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

Kazuo Koyanagi,1 Anton J. Bilchik,3 Sukamal Saha,3 Roderick R. Turner,2 David Wiese,3 Martin McCarter,4 Perry Shen,5 Linda Deacon,6 David Elashoff,6 and Dave S.B. Hoon1

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, β1-4-N-acetylgalactosaminytransferase, 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

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Lymph node and/or blood can be used as a prognostic marker that a multimarker qRT-PCR assay for assessing 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 micromtome blade was used for each specimen. Sections were deparaffinized and digested with proteinase K for 3 h before RNA extraction using a modified RINaWiz 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 Puscript BSC lysis solution (Genta), 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 oligo-dexy-thimidilate 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
probe sequences were as follows: c-MET, 5′-FAM-TGGGAGCTGATGACAGAGGAG-BHQ-1-3′; MAGE-A3, 5′-FAM-AGCTCTGGCCACACTGCCCCT-GT-BHQ-1-3′; GalNAc-T, 5′-FAM-ATGCATTACGTATTAGT-CGC-BHQ-1-3′; CK20, 5′-FAM-ATCAGTTAAGCACCCT- GCCAGAGAC-BHQ-1-3′; and glyceraldehyde-3-phosphate dehydrogenase, 5′-FAM-CAGCAATGCCTCCACCAAC- CGA-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 preincubating 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^-7 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 RNA or cDNA was excluded.

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

<table>
<thead>
<tr>
<th>Detection method</th>
<th>Detection, n (%)*</th>
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<tbody>
<tr>
<td></td>
<td>Yes</td>
</tr>
<tr>
<td>CK-IHC</td>
<td>3 (30)</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>6 (60)</td>
</tr>
<tr>
<td>CK-IHC or qRT-PCR (+)</td>
<td>7 (70)</td>
</tr>
</tbody>
</table>

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

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

<table>
<thead>
<tr>
<th>SLN</th>
<th>No. of qRT-PCR markers detected in SLN (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>≥2</td>
<td>1</td>
</tr>
<tr>
<td>Macro (n = 10)</td>
<td>5 (50)</td>
<td>3 (30)</td>
</tr>
<tr>
<td>Micro (n = 3)</td>
<td>2 (67)</td>
<td>1 (33)</td>
</tr>
<tr>
<td>ITC (n = 17)</td>
<td>5 (29)</td>
<td>1 (6)</td>
</tr>
<tr>
<td>Negative (n = 37)</td>
<td>4 (11)</td>
<td>6 (16)</td>
</tr>
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</table>

Abbreviations: SLN, sentinel lymph node; ITC, isolated tumor cells.
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 N1 disease than for patients with N0 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 N1 or N2, 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 T1, T2, and T3 primary tumors, respectively. MAGE-A3 and CK20 were not detected in blood from patients with T1 or T2 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 T3 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 node (n = 27) and 57 months (95% CI, 50-64) for qRT-PCR(+) sentinel lymph node (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>
<thead>
<tr>
<th>Table 3. Relation between qRT-PCR multimarker detection in sentinel lymph node and pN stage</th>
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<tbody>
<tr>
<td>pN stage</td>
</tr>
<tr>
<td>---------</td>
</tr>
<tr>
<td>N0 (n = 47)</td>
</tr>
<tr>
<td>N1 (n = 15)</td>
</tr>
<tr>
<td>N2 (n = 5)</td>
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<table>
<thead>
<tr>
<th>Table 4. Relation between qRT-PCR multimarker detection in sentinel lymph node and AJCC stage</th>
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<tr>
<td>AJCC stage</td>
</tr>
<tr>
<td>------------</td>
</tr>
<tr>
<td>≥2</td>
</tr>
<tr>
<td>I (n = 20)</td>
</tr>
<tr>
<td>II (n = 27)</td>
</tr>
<tr>
<td>III (n = 20)</td>
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</tbody>
</table>
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 mRNA marker 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.

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patients who have not recovered 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

D.S.B. Hoon, patent filed on detection of circulating tumor cells in colon cancer.

Acknowledgments

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References

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