a light microscope, a visual grading system was used based on the number of positively stained nuclei of the cancer cells in each tissue sample.

p53 slides were scored according to the percentage of positive tumor nuclei as follows: positive, ≥10% of the nuclei stained; negative, <10% of the nuclei stained (3). For Ki-67 immunoreactivity, staining was considered positive at >60% (9). Tumor positivity for CD10 was evaluated using a predefined cut-off of 5% (positive, >5% tumor cell staining) according to a previous study (10). CD34 slides were classified according to the microvessel count. After scanning the highly vascularized areas, we selected three areas exhibiting the most prominent neovascularization. A microvessel count was performed on a x400 field (x40 objective and x10 ocular) and the average count from the three areas was calculated (15). Patients were divided into those with a microvessel count of 0-50 and those with a microvessel count of >50. As regards metastatic lymph nodes, patients were divided into two groups according to a previous study (16), those with micrometastasis in ≥3 lymph nodes and those with micrometastasis in ≥4 lymph nodes. Written informed consent was obtained from all the patients and this study was approved by the local Ethics Committee.

**Immunohistochemistry.** Resected tumors from each of the 109 patients were fixed in 10% formalin solution and embedded in paraffin. Representative tissue sections, each containing the deepest site of cancer invasion, were cut at 4-µm. As regards the lymph node specimens, one 3-µm section was obtained for hematoxylin and eosin staining and five serial 6-µm sections for immunohistochemical staining. The avidin-biotin peroxidase complex method was used for detection of the five monoclonal antibodies in deparaffinized and rehydrated tissue sections. Antigen retrieval was performed by placing the sample in a microwave oven at 95°C for 40 min, followed by cooling for 30 min to room temperature, except for CD34 and CAM 5.2. CAM 5.2 sections were trypsinized with 0.1% calcium chloride solution. p53, Ki-67, CD10 and CD34 were incubated for 2 h at room temperature and CAM 5.2 was incubated overnight at 4°C. The slides were then incubated for 30 min with EnVision™ peroxidase mouse system (DAKO Corporation, Carpinteria, CA, USA). The color reaction was visualized by incubation for 30 min with DAB (Sigma Chemical Co., St. Louis, MO, USA) as the chromogen for 5 min. Using a light microscope, a visual grading system was used based on the number of positively stained nuclei of the cancer cells in each tissue sample.

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**Statistical analysis.** Data were statistically analyzed using SPSS statistical software (Statistical Package for Social Sciences). Univariate disease-specific survival analysis was performed using the Kaplan-Meier method and the difference was evaluated by the log-rank test. Multivariate analysis was performed using the Cox proportional hazards model. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Factors affecting patient survival.** The study included a total of 61 males and 48 females, with an average age of 67 years (range, 31-89 years). The median follow-up period for the survivors was 5.7 years (range, 1.7-11 years). At the time of analysis, 87 patients were free of disease, 7 were alive with disease, 15 had succumbed to the disease and 5 patients had succumbed due to other causes. Twenty-two patients developed recurrence or distant metastasis. Of these, 12 had liver metastases, 7 had local recurrence and 3 had lung metastasis. The 5-year disease-specific survival rate of patients with stage II colorectal cancer was 86.2%.

In the univariate analysis, bowel obstruction, lymph node micrometastasis and lymphatic invasion (P<0.01) were significant factors for determining the 5-year disease-specific survival (Table I). When all of these factors were included as independent variables in a Cox proportional hazards model, the presence of lymphatic invasion was the most powerful negative predictor of survival [hazard ratio (HR), 4.091; P=0.006], followed by lymph node micrometastasis (HR, 3.704; P=0.011) (Table II). The 5-year disease-specific survival rate was significantly lower for the group of patients with moderate to severe presence of lymphatic invasion compared to that for the group with mild to moderate presence of lymphatic invasion (55 vs. 90%, P<0.01) (Fig. 1). Similarly, the 5-year disease-specific survival rate was significantly lower for the group with ≥4 positive micrometastatic nodes compared to that for the group with 0-3 positive micrometastatic nodes (46 vs. 92%, P<0.01) (Fig. 2). When a combination of two factors, lymphatic invasion and micrometastasis was examined, the 5-year disease-specific survival rate for the group of
Table I. Univariate analysis for the 5-year disease-specific survival in patients with stage II colorectal cancer.

| Factors                          | No. of patients | 5-year survival rate (%) | P-value |
|----------------------------------|-----------------|---------------------------|---------|
| Age (years)                      |                 |                           |         |
| 0-70                             | 73              | 79                        | NS      |
| ≥71                              | 36              | 81                        |         |
| Gender                           |                 |                           |         |
| Male                             | 61              | 85                        | NS      |
| Female                           | 48              | 88                        |         |
| Location of tumor                |                 |                           |         |
| Right colon                      | 21              | 95                        | NS      |
| Left colon                       | 52              | 82                        |         |
| Rectum                           | 36              | 68                        |         |
| No. of resected lymph nodes      |                 |                           |         |
| 0-11                             | 43              | 81                        | NS      |
| ≥12                              | 66              | 79                        |         |
| Bowel obstruction                |                 |                           | <0.01   |
| Absent                           | 99              | 83                        |         |
| Present                          | 10              | 50                        |         |
| Tumor size (cm)                  |                 |                           |         |
| 0-4                              | 29              | 90                        | NS      |
| >4                               | 80              | 76                        |         |
| Depth of tumor invasion          |                 |                           |         |
| T3                               | 80              | 90                        | NS      |
| T4                               | 29              | 76                        |         |
| Differentiation                  |                 |                           |         |
| High                             | 68              | 84                        | NS      |
| Moderate                         | 36              | 75                        |         |
| Poor/mucinous                    | 5               | 60                        |         |
| Lymphatic invasion               |                 |                           | <0.01   |
| Absent, mild                     | 98              | 90                        |         |
| Moderate, severe                 | 11              | 55                        |         |
| Venous invasion                  |                 |                           |         |
|Absent                            | 80              | 89                        | NS      |
| Present                          | 29              | 79                        |         |
| Tumor budding                    |                 |                           |         |
|Absent                            | 70              | 84                        | NS      |
|Present                           | 39              | 90                        |         |
| Peritumoral lymphocytes          |                 |                           |         |
|Inconspicuous                     | 40              | 83                        | NS      |
|Conspicuous                       | 69              | 88                        |         |
|Tumor growth pattern              |                 |                           |         |
|Expansive                         | 30              | 91                        | NS      |
|Infiltrative                      | 79              | 84                        |         |
|p53                              |                 |                           |         |
|Negative                          | 49              | 84                        | NS      |
|Positive                          | 60              | 88                        |         |
|CD10                             |                 |                           |         |
|Negative                          | 70              | 83                        | NS      |
|Positive                          | 39              | 92                        |         |
patients with either one positive factor was significantly lower compared to that for the group with both factors negative (55 vs. 94%, P<0.01) (Fig. 3).

Discussion

In the present study, 20% of the stage II colorectal cancer patients presented with tumor recurrence or distant metastasis during follow-up, after curative resection. A multivariate analysis allowed us to define a subgroup of patients at high risk of recurrence, which included those with lymph node micrometastasis and those with lymphatic invasion. In addition, these factors were significantly associated with the prognosis of stage II colorectal cancer patients.

Recent advances in immunohistochemistry and molecular biology suggest that molecular changes of the primary tumor may serve as prognostic indicators for individual patients. Several studies have attempted to identify the prognostic biomarkers in patients with stage II or node-negative colorectal cancer (2-7, 12,17,18).

Although several studies have been conducted on lymph node micrometastasis in patients with colorectal cancer, the significance of the presence of lymph node micrometastasis has been a subject of debate (16,18-21). Yasuda et al (16)

![Figure 2](image1.png)

Figure 2. The 5-year disease-specific survival rate for the group of patients with ≥4 micrometastases was significantly lower compared to that for the group with 0-3 micrometastases (46 vs. 92%, P<0.01).

![Figure 3](image2.png)

Figure 3. Lymphatic invasion and micrometastasis: the 5-year disease-specific survival rate for the group of patients with either one positive factor was significantly lower compared to that for the group with both factors negative (55 vs. 94%, P<0.01).
reported that micrometastasis in ≥4 lymph nodes and micro-
metastasis to N2 or higher nodes were significantly correlated
with postoperative recurrence and prognosis in stage II
colorectal cancer patients. Bukholm et al (21) reported that
the presence of isolated tumor cells in the mesenteric lymph
nodes was independently associated with reduced relative
survival in patients with stage II colon cancer. Our study also
demonstrated that the number of lymph node micrometas-
tases was a more powerful indicator than the presence and
level of lymph node micrometastasis. Therefore, it is helpful
to investigate the number of lymph node micrometastases
with immunohistochemistry in stage II colorectal cancer
patients.

The aim of adjuvant chemotherapy is the destruction of
microscopic metastases that may already be present and the
reduction of the risk of recurrence. Postoperative chem-
otherapy for stage III colorectal cancer patients has been
shown to improve prognosis and is recommended as standard
therapy (22,23). However, the value of adjuvant chemotherapy
for patients with stage II colorectal cancer is controver-
sial (24,25). The International Multicentre Pooled Analysis
of B2 Cancer Trials (IMPACT B2) (26) and the meta-analysis
reported by Figueredo et al (27) did not demonstrate any
improvement in prognosis of stage II colon cancer patients
treated with adjuvant chemotherapy. However, the QUASAR
study demonstrated a significantly reduced recurrence rate and
improved survival of patients with stage II colorectal cancer in
favour of the adjuvant chemotherapy arm (28). Although several large studies have investigated the
subject of adjuvant chemotherapy for stage II colorectal cancer
patients, the use of adjuvant chemotherapy for all stage II
colorectal cancer patients may be inappropriate and expen-
sive (29). Therefore, there is an increasing need for accurate
stratification of stage II colorectal cancer patients in order to
identify those at high-risk of recurrence who may benefit from
adjuvant chemotherapy.

Our data suggest that two factors, lymph node microme-
tastasis and lymphatic invasion, should be included in the
high-risk group of patients with stage II colorectal cancer.
Sirop et al (30) reported improved outcomes of micrometas-
tasis after being considered as high-risk disease and treated
with chemotherapy in their pilot study. These results suggest a
trend in favour of adjuvant chemotherapy in stage II colorectal
cancer patients with high-risk factors.

In conclusion, we demonstrated that each of the two factors
investigated, lymph node micrometastasis and lymphatic
invasion, carries independent prognostic significance with
respect to the 5-year disease-specific survival rates of patients
with stage II colorectal cancer. This finding may be useful in
identifying the high-risk patients for recurrence or metastasis
among stage II colorectal cancer patients. We recommend that
stage II colorectal cancer patients with lymph node microme-
tastasis and lymphatic invasion be evaluated for the benefit of
adjuvant chemotherapy in the future, through further prospec-
tive randomized control studies.

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