

MICROMETASTASES AND SURVIVAL IN STAGE II COLORECTAL CANCER

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ABSTRACT

Background Standard treatment of colorectal cancer includes adjuvant chemotherapy for patients with stage III disease (defined by the presence of lymph-node metastases), but not for patients with stage II tumors (who have no lymph-node metastases). However, 20 percent of patients with stage II tumors die of recurrent disease. We investigated whether the detection of micrometastases can be used to identify patients with stage II disease who are at high risk for recurrence.

Methods We analyzed 192 lymph nodes from 26 consecutive patients with stage II colorectal cancer, using a carcinoembryonic antigen-specific nested reverse-transcriptase polymerase chain reaction. Five-year follow-up information was obtained on all patients. Observed and adjusted survival rates were assessed in the patients with and the patients without micrometastases.

Results Micrometastases were detected in one or more lymph nodes from 14 of 26 patients (54 percent). The adjusted five-year survival rate (for which only cancer-related deaths were considered) was 50 percent in this group, whereas in the 12 patients without micrometastases, the survival rate was 91 percent ($P=0.02$ by the log-rank test). The observed five-year survival rates were 36 percent and 75 percent, respectively ($P=0.03$). The groups were similar with respect to age, sex, tumor side (location in relation to the flexura lienalis), degree of tumor differentiation (grade), and diameter of the primary tumor.

Conclusions Molecular detection of micrometastases is a prognostic tool in stage II colorectal cancer. (N Engl J Med 1998;339:223-8.)

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DISSEMINATION to locoregional lymph nodes is an important prognostic factor in colorectal cancer. Five-year survival rates drop from 80 percent in patients with tumor-node-metastasis (TNM) stage II disease (who have no lymph-node metastases) to 45 to 50 percent in those with TNM stage III disease (in which lymph-node metastases are present).¹ Surgery and adjuvant chemotherapy are standard treatments for stage III disease but not for stage II disease, in which adjuvant therapy is recommended only in the setting of a clinical trial.²⁻⁴ Better assessment of prognosis in patients with stage II colorectal cancer could allow the selective use of adjuvant therapy and prevent unnecessary treatment.

It is known from immunohistochemical studies that

some histopathologically negative lymph nodes draining colorectal tumors and other carcinomas contain minute amounts of tumor.⁵⁻¹¹ However, the prognostic relevance of this phenomenon is not clear. Technical advances now permit the detection of micrometastases at the molecular level. For example, somatic mutations in oncogenes or tumor-suppressor genes that occur in the primary tumor are also detectable in lymph-node DNA. Detection of micrometastases by this method is not possible, however, in tumors that lack a genetic alteration suitable for amplification by the polymerase chain reaction (PCR). A more general method is the amplification of cancer-specific RNA from lymph nodes. Carcinoembryonic antigen is present in the vast majority of colorectal tumors but not in normal tissues¹² and is therefore a suitable marker of micrometastases in colorectal cancer. The development of the reverse-transcriptase-PCR assay for carcinoembryonic antigen messenger RNA (mRNA) has made it possible to detect micrometastases in the lymph nodes and bone marrow of patients with colorectal cancer.^{13,14} We used this method to evaluate whether the presence of micrometastases can be used to identify patients with stage II colorectal cancer who are at high risk for metastatic disease.

METHODS**Patients**

We studied 246 lymph nodes from 26 patients with TNM stage II colorectal cancer.¹⁵ The lymph nodes were obtained consecutively from curative resections performed at the surgical department of the Leiden University Medical Center between January 1990 and February 1992. Preoperative and perioperative examinations showed no evidence of metastatic disease. Follow-up was carried out in accordance with the department's protocol and was based on periodic evaluations of the patient. The follow-up findings were confirmed in all the patients as of November 1, 1997.

Tissue and RNA Isolation

Half of each resected node was fixed with formalin and embedded in paraffin for routine histopathological examination. The other half was snap-frozen in liquid nitrogen and stored at -80°C until the time of RNA isolation. Total cellular RNA was

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isolated from 50- μ m sections of the lymph nodes. These sections were sandwiched between two series of 5- μ m frozen sections that were used as controls for staining with hematoxylin and eosin and immunohistochemical staining with antibodies against cytokeratin and carcinoembryonic antigen. The RNA was extracted with the use of Trizol (Life Technologies, Gaithersburg, Md.) in a single-step method as described previously.¹⁶ The RNA was assessed spectrophotometrically and by electrophoresis on a 0.8 percent agarose gel to determine its integrity and quantity.

Reverse-Transcriptase PCR

Nested PCR was performed according to the method described by Gerhard et al.¹³ Briefly, complementary DNA (cDNA) was generated with avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim, Mannheim, Germany) with the use of 2 μ g of total RNA and primer B in a reaction volume of 20 μ l. For the first round of PCR, 100- μ l reactions were prepared with 15 μ l of the cDNA preparation and primers A and B.¹³ Reaction tubes were placed in a thermocycler and preheated at the denaturing temperature; after initial denaturing at 95°C for 7 minutes, 20 cycles of amplification were performed at 95°C (for 1 minute) and 72°C (for 2 minutes), with a final extension step for 10 minutes. All mixtures were prepared on ice in a PCR workstation (CBS Scientific, Del Mar, Calif.).

For the second round of PCR, the reaction mixture containing primer A and an internal primer C¹³ was prepared, and 96.5 μ l was dispensed into each tube. The tubes were overlaid with mineral oil and taken to another room, where 3.5 μ l of the first reaction mixture was pipetted through the oil with the use of self-sealing sterile filter tips (Biozyme, Landgraaf, the Netherlands) to prevent cross-contamination.

Fifteen more cycles of amplification were performed under the same conditions, except that 69°C (for one minute) was chosen as the annealing temperature. The PCR products were separated on a 2 percent agarose gel and stained with ethidium bromide. The negative control samples that were coamplified in every PCR procedure did not yield any product when this protocol was used. However, a very faint band could be detected in some negative control samples when 20 or 25 cycles were used in the second PCR analysis. Consequently, we concluded that 15 cycles were adequate in our assay.

Efficient amplification of the RNA was monitored by a control PCR of hypoxanthine phosphoribosyltransferase (HPRT) cDNA with the use of primer H1 (5'ACCGGCTTCCTCCTCCTGAG-CAGTC3') and primer H2 (5'AGGACTCCAGATGTTTCCA-ACTCAACTT3'). Seven lymph nodes from two patients, one treated for an ischemic ulcer and the other for a tubulovillous adenoma, were used as negative controls. Furthermore, we performed a negative control reaction using a confirmed metastatic lymph node by omitting the addition of reverse transcriptase to check for possible contamination of genomic DNA.

Statistical Analysis

The primary end point was survival, measured from the date of surgery to the time of the last follow-up or death. Kaplan-Meier survival curves were constructed.¹⁷ For overall survival, all deaths, irrespective of cause, were considered events. For adjusted survival, only cancer-related deaths were considered; data on the patients who died from other causes or who were still alive at the end of our study were censored. The rates of recurrence were calculated from the time of surgery to the time of relapse with the same methods. The comparison of the survival curves for the patients with and the patients without micrometastases was performed with the use of the log-rank test. We used a Cox regression analysis to estimate the simultaneous prognostic effect of the variables.¹⁸ Fisher's exact test was used to evaluate the numbers of patients with events according to various covariates; the Mann-Whitney U test was used to compare means. All tests were two-sided, and a P value of less than 0.05 was considered to indicate statistical significance.

RESULTS

Expression of Carcinoembryonic Antigen-Specific mRNA

Intact RNA was isolated from 192 lymph nodes resected from 26 patients. Fifty-four additional lymph nodes from 17 of these 26 patients were excluded from our analysis. Most of these lost nodes were too small to be divided into two parts, and the others did not yield sufficient amounts of intact RNA, on the basis of the control PCR from the HPRT gene. A 131-bp PCR product was amplified from a lymph node with histopathologically confirmed metastasis and from tumor samples. Direct sequencing of the PCR product confirmed that the 3' end of the carcinoembryonic antigen gene had been amplified (data not shown). The control samples repeatedly failed to be amplified when 15 cycles were used in the second PCR (Fig. 1).

Overall, micrometastases were present in 36 of 192 lymph nodes (19 percent). Staining with hematoxylin and eosin and immunohistochemical staining with antibodies against cytokeratin and carcinoembryonic antigen failed to identify micrometastases in any of these lymph nodes. Fourteen patients (54 percent) had one or more nodes that were positive in our assay. Table 1 presents the findings in the lymph nodes and the clinical outcomes of the 26 patients.

Characteristics of the Patients

Table 2 shows the clinical characteristics of the 26 patients whose lymph nodes were analyzed for the presence of micrometastases. The mean age was 68 years at the time of diagnosis. The mean age of the patients with micrometastases was 73 years, as compared with 63 years for the patients without nodal spread (P=0.17). There was no significant difference in the frequency of micrometastases between tumors on the left and right sides. The histologic grade of the primary tumors did not differ significantly between the patients with and the patients without micrometastases (P=0.54). The depth of invasion was not a statistically significant factor. Lymphatic invasion was observed in four patients, all of whom had evidence of micrometastatic spread of disease. Analysis of the diameter and growth pattern of the primary tumor and preoperative serum carcinoembryonic antigen levels in both groups did not show any significant differences.

Micrometastatic Disease and Survival

By the end of our study, seven cancer-related deaths had occurred in the 14 patients with evidence of micrometastases (50 percent) whereas only one cancer-related death was observed in the 12 patients without evidence of nodal spread (8 percent). No patients were lost to follow-up, and the mean length of follow-up for the patients who were still alive was 73

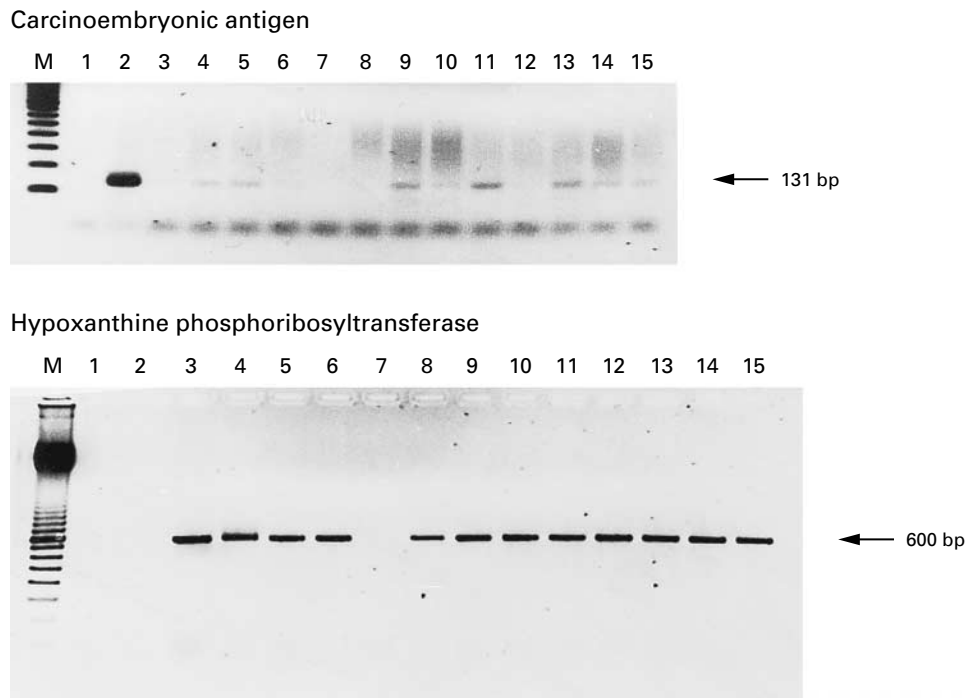


Figure 1. Nested Polymerase-Chain-Reaction (PCR) Assay for Carcinoembryonic Antigen and Hypoxanthine Phosphoribosyltransferase.

In the upper panel, lanes 4 through 15 show PCR results for lymph nodes from Patient 19. Lanes 4, 5, 9, 10, 11, 13, 14, and 15 show a PCR product of the expected length of 131 bp (carcinoembryonic-antigen gene product). This patient was therefore considered to have micrometastases. Lane 2 shows a positive control sample (a lymph node with microscopically visible metastases), and lanes 1 and 3 show negative controls: water and complementary DNA generated without reverse transcriptase, respectively. As shown in the lower panel, the same samples were analyzed for hypoxanthine phosphoribosyltransferase messenger RNA. Lanes 1 and 2 show negative controls, lane 3 a metastatic lymph node, and lanes 4 through 15 nodes from Patient 19. Lane 7 failed to show a PCR product and was therefore excluded from the final analysis. M denotes the molecular-size marker.

months (range, 66 to 85). The mean (\pm SE) observed five-year survival rate was 54 ± 10 percent and the adjusted five-year survival rate was 70 ± 10 percent.

At the end of follow-up, 9 of the 12 patients without micrometastases were still alive, as compared with 4 of the 14 patients with micrometastases. Figure 2 shows the Kaplan–Meier curves for both groups, with observed and adjusted survival. For both end points, the patients with micrometastases had a significantly worse survival rate than the patients without micrometastases. The five-year observed survival rate for the patients with micrometastases was 36 percent, as compared with 75 percent for the patients without micrometastases ($P=0.03$); the five-year adjusted survival rate was 50 percent, as compared with 91 percent ($P=0.02$ by the log-rank test), respectively.

Among the 12 patients without micrometastases, 1 had a recurrence at 15 months (8 percent); thereafter, there were no recurrences in this group. By contrast, in the group with micrometastases the recurrence rates at 36 and 60 months were 49 percent and 58 percent, respectively. According to the log-

rank test, this difference was statistically significant ($P=0.02$).

Micrometastases and Other Prognostic Variables

Analysis of micrometastases together with age, pT (pathologically confirmed tumor) category, and the presence or absence of lymphatic invasion with the Cox regression analysis showed that the differences in observed and adjusted survival were completely attributable to the presence of micrometastatic disease. Age, pT category, and lymphatic invasion were not independent prognostic factors. The relative risk of death from any cause that was associated with the presence of micrometastases was 5.0 ($P=0.03$); for cancer-related death, it was 11.7 ($P=0.03$).

DISCUSSION

Our study demonstrates that the detection of micrometastases by reverse-transcriptase–PCR amplification of carcinoembryonic antigen mRNA in lymph nodes from patients with stage II colorectal cancer may have clinical value. We found that such

TABLE 1. MOLECULAR DETECTION OF MICROMETASTASIS AND OUTCOMES OF PATIENTS WITH STAGE II COLORECTAL CANCER.

PATIENT No.	LYMPH NODES		OUTCOME		SURVIVAL	
	EXAMINED	POSITIVE	VITAL STATUS	DISEASE STATUS*	OBSERVED	ADJUSTED†
	no.				no. of mo	
1	5	0	Alive	NED	75	NA
2	5	0	Alive	NED	74	NA
3	3	0	Alive	NED	78	NA
4	5	0	Alive	NED	66	NA
5	13	0	Dead	Other	2	NA
6	8	0	Dead	Other	60	NA
7	10	0	Alive	NED	77	NA
8	12	0	Alive	NED	75	NA
9	12	0	Alive	NED	67	NA
10	11	0	Alive	NED	73	NA
11	4	0	Dead	Recurrence	21	15
12	4	0	Alive	NED	70	NA
13	10	3	Alive	NED	76	NA
14	6	1	Alive	NED	82	NA
15	3	2	Dead	Recurrence	20	13
16	6	6	Dead	Recurrence	23	16
17	14	1	Dead	Recurrence	30	29
18	16	2	Dead	Recurrence	44	30
19	11	8	Dead	Recurrence	32	29
20	4	1	Alive	NED	85	NA
21	8	1	Alive	NED	66	NA
22	10	4	Dead	Other	19	NA
23	4	2	Dead	Recurrence	28	28
24	2	1	Dead	Recurrence	65	40
25	3	3	Dead	Other	1	NA
26	3	1	Dead	Other	45	NA

*NED denotes no evidence of disease, Other death from a cause other than cancer, and Recurrence death from local or distant recurrent disease.

†NA denotes not applicable. For adjusted survival, only cancer-related deaths were considered.

micrometastases were associated with a significant reduction in the five-year survival rate, from 91 percent in patients without micrometastases to 50 percent in patients with micrometastatic disease in one or more lymph nodes.

There is a substantial literature on occult metastatic disease in colorectal cancer.⁵⁻⁸ Most studies show that micrometastases do occur, but they disagree about the prognostic significance of such a finding. In most studies, antibodies against cytokeratins and carcinoembryonic antigen were used to detect micrometastases. The differences in the choice of antibody, staining procedures, and interpretation may explain in part the discrepant results. Recent technical advances permit the detection of micrometastases at the DNA or RNA level. Known oncogenic mutations in the primary tumor can be used to detect very small amounts of tumor in lymph nodes, bone marrow aspirates, and surgical-resection mar-

TABLE 2. CLINICAL CHARACTERISTICS OF 26 PATIENTS WITH HISTOPATHOLOGICALLY NEGATIVE LYMPH NODES ANALYZED FOR THE PRESENCE OF CARCINOEMBRYONIC-ANTIGEN MESSENGER RNA.

CHARACTERISTIC	PATIENTS (N=26)	MICROMETASTASES		P VALUE*
		NEGATIVE (N=12)	POSITIVE (N=14)	
Sex (no.)				0.70
Male	10	4	6	
Female	16	8	8	
Mean age (yr)	68	63	73	0.17
Tumor side (no.)†				1.00
Right	16	7	9	
Left	10	5	5	
Differentiation (no.)				0.54
Good	7	2	5	
Moderate	17	9	8	
Poor	2	1	1	
pT category (no.)‡				0.19
pT1 and pT2	7	5	2	
pT3 and pT4	19	7	12	
Growth pattern (no.)§				0.69
Pushing	11	6	5	
Invasive	15	6	9	
Lymphatic invasion (no.)				0.10
No	22	12	10	
Yes	4	0	4	
Mean tumor size (cm)	4.42	4.12	4.67	0.27
Carcinoembryonic antigen level (no.)¶				1.00
≤6 μg per liter	19	9	10	
>6 μg per liter	5	2	3	

*P values were calculated by Fisher's exact test (two-sided) for the comparison of numbers of patients and by the Mann-Whitney U test for the comparison of means.

†Tumor side denotes the location of the tumor in relation to the flexura lienalis.

‡pT denotes pathologically confirmed tumor.

§The definitions of the growth patterns are presented in Jass et al.¹⁹

¶The carcinoembryonic antigen level refers to the preoperative serum level; levels were not determined in two cases.

gins.²⁰⁻²² Using this method, Hayashi et al. found micrometastases in lymph nodes of 52 percent of patients with Dukes' stage A and stage B tumors, in which lymph-node metastases are undetectable by routine methods.²³ However, they failed to detect mutations suitable for amplification by PCR in 49 of 120 patients, making this method unsuitable for use in a clinical setting.

A second method uses the reverse-transcriptase PCR to detect cancer-specific mRNA in tissues.^{13,24-27} Amplification of carcinoembryonic antigen mRNA has been used to detect minute amounts of tumor in the lymph nodes and bone marrow of patients with gastrointestinal tumors (at concentrations as low as 10 tumor cells in 5 × 10⁷ normal bone marrow cells).²⁶ Our study examined whether the results of this sensitive method correlate with clinical outcome.

Our data on survival are strikingly similar to those in recent studies of the loss of heterozygosity of

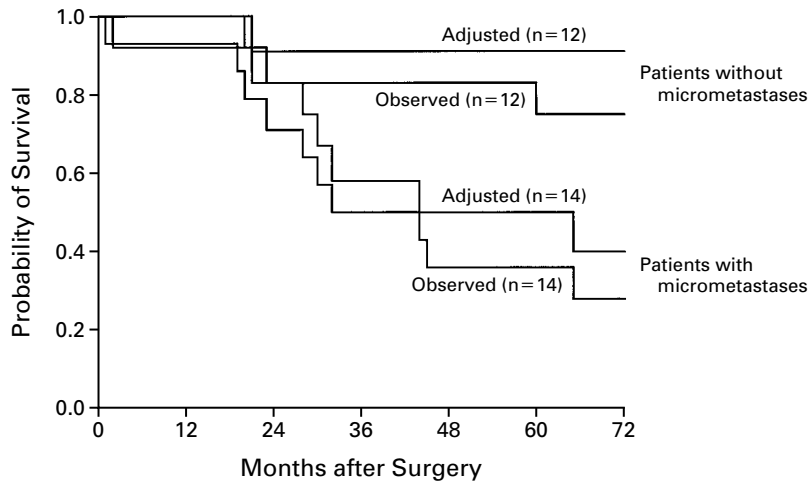


Figure 2. Kaplan-Meier Life-Table Analysis of the Observed and Adjusted Survival of Patients with Stage II Colorectal Cancer According to the Presence or Absence of Micrometastases.

For observed survival, all deaths were defined as events, whereas for adjusted survival only cancer-related deaths were considered. The patients with micrometastases had a significantly worse prognosis. The observed five-year survival rate for patients with micrometastases was 36 percent, as compared with 75 percent for those without micrometastases ($P=0.03$), and the adjusted five-year survival rate was 50 percent as compared with 91 percent ($P=0.02$).

chromosome 18q and the expression of the DCC (deleted in colorectal cancer) protein in stage II and stage III colorectal tumors.^{28,29} Survival decreased from 93 percent to 54 percent in patients with stage II tumors with loss of heterozygosity and from 94 percent to 61 percent in patients with stage II tumors with loss of DCC expression. Although these findings are clinically relevant, the biologic basis for them is unknown. The similarity of the prognostic information obtained by the analysis of DCC and the detection of micrometastases by reverse-transcriptase PCR suggests that the inactivation of the gene for DCC or an additional gene on chromosome 18q results in the early dissemination of tumor cells to locoregional lymph nodes. This idea is compatible with the association of loss of chromosome 18q with distant metastases.³⁰ In any case, it seems clear that patients with TNM stage II colorectal cancer are a heterogeneous group. Fifty percent have an excellent prognosis, whereas in the other half the prognosis is similar to that of patients with stage III disease. It is possible that these groups can be distinguished by an analysis of micrometastatic disease in lymph nodes.

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