Journal of Molecular Diagnostics, Vol. 9, No. 5, November 2007

DOI: 10.2353/jmoldx.2007.070032

Review

Is Occult Lymph Node Disease in Colorectal Cancer Patients Clinically Significant?

A Review of the Relevant Literature

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The clinical significance of micrometastasis of colorectal cancer (CRC) to regional lymph nodes remains controversial. In this review, we analyze publications that have evaluated the clinical significance of occult lymph node metastasis in CRC. An extensive literature search identified 19 publications that evaluated the clinical significance of micrometastatic CRC by various methods, including immunohistochemistry (IHC; $n = 13$) and reverse transcription-polymerase chain reaction (RT-PCR, $n = 6$). These studies were reviewed for methodology and findings. Significant limitations in methodology were identified, including inconsistent histological definitions of micrometastatic disease, poor sampling because of an inadequate number of lymph nodes or number of sections per lymph node analyzed, lack of conformity with respect to IHC antibody or RT-PCR marker, and inadequate power because of small sample size. Micrometastatic lymph node metastasis identified by RT-PCR was consistently found to be prognostically significant, but this was not true of micrometastatic disease identified by IHC. RT-PCR analysis of lymph nodes with specific markers can help identify pN0 (pathological-negative lymph node) CRC patients at increased risk for recurrence. The identification of occult disease by IHC techniques may also ultimately prove to be associated with worse outcome, but a number of inadequately powered studies have concluded conversely. ***(J Mol Diagn 2007, 9:563–571; DOI: 10.2353/jmoldx.2007.070032)***

Colorectal cancer (CRC) is the third most common malignancy and second most common cause of cancer-related death in the United States (National Cancer Institute SEER database, *http://seer.cancer.gov/*). As in other solid organ malignancies, the presence of lymph node (LN) metastasis is prognostic and impacts treatment decision making. For example, adjuvant chemotherapy confers a survival advantage in pN1 patients (LN metastasis identified by standard pathological analysis).[1,2] However, ~20 to 30% of patients with pathological-negative LNs by current methods of analysis (pN0) develop recurrent disease. Thus, these patients that seem to have localized disease, in fact, harbor occult metastatic disease that is undetected by current pathological or clinical evaluation.

Throughout the last 2 decades, techniques have been forwarded to improve the sensitivity of LN analysis including improved sampling through serial and/or step-sectioning[3] and improved sensitivity through immunohistochemistry (IHC) or RT-PCR. Multiple studies have shown that these techniques identify metastases that cannot be appreciated using standard light microscopy and limited sampling of the LN. However, the clinical significance of this micrometastatic LN disease in CRC and other malignancies remains controversial. In this article, we review publications that have evaluated the clinical significance of occult LN metastasis in CRC.

Definition of Micrometastasis and Occult Disease

There is confusion and debate over the appropriate definition of micrometastases. The American Joint Committee on Cancer has defined micrometastases as lesions

Supported in part by the National Institutes of Health (grant CA-01958 to S.J.H.).

Accepted for publication May 11, 2007.

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Table 1. IHC Studies

Study	Patients (stage)	Antibody	Mean nodes examined	% pN0 (i+)	Follow-up	Outcome	Recurrence rate	Clinical relevance
Greenson et al[6]	50 (2)	AE1/AE3 (M)	11.3	AE1/AE3: 28%	60.3 months (mean)	AE1/AE3 DSS: i−: 97%*; i+: 57%*	NA	AE1/AE3: $P < 0.0009$
		CC49 (M) (anti-TAG-72)	11.3	CC49: 76%	60.3 months (mean)	CC49 DSS: i−: ~90%*; i+: ~85%*	NA	CC49: not significant
Clarke et al[16]	100 (2)	anti-CK (stains cytokeratin 5, 6, 8, 17)	7	25%	60 months	DSS: i−: 89%; i+: 44%	NA	$P = 0.0123$
Yasuda et al[12]	42 (2)	CAM 5.2 (M)	24	76%	>5 years	0 to 3 nodes i+: 90%; >3 nodes i+: 50%	29%*	$P < 0.05$
Haboubi et al[11]	25 (2)	CAM 5.2 (M)	51†	60%†	55 months (mean)	OS†: i−: 90%†; i+: 60%†	NA	$P = 0.0652$
Jeffers et al[13]	77 (2)	AE1/AE3 (M)	7	25%	6.8 years (mean)	OS: i− (~65%*) better (at 5 years) than i+ (~50%*)	NA	$P > 0.1$
Palma et al[14]	38 (2)	AE1/AE3 (M)	10	15.7%	NA	Mean survival: i−: 75.97 months; i+: 71 months	NA	$P = 0.246$
Cutait et al[8]	46 (1,2)	CEA (P); AE1/AE3 (M)	13.1*	26%	>64 months	DFS: i−: 71%*; i+: 83%*	26%*	$P = 0.472$
Broll et al[9]	32 (1,2)	AE1/AE3 (M); BerEP4 (M)	NA	19% (stages 1 and 2 only)	84 months (median)	DFS: i−: 69%; i+: 67%	16%*	$P = 0.48$
Kronberg et al[7]	90 (1,2)	AE1/AE3 (P); PCK2 (P)	15	28.9%	90.7 months (mean)	DSS: i−: 90.4%; i+: 80.8%	21%	$P = 0.489$
Oberg et al[10]	147 (1,2)	CAM 5.2 (M)	4 (median)	32%	N/A	DSS: i−: 85%*; i+: 83%*	20%*	$P = 0.8193$
Adell et al[5]	100 (2)	anti-CK (M) (anti-8, 18, 19)	4.67	39%	49 months (mean)	DFS: i−: ~65%*; +: ~65%*	31%*	$P = 0.89$
Noura et al[17]	64 (2)	AE1/AE3 (M)	5.5	54.7%	79.5 months (mean)	i−: 85.1% OS i+: 90.8% OS	19%*	NS (no P value)
Tschmelitsch et al[15]	50‡ (2)	AE1/AE3 (M)	16.3	76%	60 months (case-control study)	NED group:§ 84% pN0(i+); Relapse: 67% pN0(i+)	51%	NS (no P value)

M, monoclonal; P, polyclonal; OS, 5-year overall survival; DFS, 5-year disease-free survival; DSS, 5-year disease-specific survival; NA, not available in the text; NS, not significant.

*Figures are calculated or estimated based on data and tables found in text.

†These figures are based on stage 2 patients only, used xylene fat clearance technique, and survival data are at 55 months as reported by Haboubi and colleagues.[11]

‡Tscmelitsch and colleagues[15] excluded five patients from the original 55 that had histologically positive LNs on the first recut by H&E staining.

§Tschmelitsch and colleagues[15] conducted this study in case-control fashion with a relapse group that all experienced recurrence and a NED group with no recurrence in 5 years.

between 0.2 and 2.0 mm in diameter, and lesions smaller than 0.2 mm are referred to as "isolated tumor cells."[4] However, these definitions cannot be applied to disease detected by molecular means. This issue was raised at a recent National Cancer Institute meeting, and the subsequent discussion resulted in the acceptance of the term "occult tumor cells" as being more inclusive. This term was defined as "disease that is not detected by standard pathological techniques."

To avoid confusion, patients identified with occult disease should now be classified as pN0(i+) if detection is negative by hematoxylin and eosin (H&E) staining but positive by IHC or pN0(mol+) if detection is positive by molecular techniques such as RT-PCR. This level of distinction will facilitate our understanding of the clinical significance of small-volume LN metastases. In this article, we review all appropriate studies that have aimed to determine the clinical significance of this previously occult disease, while recognizing that each of these studies differ subtly with regard to definitions and methodologies used to identify occult metastasis within LNs of CRC patients.

Search Methods and Article Selection

As opposed to other malignancies such as breast cancer, there is a lack of information regarding occult, hematogenous metastasis in CRC. Thus, the goal of this article is restricted to critical analysis of all studies addressing the clinical significance of occult metastatic disease to LNs in CRC. Relevant articles were identified through a PUBMED search using the terms "colorectal cancer," "lymph nodes," and "micrometastasis," Additional articles were identified through careful review of the referenced articles from these initially identified publications.[5–22] Thus, the reviewed studies consistently evaluate the pN0 LN from stage 1 and stage 2 CRC patients (in some studies referred to as Dukes A and B) for the presence of occult metastases. The following aspects were considered with regard to each study: i) size of study including number of stage 1 and 2 patients and percentage of rectal cancer patients; ii) method used for occult metastasis detection; iii) potential for sampling error (includes sectioning analysis, number of slides, sections, and levels of sections per LN, and number of LNs reviewed per patient); iv) quality control of original pathological diag-

Table 2. RT-PCR Studies

Study	Patients (stage)	Marker	Mean nodes examined	% pN0 (mol+) patients	Follow-up	Outcome	Recurrence rate	Clinical relevance
Hayashi et al[20]	71 (1,2)	MASA; K-ras, p53	14.5*	52.1%*	60 months	Recurrence within 5 years: mol−: 0%; mol+: 73%*	41%*	$P < 0.0001$
Rosenberg et al[21]	85 (1,2)	CK-20	2 (underwent RT-PCR)	52%	86 months (median)	OS: mol−: 94.7%; mol+: 77.9%	21%	$P < 0.009$
Noura et al[17]	64 (2)	CEA	5.5	29.7%	79.5 months (mean)	DFS: mol−: 88.4%; mol+: 61.4%	19%*	$P = 0.027$
Liefers et al[19]	26 (2)	CEA	7.4*	54%	60 months	DSS: mol−: 91%; mol+: 50%	31%*	$P = 0.03$
Merrie et al[18]	141 (1,2)	CK-20	15.3*	34%	42 months (median)	OS: mol−: ~85%; mol+: ~70%	NA	$P < 0.135$
						OS and # nodes: 0 mol+: ~85%; 1 to 3 mol+: ~75%; 4 mol+: ~45%	NA	$P < 0.0052$
Bustin et al[22]	32 (1,2)	GCC; CK20; CEA	7.2†	NA	47.3* months (median)	No association between mRNA expression levels and recurrence in pN0(mol+) patients	NA	NS (no *P* value)

NA, not available; NS, not significant.
*Figures are calculated or estimated based on data and tables found in the text.
†Bustin and colleagues[22] included 10 stage 3 patients in their study. This figure is calculated including all LNs from stage 1 to 3 patients.

nosis; v) percentage of patients that were upstaged; vi) mean or median follow-up (5-year survival and recurrence data are ideal); and vii) antibodies and/or markers used for IHC or PCR.

Studies that evaluated a small number of patients ($n < 25$) or those that did not correlate their findings to outcome were excluded. Because there is evidence in T3 rectal cancer patients that neoadjuvant radiation therapy has survival benefit[23] and most of these patients do receive this therapy, identifying the clinical significance of occult metastasis to regional LN in rectal cancer patients may be confounded by this additional therapy. Thus, one study[24] entirely composed of rectal cancer patients was also excluded. Attention to specimen handling, ie, warm ischemia time, could impact these studies; however, this issue is rarely addressed in the reviewed literature and could not be used as a selection criteria or for subsequent critical analysis of the studies. Using this process, 13 studies that used IHC staining and six studies that used RT-PCR were identified and subsequently reviewed.

Review of Experimental Methodology

Attention to Sampling

Inherent to improving the sensitivity of occult tumor cell detection is the reduction or elimination of sampling error through appropriate attention to the number of LNs harvested per patient, the number of sections/slides made per LN, and the region(s) of the LN sampled. Our review identified that many different approaches were used, but ultimately the majority of studies may be flawed because of inadequate attention to sampling.

First, current evidence suggests that a minimum of 12 LNs be reviewed for accurate staging,[25] and a recent report from Cancer and Leukemia Group B 80001[26] further supported this notion by demonstrating that IHC analysis of multiple levels of CRC sentinel nodes was not enough to overcome a sampling error. In the reviewed studies, the number of nodes examined ranged from 4 to 51 (Tables 1 and 2). One study did not report the mean number of nodes examined.[9] In another study, the technique of xylene fat clearance was used to increase the amount of nodes sampled per patient to 51.[11] Only 5 of 11 IHC studies examined the recommended 12 or more nodes per patient necessary for accurate staging. The number of LNs examined by molecular methods ranged between 2 and 15. Only two studies analyzed more than 12 nodes,[18,20] and both showed a significant difference in outcome. Thus, the majority of these studies do not seem to have harvested or analyzed 12 nodes per patient.

These studies are also subject to sampling error depending on volume of the LN sampled, namely the number of tissue sections evaluated by the technique. Careful sampling is labor intensive and more expensive. Optimal technique to reduce sampling error uses step-sectioning the LN so that representative sections are obtained from the entire LN. Our review identified that, in addition to the original staging by light microscopy and H&E, some of the studies evaluated only one new slide from a paraffin block for each LN, but other studies were more rigorously designed and typically evaluated four or five new slides by IHC (Table 1). In summary, the reviewed studies lack conformity on attention to sampling with respect to the number of nodes per patient evaluated and the volume of each individual LN reviewed. Less than half of the studies examined an adequate number of LNs.

Antibodies for IHC

The choice of antibody in IHC or of RNA marker in molecular studies is an important factor in the ability to accurately identify occult disease. AE1/AE3 (DAKO, Carpinteria, CA) is the most widely used antibody for IHC analysis of LN from CRC patients. This polyclonal antibody is raised against several cytokeratins, including CK19. CAM 5.2 is an antibody to CK8 and CK18, but it has been criticized as lacking specificity in a report that showed CAM 5.2 stains macrophages found in normal LNs containing phagocytosed cytokeratins.[27] Interestingly, studies that used the AE1/AE3 antibody identified occult disease in a mean of 35% and median of 28% of patients, whereas studies that used the CAM 5.2 antibody upstaged a mean of 56% and a median of 60% of patients (Table 1). This suggests that sensitivity and specificity of IHC analyses are dependent on the selected protein marker and corresponding antibody.

The Ber-EP4 antibody is an infrequently used antibody that immunostains TACSTD-1, a surface glycoprotein expressed by nearly all epithelial cells. Broll and colleagues[9] have reported complete concordance in classification between Ber-EP4- and AE1/AE3-stained specimens. The one other antibody used in these studies was CC49, an antibody against tumor-associated glycoprotein 72 (TAG-72). This protein is expressed by most colonic adenocarcinomas, as well as cancers of the breast, lung, ovaries, pancreas, stomach, and esophagus.[28] CC49 was used in only one study, concurrently with AE1/AE3.[6] Finally, we identified a methodological problem in how one study evaluated specimens by staining with two antibodies. Cutait and colleagues[8] used both an anti-CEA and AE1/AE3 antibodies. Specifically, they selected patients and LNs that stained positively for CEA and then performed an AE1/AE3 IHC test only on those LNs. Therefore, the sensitivity in that study is not reflective of the sensitivity of AE1/AE3 itself. Whenever criteria are established that a patient has to screen positive by two methods to be considered positive, this will always result in lower sensitivity than would be obtained from using either screen alone. In summary, a variety of antibodies have been used for IHC detection of occult disease in the LN of CRC patients. However, a gold standard has not been clearly established. The largest experience is clearly with the AE1/AE3 antibody; CAM 5.2 may lack specificity.

mRNA Markers for RT-PCR

A number of mRNA markers have been used for the detection of occult metastases in the LN of CRC patients by RT-PCR. In a recent publication, Xi and colleagues[29] identified the differential, relative expression of various markers between primary tumors and normal LNs in cancer patients. The six most useful markers for metastatic CRC detection were CEA, CK19, CK20, CDX1, TACSTD-1, and villin-1. One other reported marker for CRC is CK18.[30,31] Xi and colleagues[29] found this marker should be less specific because of higher background expression in normal LNs. All of the reviewed studies that used RT-PCR used CEA or CK20 mRNA markers (Table 2).

Importantly, one limitation of RT-PCR for the detection of occult disease is the potential for a lack of specificity because of low-level expression of the mRNA marker by lymphocytes or other cells present in benign LNs. This pitfall can be overcome, and a marker made specific for the presence of metastasis, by using *quantitative* techniques (ie, qRT-PCR). Xi and colleagues[29] demonstrated background expression of CEA, CK19, CDX1, and TACSTD-1 in benign LNs; thus any study that utilizes these markers and does not use qRT-PCR with expression cutoff decision rules has significant potential to falsely identify LNs as positive for metastatic disease. By comparing the expression of each of these markers to an endogenous control, Xi and colleagues[29] found that each of these six mRNA markers had a ratio of expression in tumors compared with expression in LNs from patients without cancer greater than 300 (median tumor expression of marker/highest benign LN expression). Only one study using molecular methods of occult disease detection applied qRT-PCR.[22] Thus, most of the reviewed molecular studies were subject to false-positive results.

In summary, there are a number of mRNA markers proven to be useful for the detection of occult CRC metastases within LN, provided qRT-PCR is used. Based on our review, the marker with the strongest theoretical value and empirical experimental data for occult CRC metastasis detection by molecular means is CK20. This marker seems to be expressed in virtually all CRCs[32] producing high sensitivity, and importantly, the background expression of CK20 in normal LNs is negligible, facilitating high specificity.

Issues Common to Markers and Antibodies

Finally, additional issues regarding mRNA markers and IHC antibodies warrant consideration. The majority of used markers/antibodies in the reviewed studies (Tables 1 and 2) are against epithelial cell-related markers rather than cancer-specific markers. A potential pitfall of epithelial cell-related markers is increased sensitivity at the expense of specificity. To overcome this, a pathologist must review the positively stained cells to confirm there are morphological characteristics consistent with cancer cells. This was clearly done and reported in some,[8,14] but not all, of the reviewed studies. Despite this quality control step, Noura and colleagues[17] upstaged 54.7% of patients as opposed to only 15.7% in the results of Palma and colleagues[14] despite the use of the same antibody on stage 2 patients. This disparity in the frequency of finding occult disease using the same antibody raises concerns regarding specificity.

Clinical Follow-Up

For CRC, the accepted standard for adequate follow-up is 5-year OS or 3-year disease-specific survival.[33] In this respect, all of the studies reviewed (Tables 1 and 2) seem to have had adequate follow-up, but consistent methods

Table 3. Survival Statistics for Colorectal Cancer by TNM Stage

5-Year overall survival			
Tumor	Nodestatus	Stage	Survival(%)
T1/T2	N0	1	93
T1/T2	N1	3a	83
T3	N0	2a	85
T3	N1	3b	64
T4	N0	2b	72
T4	N1	3b	64

of outcome assessment were not used. Very few of the reviewed studies reported the percentage of patients lost during follow-up. One study excluded 33 of 100 patients that were either lost to follow-up or died of an unrelated disease.[16] This could lead to bias because patients lost to follow-up may have a higher disease-specific mortality than the rest of a study's population. Another study reported specific data regarding patients that died from CRC and patients that were alive without disease, but the study did not give any data in regard to those that were alive with recurrence.[22] With respect to the frequency of encountered events as a measure of appropriate sampling, the expected recurrence rate in LN-negative patients should be ~25%. The recurrence rates of the reviewed studies typically fell within reasonable proximity to this benchmark.

Statistical Analysis and Power

We reviewed the studies for their statistical power to test the hypothesis that occult metastases to the LN of CRC patients are clinically significant. We first estimated what the anticipated effect is for a patient with pN0 disease being up-staged to pN0+ disease (Table 3). This table demonstrates 5-year OS and is organized to demonstrate the percentage change in recurrence rates associated with upstaging from N0 status to N1 for each given tumor (T) size (T1, T2, and so forth). In essence, for T1 and T2 tumors, the identification of nodal metastasis is associated with a 10% decrease in 5-year OS, and for T3 tumors the decrease is 21%.

Our first goal was to determine the number of patients necessary for adequate power to find a reasonably large effect—a 5-year OS difference at least as large as 90 versus 75% (15%). Using the method of Lee (nQuery Advisor, Statistical Solutions, Saugus, MA), we constructed two precision tables to calculate how many patients a study needs to achieve 80 or 90% power. In the first table (Table 4), we constructed a precision table assuming the groups would be divided into 50% being upstaged and 50% not. This table presents the number of patients required to have 80 or 90% power to detect differences in survival ranging from the largest (95 versus 60%) to the smallest (90 versus 85%). Table 4 examines the same variables but assumes that 30% of a study group's patients will be upstaged. This is based on the assumption that 20 to 30% of patients should recur. To consider the extreme, the fewest calculated number of patients required to have 80% power to detect a 35% difference in the percentage of patients alive at 5 years would have 48 patients by the even-split table methodology or 46 patients under the assumption of 30% of patients being upstaged.

Table 4. Precision Analysis Showing the Number of Patients Required to Demonstrate Clinical Significance of Occult Lymph Node Metastasis Using Different Estimates for the Difference in Overall Survival

Precision table (50/50)				
	5-Year overall survival		Power	
Stage	MM−	MM+	80%	90%
Size of study (*N*)	Size of study (*N*)			
T1N0	95%	65%	48	66
	95%	75%	92	122
	95%	85%	272	364
	95%	90%	860	1140
T2N0	90%	60%	62	82
	90%	70%	120	160
	90%	80%	392	522
	90%	85%	1368	1820
Precision table (30/70)				
	5-Year overall survival		Power	
Stage	MM−	MM+	80%	90%
			Size of study (*N*)	Size of study (*N*)
T1N0	95%	65%	46	69
	95%	75%	92	130
	95%	85%	296	406
	95%	90%	959	1312
T2N0	90%	60%	65	89
	90%	70%	130	176
	90%	80%	442	600
	90%	85%	1568	2108

Table 5. Power Analysis of Individual Studies

Study	Power of each negative study for the following predicted 5-year overall survival differences between Pn0+ and Pn0− groups			
	90 versus 60%	90 versus 70%	90 versus 80%	Stage 1 patients (%)
Oberg et al[10]	98	85	40	30%
Adell et al[5]	94	72	30	0
Kronberg et al[7]	90	65	27	22%*
Jeffers et al[13]	83	57	24	0
Noura et al (IHC)[17]	80	51	18	0
Cutait et al[8]	64	40	17	NA
Bustin et al[22]	56†	35†	16†	18.8%*
Palma et al[14]	52	34	18	0
Broll et al[9]	49	32	17	18.8%
Tschmelitsch et al[15]	46	20	3	0
Haboubi et al[11]	38	20	7	10.7%*

The columns represent the statistical ability of the study to appropriately test the hypothesis. Smaller differences in outcome reduce this power. NA, not available.

*Figures are calculated by author, not reported in article.

†A 30/70 split is assumed for this article.

To consider the possibility that low-volume, occult disease identified by RT-PCR or IHC is associated with an equivalent prognosis to metastatic disease readily identified using current methods of analysis, Table 4, also includes rows indicating the number of patients required to identify smaller differences in outcome. Thus, an appropriately powered study capable of 80% power to detect a 10% difference in stage 2 patients found to have occult LN metastasis would require 296 to 442 patients (Table 4).

Using the same statistical methods, we examined each negative study (Table 5) looking at the size and split of pN0+ and pN0− patients for that individual study. Our goal was to see how small an effect each study was capable of detecting. To modify this analysis to be study specific, we incorporated the percent upstaged from each study and the total number of patients in each study (Tables 1 and 2). For example, the study by Oberg and colleagues[10] had 40% power to detect a 90 versus 80% 5-year OS difference. This was based on the size of the study (147 patients) and the percentage of patients that were upstaged (32%). Based on this analysis, 5 of the 11 negative studies had 80% or greater power to detect a large (90 versus 60%) 5-year OS difference. Not a single negative study had 80% power to detect a difference in survival between 90 and 70%. Therefore, it is possible that a type 2 error (acceptance of the null hypothesis when it is false or, stated differently, a significant difference exists but is not identified because of inadequate sample size) occurred in many or all of these studies (Table 5).

Are Occult LN Metastases Clinically Significant?

IHC Studies

Overall, studies that used IHC to detect occult disease were diverse in methodology and design. The size ranged from 32 to 147 patients, and as such, none were well powered to detect smaller, but potentially significant, differences in the outcome of patients with occult LN metastasis. All were exclusively comprised of stage 1 and/or stage 2 patients with the exception of one.[9] The studies differed markedly in the percentage of upstaged patients by IHC ranging from 15 to 76%. If these analyses are to be helpful in identifying the 20 to 30% who will suffer recurrence, then one may be concerned that upstaging of 76 or 15% indicates poor specificity and sensitivity in the respective studies. It is also important to bear in mind that not all pathologically node-positive patients suffer disease recurrence (only ~50% of pN1 patients recur) and that lymphatic metastasis is not the only potential mechanism of disease dissemination and recurrence. To address these issues, it would be helpful if future studies paid close attention to the site of disease recurrence (regional or distant) and included some assessment of hematogenous spread of tumor cells either in the peripheral blood or the bone marrow.

In summary, it is unclear if there is a clinically significant difference in detecting occult LN disease by IHC. Several studies show survival trends. However, clinically significant ($P < 0.05$) differences were observed in only 3 of 11 studies. In general, most studies that analyzed a large number of nodes and upstaged a high percentage of patients did find a survival trend; only one of the five studies with ideal LN sampling did not show any survival trend. The one study that had a reverse trend [patients with pNO(i−) LNs having worse survival]) stained all LNs for CEA before using the AE1/AE3 antibody. It is possible that this approach may have adversely impacted sensitivity. Thus, we cannot strongly conclude that IHC-detected occult disease is associated with clinically significant worse outcome.

Molecular Studies

The molecular studies represent a comparatively smaller group. The size of the studies ranged from 26 to 141

patients. The studies consisted of all stage 1 and/or stage 2 CRC patients except for two. Bustin and colleagues[22] seem to have included 10 stage 3 patients in their original group of 42 patients. Similarly, Merrie and colleagues[18] list 59 patients as having positive LNs by light microscopy at the time of surgery. For the purpose of our analysis, stage 3 patients in both studies were excluded (Table 2). RT-PCR was the predominant technique used for analysis of the LNs in all of the studies—but only one study used qRT-PCR.[22] The oldest study reviewed used mutant-allele-specific amplification, with attention focused on K-ras and p53 mutations.[20] One clear criticism of this study is that mutant-allele-specific amplification was limited to only 71 of the original 120 (59%) primary tumors that actually had these mutations. Thus, the clinical utility of this approach is less clear.

With regard to the molecular studies, the percentage of upstaging patients ranged from 29.7 to 54%. The follow-up for most studies exceeded 5 years. The two exceptions had median follow-up of 42 and 47.3 months.[18,22] Neither of these two studies reported recurrence data, which would have proven more informative than OS given the shorter follow-up period. The overall recurrence rate ranged from 19 to 41% in four of six studies in which these data were reported. This is a considerably higher rate of recurrence than would be anticipated in groups of stage 1 and stage 2 CRC patients.

In the one study using qRT-PCR reported by Bustin and colleagues,[22] the markers CK20, CEA, and guanyl cyclase C (GCC) were examined for occult disease detection in LN from CRC patients (Table 2). GCC is a transmembrane receptor selectively expressed in midgut and hind-gut intestinal mucosa.[22] Using these three markers, they were able to differentiate expression levels between three categories of LNs including normal LNs from patients without cancer, histologically negative LNs from cancer patients, and histologically positive LNs from cancer patients. For all three markers, expression level differences comparing each type of LN were significant (CK20, $P < 0.001$; CEA, $P < 0.0001$; GCC, $P < 0.05$). This study was not able to determine cutoff thresholds for the differential expression that correlated with patient outcome. Overall, the molecular studies clearly suggested a clinical relevance to finding occult disease in LNs from CRC patients (Table 2). The fact that many of these studies were able to find clinical relevance while still being susceptible to sampling error, suggests that there is clinical relevance to molecular analysis of LNs in CRC.

Discussion

There is precedence for detecting clinically significant occult LN disease in other cancers. In esophageal cancer, Godfrey and colleagues[34] demonstrated clinical significance by qRT-PCR testing for CEA mRNA. These pN0(mol+) patients were significantly more likely to have recurrence and had worse OS. With non-small-cell lung cancer, Coello and colleagues[35] determined, in their review of predominantly IHC studies, that occult LN disease was a clinically significant negative prognostic factor. In addition, with breast cancer, Sakorafas and colleagues[36] concluded that the "presence of axillary sentinel lymph node (SLN) micrometastases is generally associated with a worse prognosis."

For CRC, the current data favors detection of occult disease in CRC LNs by means of RT-PCR. There are at least four studies using this method of detection showing prognostic significance of identified occult disease. In contrast, several studies aimed at identifying occult disease using IHC failed to demonstrate clinical significance. However, none of the current studies were adequately powered to definitively conclude that IHC-detected disease is not clinically significant. Realizing this limitation, despite significant differences in experimental methodology, Iddings and colleagues[37] very recently performed a meta-analysis of eight IHC-based and three RT-PCR-based studies. They concluded that occult metastases identified by RT-PCR, but not IHC, are associated with a worse clinical outcome. Thus, our review and the findings of Iddings and colleagues[37] suggest that RT-PCR, but not IHC analysis, seems to be a useful tool in identifying a higher risk group within stage 2 CRC patients. The reason(s) IHC identification of occult LN metastasis fails to convey prognostic information remains unclear but may be related to a lack of appropriate criteria to characterize the LN as positive.

Sampling error may be efficiently reduced if a technique such as SLN mapping with isosulfan blue dye is used to direct increased assessment of a subset of LNs. Saha and colleagues[38,39] routinely use this technique in colon cancer and are able to identify SLN in 99.1% of colon cancer cases. This group and others subsequently use serial sectioning with 5 to 10 sections and IHC staining of the SLN to minimize sampling errors and maximize sensitivity. However, in contrast to breast cancer and melanoma, the identification of skip metastases (metastasis is identified in other resected, nonsentinel nodes) has caused significant concern regarding the use of SLN biopsy in CRC. These skip metastases have been reported to be identified in as many as 53.8% of cases,[40] suggesting that all resected LNs need to be reviewed regardless of the status of the SLN. The recent CALGB 80001 results also argue in favor of analyzing all of the resected LNs.[26] Thus, SLN identification and analysis remains controversial and not widely used for CRC. Although review of existing studies does not facilitate a firm conclusion regarding how many LNs need to be evaluated or the value of SLN mapping, we believe that any subsequent studies of occult LN metastasis detection in CRC should be designed to evaluate at least 12 LNs.

The future directions of staging CRC will be guided by clinical significance, efficiency, and, of course, cost. One currently promising but costly technique involves the use of microarray analysis of the primary tumor. In two separate studies, microarray analysis was used in conjunction with standard histological analysis of LNs. Both studies showed utility in this technique predicting recurrence from tumor signature alone.[41,42] The study by Wang and colleagues[41] validated the relapse signature in tumors

from 36 independent patients. Although this is not currently clinically practical, the prospect of obtaining a colonoscopic biopsy and directing treatment from this small specimen cannot be ignored.

In summary, there is substantial evidence that RT-PCR analysis of LNs with specific markers can help identify pN0 CRC patients at increased risk for recurrence. The identification of occult disease identified by the less expensive and readily applicable IHC techniques may also ultimately prove to be associated with worse outcome, but a number of inadequately powered studies have concluded conversely. A meta-analysis of these methodologically disparate studies similarly failed to demonstrate that occult LN metastasis identified by IHC is clinically prognostic of worse outcome. An adequately powered and carefully designed study that determines if occult LN metastasis detected by IHC is prognostic of worse outcome is warranted. Existing data suggests that a randomized trial assessing the potential benefit of adjuvant therapy in patients with N0(mol+) or N0(i+) is also essential.

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