Detecting Micrometastases in Colon Cancer: The Sentinel Lymph Node Technique

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It's been proposed that hidden lymph node involvement accounts for a substantial proportion of recurrence and death in apparent stage I and II colon cancer. This VA pilot study explores the usefulness of the sentinel lymph node technique of targeted pathologic analysis in a veteran population.

Colorectal cancer is the third most common cancer in both men and women in the United States, with an estimated 145,290 new cases expected to occur this year.[1] It's also the second leading cause of cancer-related deaths.[1] Although about 80% of patients with colorectal cancers are diagnosed at a stage at which all gross disease can be surgically removed,[2] nearly 30% of these patients succumb to recurrent disease.[3]

A likely explanation for this high rate of recurrence is the presence of microscopic, residual cancer in draining lymph nodes. Such micrometastases often remain undetected in pathologic specimens stained by the standard hematoxylin and eosin (HE) method—but may be picked up by more advanced techniques, such as immunohistochemical staining (IHC) or polymerase chain reaction (PCR) testing. Because these tests are expensive and time consuming, however, it is not feasible to apply them to the entire harvest of lymph nodes recovered with surgical resection.

The sentinel lymph node (SLN) concept, which uses injection of a contrast dye to identify representative lymph nodes, has been well established for pathologic staging of several cancers,[4–6] and investigations of its use in colorectal cancer staging have been undertaken recently.[3,7–10] In this article, we build on previous findings by presenting the results of a pilot study of SLN techniques in a population of VA patients with colon cancer. In addition to testing the validity of the SLN concept for staging colon cancer in veterans, this study also aimed to compare the power of several pathologic tests to detect micrometastases in these representative lymph nodes.

IMPORTANCE OF LYMPH NODE INVOLVEMENT

Several studies conducted over the past decade have presented compelling evidence that adjuvant chemotherapy, using a 5-fluorouracil (5-FU)-based regimen, reduces disease recurrence and improves survival in patients with American Joint Committee on Cancer (AJCC) stage III colon cancer. In 1989, the North Central Cancer Treatment Group studied 401 patients with stage II and III colon cancer.[11] This study documented significant overall reductions in cancer recurrence ($P = .003$) with a combination of levamisole and 5-FU, but there was a survival advantage only in the stage III subset. These findings stimulated the large Intergroup trial of 1,296 patients with resected stage II and III colon cancer.[2] The results indicated that, in patients with stage III disease, levamisole plus 5-FU reduced the risk of cancer recurrence by 41% ($P < .0001$) and the death rate by 33% ($P = .006$).[2] A consensus panel convened by the National Institutes of Health in April 1990 recognized these striking findings,[12] and a recent study indicates that survival may be even higher when irinotecan is added to the chemotherapy regimen.[13]

Since adjuvant therapy is of documented survival benefit in stage III but not stage II disease, the critical factor seems to be accurate identification of lymph node metastases. Conventional methods of demarcating this watershed depend on clinical

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findings and histologic examination of HE-stained tissue. Recent studies, however, indicate that these methods are inherently imprecise, prone to sampling error, and nonsensitive.[14] These limitations probably account for significant understaging of colon cancer,[15] which leads to substantial recurrence in patients with apparent stage II disease.[14,16]

There is a growing realization that micrometastases in lymph nodes, detectable only by sensitive tests, may hold the key in determining the clinical outcome (Figure 1). Some authors conceive of micrometastases as deposits measuring less than 2 mm,[17] while others consider this term to be based on molecular as opposed to morphologic techniques of detection.[18] Our concept of micrometastases was the presence of cancer deposits not detectable by routine histologic tests.

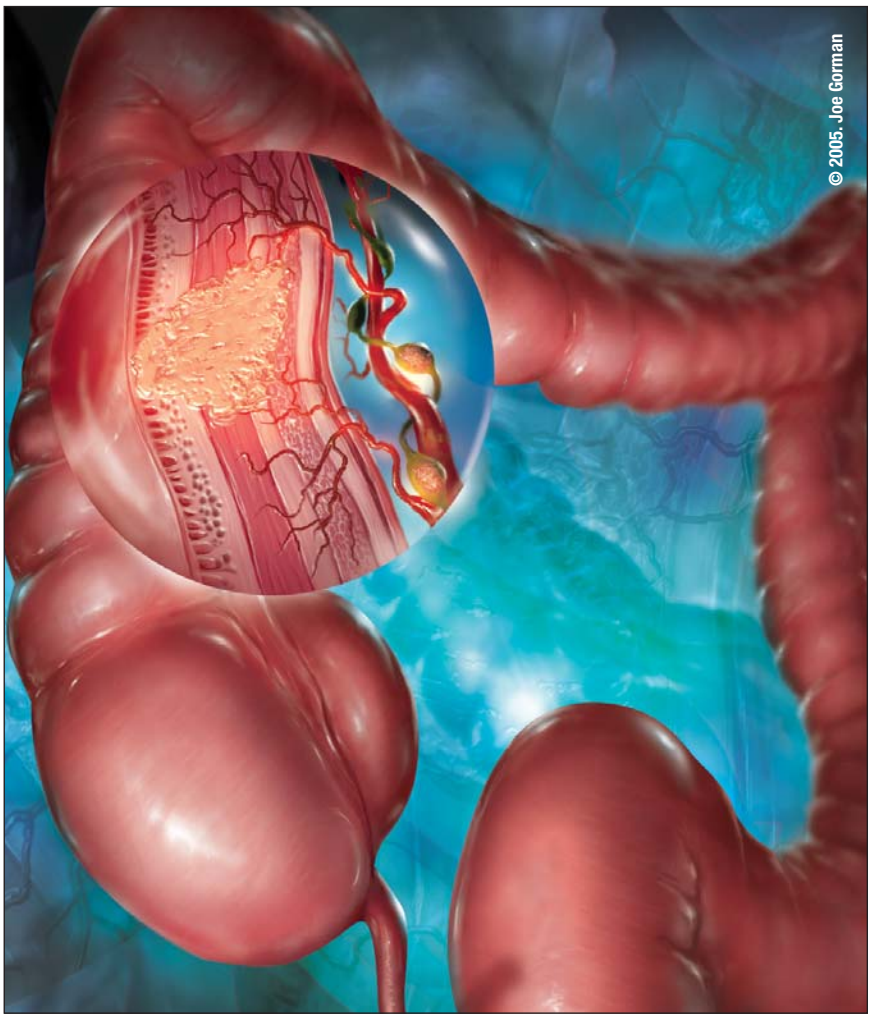

Figure 1. Colon cancer that has spread, in microscopic deposits, to the lymph nodes adjacent to the draining blood vessels—a condition that is often undetectable by conventional histologic analysis.

UNCOVERING OCCULT METASTASES

Various methods for identifying micrometastases have been described. These include IHC using antibody to cytokeratin,[13,19,20] reverse transcriptase PCR (RT-PCR),[14,15,18,21,22] and mutant-allele-specific amplification (MASA).[23,24]

RT-PCR techniques have been based on the detection of messenger RNA (mRNA) for carcinoembryonic antigen (CEA), guanylyl cyclase C, or cytokeratin.[14,15,18,21,22] Using the CEA RT-PCR method, Liefers and colleagues detected occult metastases in 54% of patients with stage II disease.[21] Waldman and colleagues were able to upstage 33% of patients with colon cancer that had been classified under the old Dukes staging system as stage B (which corresponds roughly to the AJCC stage II) using RT-PCR to detect guanylyl cyclase C.[14] A group led by Mori reported an increase in detection of nodal metastases from 26% to 66% with CEA RT-PCR.[22]

In most instances, these findings correlated with prognosis. Liefers' group found a significantly lower adjusted five-year survival in patients with micrometastases detected by PCR assay ($P = .02$).[21] Using the IHC technique, Greenson and colleagues noted a poorer prognosis for patients with Dukes stage B colorectal cancer whose lymph nodes tested positive for cytokeratin.[20] Using the MASA technique, Hayashi and colleagues reported tumor recurrence in 27 of 37 patients with genetically positive lymph nodes within five years of surgery, while none of the 34 patients with MASA-negative nodes had a recurrence.[24] These researchers concluded that genetic diagnosis of lymph node metastasis may be a useful prognostic factor in colorectal cancer.[24]

THE SLN CONCEPT

The concept of SLN is based on the premises that lymphatic spread oc-

curs in orderly progression through echelons and that a tracer could identify the few nodes to which the spread is most direct. These nodes would be expected to predict the presence or absence of metastases in the rest of the lymphatic tree. First envisaged in penile carcinoma,[25] the SLN model has been quite well established in the staging of breast cancer[4,5] and malignant melanoma.[6] While, in those situations, SLN status helps select cases for extensive dissection, within the context of colorectal cancer, this information is expected to determine the need for adjuvant therapy.

STUDY DESIGN

In undertaking this pilot study, we had three main goals: (1) to assess the technical feasibility of intraoperative identification of SLNs for colon cancer; (2) to compare the relative efficacy of IHC, PCR, and standard HE staining in identifying micrometastases; and (3) to determine the ability of SLN techniques to predict the status of the entire set of draining lymph nodes.

We enrolled 26 consecutive adult patients undergoing resection for colon cancer at the Sioux Falls VA Medical Center, Sioux Falls, SD—following approval from the University of South Dakota Institutional Review Board and the VA research department. We excluded patients with rectal cancers (defined for our purpose as those located 15 cm from the anal verge or below) because these patients usually receive adjuvant therapy even if their cancer is classified as stage II. As such, detection of micrometastases would not alter the therapeutic approach significantly.

In addition to the standard history and physical examination, we collected detailed information on previous allergic reactions to contrast agents and dyes, in order to minimize the risk of reaction to intraoperative injection of isosulfan blue, the dye used for SLN identification. Isosulfan blue is FDA approved for delineating lymphatic vessels. Its only known adverse effect is a weak allergic reaction, which occurs in 1.5% of patients.[26]

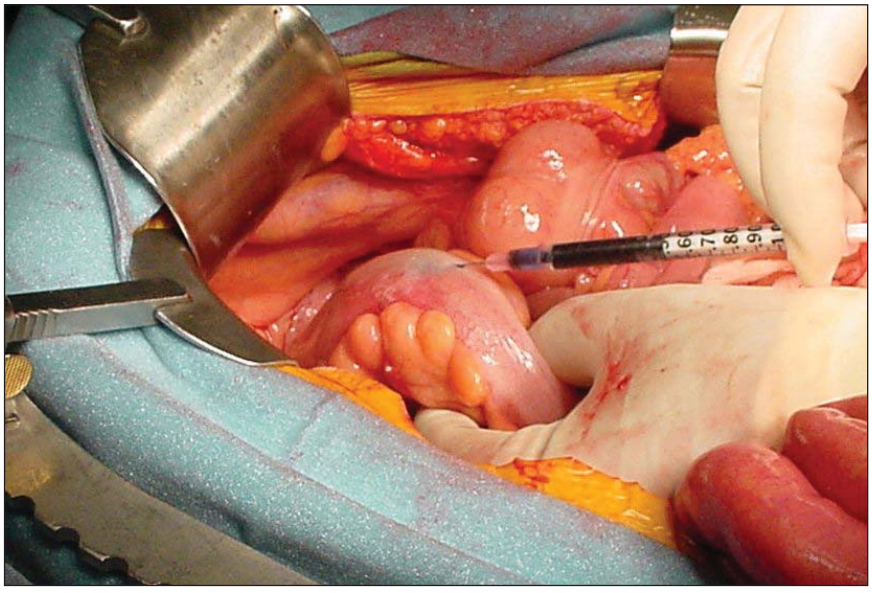

Figure 2. Intraoperative dye injection to identify sentinel lymph nodes, a technique in which three 1-mL injections of a 1% buffered sterile aqueous solution of isosulfan blue are made subserosally at the tumor site, in a circumferential manner, using a tuberculin syringe.

All patients underwent conventional preoperative workup and bowel preparation, including: measurement of complete blood count and levels of serum urea nitrogen, creatinine, electrolytes, and CEA; chest X-ray; electrocardiography; medical consultation for surgical risk; computed tomography scan of the abdomen and pelvis; colonic lavage with oral polyethylene glycol solution; and gut sterilization with oral erythromycin and neomycin.

Surgical procedure and SLN identification

During the initial surgical exploration (laparotomy), the surgical team used minimal mobilization in the mesenteric plane to avoid damage to lymphatic channels. SLN identification was performed through three 1-mL injections of a buffered, sterile, 1% aqueous solution of isosulfan blue (Figure 2). The injections were made subserosally at the tumor site, in a circumferential manner, using a tuberculin syringe.

After five minutes, the first three lymph nodes to have turned blue were tagged with distinctive sutures and marked SLN 1, SLN 2, and SLN 3. The surgeon then performed a standard colon resection according to established oncologic principles.

Pathologic examination

Upon receipt of the specimen in the histology laboratory, the pathologist examined the specimen and removed the three tagged nodes immediately. These nodes were kept separate and labeled as described earlier. Each of these three nodes was cut in half.

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One half was sent immediately to the PCR laboratory on dry ice (see "Polymerase Chain Reaction Testing Procedures" at right). The remaining half was processed according to the following procedure.

First, the half-node was cut into 3- to 5-mm sections, which were placed in separate cassettes and fixed in formalin solution. These specimens were processed overnight and embedded in paraffin in the usual manner. The paraffin blocks were then cut on the microtome into five levels at a thickness of 3 to 5 µm. Levels 1, 3, and 5 were stained with HE. Levels 2 and 4 were placed in a water bath without gelatin on coated slides for immunoperoxidase staining, and then the level 2 section was stained with pankeratin (AE1/AE3) and the level 4 section was stained with CEA.

The rest of the colonic specimen was opened, pinned on a board, fixed overnight in a fat clearing solution, and dissected in the usual manner. Other (nonsentinel) lymph nodes retrieved from the colonic specimen were stained with HE and then examined according to standard pathologic procedures.

Data analysis

In order to compare the ability of standard HE staining, IHC, and PCR to identify lymph node micrometastases, we calculated the negative predictive values (NPV) for each method. (Because there are, by definition, no false positive results when testing for lymph node involvement, the positive predictive value of all methods would be 100%.) We also compared the presence or absence of micrometastases in SLNs after PCR or IHC testing with the status of the other nodes after HE staining, calculating the number of cases in which the SLNs were positive or falsely negative. These results indicated whether

Polymerase Chain Reaction Testing Procedures

EXTRACTION OF RNA AND MESSENGER RNA

Total cellular RNA was extracted from tissues according to the method of Chomczynski and Sacchi.[1] Briefly, tissue samples were powdered in liquid nitrogen; dissolved in a 4-mol/L thiocyanate solution (pH 7) containing 25 mmol/L of sodium citrate, 0.1 mol/L of mercaptoethanol, and 0.5% sarcosyl; and homogenized on ice for five seconds, three times at full speed using a Polytron rotor-stator homogenizer (Glen Mills, Clifton, NJ). Next, 2 mol/L of sodium acetate (pH 4) and chloroform/isoamyl alcohol (ratio, 49:1) were added and mixed on a vortexer after each addition. The mixture was cooled on ice for 15 minutes and then centrifuged for 20 minutes at 10,000 times the acceleration of gravity (g). The supernatant was transferred to a sterile tube, containing an equal volume of ice-cold isopropanol, and vortexed for 30 seconds. The RNA was allowed to precipitate at –80 °C for 15 minutes. After centrifugation for 20 minutes at 10,000 times g and 4 °C, the RNA pellet was washed by centrifugation in 75% ethanol, dried in a vacuum concentrator, and dissolved in 200 µL of Tris-EDTA (pH 5.5). The concentration of the RNA was determined by optical density measurement. RNA was run on ethidium bromide-stained agarose to confirm its integrity.

REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION (RT-PCR) TESTING

First strand complementary DNA (cDNA) synthesis was performed by reverse transcription of RNA from the lymph node. Total RNA was reverse transcribed for one hour at 42 °C in 20 µL of a mixture of 20 pmol of oligo (dT) primers, 1XPCR buffer, 3 mmol/L of $MgCl_2$, 0.5 mmol/L of deoxyribonucleoside triphosphates (dNTPs), 20 U of ribonuclease inhibitor, and 200 U of Moloney murine leukemia virus reverse transcriptase, followed by incubation at 94 °C for five minutes. The resulting cDNA was amplified using a specific set (sense and antisense) of oligoprimers for either carcinoembryonic antigen (CEA) or cytokeratin.

The first strand also was also amplified by PCR of the housekeeping gene β-actin under the following conditions: 35 cycles of 94 °C for 45 seconds, 60 °C for 45 seconds, and 72 °C for two minutes, followed by a 10-minute final extension at 72 °C. Products were analyzed on a 2% agarose gel (containing ethidium bromide) and visualized under ultraviolet light. The PCR-amplified products were confirmed by DNA sequencing analysis.

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Table 1. Comparison of patients' lymph node status by method of pathologic analysis*

Lymph node status	Analysis of sentinel lymph nodes				Analysis of all lymph nodes	
	PCR† alone	IHC‡ alone	PCR + IHC	HE§ alone	Any method	All methods
Positive	8	5	10	5	11	
Negative	18	21	16	21		15

*Total number of patients = 26. †PCR = polymerase chain reaction testing. ‡IHC = immunohistochemical staining. §HE = hematoxylin and eosin staining.

the SLN status is reliable as an indicator of the metastatic status of the rest of the lymphatic basin.

SUCCESS WITH SLN

In all 26 cases, the surgeon was able to visualize and tag the first three nodes that turned blue. The feasibility of intraoperative identification of SLNs for colon cancer, therefore, was 100%.

A total of 11 patients had at least one lymph node (sentinel or nonsentinel) that tested positive for cancer cells by any method of pathologic analysis. In the remaining 15 patients, all lymph nodes tested negative by all methods.

When comparing the various methods of SLN analysis, we found that the combination of PCR and IHC yielded the most accurate results, detecting 10 of the 11 patients with positive lymph nodes (Table 1). PCR alone identified eight positive patients, while IHC and HE each found only five. The NPVs ranged from a high of 93.7% for PCR plus IHC to a low of 71.4% for either IHC or HE alone (Table 2).

Of the 11 patients with positive lymph nodes by any method, one had negative SLNs—meaning that the false-negative rate for SLN evaluation was 3.8% (Table 3). For the rest of the sample (96.2%), the SLN status appeared to reflect the status of the nodal basin.

In five patients, SLN evaluation yielded positive results while non-SLN evaluation revealed no metastases. Therefore, these five patients (19.2% of the total sample) would have been understaged without the use of SLN testing (Table 4). Despite this noticeable trend toward an advantage for SLN study over conventional analysis, the difference fell just short of statistical significance (P = .063 by Fischer's exact test). This was most likely due to the small sample size.

PUTTING THE RESULTS IN CONTEXT

In a landmark 2000 publication, Saha and colleagues first described the technical details of SLN biopsy for colorectal cancer.[3] They established that the procedure was technically feasible and described an accuracy of more than 95% in their series of 86 patients. In our veteran sample, we also found SLN identification to be highly feasible and to provide a useful representative sample for the entire nodal basin in the vast majority of cases (over 96%).

Tsioulias and colleagues performed lymphatic mapping in 65 patients with gastrointestinal neoplasms and analyzed SLNs by HE staining, multiple sectioning, and cytokeratin-based IHC.[7] They found that the tumor status of the SLNs accurately predicted the tumor status of the locoregional lymph nodes in 95% of cases. In 89% of the cases, negative SLNs accurately predicted the absence of tumor metastases in all other regional lymph nodes. The false-negative rate, excluding rectal tumors, was 4%. Of their 50 large bowel cancer cases, they found that 20% could be upstaged by this technique. Our pilot study yielded similar rates of false-negative results (3.8%) and upstaging opportunities (19.2%).

Table 2. Relative efficacy of pathologic methods in identifying micrometastases

Method	Negative predictive value (%)
PCR* alone	83.3
IHC† alone	71.4
PCR + IHC	93.7
HE‡ alone	71.4
PCR + HE	93.7

*PCR = polymerase chain reaction testing. †IHC = immunohistochemical staining. ‡HE = hematoxylin and eosin staining.

Wiese and colleagues reported a series of 83 patients with colorectal carcinoma for whom the SLN identification rate was 99%.[8] In this study, designated SLNs were sectioned at 10 levels through the block, and cytokeratin immunostaining (AE1) was performed. The authors concluded that focused pathologic evaluation of SLNs improved pathologic staging.

Other researchers, however, have had less favorable results. Although Cserni and colleagues reported successful lymphatic mapping in 96% of their colorectal cancer cases, they encountered a relatively high false-negative rate (38%) and were unable to find any staging advantage with this technique.[9] In a series of 50 patients, Joosten and colleagues were able to identify SLNs in only 70%, with a false-negative rate of 60%—even using IHC.[10] Chin and colleagues sounded a word of caution in their case report of a patient with appendiceal carcinoid whose SLN status was falsely negative for malignancy.[27]

In comparing techniques for detecting micrometastases in the representative lymph nodes identified through the SLN procedure, we found that the addition of PCR improves the accuracy of detection of nodal metastases over conventional staining techniques. IHC alone was comparable to traditional HE staining, whereas PCR combined with either IHC or HE improved the NPV substantially.

Table 3. Ability of sentinel lymph node (SLN) status to predict the status of the entire set of draining nodes

Total no. of patients	26
No. of patients in whom SLNs were positive by any method	10
No. of patients in whom SLNs were positive by any method but non-SLNs were negative	5
No. of patients in whom SLNs were negative by all methods but non-SLNs were positive	1
False-negative rate	3.8%

Table 4. Ability of detailed study of sentinel lymph nodes (SLNs) to upstage nodal status for cancer spread

Total no. of patients	26
No. of patients in whom SLNs were positive by any method	10
No. of patients in whom SLNs were positive by any method but non-SLNs were negative	5
% of patients upstaged due to focused examination of SLNs	19.2

STUDY LIMITATIONS

This study had several limitations, the most notable of which was its small sample size, which probably accounts for the lack of statistical significance of the findings. In addition, PCR detection of appropriate antigenic moiety may not be specific for neoplastic disease. Furthermore, since half of each node underwent PCR testing and the other half underwent IHC staining, there is a possibility that one half actually might have been free of metastases while the other had malignant deposits. Such an occurrence, though expected to be rare, would introduce an error in assessment of relative efficacies of the two methods. Niemann and colleagues have studied the issue of analysis of half versus whole nodes in detail.[28] They found that, in a sample of 149 patients, submitting the entire lymph node identified metastases in seven additional patients—at an estimated cost of $5,936.

Finally, while most authors have demonstrated a positive correlation between micrometastases and clinical outcome, the prognostic value of micrometastases is not firmly established. Jeffers and colleagues, for example, found no difference in 10-year survival rates between patients with micrometastases and those without.[29] When Cutait and colleagues analyzed 603 lymph nodes from 46 lesions stained by the peroxidase-antiperoxidase technique, they found no impact of micrometastases on five-year survival.[30] And Adell's group from Sweden used cytokeratin-based IHC and were unable to find any difference in outcome between the two groups.[19]

A STEP FORWARD

The results of this pilot study suggest the validity and feasibility of the SLN principle for staging colon cancer in a veteran population. In our sample, it was possible to apply ad-

vanced tests to a few selected lymph nodes for more accurate detection of metastases, a process that can be termed "ultrastaging." In spite of these encouraging findings, however, statistical significance could not be achieved—most likely due to the small sample size.

Since colon cancer disproportionately affects geriatric patients, the potential positive impact of improved colon cancer staging on survival and health care costs is significant for the predominantly older population of veterans treated in the VA health care system. Because it was conducted in a veteran sample, our study findings begin to shed some light on the applicability of the SLN concepts to the VA patient population with colon cancer and suggest a detailed protocol that may be feasible in a community veterans hospital. Assuming that larger studies validate our findings, this protocol has the potential to enhance detection of node-positive disease that otherwise might go undiscovered and to facilitate appropriate management of these cases.

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