PERGAMON

European Journal of Cancer 38 (2002) 977–985

European Journal of Cancer

www.ejconline.com

Ultrastaging of early colon cancer using lymphatic mapping and molecular analysis☆

A.J. Bilchik[a,*], D. Nora[a], R.A.E.M. Tollenaar[b], C.J.H. van de Velde[b], T. Wood[a], R. Turner[a], D.L. Morton[a], D.S.B. Hoon[a]

[a]*The John Wayne Cancer Institute, 2200 Santa Monica Boulevard, Santa Monica, CA 90404, USA*
[b]*Leiden University Medical Center, Leiden, The Netherlands*

Received 18 December 2001; accepted 11 February 2002

Abstract

Approximately one-third of node-negative colon cancers will recur, possibly due to understaging and inadequate pathological examination of lymph nodes (LNs). We evaluated the sensitivity, accuracy and feasibility of staging based on lymphatic mapping, focused examination, and molecular analysis of the sentinel node (SN) in patients with primary colorectal carcinoma. Between 1996 and 2000, 100 patients with colon carcinoma (CRC) underwent lymphatic mapping immediately after peritumoral injection of 1.0 cc of isosulphan blue dye. All LNs in the CRC specimen were examined by routine haematoxylin and eosin (H&E) staining. Sentinel nodes were examined by step serial sectioning, cytokeratin immunohistochemistry (CK-IHC) and/or reverse transcriptase-polymerase chain reaction (RT-PCR) analysis in an attempt to identify occult micrometastatic disease. Lymphatic mapping was successful in 97% of the cases. There were 5 false-negative cases, predominately associated with T3/T4 tumours. Aberrant lymphatic drainage was identified in 8 patients (8%) altering the operative approach. 26 patients had H&E-positive LNs. In 74 patients who were node-negative by routine H&E, 18 (24%) had occult nodal micrometastases missed on routine H&E examination, but detected by focused analysis of the SN. RT-PCR analysis of the SN was performed in 40 patients, 26 of which were negative by H&E and CK-IHC. In 12/26 (46%) of these patients, there was additional evidence of micrometastatic disease. In this study, focused examination of the SN in conjunction with RT-PCR analysis identified micrometastatic disease in a significant number of node-negative patients. This may have important implications when selecting patients for adjuvant treatment protocols.

1. Introduction

Colorectal carcinoma (CRC) is the most common gastrointestinal cancer in the United States with an estimated 135 400 new cases and 56 700 deaths in 2001 [1]. Lymph node (LN) status is the most important predictor of outcome [2]: the 5-year survival is 80% for node-negative patients, but 45–50% for node-positive [3]. Adjuvant chemotherapy can significantly improve 5-year survival for patients with stage III (node-positive) CRC [4,5]. Thus, accurate nodal staging is critical to determine prognosis and select patients for adjuvant chemotherapy.

Pathological assessment provides the basis for staging CRC, but routine gross dissection of the mesentery may miss crucial LNs that contain occult metastases [6]. The number of harvested LNs varies among surgeons [7] and the disproportionate number of stage I and II cases (71%) compared with stage III cases (29%) suggests that many patients are being understaged. Recently, Tang and colleagues reported inaccurate staging when fewer than 10 LNs are harvested from the mesentery [8]. Koren and colleagues described a LN-revealing solution that allowed the identification of additional LNs which upstaged 27% of stage II patients to stage III [9]. There are other reports of potential upstaging with careful mesenteric clearing methods [10]. Unfortunately, these techniques to identify more nodes are cumbersome, labour-intensive and toxic.

The number of sections examined in each node is important as well. A 6-mm node can be sliced into 1000 6-μ sections, but typically only one or two of these

☆ Presented in part at the First Multidisciplinary ColoRectal Cancer Congress, Noordwijk, The Netherlands, 17–20 April 2001.

* Corresponding author. Tel.: +1-310-449-5206; fax: +1-310-449-5261.

E-mail address: bilchika@jwci.org (A.J. Bilchik).

0959-8049/02/$ - see front matter
PII: S0959-8049(02)00058-8

sections will be selected for evaluation. This inspects less than 0.5% of the entire node. Serial sectioning to extinction would minimise the risk of missing metastasis, but is prohibitively expensive [11].

Sensitive tests such as immunohistochemistry (IHC) and reverse transcriptase-polymerase chain reaction (RT-PCR) technology can enhance tumour detection. However, IHC depends upon random microsectioning of a LN and is impractical to perform on numerous nodes. RT-PCR is more sensitive and specific than standard pathological evaluation for detecting nodal disease [12,13], but it too is impractical for routine use.

Sentinel lymph node mapping (LM) can identify the LNs that have the highest potential to harbour metastasis if present. First popularised by Morton and colleagues [14], SLN is now applicable in a variety of solid malignancies including colon cancer [15]. We examined whether SLN mapping in combination with serial sectioning, IHC and RT-PCR could augment the sensitivity of detecting occult disease in patients with CRC.

2. Patients and methods

Between August 1996 and November 2000, 100 consecutive patients undergoing resection for clinically localised CRC at the John Wayne Cancer Institute were enrolled. Informed consent was obtained preoperatively from the patients in accordance to an investigational protocol approved by the Institutional Review Board (IRB) of Saint John's Health Center, Santa Monica, CA, USA. The operative and pathological approaches were previously described [16].

2.1. Colon resection technique

After resectability had been determined by abdominal exploration, the involved segment of colon was mobilised. One millilitre of isosulphan blue dye (Lymphazurin, United States Surgical Corporation, Ben Venue Laboratories, Inc., Bedford, OH, USA) was carefully injected subserosally in four quadrants around the periphery of the tumour using a tuberculin syringe. The dye travelled from the injection site along the lymphatics to the sentinel node(s) (SN(s)) typically within 30–60 seconds (Fig. 1). Occasionally gentle dissection of the mesentery was performed to trace the lymphatic path to the SN. Each blue-stained node was marked with a suture, and the colectomy performed with a standard open (OCR) or laparoscopic (LCR) method, including all blue nodes in the mesenteric resection.

2.2. Ex-vivo *SLN mapping*

The *ex-vivo* technique was used primarily, or secondarily for failed *in-vivo* attempts. Our technique was

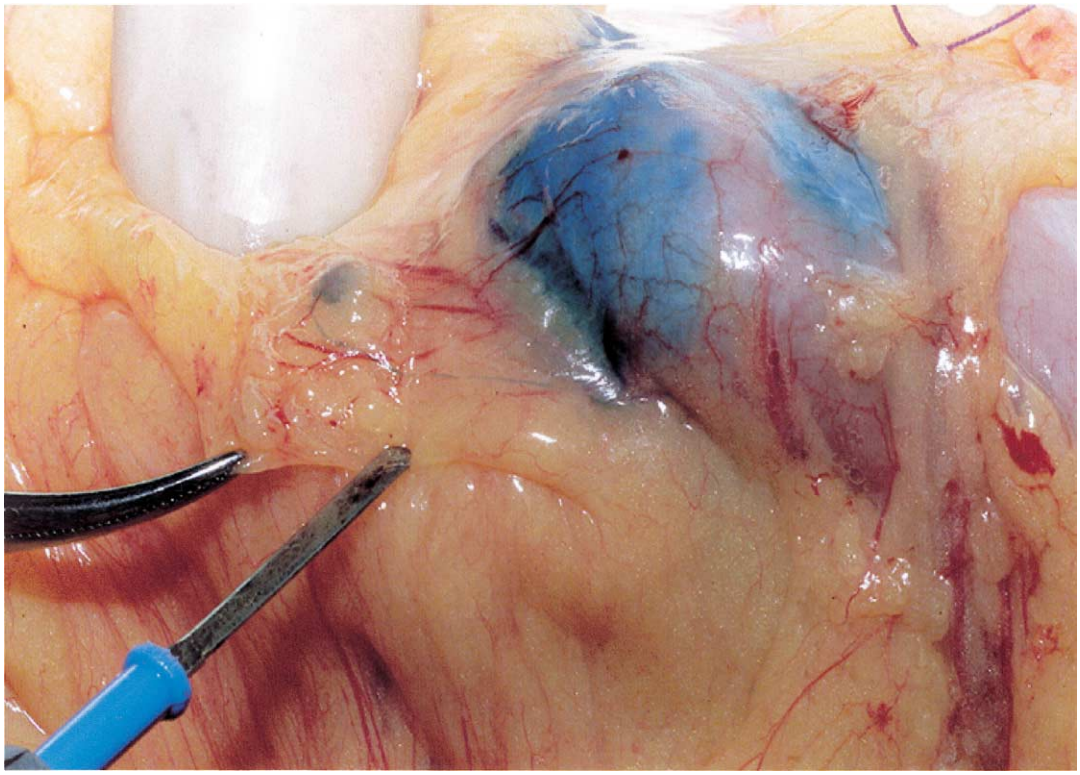

Fig. 1. Shortly after a subserosal injection of isosulphan blue dye, a lymphatic channel can be followed to the sentinel node(s) (SN(s)). Typically, 1–4 SNs are mapped during each lymph node mapping (LM) procedure.

modified from that of Wong and colleagues [17]. Upon completion of the colectomy, the specimen was immediately taken to a side table. One to two millilitres of isosulphan blue dye was injected subserosally or submucosally around the tumour after the bowel had been opened. The dye was visualised as it travelled along the lymphatic channels to the respective SNs. Each SN was marked with a suture.

2.3. Histopathology protocol

Pathological review analysed the tumour, margins and all LNs via haematoxylin and eosin (H&E) staining. Each SN was specifically examined by a focused technique originally developed in breast carcinoma [18]. The pathologist bisected each SN into slices no thicker than 2–3 mm. Paraffin sections, each approximately 4 μm thick, were cut at two levels separated by 200 μm. One section from each level was stained with H&E and another with cytokeratin (CK-IHC) (Fig. 2).

2.4. Immunohistochemical staining

Paraffin sections for CK-IHC were placed on charged slides (Superfrost Plus M6416-plus, Baxter Diagnostics Inc, McGaw Park, IL, USA). An automated immunostainer (Ventana ES, Ventana Medical Systems, Tucson, AZ, USA) with enzyme digestion (Protease 1) of tissue sections for 8 min and AE-1/AE-3 CK antibody (Dako Corporation, Carpinteria, CA, USA) staining (1:200 dilution) for 32 min was used. Diaminobenzidene was the chromogen. IHC stains were interpreted according to strict criteria combining pathological and immunoreactivity features.

2.5. RT-PCR analysis

Expression of mRNA tumour markers *β-hCG*, *c-Met*, and universal MAGE (*uMAGE*) was evaluated in CRC cell lines, donor peripheral blood lymphocytes (PBL) from healthy volunteers, and benign LNs. Non-malignant nodal tissue was obtained from 5 patients undergoing colon operations for non-cancerous conditions. Colon cancer cell lines HT-29 and SW480 were obtained from the American Tissue Culture Collection (ATCC) (Rockville, MD, USA). JAR choriocarcinoma cell line was obtained from ATCC. CRC cell lines JWCI-0044 (A), JWCI-0044 (B), JWCI-0361, JWCI-0427, JWCI-0485, JWCI-1100 and JWCI-1203 were established and characterised at JWCI. These were cultured in Roswell Park Memorial Institute (RPMI) 1640 media supplemented with 10% heat-inactivated fetal calf serum (FCS) (Gemini, Calabasas, CA, USA), penicillin and streptomycin. Cells were washed with sterile physiological phosphate-buffered solution (PBS). Total RNA was extracted from cells when cultures reached 70–80% confluence.

Reverse transcription (RT) was performed using Moloney murine leukaemia virus (MMLV) reverse

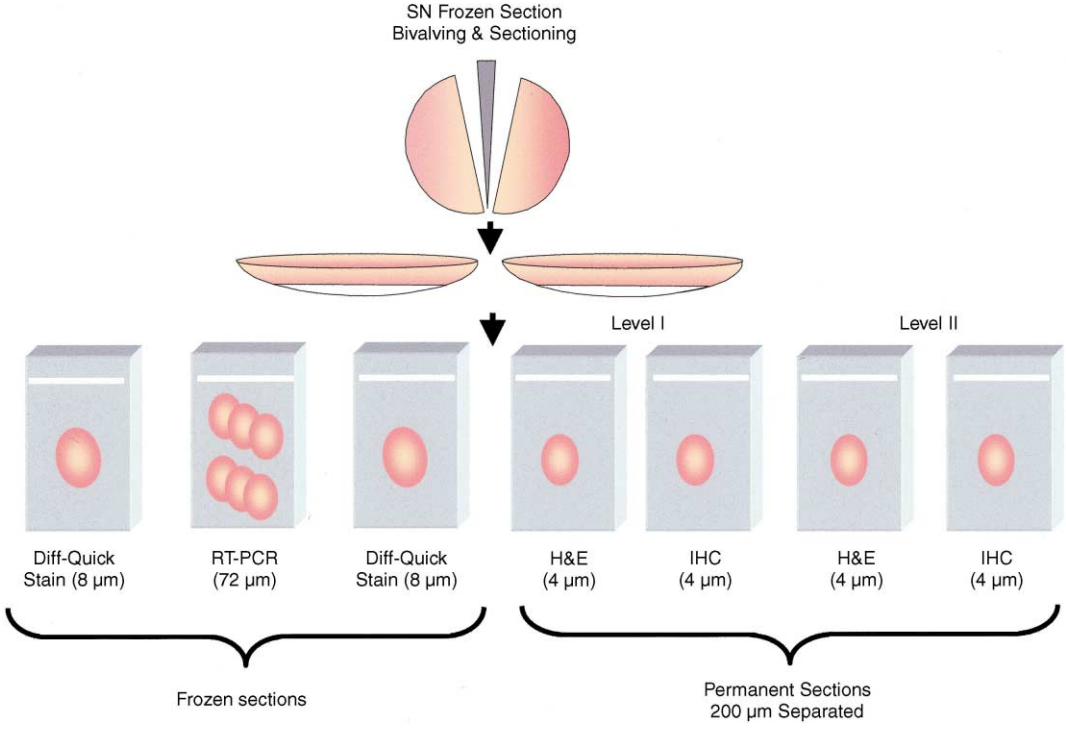

Fig. 2. Each sentinel node (SN) is subjected to a focused pathological examination based upon the protocol used at our institution for studying breast cancer SNs [14]. After the SN is bisected, sections separated by 200 μm are cut. One section from each level is stained with haematoxylin and eosin (H&E), while another is studied by cytokeratin immunohistochemistry (CK-IHC). RT-PCR, reverse transcriptase-polymerase chain reaction.

transcriptase (Promega, Madison, WI, USA). The same amount of RNA (1 μg) was used for all samples in the study, including controls. Polymerase chain reaction (PCR) was then performed on the cDNA as follows: denaturing at 95 °C for 5 min, followed by 35 cycles at 95 °C for 1 min, annealing at 65 °C for *β-hCG*, 55 °C for *c-Met*, and 62 °C for *uMAGE* for 1 min, and 72 °C for 1 min before a final primer sequence extension incubation at 72 °C for 10 min. RT-PCR was performed using a Hybaid thermocycler (Hybaid, Middlesex, UK).

PCR products were evaluated by enhanced chemiluminescence (ECL) analysis on the ORIGEN Analyzer (IGEN, Gaithersburg, MD, USA). For each assay, at least two positive controls (CRC lines), four negative controls (normal donor PBL and normal LNs), and reagent controls (reagents alone without RNA or cDNA) for the RT-PCR/ECL assay were included. Each assay contained its own set of controls for establishing background levels. Any sample ECL U value above the cut-off was considered as a positive result in each assay. RT-PCR analysis of tumour and nodal tissue was performed at least twice to verify the results. From this point on, RT-PCR analysis refers to RT-PCR assay and ECL detection. For RT-PCR analysis, expression of at least two of the three mRNA markers was defined as a positive result based on our previous RT-PCR studies of blood and SN specimens [12].

3. Results

The 49 males and 51 females had an average age of 68 years (range 28–97 years). Primary tumours were in the right colon ($n=47$), transverse colon ($n=6$), left colon ($n=5$), sigmoid colon ($n=20$), and rectum ($n=22$). *In-vivo* LM was undertaken during 79 OCRs and 15 LCRs. *Ex-vivo* LM was undertaken in 12 cases, 6 of which were primary procedures. In the other 6 cases, an *ex-vivo* technique was attempted as a salvage LM procedure for a failed open, *in vivo* mapping; therefore, 106 LM procedures were undertaken in the 100 patients.

LM demonstrated at least one SN in 97/100 cases (Table 1). However, in 8 patients, a primary LM procedure failed to demonstrate a SN (Fig. 3). These failures occurred during the first 50 cases of our series. In 5 patients, a SN was identified using *ex-vivo* LM as a salvage procedure. 2 of the 3 cases in which a SN was never identified occurred in patients with low rectal tumours. The single case in which both a primary and a secondary (*ex vivo*) LM procedure failed to map a SN occurred in a patient who was found to have 13/15 LNs positive for metastases, with many replaced with tumour. Overall, an average of two SNs were identified and the average number of nodes harvested from each CRC specimen was 15. There were no failures in identifying SNs in our last 50 cases, and no *ex-vivo* salvage mapping procedures were necessary during this period.

In the majority of cases, the SNs were located close to the primary tumour. However, some variations in lymphatic drainage were found. In 8 cases, the primary lymphatic drainage to the SNs was outside the margins of conventional resection. In these cases, the operation performed was altered to include the aberrant SNs within the field of resection. In 5 cases, the SNs were mapped to the base of the mesentery, requiring an extended mesenteric resection. In 3 other cases, the SN was mapped to the left of the middle colic vessels in right-sided primaries. In 2 of these cases, the aberrantly mapped SN was the only positive node. In 1 of these cases, the metastases were detected only by CK-IHC.

The tumour status of the SN accurately reflected the status of the nodal basin in 92/97 (95%) patients (Table 1). All 5 false-negative cases were associated with T3 or T4 tumours and 3 of these occurred during the first 30 cases. A critical review of the false-negative cases revealed possible technical explanations for four of the five failures. In 2 of these cases, the H&E-positive nodes were replaced with tumour (Fig. 4). In another case, the only 'positive' node was a non-sentinel node involved with tumour by direct extension (T4), while the SN was negative. This case could be arguably classified as negative for nodal metastasis. In the fourth case, a technical

Table 1
Success and accuracy rates of lymphatic mapping in 100 consecutive cases of CRC

Approach	*n*	Mapping successful	Average no. of nodes (range)		Accuracy[a]
			Total	Sentinel	
In vivo					
Open	79	71 (90%)	15 (3–28)	2 (1–4)	68/71 (96%)
Laparoscopic	15	15 (100%)	12 (2–20)	2 (1–3)	14/15 (93%)
Ex vivo	12	11 (92%)	16 (8–24)	2 (1–3)	10/11 (91%)
Overall	106[b]	97/100 patients (97%)	15 (2–28)	2 (1–4)	92/97 (95%)

[a] Accuracy correspondence between the sentinel node (SN) status (metastasis positive or negative) and the regional lymph node (LN) basin status as a whole.

[b] Overall, 106 lymph node mapping (LM) procedures were performed in the 100 patients. 6 patients in whom *in-vivo* mapping failed to map a SN were attempted via the *ex-vivo* approach. 5 of these were salvaged by the *ex-vivo* approach, i.e. 1 or more SNs were successfully mapped.

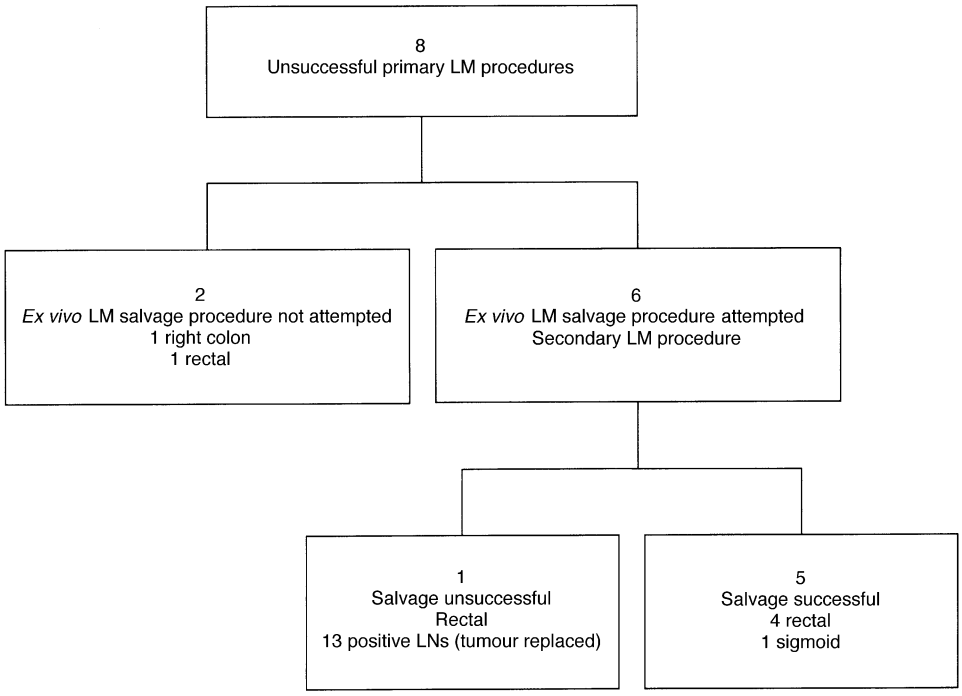

Fig. 3. During our first 50 cases, we experienced 8 patients in whom our primary attempt at lymph node mapping (LM) undertaken at standard open method (OCR) was unsuccessful, i.e. a sentinel node (SN) was not mapped. Six of these failures occurred in rectal tumours. *Ex-vivo* LM was attempted in 6 patients as a secondary, salvage LM procedure. One or more SNs were successfully mapped in 5 of the patients. The 1 case in which both primary and secondary LM attempts failed was a rectal cancer with extensive nodal and lymphatic disease.

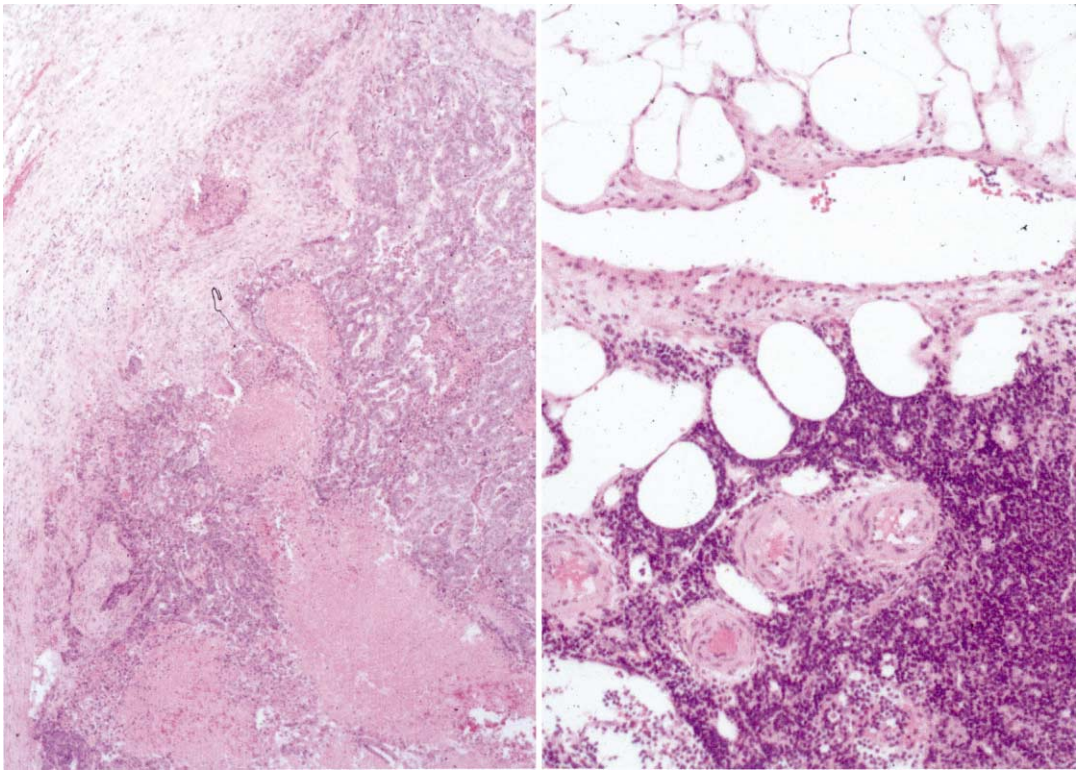

Fig. 4. In this case, a tumour-replaced lymph node (LN) likely leading to a false-negative sentinel node (SN). The photomicrograph (haematoxylin and eosin (H&E)) on the left demonstrates a mesenteric LN that has been extensively replaced with metastatic colon cancer. The normal nodal architecture has been distorted and the subcapsular sinus has been obliterated by the metastatic tumour. Because of the degree of lymphatic involvement with tumour, the blue dye bypassed the true-SN, which was replaced with tumour, and flowed to a false-negative SN. The normal appearing nodal architecture of the false-negative SN demonstrated in the photomicrograph on the right.

Table 2
Relationship of tumour (T) stage to LN status

Tumour stage	*n*	Node-negative	Node-positive	Positive nodes (s) confined to SNs	Cases upstaged by detection of occult micrometastases in SN
T1	25	24	1	1	1
T2	23	16	7	6	5
T3	46	15	31	15	12
T4	6	1	5	0	0
Total	100	56[a]	44[a]	22	18

LN, lymph node; SN, sentinel node.
[a] LN status after focused pathological examination. By routine haematoxylin and eosin (H&E) staining, 26 cases were node-positive.

error was made during percutaneous injection of the blue dye during a laparoscopic resection. In this patient, a preoperatively placed colonoscopic tattoo with carbon dye was inadvertently placed in an area away from the tumour itself. The tumour was not visible laparoscopically so the blue dye was injected circumferentially around the tattoo. Although a blue node was found and marked as a SN, it was falsely negative.

26 patients were node-positive by routine H&E staining and 74 were node-negative. 18 patients with negative nodes by routine H&E examination were found to have evidence of occult micrometastatic disease by a focused examination of the SNs, thereby upstaging an additional 24% (18/74) to the node-positive group. In 5 of these patients, micrometastases were detected by H&E/multi-level serial sectioning, while in the remaining 13 patients micrometastases were documented only by CK-IHC. As discussed above, 1 patient was upstaged not by focused pathological examination, but rather by mapping of an aberrant SN that was found to be positive by routine H&E staining. This node would not have been resected and analysed had the LM procedure not been performed.

Increasing tumour (T) stage was inversely related to the probability of isolated metastases in the SN. In 100% (1/1) of node-positive T1 tumours, 86% (6/7) of node-positive T2 tumours, 47% (15/32) of node-positive T3 tumours, and 0% (0/5) of node-positive T4 tumours, the SNs were the only nodes to contain tumour (Table 2). In the majority of cases in each tumour stage (excluding T4), isolated nodal disease in the SNs was in the form of micrometastatic disease detected only by a focused pathological examination (1/1 T1, 5/6 T2, 12/15 T3).

3.1. RT-PCR analysis of the SN

The SNs from the 40 most recent patients undergoing SN mapping were evaluated by H&E, CK-IHC, and multi-marker RT-PCR. Table 3 shows the number of markers detected in the SN of all 40 patients. Among the markers, only *c-MET* expression correlated with *uMAGE* expression (Kappa coefficient; $P < 0.02$). In the 14 patients whose SN metastasis was identified by H&E and/or CK-IHC, 12 (86%) had RT-PCR evidence of SN metastasis. In the 10 SNs positive by H&E, 9 (90%) were RT-PCR positive. Of the four SNs positive only by CK-IHC, 3 (75%) were RT-PCR positive (Fig. 5). The two false-negative results may have been due to sampling error; tumour cells may have been in the sections for histopathology and not in the RT-PCR sections. Alternatively, there may not have been sufficient marker mRNA in the RT-PCR sections, or the tumour cells may not have expressed any of the markers. Overall, CK-IHC and RT-PCR identified occult micrometastasis in 53% (16 of 30) of patients whose SNs were negative by H&E.

In the remaining 26 patients with histopathologically-negative SNs, 12 had T1 tumours, 5 had T2 tumours, and 9 had T3 tumours. 12 (46%) of the 26 patients had positive RT-PCR results; these were associated with 4 T1, 2 T2, and 6 T3 lesions. Expression of two or more markers was found in 4 T1, 2 T2, and 6 T3 lesions thereby upstaging these patients. T3 patients had the

Table 3
Expression of mRNA markers in sentinel node (SN)

Primary tumour	Patients (*n*)	RT-PCR Markers			Number of markers				
		β-hCG	*c-Met*	*uMAGE*	0	1	2	3	⩾2
T1	11	3	2	4	6	1	1	0	4
T2	9	5	4	2	4	1	2	2	4
T3/T4	20	11	10	8	2	7	4	4	11
Total	40	19	16	14	12	9	7	6	19

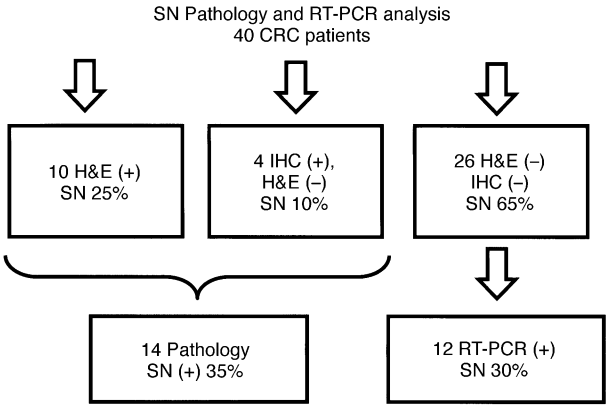

Fig. 5. Of the 40 patients analysed, the sentinel lymph node (SN) was positive by haematoxylin and eosin (H&E) in 25%, negative by H&E, but positive by IHC in 10%. Reverse transcriptase-polymerase chain reaction (RT-PCR) upstaged an additional 30%.

highest number of patients upstaged (67%) followed by T2 (40%). There was a significant correlation of the number of markers detected in SN (Spearman correlation coefficient = 0.33; $P < 0.04$) with higher T stages. For individual marker expression in histopathologically-negative SNs, there was a significant correlation with *β-hCG* detection and higher T stage (Wilcoxon rank sum test, $P = 0.02$).

The concordance for paired tumour and SN marker expression was 67% for *β-hCG*, 55% for *uMAGE*, and 50% for *c-Met*. *UMAGE* mRNA expression demonstrated the most significant ($P < 0.03$) concordance in paired specimens. As expected, there was an overall lower detection of mRNA markers in the paired SN.

4. Discussion

The LM procedure identifies the node(s) most likely to contain metastatic disease if a primary tumour has spread to the regional LNs. Studies in different solid malignancies including CRC demonstrate that if the SN is negative for metastasis, then the likelihood of skip metastasis is low (<4%) [15,19]. We have shown that meticulous and focused analysis of the SN can increase the identification of micrometastatic disease. However, as with any new operative manoeuvre, there is always an appreciable learning curve [20]. Joosten and colleagues described only a 70% success rate of mapping the SN with blue dye [21]. The authors described technical problems such as intraluminal instead of subserosal injection. The series also included rectal cases in which *in-vivo* SLN was not always successful. Standardised training modules and skills verification programmes should be established before large-scale trials of SLN in CRC are begun.

In this study, SLN was logistically feasible, accurate, fast and inexpensive. The technique is technically simpler than that performed for melanoma or breast cancer because the tumour, the lymphatic channels and the SN are directly visible. The findings of this study were similar to Saha and colleagues who reported a 99% rate of SN identification, a 96% rate of accuracy for the SN as an indicator of regional LN status, and a 17% rate of upstaging [15]. Moreover, in the current study, unexpected lymphatic drainage was demonstrated in 3 patients, altering the operative approach. We have previously demonstrated that false-negatives can occur when the SN is replaced by tumour [20]. Tumour can occlude the lymphatic vessels resulting in aberrant drainage. Since these nodes are large and firm it is unlikely that SLN will be of value in this group of patients.

LM and SN identification theoretically reduces the number of nodes that need to be examined by the pathologist. Advanced techniques such as step sectioning, IHC and RT-PCR are thus more accessible and less cost-prohibitive. This is important because these tests greatly increase the yield of detecting occult metastasis. Mori and colleagues reported that LN positivity increased when RT-PCR was used to detect carcinoembryonic antigen (*CEA*) mRNA [22]. Nakamori and colleagues also reported that RT-PCR assay could detect *CEA* mRNA in otherwise histologically-negative LNs [23]. Several other investigators have reported that histologically-negative LNs contained evidence of occult metastases by RT-PCR using *CK20* [24] or guanylyl cyclase C (*GCC*) [25] in qualitative assay systems. However, *GCC*, *CEA* and *CK20* are expressed by normal tissues and therefore may introduce false-positive results. Our group and others have questioned their utility for the detection of micrometastases.

At the JWCI, we developed novel RT-PCR applications for the detection of micrometastases using a multimarker (MM) RT-PCR assay as opposed to a single marker assay [26]. The rationale is that a multimarker system eliminates some of the inherent problems associated with single marker techniques such as tumour heterogeneity, clonal selection and variable expression of individual genes. Furthermore, we believe that the markers selected should not be expressed in non-malignant tissue [13]. Specificity and sensitivity are considerably increased and the chances of detecting occult metastases are greatly improved.

Almost half (46%) of the 26 patients with histopathologically-negative SNs had positive RT-PCR results. Of the three mRNA markers, *β-hCG* was the most frequently expressed in both tumour and nodal tissue, followed by *c-Met* and then by *uMAGE*. The pathophysiological role of *β-hCG* and its significance in carcinomas are still unknown; it may function as a suppressive factor of immune responses. *C-Met* may be important as a molecular phenotypic marker for metastasis and as a marker for early detection. The physiological role of *MAGE-A* gene expression is also

unclear, but both MAGE-A-1 and MAGE-A-3 are immunogenic in humans and potential targets for active-specific immunotherapy.

The significance of micrometastatic disease detected by IHC is yet to be defined in CRC. However, it is likely to be an important stratifying factor in choosing adjuvant chemotherapy. Cutait and colleagues re-examined 46 node-negative patients using CK-IHC and CEA-IHC and demonstrated evidence of micrometastases in 12 patients (26%). However, this did not significantly affect 5-year survival [27]. Jeffers and colleagues had similar results, but random microsectioning may have missed tumour cells, thereby causing non-significant survival differences between the two groups [28]. Greenson and colleagues examined regional mesenteric LNs in stage II CRC with anti-CK-IHC. This group found tumour cells in 28% of cases and published a significant 5-year survival in CK-IHC-negative patients. This lack of consensus in the literature in part reflects the absence of standard antibody titres and staining techniques; there are considerable inter-institutional variations in the analysis of CRC LNs by CK-IHC.

Similarly, clinical outcome studies of single marker expression in CRC are also limited. Hayashi and colleagues demonstrated decreased survival in patients with *TP53* or K-*ras* mutations in colonic LNs [29]. In another study of Dukes' B patients, Liefers and co-workers reported a 5-year survival rate of 50% for patients whose nodes expressed CEA, versus 91% for those whose nodes did not express CEA [30].

Clearly, better staging methods are needed for patient stratification. To better elucidate the prognostic significance of phenotypic and genotypic characteristics of nodal micrometastases in CRC and to determine how these factors might play a part in a comprehensive staging schema, we have initiated a prospective trial for in-depth analysis of SNs with IHC and multimarker molecular studies (NCI 1R01CA90848–01).

Acknowledgements

This work was supported in part by grant CA 090848 from the National Cancer Institute and by funding from the Rogovin-Davidow Foundation, Los Angeles, USA and the Rod Fasone Memorial Cancer Fund, Indianapolis, IN, USA.

References

1. Greenlee RT, Hill-Harmon MB, Murray T, Thun M. Cancer statistics, 2001. *CA Cancer J Clin* 2001, **51**, 15–36.
2. Fielding P. Staging systems. In Cohen A, Winawer S, eds. *Cancer of the Colon, Rectum and Anus*. New York, McGraw-Hill, 1995, 207.
3. O'Connell MJ, Mailliard JA, Kahn MJ, *et al*. Controlled trial of fluorouracil and low-dose lecucovorin given for 6 months as postoperative adjuvant therapy for colon cancer. *J Clin Oncol* 1997, **15**, 246–250.
4. Moertel CG, Fleming TR, MacDonald JS, *et al*. Levamisole and fluorouracil for adjuvant therapy of resected colon cancer. *N Engl J Med* 1990, **322**, 352–358.
5. IMPACT B2 investigators. Efficacy of adjuvant fluorouracil and folinic acid in B2 colon cancer. *J Clin Oncol* 1999, **17**, 1356–1363.
6. Herrera-Ornelas L, Justiniano J, Castillo N, Petrelli NJ, Stulc JP, Mittelman A. Metastases in small lymph nodes from colon cancer. *Arch Surg* 1987, **122**, 1253–1256.
7. Bernick PE, Wong WD. Staging: in colorectal cancer. *Surg Clin N Amer* 2000, **9**, 703–720.
8. Tang R, Wang JY, Chen JS, *et al*. Survival impact of lymph node metastasis in TNM stage III carcinoma of the colon and rectum. *J Am Coll Surg* 1995, **180**, 705–712.
9. Koren R, Siegal A, Klein B, *et al*. Lymph node-revealing solution: simple new method for detecting minute lymph nodes in colon carcinoma. *Dis Colon Rectum* 1997, **40**, 407–410.
10. Scott KW, Grace RH, Gibbons P. Five-year follow-up study of the fat clearance technique in colorectal carcinoma. *Dis Colon Rectum* 1994, **37**, 126–128.
11. Cochran AJ. The pathologist's role in sentinel lymph node evaluation. *Semin Nucl Med* 2000, **30**, 11–17.
12. 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**, 1128–1136.
13. Bostick PJ, Morton DL, Turner RR, *et al*. Prognostic significance of occult metastases detected by sentinel lymphadenectomy and reverse transcriptase-polymerase chain reaction in early-stage melanoma patients. *J Clin Oncol* 1999, **17**, 3238–3244.
14. Morton DL, Wen DR, Wong JH, *et al*. Technical details of intraoperative lymphatic mapping for early stage melanoma. *Arch Surg* 1992, **127**, 392–399.
15. 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**, 120–124.
16. Wood TF, Saha S, Morton DL, *et al*. Validation of lymphatic mapping in colorectal cancer: in vivo, ex vivo, and laparoscopic techniques. *Ann Surg Oncol* 2001, **8**, 150–157.
17. Wong JH, Steineman S, Calderia C, Bowles J, Namiki T. Ex vivo sentinel node mapping in carcinoma of the colon and rectum. *Ann Surg* 2001, **233**, 515–521.
18. Turner RR, Ollila DW, Stern S, Giuliano AE. Optimal histopathologic examination of the sentinel lymph node for breast carcinoma staging. *Am J Surg Pathol* 2000, **24**, 307–308.
19. Chu KU, Turner RR, Hansen NM, Brennan MB, Giuliano AE. Sentinel node metastasis in patients with breast carcinoma accurately predicts immunohistochemically detectable nonsentinel node metastasis. *Ann Surg Oncol* 1999, **6**, 756–761.
20. Saha S, Nora D, Wong JH, Weise D. Sentinel lymph node mapping in colorectal cancer—a review. *Surg Clin North Am* 2000, **80**, 1811–1819.
21. Joosten JJ, Strobbe LJ, Wauters CA, Pruszczynski M, Wobbes T, Ruers TJ. Intraoperative lymphatic mapping and the sentinel node concept in colorectal carcinoma. *Br J Surg* 1999, **86**, 1225–1226.
22. Mori M, Mimori K, Inoue H, *et al*. Detection of cancer micrometastases in lymph nodes by reverse transcriptase-polymerase chain reaction. *Cancer Res* 1995, **55**, 3417–3420.
23. Nakamori S, Kameyama M, Furukawa H, *et al*. Genetic detection of colorectal cancer cells in circulation and lymph nodes. *Dis Colon Rectum* 1997, **40**(Suppl.), S29–S36.
24. Rosenberg R, Hoos A, Mueller J, Nekarda H. Impact of cytokeratin-20 and carcinoembryonic antigen mRNA detection by RT-PCR in regional lymph nodes of patients with colorectal cancer. *Br J Cancer* 2000, **83**, 1323–1329.

25. Cagir B, Gelmann A, Park J, *et al*. Guanylyl cyclase C messenger RNA is a biomarker for recurrent stage II colorectal cancer. *Ann Int Med* 1999, **131**, 805–812.
26. Hoon DSB, 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**, 2109–2116.
27. Cutait R, Alves VA, Lopes LC, *et al*. Restaging of colorectal cancer based on the identification of lymph node micrometastases through immunoperoxidase staining of CEA and cytokeratins. *Dis Colon Rectum* 1991, **34**, 917–920.
28. Jeffers MD, O'Dowd GM, Mulcahy H, Stagg M, O'Donoghue DP, Toner M. The prognostic significance of immunohistochemically detected lymph node micrometastases in colorectal carcinoma. *J Pathol* 1994, **172**, 183–187.
29. Hayashi N, Ito I, Yanagisawa A, *et al*. Genetic diagnosis of lymph-node metastasis in colorectal cancer. *Lancet* 1995, **345**, 1255–1256.
30. Liefers GJ, Cleton-Jansen AM, van de Velde CJ, *et al*. Micrometastases and survival in stage II colorectal cancer. *N Engl J Med* 1998, **339**, 223–228.