Imaging, Diagnosis, Prognosis

Prognostic Relevance of Occult Nodal Micrometastases and Circulating Tumor Cells in Colorectal Cancer in a Prospective Multicenter Trial

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Abstract

Purpose: Nodal micrometastasis and circulating tumor cells detected by multimarker quantitative real-time reverse transcription-PCR (qRT-PCR) may have prognostic importance in patients with colorectal cancer.

Experimental Design: Paraffin-embedded sentinel lymph nodes from 67 patients and blood from 34 of these patients were evaluated in a prospective multicenter trial of sentinel lymph node mapping in colorectal cancer. Sentinel lymph nodes were examined by H&E staining and cytokeratin immunohistochemistry. Sentinel lymph nodes and blood were examined by a four-marker qRT-PCR assay (c-MET, melanoma antigen gene-A3 family, $\beta 1 \rightarrow 4$-*N*-acetylgalactosaminyltransferase, and cytokeratin-20); qRT-PCR results were correlated with disease stage and outcome.

Results: In H&E-negative sentinel lymph node patients that recurred, cytokeratin immunohistochemistry and qRT-PCR detected metastasis in 30% and 60% of patients, respectively. Disease-free survival differed significantly by multimarker qRT-PCR upstaged sentinel lymph node ($P = 0.014$). qRT-PCR analysis of blood for circulating tumor cells correlated with overall survival ($P = 0.040$).

Conclusion: Molecular assessment for micrometastasis in sentinel lymph node and blood specimens may help identify patients at high risk for recurrent colorectal cancer, who could benefit from adjuvant therapy.

Colorectal carcinoma is a common gastrointestinal malignancy in the United States and remains the second most common cause of cancer mortality (1). The 5-year survival rate is ~90% for patients with localized disease and ~66% with regional disease as determined at diagnosis (2). There is a 25% incidence of disease recurrence in the absence of regional node involvement, suggesting that conventional pathology may fail to detect occult nodal metastases (3). Adjuvant therapy improves survival in as many as one third of patients with stage III disease (4); however, there is no consistent evidence that adjuvant therapy improves survival in node-negative (stage I or II) disease (5). Lymph node evaluation is essential for accurate staging and improves the selection of patients for adjuvant therapy (6).

Current techniques for nodal evaluation are inadequate for the detection of micrometastases; more sensitive techniques, such as multilevel step sectioning and intraoperative sentinel lymph node mapping, have therefore been applied to solid malignancies. The sentinel lymph node is the lymph node that has the highest probability of harboring metastatic tumor cells from a primary tumor (7–10); this concept has been validated in melanoma and breast cancers. Sentinel lymph node evaluation allows a focused examination for the detection of occult metastases not detected by conventional techniques. In colorectal cancer, we have shown that the assessment of sentinel lymph nodes using multilevel sectioning and cytokeratin immunohistochemistry detects micrometastasis in 23% of patients whose nodal specimens are tumor-free as determined by routine H&E staining (11, 12). We have previously shown the utility of molecular markers for the detection of occult metastasis in colorectal cancer histopathology–negative frozen sentinel lymph nodes (10, 13). Focused analysis of the sentinel lymph node can therefore provide a more efficient approach for the detection of micrometastases and improve patient management (10, 13, 14).

The development of a quantitative real-time reverse transcription-PCR (qRT-PCR) assay has allowed rapid quantitative detection of occult metastatic tumor cells in lymph nodes and blood (15–17). A multimarker assay is more sensitive than

Translational Relevance

The study described provides, for the first time in colorectal cancer, sentinel lymph node analysis that molecular upstaging has significant prognostic value in disease outcome. The novelty of the study is that the molecular upstaging was done by multimarker mRNA markers on paraffin-embedded tissue sections of sentinel lymph node. The study also showed that detection of circulating tumor cells in sentinel lymph node patients by multiple mRNA markers had prognostic utility for disease outcome. The importance of the study is that molecular upstaging with specific mRNA markers is of clinical utility in the sentinel lymph node and blood of colorectal cancer patients. Our approach shows that molecular diagnosis of colorectal cancer patients' sentinel lymph node may be of significance in improving staging. Identification of circulating tumor cells in colorectal cancer patients during primary tumor removal may be of utility in determining those patients likely to have distant disease recurrence. The diagnosis of early-stage occult disease in sentinel lymph node by molecular approaches may improve overall management of colorectal cancer patients and identify patients not likely to recur.

single-marker assays for the detection of occult tumors (18–21). In this study, we evaluated four mRNA markers: c-MET (hepatocyte growth factor receptor), MAGE-A3 (melanoma antigen gene-A3 family), GalNAc-T (β1→4-*N*-acetylgalactosaminyltransferase), and CK20 (cytokeratin-20). c-MET is a proto-oncogene encoding the receptor for hepatocyte growth factor; c-MET mRNA overexpression is associated with tumor invasion and regional lymph node metastasis in colorectal cancer (15, 22). MAGE-A3 is a commonly expressed tumor antigen in various tumors but not in normal tissue, except male germline cells (23, 24). GalNAc-T is an enzyme involved in specific carbohydrate molecule synthesis (17, 25). CK20, a member of the CK family, has been used for detection of occult tumor cells in lymph nodes and blood (26–28).

Circulating tumor cells (CTC) in the blood are essential for the formation of distant metastasis, and the analysis of these CTC can be used to depict real-time tumor spreading (16–18, 20, 21). We introduced the multimarker qRT-PCR assay for detection of circulating tumor cells in blood and showed the relation of circulating tumor cell detection, disease stage, and treatment outcome in various cancers (16–18, 20, 21, 29). In this study, we conducted a multimarker qRT-PCR assay to detect metastasis, hypothesizing that a multimarker qRT-PCR assay for assessing sentinel lymph node and/or blood can be used as a prognostic surrogate for micrometastasis and disease outcome.

Patients and Methods

Patients. The 67 patients included in the qRT-PCR analysis were a subset of 148 patients enrolled in a prospective multicenter trial of sentinel lymph node mapping for colorectal cancer. A more detailed description of the clinical study was recently reported (3). The sentinel lymph node was identified in 146 (99%) patients. However, only those patients with available and confirmed paraffin-embedded sentinel lymph node were included in the qRT-PCR analysis. Patients were seen at one of five participating centers (John Wayne Cancer Institute, California; Century City Hospital, California; University of Colorado Health Science Center, Colorado; Wake Forest University, North Carolina; and Michigan State University, Michigan), and all had given signed consent for the specimen accrual and analysis in accordance with institutional review board guidelines.

Sentinel lymph node mapping and histopathologic examination. Sentinel lymph node mapping was done during standard surgical resection, as previously described (3, 11, 12). The colon was mobilized, and isosulfan blue dye (Lymphazurin, U.S. Surgical Corporation) was injected subserosally around the tumor. Each blue-stained lymph node was identified as a sentinel lymph node. A colectomy that included all blue-stained lymph nodes was done in standard fashion, and the en bloc specimen was sent to pathology.

Each tagged sentinel lymph node was harvested and paraffin embedded individually in a tissue block cassette, and multiple sections of each paraffin-embedded specimen were examined by routine H&E and cytokeratin immunohistochemistry (12). Cytokeratin immunohistochemistry was done with a pan-specific antibody cocktail (AE1/AE3, DAKO).

Tumor deposits within lymph nodes were classified and staged according to American Joint Committee on Cancer (AJCC) guidelines (30). Macrometastases were >2 mm; micrometastases were ≤2 mm; and isolated tumor cells were single tumor cells or cell clusters measuring ≤0.2 mm that were almost always detected by immunohistochemistry. Pathologic examination was done in a blinded fashion without clinicopathology knowledge.

Colorectal cancer cell lines and control specimens for qRT-PCR. Eight colorectal cancer cell lines were used: HT-29, SW480, and SW620 were obtained from American Type Culture Collection, and cell lines of colorectal cancer A, B, C, D, and E were established at John Wayne Cancer Institute. Cells were grown and harvested for mRNA analysis, as previously described (16). Other specimens used for optimization of qRT-PCR assay included 10 paraffin-embedded primary colorectal cancer tumors, 6 paraffin-embedded colorectal cancer–involved lymph nodes, benign lymph nodes from 8 noncancer patients, and peripheral blood leukocytes from 47 healthy donors.

RNA isolation and qRT-PCR assay. All sentinel lymph node and blood specimens were coded by a computer-generated number. Processing of sentinel lymph node and blood specimens, RNA extraction, reverse transcription-PCR setup, and post–reverse transcription-PCR product analysis were set up as previously described (15, 24).

For sentinel lymph node multimarker qRT-PCR assay, five to eight 10-μm sections were cut from paraffin-embedded sentinel lymph node; a sterile microtome blade was used for each specimen. Sections were deparaffinized and digested with proteinase K for 3 h before RNA extraction using a modified RNAWiz protocol (Ambion; ref. 31).

For circulating tumor cell assay, peripheral blood specimens were collected in 2×4.5 mL sodium citrate–containing tubes before surgery (16). Total cells in blood were collected using Purescript RBC lysis solution (Gentra), and Tri-Reagent (Molecular Research Center) was used to isolate total cellular RNA (16). Total cellular RNA was quantified and assessed for purity by UV spectrophotometry and RiboGreen assay (Molecular Probes).

Reverse transcription reactions were done using Moloney murine leukemia virus reverse transcriptase (Promega) with both oligodeoxythimidylate and random primers (24). The qRT-PCR assay was done to assess the presence of c-MET, MAGE-A3, GalNAc-T, and CK20 mRNA. The sensitivity and specificity of each marker for occult metastasis in sentinel lymph node and blood have previously been described (10, 16, 24). ABI Prism 7900HT Detection System (Applied Biosystems) was used for detection of markers. Primer and probe sequences were designed for qRT-PCR assay. Fluorescence resonance energy transfer

Table 1. Upstaging by cytokeratin immuno-histochemistry and qRT-PCR within H&E-negative sentinel lymph node

Detection method	Detection, *n* (%)*	
	Yes	**No**
CK-IHC	3 (30)	7 (70)
qRT-PCR	6 (60)	4 (40)
CK-IHC or qRT-PCR(+)	7 (70)	3 (30)

Abbreviation: CK-IHC, cytokeratin immunohistochemistry.
*Ten of 58 (17%) H&E-negative sentinel lymph node patients who recurred.

probe sequences were as follows: c-MET, 5′-FAM-TGGGAGCTGATGA-CAAGAGGAG-BHQ-1-3′; MAGE-A3, 5′-FAM-AGCTCCTGCCCACACTC-CCGCCTGT-BHQ-1-3′; GalNAc-T, 5′-FAM-ATGAGGCTGCTTTCACTAT-CCGCA-BHQ-1-3′; CK20, 5′-FAM-ATCAGTTAAGCACCCT-GGAAGAGAG-BHQ-1-3′; and glyceraldehyde-3-phosphate dehydrogenase, 5′-FAM-CAGCAATGCCTCCTGCACCACCAA-BHQ-1-3′. cDNA (200 ng RNA) was added to a 384-well PCR microplate containing 0.5 μmol/L primer, 0.3 μmol/L probe, and 5 μL of iTaq custom supermix with ROX (Bio-Rad). Samples were amplified with a precycling hold at 95°C for 10 min, followed by denaturation at 95°C for 15 s. Annealing or extension was carried out for 1 min at the following temperatures: 55°C for c-MET and glyceraldehyde-3-phosphate dehydrogenase, 56°C for CK20, 58°C for MAGE-A3, and 62°C for GalNAc-T. The standard curve was generated using threshold cycles of the plasmid template dilutions for each gene (10^6-10^1 copies). Sample threshold cycle was interpolated from the standard curve to calculate mRNA copies.

Each qRT-PCR assay was done at least twice and included marker-positive, marker-negative, and reagent controls (reagent without RNA or cDNA). If only one of the duplicates gave a positive result, we conducted a third qRT-PCR assay to confirm the results. *GAPDH* gene was used as a housekeeping gene. Any specimen with inadequate glyceraldehyde-3-phosphate dehydrogenase mRNA was excluded.

Statistical analysis. χ^2 analysis (Cohen's κ) was used to assess agreement between any two of the four qRT-PCR markers and between marker detection in sentinel lymph nodes and marker detection in blood. Mantel-Haenszel χ^2 was used to assess the correlation between marker detection and the size of sentinel lymph node metastasis, the tumor pathology stage, and AJCC stage. Age, gender, and tumor-node-metastasis (TNM) staging were compared between the study sample and the remaining patients in the prospective multicenter trial using χ^2 analyses and a Wilcoxon test.

Multinomial logistic regression was used to assess the ability of qRT-PCR detection in sentinel lymph node to predict TNM staging after controlling for age and gender. The log-rank test was used to examine disease-free survival and overall survival according to marker detection

in sentinel lymph node and blood. Survival curves were generated using the Kaplan-Meier method. Cox proportional hazards models were generated for overall survival and disease-free survival to estimate the prognostic significance of marker detection in sentinel lymph node and blood after controlling for age, gender, and disease characteristics. Analyses were done using SPSS statistical software, and all tests were two-sided with a significance level of ≤0.05.

Results

Patients for qRT-PCR study. There were 74 patients from the trial who were eligible and consented for molecular studies based on sentinel lymph node availability from participating investigators. Seven patients were excluded from the current analysis: six patients had benign tumors and one patient's sentinel lymph node was not identified. The 67 remaining patients included 33 males and 34 females with a median age of 74 years (range, 35-95). The tumor stage distribution was as follows: T_1, 10 patients (15%); T_2, 12 patients (18%); T_3, 45 patients (67%). Blood samples from 34 of 67 patients were available for the circulating tumor cell assay. Age, gender, and TNM staging did not differ significantly between the 67 patients, and the remainder of the patients in the prospective multicenter trial was not studied.

Standard curves and specificity of multimarker qRT-PCR assay. The standard curves showed the expected linear increase of signal with logarithm of the copy number (data not shown). PCR efficiency assessed from the standard curves was between 90% and 100%. The *r* for all standard curves (threshold cycle versus log copy number) in the study was ≥0.99. Each of the eight colorectal cancer lines expressed all four markers. Each of the 10 primary tumor specimens and tumor-involved lymph nodes expressed the individual markers. No markers were detected in peripheral blood leukocytes from 47 healthy donors or in control tissues under the optimized conditions. Individual marker sensitivity: detection in one to five colorectal cancer cells diluted in 10^7 healthy donor peripheral blood leukocytes.

Metastasis detection in sentinel lymph node by qRT-PCR. Nodal qRT-PCR assay detected ≥1 marker in 27 of 67 (40%) patients; 11 (16%) patients had 1 marker and 16 (24%) had >1 marker. Patients whose sentinel lymph nodes expressed a specific marker was 9 (13%) for c-MET, 12 (18%) for MAGE-A3, 13 (19%) for GalNAc-T, and 13 (19%) for CK20. The predictive value of the qRT-PCR assay was 90%; 4 (10%) recurrences were evidenced among 40 qRT-PCR(-) patients. Concurrent detection was significant for c-MET and CK20 ($P < 0.001$) and for MAGE-A3 and GalNAc-T ($P < 0.001$).

The prognostic utility of cytokeratin immunohistochemistry and qRT-PCR detection in the sentinel lymph node, based on

Table 2. Relation between qRT-PCR multimarker detection and metastasis size in sentinel lymph node

SLN	No. of qRT-PCR markers detected in SLN (%)			*P*
	≥2	**1**	**0**	
Macro (*n* = 10)	5 (50)	3 (30)	2 (20)	<0.001
Micro (*n* = 3)	2 (67)	1 (33)	0 (0)	
ITC (*n* = 17)	5 (29)	1 (6)	11 (65)	
Negative (*n* = 37)	4 (11)	6 (16)	27 (73)	

Abbreviations: SLN, sentinel lymph node; ITC, isolated tumor cells.

Table 3. Relation between qRT-PCR multimarker detection in sentinel lymph node and pN stage

pN stage	No. of qRT-PCR markers detected in SLN (%)			P
	≥2	1	0	
N_0 ($n = 47$)	7 (15)	6 (13)	34 (72)	<0.001
N_1 ($n = 15$)	6 (40)	3 (20)	6 (40)	
N_2 ($n = 5$)	3 (60)	2 (40)	0 (0)	

upstaging within those found negative by conventional pathologic examination (H&E), is shown in Table 1. Of 12 total recurrences, 2 (17%) were detected by H&E; 3 (30%) of the remaining 10 recurrences were detected by cytokeratin immunohistochemistry, 6 (60%) were detected by the qRT-PCR assay, and a total of 70% upstaging was evidenced using cytokeratin immunohistochemistry and qRT-PCR detection methods.

To show the relationship between detection by the qRT-PCR assay and standard clinical indicators, we compared the number of markers detected by qRT-PCR with sentinel lymph node metastasis size and TNM staging. The number of markers detected by qRT-PCR increased with the size of sentinel lymph node metastasis ($P < 0.001$; Table 2). Of 13 patients with macrometastasis ($n = 10$) or micrometastasis (3 patients), 11 (85%) had ≥1 marker and 7 (54%) had ≥2 markers. The marker detection rate was significantly higher for patients with AJCC N_1 disease than for patients with N_0 disease ($P < 0.001$). Table 3 shows the correlation between nodal stage and the number of markers detected by qRT-PCR assay ($P < 0.001$). Marker detection in sentinel lymph nodes significantly increased in patients with regional lymph node metastasis ($P < 0.001$).

Next, we assessed the correlation between markers detected in sentinel lymph node and AJCC stage (Table 4). Of 20 stage I patients, 16 (80%) showed no markers and 2 (10%) had ≥2 markers. In contrast, 14 (70%) patients with stage III disease had ≥1 marker and 6 (30%) had no markers. Marker detection in sentinel lymph nodes significantly increased with AJCC stage of disease ($P < 0.01$) but was not correlated with pT stage. Finally, multivariate analysis indicated that, after controlling for age and gender, qRT-PCR detection in sentinel lymph node was significantly predictive of N stage (odds ratio for stage N_1 or N_2, 8.1; $P < 0.01$) and AJCC stage (stage III odds ratio, 13.0; $P < 0.01$).

Circulating tumor cell detection in blood by qRT-PCR. Of 67 patients, 34 had blood available for the multimarker qRT-PCR assay. Overall, 16 (47%) patients expressed ≥1 mRNA marker, and 18 (53%) expressed no markers. The number of specimens that expressed a marker was 6 (18%) for c-MET, 4 (12%) for MAGE-A3, 7 (21%) for GalNAc-T, and 2 (6%) for CK20. There was no significant concordance for any two markers.

Results of blood qRT-PCR showed no correlation with pN, pT, or AJCC stage (data not shown). The number of blood specimens that expressed a marker was 3 (19%), 1 (6%), and 12 (75%) for patients with T_1, T_2, and T_3 primary tumors, respectively. MAGE-A3 and CK20 were not detected in blood from patients with T_1 or T_2 tumors. Three of a total of six patients identified with primary tumor vascular invasion had markers detected in the blood.

Histopathologic assessment of sentinel lymph nodes identified macrometastasis, micrometastasis, and isolated tumor cells in 4 (12%), 1 (3%), and 3 (9%), respectively, of the 34 patients whose blood was assessed by qRT-PCR. Of 14 patients without nodal metastasis, 6 (43%) had blood that expressed none of the markers and 8 (57%) had blood that expressed ≥1 mRNA marker. Of 6 patients, 4 (67%) patients with macrometastases and one third (33%) of patients with micrometastases expressed ≥1 marker. The number of markers in blood showed no correlation with sentinel lymph node metastasis size.

Of 16 patients whose blood expressed ≥1 marker, 7 (44%) had sentinel lymph nodes that also expressed ≥1 marker; five of these patients had T_3 tumors and were cytokeratin immunohistochemistry positive, and the other two had stage I colorectal cancer and were cytokeratin immunohistochemistry negative. Of 18 patients whose blood expressed no markers, 6 (33%) had sentinel lymph nodes that expressed ≥1 marker. There was no correlation between marker expression in sentinel lymph nodes and blood.

Relation between marker detection and survival. At a median follow-up of 34 months, a log-rank test indicated no significant difference in overall survival curves by marker detection in sentinel lymph node. The mean overall survival was 43 months [95% confidence interval (95% CI), 37-50] for qRT-PCR(+) sentinel lymph nodes ($n = 27$) and 57 months (95% CI, 50-64) for qRT-PCR(-) sentinel lymph nodes ($n = 40$; Fig. 1A). In addition, qRT-PCR(+) ($n = 27$) in sentinel lymph node was not found to be prognostic of overall survival after controlling for age, gender, and TNM staging (hazard ratio, 1.0; $P > 0.050$). However, a log-rank test revealed a significant difference in disease-free survival by marker detection in sentinel lymph node ($P = 0.014$; Fig. 1B). The mean disease-free survival was 37 months (95% CI, 29-45) for qRT-PCR(+) sentinel lymph node and 61 months (95% CI, 56-67) for qRT-PCR(-) sentinel lymph node. Detection of qRT-PCR(+) in sentinel lymph node was further shown to have prognostic value for disease-free survival independent of age, gender, and TNM staging (hazard ratio, 4.9; $P = 0.027$). Of the four markers, two were also found to be independently prognostic (MAGE-A3 hazard ratio, 5.1; $P = 0.013$; GalNAc-T hazard ratio, 4.6; $P = 0.012$).

A significant difference was found in overall survival curves by qRT-PCR detection of circulating tumor cells in blood ($P = 0.040$; Fig. 2). The mean overall survival was 36 months (95% CI, 26-46) for qRT-PCR(+) blood ($n = 16$), as compared with 50 months (95% CI, 44-56) for qRT-PCR(-) blood ($n = 19$). A Cox proportional hazards model indicated that marker detection in blood has independent prognostic value for overall survival beyond age, gender, and TNM staging (hazard ratio, 12.3; $P = 0.045$).

Table 4. Relation between qRT-PCR multimarker detection in sentinel lymph node and AJCC stage

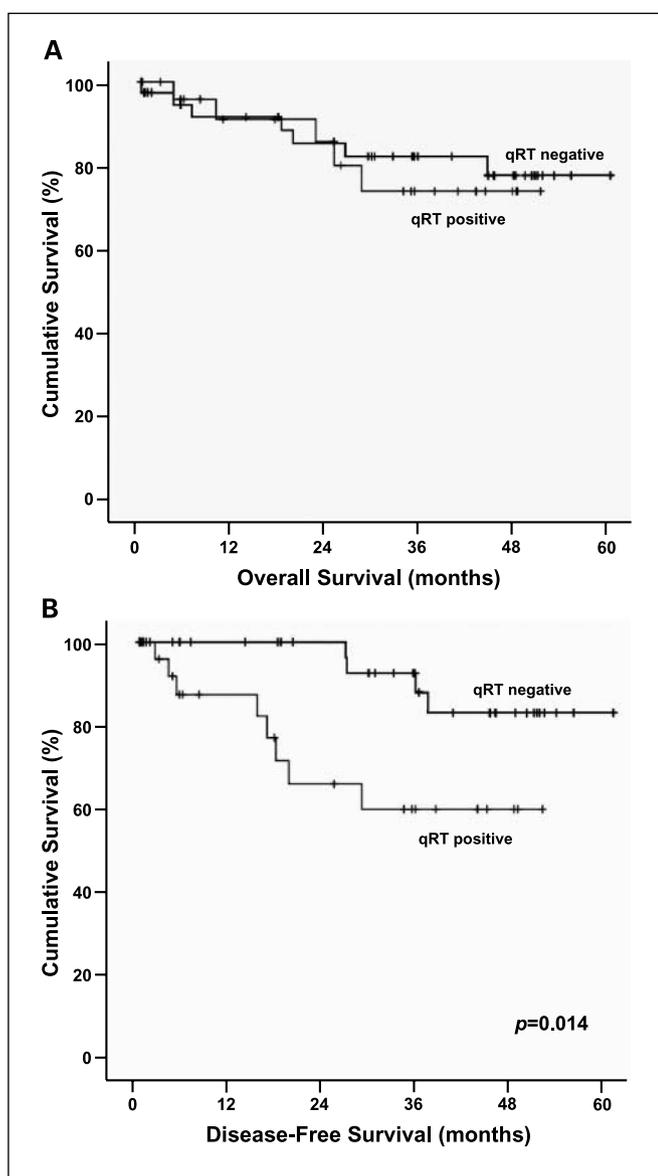| AJCC stage	No. of qRT-PCR markers detected in SLN (%)			P
	≥2	1	0	
I ($n = 20$)	2 (10)	2 (10)	16 (80)	<0.01
II ($n = 27$)	5 (19)	4 (15)	18 (67)	
III ($n = 20$)	9 (45)	5 (25)	6 (30)	

A

B

Fig. 1. Kaplan-Meier curves for disease outcome according to detection of at least one qRT-PCR (*qRT*) marker in sentinel lymph nodes. *A,* overall survival by qRT-PCR(+) in sentinel lymph nodes (not significant). *B,* disease-free survival by qRT-PCR(+) in sentinel lymph nodes (*P* = 0.014).

Discussion

Distant metastasis of node-negative colorectal cancer may be explained by failure to detect occult nodal metastasis in the resected colorectal cancer specimen (32, 33). This problem has been mitigated by introduction of sentinel lymph node mapping, which targets a few tumor-draining nodes for focused analysis, thereby increasing the accuracy and decreasing pathology costs. Although sentinel lymph node mapping does not decrease the extent of nodal resection in colorectal cancer, its use can improve nodal staging and identify patients who are likely to benefit from adjuvant chemotherapy (32).

Marker mRNA expression in the sentinel lymph node was correlated with the size of sentinel lymph node metastasis, the histopathologic status of the regional lymph nodes, AJCC stage, and disease-free survival. In contrast, mRNA marker circulating

tumor cell detection was correlated with stage of the primary tumor and with overall survival but not with nodal stage. The absence of a significant correlation between results of nodal and blood qRT-PCR assays suggests that metastasis from primary colorectal cancer may occur via lymphatic and/or hematogenous pathways. This further suggests that the qRT-PCR assessment of both nodal and blood specimens may have more clinical relevance. However, there was a 50% correlation between primary tumor vascular invasion and circulating tumor cell detection.

Because metastatic tumors are heterogeneous in marker expression, cytokeratin immunohistochemical staining may have limited sensitivity for the detection of occult tumor cells. As we previously showed, the combination of markers can compensate for variations in individual marker expression, increasing the detection of tumor cells (18). qRT-PCR positivity based on four markers in nodal or blood specimens was higher than qRT-PCR positivity based on any single marker. These findings confirm the limited clinical utility of assays using a single circulating tumor cell marker (18, 34, 35).

Results of nodal qRT-PCR assay revealed expression of ≥1 marker in 29 of 67 (43%) patients. Of the 14 patients with histopathologic evidence of sentinel lymph node metastasis, 12 had positive nodal qRT-PCR results, and the number of markers increased with the size of sentinel lymph node metastasis. In 17 patients with isolated tumor cells, only 6 (35%) had positive qRT-PCR markers. Negative results in the remaining 11 cases may have reflected a failure of isolated tumor cells to express any of the four mRNA markers; alternatively, the portion of the node submitted for molecular analysis might not have been the portion that contained isolated tumor cells.

The qRT-PCR assay may be of value when cytokeratin immunohistochemistry fails to detect sentinel lymph node micrometastasis. Although the clinical significance of immunohistochemistry-detected micrometastases remains subject to question, we found that the results of qRT-PCR were correlated not only with pN and AJCC stage but also with disease-free survival. The number of qRT-PCR(+) sentinel lymph node
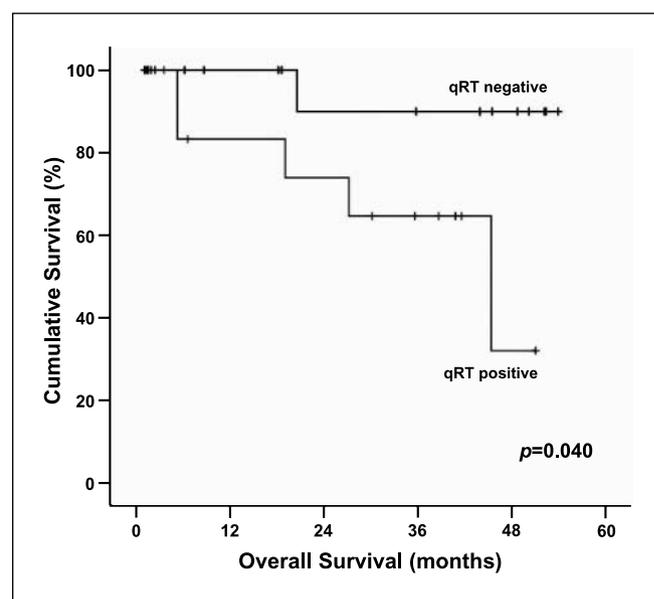

Fig. 2. Kaplan-Meier curves: qRT-PCR marker detection in blood. Overall survival by qRT-PCR(+) in blood (*P* = 0.040).

patients who have not recurred may reflect the median duration of follow-up and/or the clinical effect of early resection. For this stage of disease, a longer follow-up may be needed to obtain the true value of the molecular upstaging as shown in the melanoma sentinel lymph node studies (24).

Recent studies suggest the promise of qRT-PCR assay for assessment of subclinical disease and response to treatment (16, 36). In general, vascular invasion of the primary tumor is the first step in the metastatic cascade, and the degree and intensity of vascular invasion usually depend on the characteristics of the primary lesion. This would explain the correlation between qRT-PCR evidence of circulating tumor cells and pathologic stage of the primary tumor. Results of blood qRT-PCR also confirmed recent reports of a correlation between circulating tumor cells and disease outcome (17, 36). Circulating tumor cells had a significant inverse correlation with overall survival, suggesting that the assessment of blood may have prognostic importance. Larger studies with long-term follow-up are needed to determine the clinical significance of circulating tumor cells in early-stage colorectal cancer.

In conclusion, qRT-PCR assessment of sentinel lymph nodes that drain primary colorectal cancer can detect nodal micrometastases missed by conventional pathologic examination. In addition, the multimarker qRT-PCR assay has independent prognostic significance for circulating tumor cell detection in blood of patients with colorectal cancer. The concurrent assessment of blood and sentinel lymph node may help identify candidates for adjuvant therapy. Subsequently, serial sampling and assessment of blood could prove useful for monitoring the response to therapy.

Disclosure of Potential Conflicts of Interest

Acknowledgments

References

1. Jemal A, Siegel R, Ward E, Murray T, Xu J, Thun MJ. Cancer statistics, 2007. CA Cancer J Clin 2007;57:43–66.
2. Cancer Stat Fact Sheet. Surveillance Epidemiology and End Results, 2007.
3. Bilchik AJ, Hoon DS, Saha S, et al. Prognostic impact of micrometastases in colon cancer: interim results of a prospective multicenter trial. Ann Surg 2007;246:568–77.
4. Punt CJ, Buyse M, Kohne CH, et al. Endpoints in adjuvant treatment trials: a systematic review of the literature in colon cancer and proposed definitions for future trials. J Natl Cancer Inst 2007;99:998–1003.
5. Wolpin BM, Meyerhardt JA, Mamon HJ, Mayer RJ. Adjuvant treatment of colorectal cancer. CA Cancer J Clin 2007;57:168–85.
6. 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;99:433–41.
7. Morton DL, Wen DR, Wong JH, et al. Technical details of intraoperative lymphatic mapping for early stage melanoma. Arch Surg 1992;127:392–9.
8. Giuliano AE, Kirgan DM, Guenther JM, Morton DL. Lymphatic mapping and sentinel lymphadenectomy for breast cancer. Ann Surg 1994;220:391–8.
9. Saha S, Wiese D, Badin J, et al. Technical details of sentinel lymph node mapping in colorectal cancer and its impact on staging. Ann Surg Oncol 2000;7:120–4.
10. Bilchik AJ, Saha S, Wiese D, et al. Molecular staging of early colon cancer on the basis of sentinel node analysis: a multicenter phase II trial. J Clin Oncol 2001;19:1128–36.
11. Bilchik AJ, Nora DT, Sobin LH, et al. Effect of lymphatic mapping on the new tumor-node-metastasis classification for colorectal cancer. J Clin Oncol 2003;21:668–72.
12. Bilchik AJ, DiNome M, Saha S, et al. Prospective multicenter trial of staging adequacy in colon cancer: preliminary results. Arch Surg 2006;141:527–33; discussion 533–524.
13. Bilchik AJ, Nora D, Tollenaar RA, et al. Ultrastaging of early colon cancer using lymphatic mapping and molecular analysis. Eur J Cancer 2002;38:977–85.
14. Martinez SR Bilchik AJ. Lymphatic mapping and sentinel node analysis in colon cancer. Clin Colorectal Cancer 2005;4:320–4.
15. Takeuchi H, Bilchik A, Saha S, et al. c-MET expression level in primary colon cancer: a predictor of tumor invasion and lymph node metastases. Clin Cancer Res 2003;9:1480–8.
16. Koyanagi K, Kuo C, Nakagawa T, et al. Multimarker quantitative real-time PCR detection of circulating melanoma cells in peripheral blood: relation to disease stage in melanoma patients. Clin Chem 2005;51:981–8.
17. Koyanagi K, O'Day SJ, Gonzalez R, et al. Serial monitoring of circulating melanoma cells during neoadjuvant biochemotherapy for stage III melanoma: outcome prediction in a multicenter trial. J Clin Oncol 2005;23:8057–64.
18. Hoon DS, Wang Y, Dale PS, et al. Detection of occult melanoma cells in blood with a multiple-marker polymerase chain reaction assay. J Clin Oncol 1995;13:2109–16.
19. Sarantou T, Chi DD, Garrison DA, et al. Melanoma-associated antigens as messenger RNA detection markers for melanoma. Cancer Res 1997;57:1371–6.
20. Hoon DS, Bostick P, Kuo C, et al. Molecular markers in blood as surrogate prognostic indicators of melanoma recurrence. Cancer Res 2000;60:2253–7.
21. Wascher RA, Morton DL, Kuo C, et al. Molecular tumor markers in the blood: early prediction of disease outcome in melanoma patients treated with a melanoma vaccine. J Clin Oncol 2003;21:2558–63.
22. Trusolino L Comoglio PM. Scatter-factor and semaphorin receptors: cell signalling for invasive growth. Nat Rev Cancer 2002;2:289–300.
23. Miyashiro I, Kuo C, Huynh K, et al. Molecular strategy for detecting metastatic cancers with use of multiple tumor-specific MAGE-A genes. Clin Chem 2001;47:505–12.
24. Takeuchi H, Morton DL, Kuo C, et al. Prognostic significance of molecular upstaging of paraffin-embedded sentinel lymph nodes in melanoma patients. J Clin Oncol 2004;22:2671–80.
25. Kuo CT, Bostick PJ, Irie RF, Morton DL, Conrad AJ, Hoon DS. Assessment of messenger RNA of β1→4-N-acetylgalactosaminyl-transferase as a molecular marker for metastatic melanoma. Clin Cancer Res 1998;4:411–8.
26. Merrie AE, van Rij AM, Dennett ER, Phillips LV, Yun K, McCall JL. Prognostic significance of occult metastases in colon cancer. Dis Colon Rectum 2003;46:221–31.
27. Rosenberg R, Hoos A, Mueller J, et al. Prognostic significance of cytokeratin-20 reverse transcriptase polymerase chain reaction in lymph nodes of node-negative colorectal cancer patients. J Clin Oncol 2002;20:1049–55.
28. Schuster R, Max N, Mann B, et al. Quantitative real-time RT-PCR for detection of disseminated tumor cells in peripheral blood of patients with colorectal cancer using different mRNA markers. Int J Cancer 2004;108:219–27.
29. Nakagawa T, Martinez SR, Goto Y, et al. Detection of circulating tumor cells in early-stage breast cancer metastasis to axillary lymph nodes. Clin Cancer Res 2007;13:4105–10.
30. Greene FL, Page DL, Fleming ID, et al. Cancer staging manual. 6th ed. New York: Springer; 2002.
31. Kim J, Reber HA, Hines OJ, et al. The clinical significance of MAGEA3 expression in pancreatic cancer. Int J Cancer 2006;118:2269–75.
32. Saha S, Seghal R, Patel M, et al. A multicenter trial of sentinel lymph node mapping in colorectal cancer: prognostic implications for nodal staging and recurrence. Am J Surg 2006;191:305–10.
33. Koyanagi K, Mori T, O'Day SJ, Martinez SR, Wang HJ, Hoon DS. Association of circulating tumor cells with serum tumor-related methylated DNA in peripheral blood of melanoma patients. Cancer Res 2006;66:6111–7.
34. Jung FA, Buzaid AC, Ross MI, et al. Evaluation of tyrosinase mRNA as a tumor marker in the blood of melanoma patients. J Clin Oncol 1997;15:2826–31.
35. Glaser R, Rass K, Seiter S, Hauschild A, Christophers E, Tilgen W. Detection of circulating melanoma cells by specific amplification of tyrosinase complementary DNA is not a reliable tumor marker in melanoma patients: a clinical two-center study. J Clin Oncol 1997;15:2818–25.
36. Keilholz U, Goldin-Lang P, Bechrakis NE, et al. Quantitative detection of circulating tumor cells in cutaneous and ocular melanoma and quality assessment by real-time reverse transcriptase-polymerase chain reaction. Clin Cancer Res 2004;10:1605–12.