Clinical Cancer Research

Micrometastasis Volume in Lymph Nodes Determines Disease Recurrence Rate of Stage II Colorectal Cancer: A Prospective Multicenter Trial

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Abstract

Purpose: We reported in a retrospective study that the presence of micrometastasis in lymph nodes, when assessed by carcinoembryonic antigen (*CEA*)-specific RT-PCR, is a significant prognostic factor in stage II colorectal cancer. The aim of this study was to clarify the clinical value of micrometastasis in a prospective multicenter trial.

Experimental Design: From November 2001 to December 2005, a total of 419 colorectal cancer cases were preoperatively registered at a central data center. Of them, 315 node-negative stage II colorectal cancer cases were enrolled. After RNA quality check, 304 colorectal cancer cases were analyzed for *CEA* mRNA in lymph nodes by both conventional RT-PCR (a band method) and quantitative RT-PCR. Long-term prognosis of the patients was determined by each method.

Results: A positive band for *CEA* mRNA was detected in 73 (24.0%) of 304 patients. Postoperative adjuvant chemotherapy was applied in 31 *CEA* band-positive cases with an oral 5-fluorouracil derivative HCFU (1-hexylcarbamoyl-5-fluorouracil) for 1 year, whereas chemotherapy was not administered to *CEA* band-negative group. Multivariate Cox regression analyses revealed that a high micrometastasis volume (high MMV, $n = 95$) was an independent poor prognostic factor for 5-year disease-free survival (DFS; $P = 0.001$) and 5-year overall survival (OS; $P = 0.016$).

Conclusions: This prospective clinical trial demonstrates that micrometastasis volume is a useful marker in identifying patients who are at high or low risk for recurrence of stage II colorectal cancer. *Clin Cancer Res; 22(13); 3201–8. ©2016 AACR.*

Introduction

Metastasis to regional lymph nodes is a reliable prognostic factor, adopted globally in the tumor–node–metastasis (TNM) staging system and used for clinical decision-making regarding postoperative adjuvant chemotherapy (1–3). Studies have shown the presence of occult cancer metastasis (also designated as micrometastasis) in the lymph nodes of colorectal cancer patients (4).

With IHC and molecular genetics methods, occult tumor cells in regional lymph nodes are detected in 25% to 50% of patients with node-negative colorectal cancer (5–18). Because up to 25% of patients with node-negative colorectal cancer ultimately die as a result of disease relapse, occult cancer metastasis has been suspected as a potential marker for systemic spread of tumor cells (1). However, the prognostic value of molecular tumor cell detection in patients with node-negative colorectal cancer has remained uncertain because of lack of evidence from prospective studies (5–18).

National Comprehensive Cancer Network (NCCN) clinical practice guidelines in oncology (version 4, 2013; ref. 19) recommends that detection of cancer cells by IHC or by the molecular detection of cancer cells in regional lymph nodes should be considered investigational because most of

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

This study was carried out on behalf of the Clinical Study Group of Osaka University (CSGO), Colorectal Group.

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Translational Relevance

Evidence from prospective studies lacks as to the clinical significance of micrometastasis in lymph nodes of stage II colorectal cancer. In this study, we measured carcinoembryonic antigen (*CEA*) mRNA levels in lymph nodes of stage II colorectal cancer cases. We found that micrometastasis volume (MMV) determined by qRT-PCR of *CEA* mRNA was essentially important to predict prognosis of the patients with stage II colorectal cancer. Our data also indicate that stage II is a transitional place between localized stage I and expanding stage III, where colorectal cancer tumors continuously increase MMV in lymph nodes and the risk for tumor recurrence.

evidence cited in this guideline is constructed by retrospective studies which include our works on distribution and prognostic impact of micrometastases in node-negative colorectal cancers by IHC for cytokeratins or carcinoembryonic antigen (*CEA*)-targeted RT-PCR (7, 20). In addition, a recent meta-analysis by Rahbari and colleagues emphasized that prospective studies are required to confirm their results that micrometastasis was associated with poor overall survival (OS) and shorter disease-free survival (DFS) in node-negative colorectal cancers (21).

In this study, we conducted a prospective multicenter clinical trial for a definite prognostic marker in patients with node-negative stage II colorectal cancer. To search for the clinically appropriate threshold of high risk for disease recurrence, we employed molecular detection of *CEA* mRNA by qRT-PCR in addition to conventional qualitative RT-PCR (the so-called "a band" method). A total of 296 patients with pathologic stage II colorectal cancer were eventually analyzed for their prognosis. After a long follow-up period (median follow-up, 82.3 months), we uncover the prognostic impact of micrometastases in stage II colorectal cancer.

Patients and Methods

Patients

After obtaining written informed consent on lymph node sampling and the subsequent clinical trial, lymph node samples were collected during surgery from 419 potential candidates who were preoperatively evaluated as stage II or stage III [International Union Against Cancer (UICC) TNM Classification - the 7th version; ref. 3] by examination of colonoscopy and CT scan, with performance status 0-2 (age, 20–75) from November 2001 to December 2005. After surgery, 315 patients diagnosed with pathologically node-negative stage II colorectal cancer were enrolled (Fig. 1). Experienced surgical staff from the 21 hospitals participated in this study. The study protocol was approved by each local institutional review board and this study was conducted in accordance with the Good Clinical Practice guidelines and the Declaration of Helsinki.

Lymph node sampling

Lymph nodes were collected, fixed in 10% buffered formalin, and embedded in paraffin for routine histopathologic examination. Mean lymph node sampling number inspected in each colorectal cancer case was 16.0. Of them, 5 pericolic nodes adjacent to the tumor were further examined for micrometastasis to detect initial cancer spread. For this purpose, a half of lymph node was collected using a RNase-free disposable lymph node sampling kit within 3 hours after surgical resection as concluded by our early work (22), being preserved in a tube containing RNA later (Ambion) at −20°C until RNA extraction (Supplementary Fig. S1).

RNA extraction and RNA quality of lymph node samples

Once pathologic tests indicated node-negative stage II colorectal cancer, lymph nodes were minced in TRIzol Reagent (Invitrogen). RNA extraction was performed as described previously (23, 24).For assessment of RNA quality, RNA was electrophoresed on a 0.8% agarose mini-gel, and ribosomal RNAs at 28S and 18S were visualized with ethidium bromide (Supplementary Fig. S1). Among 315 enrolled stage II colorectal cancer patients, lymph node samples from 11 patients showed degradation (3.5%; Fig. 1).

RT reaction

Reverse transcription (RT) was performed as described previously (22–24).

Conventional PCR. Conventional PCR and the primer sequences for PCR amplification of *CEA* and the housekeeping gene porphobilinogen deaminase *(PBGD)* were described previously (7, 22–23, 25–27). PCR products were electrophoresed on a 2% agarose gel and visualized with ethidium bromide (Supplementary Fig. S1). To ensure the reproducibility of PCR, the *CEA* control panel was produced using a serial dilution of cDNA from MKN45 and LoVo cells (22) and referenced to estimate the positivity of the *CEA* band in colorectal cancer cases (Supplementary Fig. S1).

Quantitative PCR assay with Light Cycler

Fluorescence PCR was performed using the Light Cycler (Roche Diagnostics), as described previously (22, 24, 26).The standard curves for quantification of *CEA* or *PBGD* mRNAs were drawn using 10-fold dilutions of cDNA from MKN45 cells (22, 26).We confirmed that *CEA* mRNA was reproducibly measurable in the range of a dilution of 1×10^{-1} to 1×10^{-4} (Supplementary Fig. S2).

Postoperative adjuvant chemotherapy

Postoperative adjuvant chemotherapy was randomly applied to the colorectal cancer patients with positive band for *CEA* mRNA using the oral 5-fluorouracil derivative HCFU (1-hexylcarbamoyl-5- fluorouracil; refs. 28, 29). Thus, if the patients agreed, they were randomly allocated to either chemotherapy (HCFU 300 mg/day for 1 year) or no treatment (Fig. 1). If they did not agree to allocation, we followed each patient's desire and the study protocol was fixed that they were not excluded from the study. The protocol prohibited use of chemotherapy in CEA band-negative cases.

Follow-up of patients

Among 304 patients whose *CEA* levels in lymph nodes were determined, several patients were judged as not meeting criteria for the purpose of this study. These included protocol violation (applied chemotherapy in *CEA* band-negative case, $n = 5$), insufficient follow-up period (5 months; *CEA* band-positive

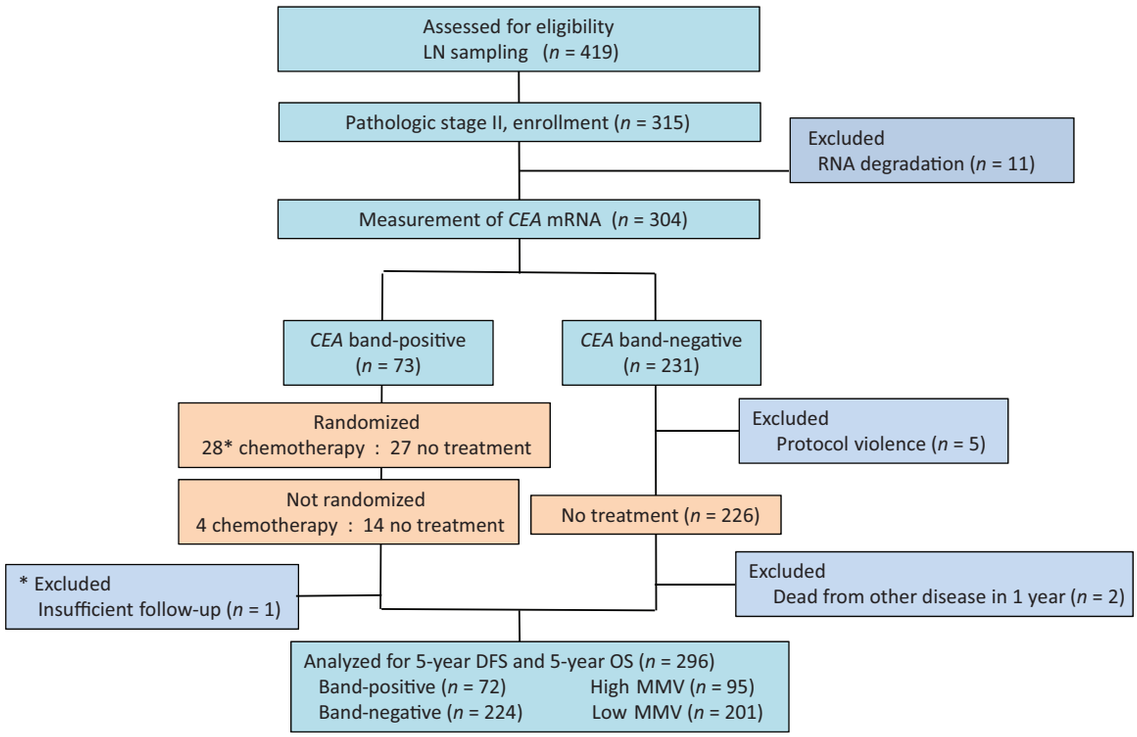

Figure 1.
Flow chart of patients. Lymph node (LN) samples were collected from 419 patients who were preoperatively diagnosed as stage II and III colorectal cancers and 315 pathologic stage II patients were enrolled in the study. After exclusion for RNA degradation ($n = 11$), 304 patients were determined for the presence of the *CEA* band and for their *CEA* mRNA value. Postoperative adjuvant chemotherapy (HCFU 300 mg/day for 1 year) was adopted in 32 *CEA* band-positive patients among whom 28 cases were randomly allocated to chemotherapy and 4 patients refused to be allocated and desired to receive chemotherapy. Among 41 cases with no treatment, 27 cases were allocated to no treatment and 14 cases desired no treatment. One patient lost to follow-up at 5 months (*this patient was allocated to adjuvant chemotherapy by randomization) and *CEA* band-negative 2 patients who died at 2 months and 10 months from other diseases were excluded for survival analyses. Ultimately, data for 296 stage II patients were analyzed for disease recurrence and survival. LN, lymph node.

case, $n = 1$), and early death from other disease (2 months and 10 months, respectively; *CEA* band-negative cases, $n = 2$). As a result, 296 colorectal cancer patients were analyzed for their disease recurrence and survival (Fig. 1). The patients were periodically monitored by physiologic examination, CT scan, chest x-ray, colonoscopy, and blood tests. The median follow-up period was 82.3 (5.3–133.2) months.

Clinical trials registry

This study was registered with University Hospital Medical Information Network Clinical Trials Registry (UMIN-CTR). Clinical trial information is referenced by UMIN C000000185.

Statistical analysis

Statistical analysis was performed using the StatView J-5.0 program (Abacus Concepts, Inc). The Kaplan–Meier method was used to estimate tumor recurrence and cancer-specific survival, and the log-rank test was used to examine statistical significance. A Cox proportional hazards model was used to assess the risk ratio under simultaneous contribution from several covariates. The associations between the discrete variables were assessed using the χ^2 test. Mean values were compared using the Mann–Whitney test. All data were expressed as the mean ± SD. Values of $P < 0.05$ denoted the presence of a statistically significant difference.

Results

Conventional RT-PCR for *CEA* mRNA

The positivity for the *CEA* band was assessed in reference to the control panel using *CEA*-expressing cells (Supplementary Fig. S1). Of 304 stage II colorectal cancer patients, 73 cases (24.0%) displayed a clear *CEA* band with expression of the *PBDG* gene, and the remaining 231 cases did not by conventional RT-PCR assays (Fig. 1).

qRT-PCR for *CEA* mRNA

CEA transcripts were measured by qRT-PCR and normalized by *PBDG* gene expression. The *CEA*/*PBDG* value, designated as the micrometastasis volume (MMV) ranged widely from 0 (group a, $n = 90$) to $>1 \times 10^{-4}$ (group b, $n = 111$), $>1 \times 10^{-3}$ (group c, $n = 62$), and $>1 \times 10^{-2}$ (group d, $n = 33$). We grouped categories c and d as the high MMV group ($n = 95$, 32.1%) and categories a and b into the low MMV group ($n = 201$, 67.9%). A clinicopathologic survey indicated that high MMV was significantly associated with the presence of lymphatic invasion ($P = 0.030$) and smaller tumor size ($P = 0.009$). MMV was not associated with the presence of ASCO risk factors (Table 1).

Survival analyses

Conventional RT-PCR. After exclusion of inappropriate cases ($n = 8$, Fig. 1), 72 *CEA* band-positive cases and 224 *CEA* band-negative cases were analyzed for DFS and OS. We found

Table 1. Relationship between MMV and clinicopathologic parameters

Characteristic	High MMV ($n = 95$)	Low MMV ($n = 201$)	P
Age, years	63.7 ± 7.58	64.7 ± 8.01	0.174
Sex			
Male	56	116	0.840
Female	39	85	
Tumor location			
Colon	77	154	0.389
Rectum	18	47	
T stage			
T3	77	169	0.516
T4a, T4b	18	32	
Histology			
Well, Mod	94	191	0.095
Por, Muc	1	10	
Lymphatic invasion			
Present	51	81	0.030*
Absent	44	120	
Venous invasion			
Present	35	76	0.872
Absent	60	125	
Tumor size, mm	46.6 ± 19.1	52.6 ± 19.6	0.009[a]
ASCO risk factor[b]			
Positive	50	87	0.136
Negative	45	114	

Abbreviations: Mod, moderately differentiated adenocarcinoma; Muc, mucinous carcinoma; Por, poorly differentiated adenocarcinoma; Well, well-differentiated adenocarcinoma.

[a]*P* value from the χ^2 test.

[b]Perforation, T4 lesions, poorly differentiated histology, inadequately sampled nodes (<12 LNs).

that the *CEA* band-positive cases had significantly worse 5-year DFS and final OS than the *CEA* band-negative cases ($P = 0.021$, 0.030, respectively, Fig. 2A and C), but there was no significant difference in 5-year OS ($P = 0.287$, Fig. 2B). Of *CEA* band-positive cases, 27 cases received postoperative chemotherapy by random allocation using HCFU for 1 year, whereas 27 cases were randomly allocated to no treatment. There was no significant difference in 5-year DFS and 5-year OS between the two groups (Supplementary Fig. S4). There was no difference in mean follow-up period in the *CEA* band-positive and band-negative groups ($P = 0.660$; mean ± SD, 79.2 ± 24.4 vs. 79.9 ± 22.9 months).

qRT-PCR. Survival analyses indicated that the high MMV group had significantly worse 5-year DFS, 5-year OS, and final OS as compared with the low MMV group ($P = 0.001$, 0.016, 0.003; Fig. 3A–C). Irrespective of chemotherapy applied to high MMV cases, high MMV group consistently showed significantly worse 5-year DFS as compared with the low MMV group with no treatment ($P = 0.009$, 0.007; Supplementary Fig. S5). Similar results were observed in analyses for OS ($P = 0.003$, 0.026; data not shown). There was no difference in mean follow-up period in high MMV and lowMMV groups ($P = 0.8471$; mean ± SD, 86.0 ± 25.8 vs. 79.9 ± 22.1 months).

Among various clinical and pathologic characteristics, presence of lymphatic invasion was a significant risk factor for shorter 5-year DFS ($P = 0.033$) in addition to high MMV ($P = 0.001$), but ASCO risk factor was not (Table 2 and 3). Multivariate analyses indicated that high MMV alone was retained as an independent risk factor ($P = 0.004$, Table 2). As for 5-year OS and final OS, high MMV alone was significantly associated with poor prognosis ($P = 0.020$, 0.004; risk ratio 3.097, 3.340, respectively; Table 3).

In addition, the micrometastasis-free colorectal cancer group (group a) had a favorable prognosis. Five-year DFS was 93.3%; 5-year OS and final OS were both 97.8% (Supplementary Fig. S3A–S3C).

Disease recurrence mode

Among 296 patients, 51 developed disease recurrence after surgery. The recurrent organ sites are listed in Supplementary Fig. S6. Like more advanced-stage patients, stage II patients often had hematogenous metastasis to liver and lung.

Discussion

We have demonstrated clinical significance of micrometastasis in stage II colorectal cancer as a result of long-term follow-up. We are not aware of other reports on prospective multicenter studies that followed patient outcomes for a duration as long as our clinical trial (median, 82. 2 months). We found that MMV was a particularly useful biomarker for discriminating both high risk (group c+d) and low risk (group a) for disease recurrence in stage II colorectal cancer. The recurrent mode was mainly hematogenous metastasis to liver and lung in stage II colorectal cancer patients, which is the serious problem as is the case with stage III colorectal cancer.

To detect occult tumor cells in lymph nodes, we employed *CEA*-targeted molecular detection by RT-PCR because our retrospective study showed that micrometastasis detected by RT-PCR, but not by IHC, was associated with poor prognosis in stage II colorectal cancer (7). In routine practice, RT-PCR is more convenient than IHC because IHC of occult cancer cells requires the extraordinary labor of preparing slides and searching minimal tumor cells under the microscopy. Moreover, the advantage of the molecular genetic technique is that it allows examination of a large quantity of lymph nodes; in this way, unstable features of IHC caused by the number of inspected slices can be avoided (20).

In addition to the routine pathologic lymph node examination (mean inspection number, 16.0 lymph nodes per case), we

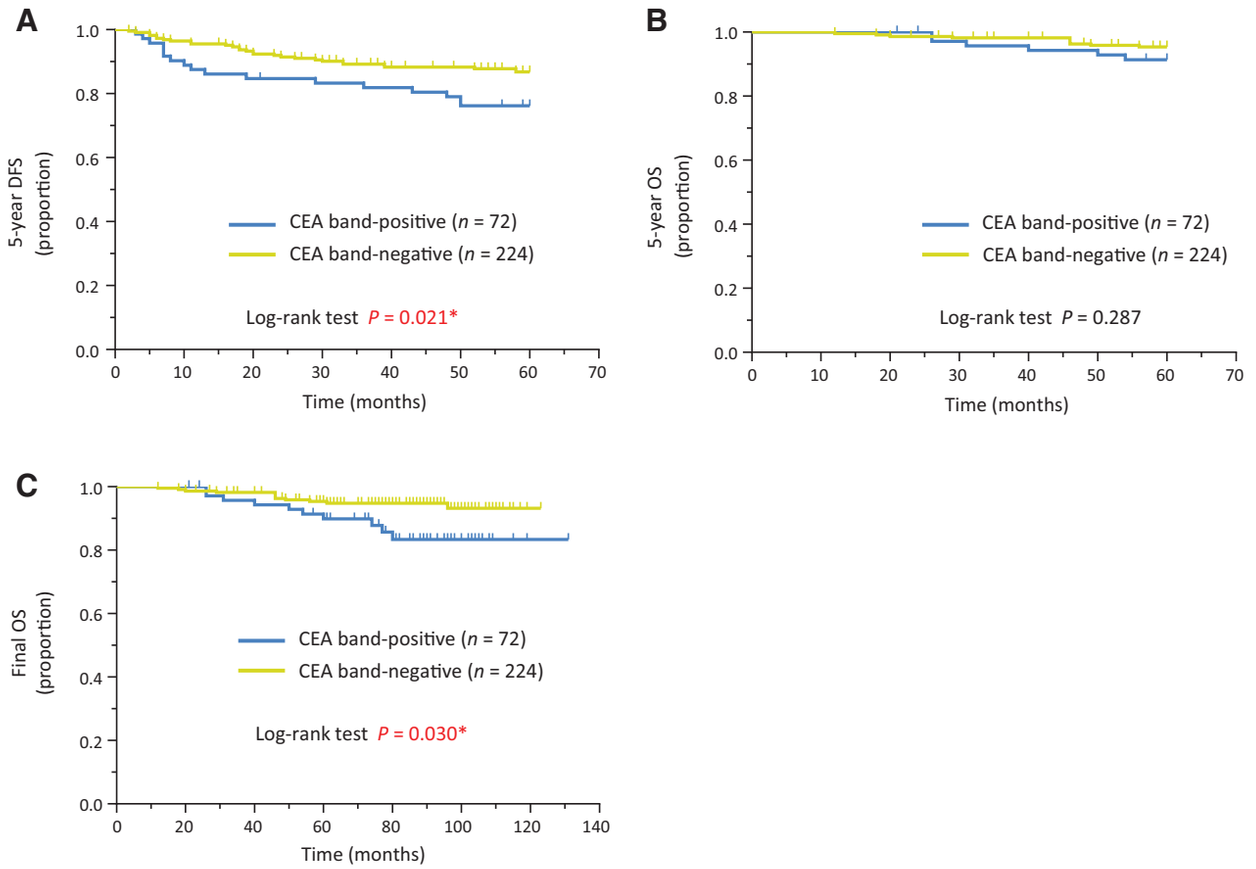

Figure 2.
Survival curves stratified by positive and negative *CEA* bands. A, the *CEA* band-positive cases ($n = 72$) had a significantly worse 5-year DFS (76.4%) than the *CEA* band-negative cases (87.5%; $n = 224$, $P = 0.021$). B, there was no significant difference in 5-year OS between the two groups ($P = 0.287$). The 5-year OS was 91.7% versus 95.1%, respectively. C, there was a significant difference in final OS between the two groups ($P = 0.030$). The final OS was 86.1% versus 94.2%, respectively. There was no difference in mean follow-up period in the *CEA* band-positive and band-negative groups ($P = 0.660$; mean $\pm$ SD, 79.2 $\pm$ 24.4 vs. 79.9 $\pm$ 23.0 months). Median follow-up period was 81.8 (22.0–133.2) months and 82.5 (5.3–124.9) months, respectively. DFS, disease-free survival; OS, overall survival.

examined representative 5 lymph nodes near the primary tumor for detection of micrometastasis. We made this choice based on detailed lymph node mapping of micrometastasis by IHC that showed that the initial occult tumor spread was found at pericolic nodes adjacent to the tumor (level 1) in 25 of 26 node-negative colorectal cancer patients (96.2%; Supplementary Fig. S7), which suggests that micrometastasis is initially generated around the tumor. We also considered that molecular inspection of additional 5 lymph nodes on current pathologic examination would be clinically applicable as a routine practice.

Through our previous translational studies (22), we decided that lymph nodes should be collected within 3 hours after tumor resection to maintain the quality of RNA materials. Surgeons were instructed before the start of this clinical trial to retrieve lymph nodes using the RNase-free disposable lymph node sampling kit (Supplementary Fig. S1; ref. 22). As a result, RNA quality was preserved in 304 of 315 colorectal cancer cases (96.5%), which we think is a sufficiently acceptable range in clinics. For clinical application, qRT-PCR is suitable because the clinical useful threshold was better provided by qRT-PCR, than the conventional RT-PCR, suggesting that adjustment of *CEA* value by the appropriate internal control is essentially important.

Studies have not supported a rationale for application of chemotherapy for all stage II colorectal cancer patients (30, 31).However, 25% of stage II patients ultimately die from the disease recurrence (1).This dilemma has been carried over for decades. ESMO (European Society for Medical Oncology) guideline and a proposal from ASCO in 2004 introduced several prognostic factors such as perforation, T4 lesions, poorly differentiated histology, inadequately sampled nodes to identify high-risk stage II colon cancer (30, 31). Univariate analysis showed that high MMV and lymphatic invasion were indicative of shorter 5 year, but ACSO risk factor was not a significant prognostic parameter. Multivariate analysis indicated that high MMV alone was an independent prognostic factor in stage II colorectal cancer.

A study by O'Connor and colleagues showed that 75% of the 24,847 stage II cancers had one or more poor prognostic features of perforation, T4 stage, poor histology, or others, and that survival benefit from adjuvant chemotherapy was not observed even with any poor prognostic features (32). The findings indicate that prognostic factors do not always predict patients' survival benefit by adjuvant chemotherapy. Although we applied

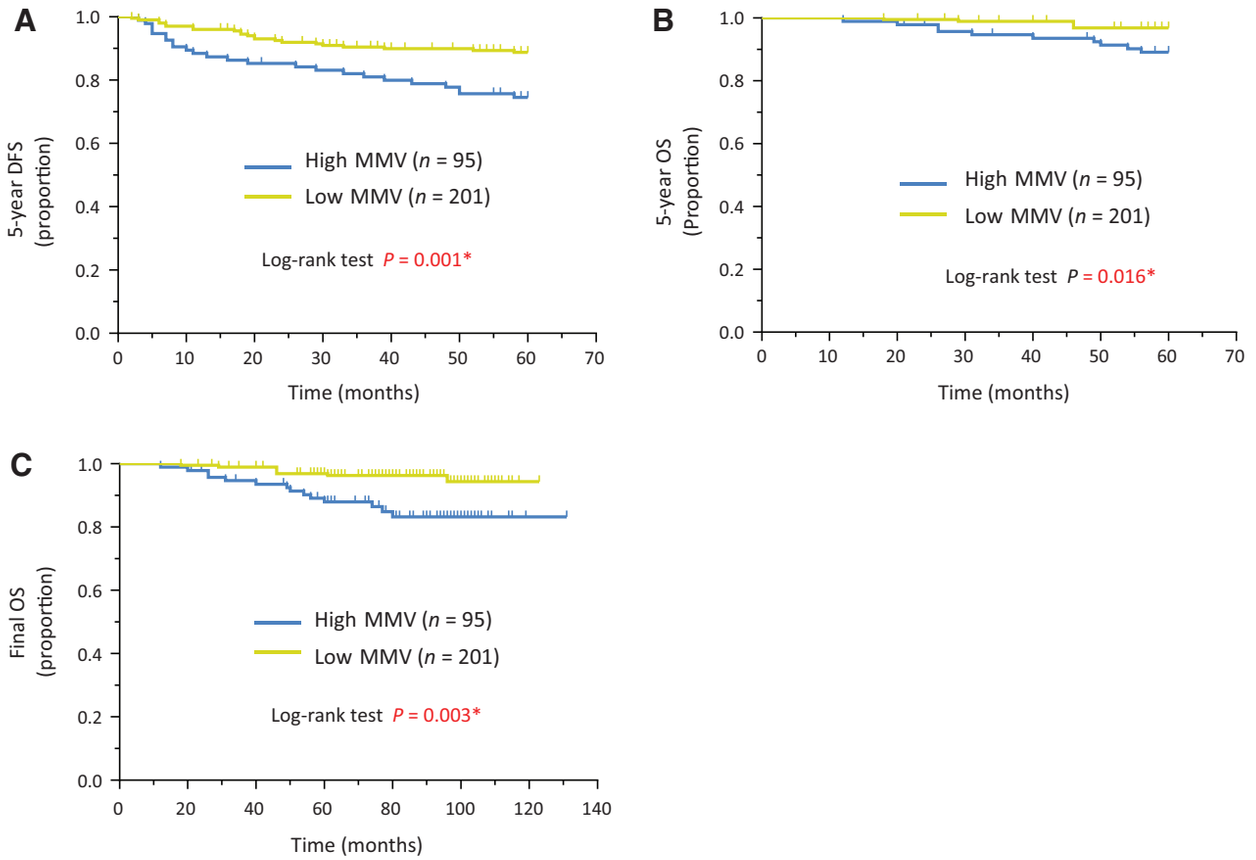

Figure 3.
Survival curves stratified by high MMV and low MMV. Survival analyses indicated that the high MMV group had significantly (A) worse 5-year DFS (74.7% vs. 88.6%), (B) 5-year OS (89.5% vs. 95.5%), and (C) OS (86.3% vs. 95.5%) as compared with the low MMV group ($P = 0.001$, 0.016, 0.003). There was no difference in mean follow-up period in high MMV and low MMV groups ($P = 0.8471$; mean ± SD, 86.0 ± 25.8 vs. 79.9 ± 22.1 months). Median follow-up period was 82.9 (12.6–133.2) months and 81.4 (5.3–124.9) months, respectively. DFS, disease-free survival; OS, overall survival.

postoperative chemotherapy by randomization to *CEA* band-positive groups, no significant difference was noted in patients' prognosis (Supplementary Fig. S4). In addition, survival analyses of the entire *CEA* band-positive groups (31 chemotherapy vs. 41 no treatment) showed similarly insignificant results (Supplementary Fig. S8). This is because of small size of the PCR-positive group and more likely to be due to a sort of adopted therapeutics. The current standard adjuvant therapy including FOLFOX, capecitabine, or UFT tegafur-uracil/UZEL (33–35) did not emerge in clinics those days when this study started. We used HCFU which was later proved to be less effective even than UFT alone (36)and it is not available today. Therefore, to obtain the correct answer, a standard chemotherapy should be applied to the more numbers of PCR-positive cases in the next stage.

Table 2. Univariate and multivariate analysis for 5-year DFS

5-year DFS: Univariate analysis	Risk ratio (95% CI)	*P*
CEA mRNA in LNs (high MMV vs. low MMV)	2.576 (1.433–4.665)	0.001*
Age (>64 years vs. ≤64 years)	0.999 (0.555–1.829)	0.997
Sex (male vs. female)	1.666 (0.904–3.230)	0.103
Tumor location (colon vs. rectum)	1.185 (0.573–2.264)	0.628
Tumor size (>51 mm vs. ≤51 mm)	0.730 (0.382–1.335)	0.313
Differentiation (well, mod vs. muc, por)	0.591 (0.033–2.707)	0.571
Lymphatic invasion (present vs. absent)	1.893 (1.050–3.495)	0.033*
Venous invasion (present vs. absent)	1.380 (0.758–2.479)	0.286
T stage (T3 vs. T4a, T4b)	1.226 (0.554–2.435)	0.591
Retrieved LN number (<12 vs. 12, >12)	0.719 (0.398–1.293)	0.269
ASCO risk factor (positive vs. negative)	1.241 (0.690–2.239)	0.468
5-year DFS: Multivariate analysis	**Risk ratio (95% CI)**	***P***
CEA mRNA in LNs (high MMV vs. low MMV)	2.380 (1.315–4.339)	0.004*
Lymph invasion (present vs. absent)	1.661 (0.915–3.088)	0.095

Abbreviations: Mod, moderately differentiated adenocarcinoma; Muc, mucinous carcinoma; Por, poorly differentiated adenocarcinoma; LN, lymph node; Well, well-differentiated adenocarcinoma. *, statistically significant.

Although we could not determine an efficacy of chemotherapy in high MMV cases, we still consider that such group may be an appropriate target for a postoperative adjuvant chemotherapy for the following reasons. First of all, lymph node metastasis in stage III colorectal cancer is a well-established predictive marker to ensure survival benefit by chemotherapy (33, 37–38). We previously found that one-step nucleic acid amplification (OSNA) of cytokeratin 19 (*CK19*) mRNA provided a judgment performance equivalent to a 2-mm-interval histopathologic examination of lymph nodes of colorectal cancer (39). In our separate multicenter prospective study, we found by OSNA that tumor volume in lymph nodes of colorectal cancers upstaged from stage II to stage III (17.6%, 13/76) ranged widely. Of interest was that the values were

Table 3. Univariate analysis for 5-year OS and OS

5-year OS: Univariate analysis	Risk ratio (95% CI)	*P*
CEA mRNA in LNs (high MMV vs. low MMV)	3.097 (1.189–8.531)	0.020*
Age (>64 years vs. <64 years)	1.026 (0.394–2.826)	0.958
Sex (male vs. female)	1.038 (0.399–2.861)	0.938
Tumor location (colon vs. rectum)	2.047 (0.705–5.381)	0.176
Tumor size (>51 mm vs. <51 mm)	1.055 (0.383–2.747)	0.913
Differentiation (well, mod vs. muc, por)	1.839 (0.101–9.015)	0.587
Lymphatic invasion (present vs. absent)	1.778 (0.683–4.898)	0.237
Venous invasion (present vs. absent)	0.934 (0.321–2.454)	0.892
T stage (T3 vs. T4a, T4b)	1.545 (0.435–4.368)	0.464
Retrieved LN number (<12 vs. 12, >12)	0.539 (0.195–1.405)	0.206
ASCO risk factor (positive vs. negative)	1.674 (0.643–4.610)	0.291
OS: Univariate analysis	**Risk ratio (95% CI)**	***P***
CEA mRNA in LNs (high MMV vs. low MMV)	3.340 (1.464–8.019)	0.004*
Age (>64 years vs. ≤64 years)	0.688 (0.298–1.572)	0.372
Sex (male vs. female)	1.170 (0.513–2.812)	0.711
Tumor location (colon vs. rectum)	1.996 (0.803–4.596)	0.130
Tumor size (>51 mm vs. ≤51 mm)	0.825 (0.332–1.901)	0.659
Differentiation (well, mod vs. muc, por)	1.339 (0.074–6.377)	0.784
Lymphatic invasion (present vs. absent)	1.562 (0.686–3.663)	0.286
Venous invasion (present vs. absent)	0.712 (0.273–1.672)	0.446
T stage (T3 vs. T4a, T4b)	1.017 (0.294–2.711)	0.974
Retrieved LN number (<12 vs. 12, >12)	0.682 (0.296–1.558)	0.361
ASCO risk factor (positive vs. negative)	1.314 (0.575–3.028)	0.512

Abbreviations: Mod, moderately differentiated adenocarcinoma; Muc, mucinous carcinoma; Por, poorly differentiated adenocarcinoma; LN, lymph node; Well, well-differentiated adenocarcinoma. *, statistically significant.

largely overlapped with those in lymph nodes of stage III colorectal cancers (40). The prognosis of these upstaged colorectal cancer patients is uncertain until 3 to 5 years ahead. Importantly, however, the current study uncovered that MMV determined by qRT-PCR for *CK19* mRNA was also useful to discern high-risk stage II colorectal cancer patients in the same clinical settings (Supplementary Fig. S9). In this context, OSNA could substitute *CEA* targeted RT-PCR owing to its simple and rapid system and would be helpful to confirm efficacy of chemotherapy in colorectal cancers upstaged from stage II to stage III in a large-scale trial in the future.

In conclusion, we prospectively demonstrated that molecular detection of MMV adds prognostic value to the conventional histologic survey in stage II colorectal cancer. Our data also provide a concept that stage II is a transitional place between localized stage I and expanding stage III, where colorectal cancer tumors continuously increase MMV in lymph nodes and the risk for tumor recurrence.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: H. Yamamoto, K. Murata, T. Ohnishi, T. Kato, M. Ikeda, M. Sekimoto, N. Matsuura, M. Monden, M. Mori

Development of methodology: H. Yamamoto, T. Ohnishi, T. Kato, Y. Nakamura, M. Ikeda

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