Association Between a High Number of Isolated Lymph Nodes in T1 to T4 N0M0 Colorectal Cancer and the Microsatellite Instability Phenotype

Clarisse Eveno, MD; Judith Nemeth, MD; Hany Soliman, MD; Françoise Praz, MD, PhD; Hugues de The, MD, PhD; Patrice Valleur, MD; Ian C. Talbot, MD, PhD; Marc Pocard, MD, PhD

Hypothesis: Stage I or II colorectal carcinomas with microsatellite instability (MSI) are characterized by more isolated lymph nodes in the resected specimen than their counterparts with microsatellite stability (MSS).

Design: Prospective study.

Setting: Academic research.

Patients: Using a pentaplex polymerase chain reaction assay, MSI status was determined prospectively for 135 operative patients.

Main Outcome Measures: Mismatch repair defects were investigated by immunohistochemistry on tumors demonstrating MSI.

Results: Among 82 stage I or II colorectal carcinomas, 11 had MSI, and 71 had MSS, with a mean (SD) number of 23.6 (3.1) and 13.7 (1.0) negative lymph nodes, respectively (P = .001). The mean number of lymph nodes for all resected stage I or II colorectal carcinomas analyzed at our hospital was 15. The prevalence of MSI among tumors with more than 15 lymph nodes in the specimen was 25% (9 of 36), and 82% (9 of 11) of MSI tumors belonged to this group.

Conclusions: A high number of isolated lymph nodes in stage I or II colorectal carcinomas was associated with the MSI phenotype. Good prognosis that is usually associated with tumors having a high number of uninvolved lymph nodes might reflect the high prevalence of MSI among these tumors. The number of examined lymph nodes as a quality criterion should be used with caution. For stage I or stage II colorectal carcinomas, restricting MSI phenotyping to tumors with more than the mean number of lymph nodes identifies almost all MSI tumors.

Arch Surg. 2010;145(1):12-17

Colorectal cancer is the second most common cause of cancer death in North America.¹ ² The mesocolon lymph node status at presentation predicts long-term survival and is the most important prognostic indicator for primary colon cancer identified so far.³ Although adjuvant systemic treatment is no longer solely based on lymph node status, the presence of histologically involved lymph nodes is still a major indication.⁴ Therefore, correctly identifying patients with positive regional lymph nodes has important prognostic and therapeutic implications.

Among patients with node-negative colon cancer, studies⁵-⁷ have shown that pathology reports of few lymph nodes in the resected specimen correlate with shorter survival. Swanson et al⁸ stratified a cohort of patients with T3N0 disease into 3 groups according to the number of examined lymph nodes (1-7, 8-12, and ≥13); these groups had significantly different 5-year survival (49.8%, 56.2%, and 63.4%, respectively) (P < .001). A recent systematic review⁹ confirmed that the number of lymph nodes evaluated after surgical resection was positively associated with survival among patients with stage II tumors.

At our hospital, this has also been reported for survival and local relapse rates associated with rectal cancer:¹⁰ the actuarial survival rate at 10 years was significantly lower for patients having T3 tumors with fewer than 10 vs 10 or more lymph nodes in the resected specimen (P < .05). The local recurrence rate was significantly higher for patients with fewer than 10 (20%) vs 10 or more (7%) lymph nodes analyzed in the resected specimen (P < .02). Together, these studies demonstrate that a higher number of examined lymph nodes is associated with a better prognosis. To date, there has been no clear biological explanation for this observation.¹¹⁻¹⁵

See Invited Critique at end of article

Author Affiliations are listed at the end of this article.
It has often been suggested that poor prognosis is due to tumor understaging of patients who are node positive but are deemed node negative because of inadequate surgical removal of lymph node-bearing mesentry. These understaged patients are then erroneously assigned to a better prognostic category because of their node-negative status and subsequently have poorer survival than actual node-negative patients. Accordingly, the number of examined lymph nodes may be considered a quality criterion. In an effort to improve the staging of patients undergoing colorectal resection, national and international organizations have recommended to surgeons and pathologists that a minimum of 12 lymph nodes should be identified during pathologic review.

There are 2 major distinct colorectal oncogenic pathways, namely, microsatellite instability (MSI) and chromosomal instability. Microsatellite instability status is associated with better prognosis for each specific tumor stage compared with microsatellite stability (MSS) status. In a French study that included 142 patients, Parc et al reported longer overall and recurrence-free survival among patients with MSI tumors than among patients with MSS tumors (P = .002). In a recent article, the presence of a Crohn disease–like infiltrate in Dukes B rectal cancers was associated with a higher mean number of lymph nodes in the resected specimen (14.7 vs 11.0 if absent) (P < .001). This Crohn disease–like infiltrate is characteristic of MSI.

We postulated that these data might be linked and that stage I or II MSI colorectal carcinomas are characterized by higher numbers of lymph nodes in the resected specimen than their MSS counterparts. Confirmation of this hypothesis would lead to the following implications: (1) the hypothesis may account for the better prognosis in patients with stage I or II colorectal carcinomas having more lymph nodes in the resected specimen, (2) the number of examined lymph nodes as a quality criterion should be used with caution, and (3) it is worth exploring whether the mean number of lymph nodes retrieved by a surgical unit may be used to select patients for whom MSI analysis should be performed.

This study includes a population-based series of patients with colorectal cancer from Lariboisière Hospital, Paris, France. During a 3-year period from January 15, 2003, to December 15, 2005, a total of 135 patients who underwent surgical resection for a colorectal adenocarcinoma were studied prospectively. Patients undergoing emergency surgery were excluded. All patients were operated on by the same surgical team and underwent the same operative procedures, including total mesorectal excision for low rectal carcinomas as previously reported. No rectal tumors were irradiated before surgery.

Eighty-two patients (52 with colon tumors and 30 with rectal tumors) without lymph node invasion were included in the study. The tumors were classified as T1 (n = 6), T2 (n = 21), T3 (n = 54), or T4 (n = 1) according to the TNM system.

**STANDARD PATHOLOGIC EXAMINATION**

All resected specimens were examined by the same pathologist (J.N.) using a standard technique. After adequate fixation, the entire mesocolon or mesorectum is separated as soon as possible from the bowel into a monobloc. If there is substantial subserosal paratumoral invasion, the bloc is dissected with special care. Then, atypical lymph nodes are marked with a thread, and the mesocolon or mesorectum is cut into sections of less than 1 mm. Macroscopic examination of the lymph nodes is completed by palpation to detect invisible nodes. Pathologic staging was based on the 1997 TNM system by the International Union Against Cancer.

**MICROSATELLITE ANALYSIS**

Ten-micrometer sections of formalin-fixed paraffin-embedded blocks of surgically resected, pathologically confirmed cancerous tissue were used to extract DNA just after fixation (QiAamp tissue kit; Qiagen, Courtaboeuf, France). The revised Bethesda Guidelines were used to determine MSI status. Analysis of MSI was performed using panel 18 of 5 quasi-monomorphic mononucleotide repeat markers (BAT26, BAT25, NR21, NR22, and NR24) that map to intron 15, intron 16, 5′ untranslated region (UTR), 3′ UTR, and 3′ UTR of the MSH2, c-kit, SLC7A8, transmembrane protein precursor B5, and 2NF2 genes, respectively. A single pentaplex polymerase chain reaction (PCR) allows coamplification of all 5 markers, which are subsequently analyzed using a genetic analyzer (ABI PRISM 310; Applied Biosystems, Courtaboeuf, France) and computerized fragment analysis. For each of 5 primer pairs used in the PCR, 1 primer was labeled with fluorescent dye FAM, HEX, or NED as follows: BAT26 (5′-TGACTCTTTTGACCTAGGC-3′ and FAM-5′-AACATTCAACATTTTTAACC-3′), BAT25 (5′-TCTGATTTTAACTACTGGCTC-3′ and NED-5′-TCGTTCCAAAGAATGTAAGT-3′), NR21 (3′-TAAATGATGTCCTCCCTGGG-3′ and HEX-5′-ATTCTACTCCGCATTCA-3′), NR22 (5′-AGGCCCTTGTCAAGGACATAA-3′ and FAM-5′-AATTGCGGACTGCATCCATGTTG-3′), and NR24 (5′-CCATTTGCAATTCTACCT-3′ and HEX-5′-ATTGTGCTGCCATTGACCA-3′). The PCR contained 40 to 50 ng of DNA, 200 μM deoxyribonucleotide triphosphate, 2.5 mM magnesium chloride, 1 μM of each primer, and 1 U of DNA polymerase (AmpliTag Gold, Applied Biosystems) in a total volume of 20 μL. Cycling variables were 7 minutes at 95°C, 45 seconds at 95°C, and 1 minute at 60°C for 40 cycles, with a final extension step for 10 minutes at 72°C. Two microliters of the completed PCR was added to 20 μL of HiDi formamide (Applied Biosystems) containing 1 μL of ROX (GeneScan 500, Applied Biosystems) in a water bath (pH 9) at 95°C to 98°C for 40 minutes to target retrieval solution (DakoCytomation; Dako, Carpinteria, California [when the NexES autoanalyzer was used; when the Bench-Mark autoanalyzer was used, target retrieval solution for antigenic restoration was included in the autoanalyzer]). Anti-MLH1 antibody (G168-615; Pharmingen International, San Diego, Cali-
California) was diluted 1:50 and incubated for 30 minutes. Anti-MSH2 antibody (FE11; Oncogenic Research, Cambridge, Massachusetts) was diluted 1:50 and incubated for 30 minutes. Anti-MSH6 antibody (clone 44, Pharmingen International) was diluted 1:100 and incubated for 30 minutes. Anti-PMS2 antibody (A16-4, Pharmingen International) was diluted 1:50 and incubated for 30 minutes. Labeling was interpreted as follows: Protein expression was defined as the presence of nuclear labeling in tumor cells and internal controls (Figure 1A). Loss of MLH1, MSH2, MSH6, or PMS2 expression was defined as the absence of nuclear labeling in tumor cells in the presence of positive controls (basal cells from the colonic mucosal crypts or lymphocytes) (Figure 1B). Noninterpretable labeling was defined as section detachment or the absence of labeling in tumor cells and internal controls, all performed in duplicate.

**STATISTICAL ANALYSIS**

The Fisher exact test and Mann-Whitney test were used to compare frequencies and medians. The prognostic factors studied were sex, age at tumor diagnosis, tumor site, perineural invasion, lymphovascular invasion, and MSI status as determined by immunohistochemical analysis and PCR. Statistical significance was set at $P < .05$. Analyses were performed using commercially available statistical software (StatView, 1992-1998; SAS Institute Inc, Cary, North Carolina).

**RESULTS**

**PATIENT INCLUSION IN THE STUDY**

Eleven of 82 patients (13%) showed MSI both in the molecular and immunohistochemical analyses. Characteristics of these patients are given in Table 1. Patient 11 was classified as having an MSI tumor by analysis with 3 of 5 unstable molecular markers but retained expression of all mismatch repair proteins (MLH1, MSH2, MSH6, and PMS2). The power analysis showed that 82 resected specimens provided 88% power for the conclusions reached in this study.

**TUMOR CHARACTERISTICS**

Microsatellite instability was more likely to be found in tumors located in the proximal colon than in tumors located in the distal colon or rectum ($P < .001$); lymphovascular or perineural invasion did not correlate with MSI status (Table 2). No association was found between MSI status and sex or age at tumor diagnosis. The youngest patient with MSI was 35 years; the mean age of patients with MSI...
was 63.5 years compared with 68.0 years in the remainder of the cohort. No rectal cancers received neoadjuvant treatment. No patient had hereditary nonpolyposis colorectal cancer or family history of colorectal cancer.

Analysis of the mean (SD) lymph node counts according to MSI status showed that a higher number of detected lymph nodes among patients with N0 disease was associated with MSI (23.6 [3.1] for MSI and 13.7 [1.0] for MSS) (P = .001). These results are shown in Figure 2.

No MSI tumors were found among patients in the cohort with fewer than 7 examined lymph nodes. Because the MSI phenotype was associated with a high number of lymph nodes in the resected specimen, our aim was to develop a score that allows the selection of patients with a high probability of MSI using solely the number of lymph nodes in the resected specimen. Among all resected stage I or II colorectal carcinomas analyzed at Lariboisière Hospital, the mean number of lymph nodes in the specimen was 15.

Among tumors with more than the mean number of lymph nodes in the specimen (ie, >15), the prevalence of MSI was 25% (9 of 36). Use of this score (>15 lymph nodes in the specimen) detected 82% (9 of 11) of MSI tumors.

The most important determinant of patient outcome following diagnosis of colorectal cancer is the stage of disease at the time of treatment. Particularly, this includes the TNM classification and the number of involved lymph nodes.

Analysis of lymph node counts according to MSI status suggests that MSI is associated with a high number of lymph nodes in the resected specimen among patients with N0 disease. This observation allows us to link a specific cancer pathway (MSI) to a specific pathologic result, leading to several particular implications.

Confirmation of our study hypothesis explains the better prognosis observed in patients with stage I or II colorectal carcinomas who have more lymph nodes in the resected specimen. The authors of a recent article concluded that prognosis (overall survival and disease-free survival) was significantly better among patients with tumors containing a high density of inflammatory cells, as reported in rectal cancer.20,24 Dense peritumoral inflammatory cell infiltration (so-called Crohn disease–like reaction) is characteristic of MSI colon cancer and has a cytotoxic effect on tumor cells.23 This may explain why tumors developing through the MSI colon cancer–specific pathway generally have a better prognosis than their MSS counterparts at the same TNM stage.26 Together, these observations prompted us to test whether the number of examined lymph nodes (which is further related to the immune status and to the density of inflammatory cells) in node-negative patients26 may be a prognostic factor related to MSI status, as the mean number of lymph nodes is high in MSI tumors and low in MSS tumors.

The number of examined lymph nodes as a quality criterion should be used with caution.10,17 Previous studies demonstrated that the mean number of lymph nodes may vary based on patient characteristics and on the use of preoperative radiotherapy, which results in fewer lymph nodes being found on pathologic examination.

For some other primary tumors, additional factors affect the mean number of analyzed lymph nodes. In breast cancer, the number of examined axillary lymph nodes is influenced by age. In a study30 of patients with node-negative results among 5314 consecutive procedures, 73% (561 of 765) of women younger than 50 years had 10 or more examined lymph nodes, which was significantly different from the 64% (496 of 776) of women older than 70 years who had 10 or more examined lymph nodes. A similar result was found among 1081 patients with colon cancer in a study30 that dem-

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>MSI (n=11)</th>
<th>MSS (n=71)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at tumor diagnosis, mean, y</td>
<td>63.5</td>
<td>67.8</td>
<td>.34</td>
</tr>
<tr>
<td>Sex, No.</td>
<td></td>
<td></td>
<td>.11</td>
</tr>
<tr>
<td>Female</td>
<td>8</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>3</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>Tumor site, No. (%)</td>
<td></td>
<td></td>
<td>.04</td>
</tr>
<tr>
<td>Colon</td>
<td>10 (91)</td>
<td>42 (59)</td>
<td></td>
</tr>
<tr>
<td>Rectum</td>
<td>1 (9)</td>
<td>29 (41)</td>
<td>.04</td>
</tr>
<tr>
<td>Tumor stage, No. (%)</td>
<td></td>
<td></td>
<td>.27</td>
</tr>
<tr>
<td>T1 to T2</td>
<td>2 (18)</td>
<td>25 (35)</td>
<td>.27</td>
</tr>
<tr>
<td>T3 to T4</td>
<td>9 (82)</td>
<td>46 (65)</td>
<td>.27</td>
</tr>
<tr>
<td>Invasion, No. (%)</td>
<td></td>
<td></td>
<td>.31</td>
</tr>
<tr>
<td>Perineural</td>
<td>1 (9)</td>
<td>16 (23)</td>
<td>.31</td>
</tr>
<tr>
<td>Lymphovascular</td>
<td>3 (27)</td>
<td>18 (25)</td>
<td>.31</td>
</tr>
<tr>
<td>Negative lymph nodes, mean (SD), No.</td>
<td>23.6 (3.1)</td>
<td>13.7 (1.0)</td>
<td>.001</td>
</tr>
</tbody>
</table>

Figure 2. Distribution of the number of detected negative lymph nodes relative to microsatellite instability status. Solid circles indicate patient with microsatellite instability; open circles, patient with microsatellite stability; and horizontal line, the mean number (15) of lymph nodes.
onstrated in a monovariate analysis that age influenced the number of examined lymph nodes. The mean (SD) number of examined lymph nodes was fewer in patients older than 75 years (6.7 [0.4]) than in younger patients (8.2 [0.4]) (P <.01). In our study, the only MSI tumor with very few examined lymph nodes (n = 9) was in a 98-year-old patient.

These results underline that the number of examined lymph nodes cannot be considered a quality criterion for an isolated case, as there are other factors affecting this number. However, the mean number of examined lymph nodes obtained for a series of cases probably represents a quality criterion. In a recent French study of public university hospitals (Assistance Publique des Hôpitaux de Paris) in a Paris metropolitan area, fewer than 8 examined lymph nodes were reported in 8.6% (31 of 362) of resected specimens, which was statistically different from that reported in private hospitals during the same period, in which fewer than 8 examined lymph nodes were reported in 32.6% (248 of 760) of resected specimens.

There are no major guidelines regarding patient selection for MSI phenotyping. The cost of this research relative to its clinical benefit remains to be evaluated. Although the Amsterdam and Bethesda criteria have been developed to detect hereditary nonpolyposis colorectal cancer, indications for routine MSI testing among patients with sporadic colon cancer are not clearly established. In some academic centers in France, all resected colorectal cancer specimens are tested for MSI, but this procedure is too expensive for routine use. To define the group of patients who are more likely to develop MSI tumors, we investigated whether a high number of isolated lymph nodes is associated with the MSI phenotype.

Because clinical practice varies among surgical units, it is necessary to consider the usual mean number of lymph nodes analyzed in a particular situation to adequately establish a protocol that allows selection of patients for whom MSI analysis is likely to result in a high rate of detection. We recommend MSI analysis of sporadic N0 tumors in which the mean number of regional lymph nodes detected exceeds the usual mean number. This value needs to be established based on local information because the mean number of detected lymph nodes varies based on the region of practice.

Our work is based on a cohort of 82 patients from Lariboisiere Hospital. The study was limited to a single medical center. Although the small cohort size is a limitation of this study, the operative procedures and pathologic examinations were homogeneous. All patients were operated on under the supervision of 2 senior surgeons, and the histologic slides of all resected specimens were examined by the same pathologist (J.N.).

Microsatellite instability testing was performed using a single method recently recommended by the National Cancer Institute, with immunohistochemistry performed only in tumors demonstrating MSI. Molecular detection of MSI can be nonproductive if there is massive normal tissue contamination giving a false-negative result in the PCR. However, several authors emphasize that immunohistochemistry results should be interpreted with caution because they are less reproducible than results of molecular analyses. Notably, immuno-

histrochemistry relies on tumor fixation and antigen retrieval procedures that are poorly controlled.

Finally, it would be relevant to confirm our results in a larger cohort of patients undergoing surgery at Lariboisiere Hospital and at other medical centers. A definitive conclusion regarding the biological association between lymphoid reaction to the tumor and MSI status is confounded by the fact that MSI tumors are more common in the proximal colon, where mesentery and lymph nodes tend to be quantitatively greater. However, our data suggest that the number of examined lymph nodes cannot be used as a reliable criterion of the quality of surgical or pathologic dissection.

Accepted for Publication: January 30, 2009.

Author Affiliations: Departement Medico-Chirurgical de Pathologie Digestive (Dr Eveno, Valleur, and Pocard) and Departement d’Anatomopathologie (Dr Nemeth), Assistance Publique des Hôpitaux de Paris, Hôpital Lariboisiere, and Centre National de la Recherche Scientifique Unité, Mixte de Recherche 7151, Hôpital St Louis (Drs Soliman and de The), Université Paris 7, and Institut National de la Santé et de la Recherche Médicale, Unité Mixte de Recherche S762, Instabilité des Microsatellites et Cancers, Université Pierre et Marie Curie–Paris 6 (Dr Praz), Paris, France; and Academic Department of Pathology, Cancer Research–United Kingdom Colorectal Cancer Unit, St Mark’s Hospital, Harrow, London, England (Dr Talbot).

Correspondence: Marc Pocard, MD, PhD, Département Medico-Chirurgical de Pathologie Digestive, Assistance Publique des Hôpitaux de Paris, Hôpital Lariboisiere, Université Paris 7, 2 Rue Ambroise Paré, 75010 Paris, France (marc.pocard@lrb.aphp.fr).

Author Contributions: Study concept and design: Eveno and Pocard. Acquisition of data: Eveno, Soliman, Valleur, and Pocard. Analysis and interpretation of data: Eveno, Nemeth, Soliman, Praz, de The, Talbot, and Pocard. Drafting of the manuscript: Eveno and Pocard. Critical revision of the manuscript for important intellectual content: Eveno, Nemeth, Soliman, Praz, de The, Valleur, Talbot, and Pocard. Obtained funding: Eveno. Administrative, technical, and material support: Eveno, Nemeth, Soliman, and Praz. Study supervision: Nemeth, de The, Valleur, Talbot, and Pocard.

Financial Disclosure: None reported.

Additional Contributions: Carole Sanchez and Malika Bennis assisted with the study.

REFERENCES

6. Law CH, Wright FC, Rapanos T, et al. Impact of lymph node retrieval and patho-
Advances in the Relationship Between Lymph Node Status and Prognosis

Although significant advances in the biology of colorectal cancer have been achieved in the past decade, lymph node status remains the strongest predictor of prognosis. Furthermore, the presence of lymph node metastasis dictates the need for adjuvant therapy. Inconsistencies in the quality of lymph node dissection and examination threaten the usefulness of this marker not only as a prognostic factor but also as the basis for therapeutic decisions. Significant controversy surrounds the concept of what constitutes an adequate number of lymph nodes. Based on a combination of statistical and observational studies, the National Cancer Institute1 (among others) recommended the examination of a minimum of 12 lymph nodes to ensure accurate staging. Several investigations have demonstrated a survival advantage in patients with stage II disease when an increased number of lymph nodes is evaluated.2 The explanation behind this association is unclear; however, factors other than accurate staging likely account for the improvement in survival.

The number of lymph nodes retrieved from colorectal specimens resected for cancer depends on a group of variables, some of which are more amenable to modifications than others. The surgeon-pathologist team (management factor) is responsible for harvesting all the positive nodes from the specimen. Only 10% of patients with Dukes’ B (TNM stage II) colorectal carcinoma, examination of six or fewer lymph nodes is related to poor prognosis. Cancer. 1998;83(4):666-672.

S. Caplin, S. Cerottini JP, Bosman FT, Constanda MT, Givel JC. For patients with Dukes’ B (TNM stage II) colorectal carcinoma, examination of six or fewer lymph nodes is related to poor prognosis. Cancer. 1998;83(4):666-672.