Combination of Microsatellite Instability and Lymphocytic Infiltrate as a Prognostic Indicator for Adjuvant Therapy in Colon Cancer

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Background: Microsatellite instability (MSI) and lymphocytic infiltrate (LI) in colon cancer are associated with less aggressive biological characteristics. Patients with stage II disease who are negative for MSI and LI have been found to have a less favorable prognosis. These patients may be candidates for more aggressive adjuvant therapy.

Objective: To evaluate the outcomes of patients with colon cancer treated with and without adjuvant chemotherapy on the basis of stage, MSI, and LI.

Design: Prospective evaluation of MSI and LI status with retrospective analysis of chemotherapy regimen.

Setting: Community hospital system.

Patients: A total of 167 patients with colon cancer.

Intervention: Definitive resection of colorectal cancer with or without chemotherapy.

Main Outcome Measure: Disease-free survival (DFS) with and without chemotherapy according to combined MSI and LI status.

Results: Data on MSI and LI status and chemotherapeutic regimens were available for 140 patients. The 5-year DFS was 50% for patients with stage II disease who underwent chemotherapy vs 76% for those who did not (P = .02). In the group negative for MSI and LI, 5-year DFS was 29% for those undergoing chemotherapy and 91% for those who did not (P = .001).

Conclusions: Forgoing adjuvant chemotherapy should be considered in patients with stage II colon cancer who are negative for MSI and LI. The MSI and LI status shows promise as a combined prognostic marker and may prove particularly useful in selecting patients with stage II disease for adjunctive therapy.

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The colon is the third leading cancer site, with an estimated 112,340 cases diagnosed per year in the United States. Significant progress has been made in the realm of treatment with new chemotherapeutic regimens, thereby improving response rates. However, despite therapeutic improvements, prognostic markers have yet to be developed to select patients who would benefit from early adjunctive therapy. To date, clinicians still base that decision mainly on nodal involvement; nevertheless, approximately 25% of patients with node-negative disease will have recurrences. This raises concern for illuminating other factors that would allow better selection of high- or low-risk groups of patients. Our group has previously published a report on a group of patients with colorectal cancer (CRC) in whom we prospectively evaluated microsatellite instability (MSI) and lymphocytic infiltrate (LI) status and observed their outcome. It was clear that absence of both MSI and LI portended a worse outcome for all stages. We theorized that patients with stage II disease who are negative for MSI and LI may be a group for whom adjuvant therapy would offer an advantage. In the present study, we evaluate patient outcomes on the basis of recorded adjuvant chemotherapy.

Microsatellite instability is a phenomenon in CRC that has attracted attention because of the prognostic implications associated with it. Almost all cases of hereditary nonpolyposis colorectal cancer (HNPCC) and approximately 15% to 30% of cases of sporadic colon cancer demonstrate MSI. A number of studies have demonstrated an improved survival in patients with CRC whose tumors demonstrate MSI. The mechanism by which these tumors lead to a more favorable prognosis has been unclear.

Intratumoral LI, which is often seen in association with a peritumoral stromal
lymphocytic inflammatory infiltrate, is another feature often identified.\textsuperscript{5-8} CD3 lymphocyte counts in excess of 40 per 1000 epithelial cells occur in 70% of high-frequency MSI, 33% of low-frequency MSI, and 17.5% of microsatellite-stable tumors.\textsuperscript{9} CD3 counts in excess of 100 per 1000 epithelial cells are highly sensitive for high-frequency MSI tumors.\textsuperscript{9} Lymphocytic infiltration is thought to contribute to the improved survival seen in patients with MSI-positive tumors, perhaps as a marker of a heightened immune response against the malignant neoplasm.\textsuperscript{10} We sought to evaluate the combined prognostic significance of MSI and LI for adjuvant chemotherapy in our colon cancer population.

### METHODS

All patients undergoing definitive resection of CRC tumors in a community hospital system were prospectively identified during a 3-year period from 2001 to 2004. No restrictions were imposed on patient age, tumor location, or family history of CRC. Tumors of consenting patients were submitted for evaluation for MSI and LI. For the evaluation of MSI, genomic DNA from the tumor was compared with that of adjacent, histologically normal tissue with respect to a standard panel of LI markers, including those defined by the 1997 National Cancer Institute guidelines (Clinical Molecular Diagnostic Laboratories, City of Hope Medical Center, Duarte, California).\textsuperscript{11} Tumors in which none of the loci demonstrated instability were classified as microsatellite stable. Those with a single unstable locus were classified as having low-frequency MSI. Tumors with 2 or more unstable loci were classified as having high-frequency MSI. Patients with high-frequency MSI tumors are hereinafter referred to as MSI++; those with microsatellite-stable and low-frequency MSI tumors are referred to as MSI−. Tumors were examined for intratumoral LI under light microscopy and staining with hematoxylin-eosin. Presence of LI (referred to as LI+) was defined as greater than 5 lymphocytes per 10 high-power (×40) fields of the tumor epithelium. Patients were prospectively followed up for development of recurrence and death. Data were collected regarding age, sex, pathological stage of disease, tumor grade, and lymph node involvement. All procedures were performed under a protocol approved by the institutional review board of the Legacy Health System, Portland, Oregon.

Comparisons were made between patients with LI+ and LI− tumors as well as MSI+ and MSI− tumors. The unpaired t test was used to compare age. Categorical data, such as sex, tumor grade, pathological stage of disease, and nodal involvement were compared and P values were generated by means of the Fisher exact test. Patients with stage II disease were categorized into 2 groups according to whether they had received chemo-

### RESULTS

One hundred sixty-seven patients were enrolled in the study. Testing for MSI was inconclusive in 6 patients. In 11 patients, there was insufficient tumor for analysis. Nine patients were excluded for insufficient chemotherapy information. One patient had recurrent disease and was also excluded, leaving 140 patients for the complete analysis. Of the 149 patients in whom LI and MSI data were available, the tumors in 25 (17%) demonstrated LI. Of these, 1/16 (6%) were MSI+. Among the 124 patients without LI, 29 patients (24%) had MSI+ tumors (Table 1).

Forty-three of 149 patients (29%) had stage I disease, 56 (38%) had stage II, 34 (23%) had stage III, and 16 (11%) had stage IV. Stage distribution, lymph node involvement, and tumor grade were similar between LI+ and LI− patients.

Patients with stage II tumors were well matched between the chemotherapy and no-chemotherapy groups (Table 2). The average age was 64.3 years for the chemotherapy group and 71.5 years for the no-chemotherapy group (P = .46). The harvested node count was greater than or equal to 12 in 49% of the no-chemotherapy group and 25% of the chemotherapy group (P = .15). The grade was well matched, with poorly differentiated tumors composing 24% of the no-chemotherapy group and 25% of the chemotherapy group (P = .62). There was lymphovascular invasion in 8% of the no-chemotherapy group and 25% of the chemotherapy group (P = .15). Of those with perforated or obstructed tumors, 33% received chemotherapy, whereas such patients composed only 8% of those who did not receive chemotherapy (P = .05).

The median length of follow-up was 48.7 months (range, 2.7-66.6 months). During the course of follow-

### Table 1. Disease Recurrence and Death According to MSI and LI Status for All Patients Including Unknown Chemotherapy Status

<table>
<thead>
<tr>
<th>Stage of Disease</th>
<th>No. (%) of Patients Who Developed Disease Recurrence or Died</th>
<th>No. (%) of Patients Who Developed Disease Recurrence or Died</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MSI+/LI+</td>
<td>MSI+/LI−</td>
</tr>
<tr>
<td>I</td>
<td>0/6 (0)</td>
<td>0/7 (0)</td>
</tr>
<tr>
<td>II</td>
<td>0/5 (0)</td>
<td>7/16 (44)</td>
</tr>
<tr>
<td>III</td>
<td>1/5 (20)</td>
<td>0/6 (0)</td>
</tr>
<tr>
<td>IV</td>
<td>0</td>
<td>1/1 (100)</td>
</tr>
<tr>
<td>Overall</td>
<td>1/16 (6)</td>
<td>7/29 (24)</td>
</tr>
</tbody>
</table>

Abbreviations: LI−, lymphocytic infiltration absent; LI+ , lymphocytic infiltration present; MSI−, microsatellite stable or low-frequency microsatellite instability; MSI+, high-frequency microsatellite instability.

These were further categorized into 4 groups according to MSI and LI status. Time-to-event analyses of disease-free survival (DFS) and overall survival after surgery were performed between the chemotherapy and no-chemotherapy groups as well as MSI+/LI+ and MSI−/LI− groups. Kaplan-Meier survival estimates were plotted and stratified according to MSI+/LI+ and MSI−/LI− groups. The log-rank test was used to compare the survival estimates. Multivariate analysis of survival according to MSI status, LI status, and use or nonuse of chemotherapy was performed by means of a Cox regression analysis. Statistical calculations were performed in SPSS 13 (SPSS Inc, Chicago, Illinois).
up, 49 of all patients developed recurrence or died (Table 1).

The 5-year DFS is shown in Figure 1. For patients with stage I tumors the DFS was 83%; for stage II, 74%; for stage III, 76%; and for stage IV, 6%. When patients were grouped by whether they received chemotherapy, those who received chemotherapy had a 5-year DFS of 51%, compared with 75% for those who did not receive it (P=.001). When patients were grouped according to both MSI and LI status, those with MSI−/LI− tumors had a 5-year DFS of 37%, compared with 72% for those with MSI+/LI+/LI+/H11001 (P=.01) (data not shown). Five-year DFS for patients with MSI−/LI+ tumors and for those with MSI+/LI− tumors was 75% (data not shown).

In patients diagnosed as having stage II disease, the 5-year DFS was 50% for those who underwent chemotherapy, compared with 76% for those who did not (P=.02) (Figure 2). For patients with stage III disease, the 5-year DFS was 77% for those undergoing chemotherapy compared with 66% for those with no chemotherapy (P=.70) (Figure 3). The overall DFS for the patients according to MSI/LI status is shown in Figure 4. For MSI−/LI− the DFS was 59%; for MSI+/LI−, 78%; for MSI−/LI+, 74%; and for MSI+/LI+, 79%.

The difference in patients with stage II tumors was driven primarily by the MSI−/LI− group, where DFS was 91% and 29% for the no-chemotherapy and chemotherapy subgroups, respectively (P=.001) (Figure 5). The remainder of the patients with stage II tumors did not show significant differences in DFS. The DFS in the MSI−/LI+ group was 100% for the no-chemotherapy subgroup and unknown for the chemotherapy subgroup. In the MSI+/LI− group, DFS was 60% for the no-chemotherapy subgroup and 67% for the chemotherapy subgroup (P=.70). The DFS in the MSI+/LI+ group was 0% and 100% for the no-chemotherapy and chemotherapy subgroups, respectively (P=.36). However, this is difficult to interpret given a sample size of 3.

Table 2. Distribution of Patients With Stage II Disease by Chemotherapy Status

<table>
<thead>
<tr>
<th>Group</th>
<th>No Chemotherapy (n=37)</th>
<th>Chemotherapy (n=12)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age, y</td>
<td>71.5</td>
<td>64.3</td>
<td>.46</td>
</tr>
<tr>
<td>No. of nodes</td>
<td>&lt;12</td>
<td>19 (51)</td>
<td>.15</td>
</tr>
<tr>
<td></td>
<td>≥12</td>
<td>18 (49)</td>
<td>.25</td>
</tr>
<tr>
<td>Tumor differentiation</td>
<td></td>
<td></td>
<td>.62</td>
</tr>
<tr>
<td>Well/moderately differentiated</td>
<td>28 (76)</td>
<td>9 (75)</td>
<td>.62</td>
</tr>
<tr>
<td>Poorly differentiated</td>
<td>9 (24)</td>
<td>3 (25)</td>
<td>.15</td>
</tr>
<tr>
<td>Lymphovascular invasion</td>
<td></td>
<td></td>
<td>.15</td>
</tr>
<tr>
<td>No</td>
<td>34 (92)</td>
<td>9 (75)</td>
<td>.62</td>
</tr>
<tr>
<td>Yes</td>
<td>3 (8)</td>
<td>3 (25)</td>
<td>.36</td>
</tr>
<tr>
<td>Perforation/obstruction</td>
<td></td>
<td></td>
<td>.05</td>
</tr>
<tr>
<td>No</td>
<td>34 (92)</td>
<td>8 (67)</td>
<td>.01</td>
</tr>
<tr>
<td>Yes</td>
<td>3 (8)</td>
<td>4 (33)</td>
<td>.36</td>
</tr>
</tbody>
</table>

a Includes only patients with known chemotherapy status.

Figure 1. Kaplan-Meier estimates of disease-free survival for all patients grouped by stage. Disease-free survival was 83% for stage I, 74% for stage II, 76% for stage III, and 6% for stage IV.

Figure 2. Kaplan-Meier estimates of disease-free survival for patients with stage II disease grouped by whether they received chemotherapy. Disease-free survival was 76% without chemotherapy and 50% with it (P=.02).

Figure 3. Kaplan-Meier estimates of disease-free survival for patients with stage III disease grouped by whether they received chemotherapy. Disease-free survival was 66% without chemotherapy and 77% with it (P=.70).
In 1990 the National Institutes of Health consensus conference advised that adjuvant chemotherapy be offered to stage II patients only as part of a trial. This is where the evidence remains almost 20 years later.

The American Society of Clinical Oncology continues to point out a lack of evidence to support routine use of adjuvant therapy in stage II disease, the exceptions for consideration being perforation/obstruction, poor differentiation, T4 disease, or fewer than 10 lymph nodes sampled. Despite the initial excitement generated by the findings of the Multicenter International Study of Oxaliplatin/5-Fluorouracil/Leucovorin in the Adjuvant Treatment of Colon Cancer (MOSAIC) in 2004 showing improved DFS for both stage II and III CRC, many clinicians are hesitant to recommend adjuvant therapy to patients with stage II disease because of the marginal benefit derived in that subset of patients.4

In fact, the longer-term follow-up of this patient group shows no benefit for stage II adjuvant therapy. To date, no consensus on biological markers to select patients likely to benefit from adjuvant therapy in this setting has been found. We have done little to advance our understanding of the selection process for this cohort of patients, which constitutes one-quarter of the approximately 112,000 patients diagnosed as having CRC annually in the United States. We believe that, similar to breast cancer, the treatment of CRC may stand to gain from the ability to develop molecular markers that suggest a risk profile and response over and beyond the current TNM tumor staging system.

Microsatellite instability is a phenomenon in CRC that has attracted attention because of the prognostic implications associated with it. Almost all cases of HNPCC9 and approximately 15% to 30% of cases of sporadic colon cancer demonstrate MSI. A number of studies have demonstrated an improved survival in patients with CRC whose tumors demonstrate MSI. The mechanism by which these tumors lead to a more favorable prognosis has been unclear.

Intratumoral LI, which is often seen in association with a peritumoral stromal lymphocytic inflammatory infiltrate, is another feature often identified. CD3 lymphocyte counts in excess of 40 per 1000 epithelial cells occur in 70% of high-frequency MSI, 33% of low-frequency MSI, and 17.5% of microsatellite-stable tumors.9 CD3 counts in excess of 100 per 1000 epithelial cells are highly sensitive for high-frequency MSI tumors.9 Lymphocytic infiltration is thought to contribute to the improved survival seen in patients with MSI+ tumors, perhaps as a marker of a heightened immune response against the malignant neoplasm.10

As our group reported previously, combining MSI and LI status can stratify CRC into high-risk (MSI−/LI−) and low-risk (MSI+/LI+) groups. This study would suggest that this information may be valuable in selecting patients for adjuvant therapy as well. Although we hypothesized that patients with stage II MSI−/LI− CRC may benefit from adjuvant chemotherapy to improve their outcome, our data suggested that the addition of a chemotherapy regimen actually worsened their outcomes. During the initial phase of this study, fluorouracil–leucovorin calcium was the standard regimen being given in the community. Toward the end of the study accrual, 9 patients who received oxaliplatin added to fluorouracil and leucovorin were enrolled.

The results of the current study suggest that adjuvant chemotherapy may actually harm patients with stage II CRC, as those treated had a poorer DFS. One explanation is that more patients with perforation or obstruction were offered adjuvant therapy, thus adding to the evidence that they are truly at high risk. Subgroup analysis of other factors such as age, tumor differentiation, lymphovascular invasion, and harvesting fewer than 12 nodes did not show statistical relevance in trying to explain this finding, although the numbers were insufficient to draw any definitive conclusions. Our findings are difficult to interpret within the context of previous articles such as that by Ribic et al, which showed no benefit of fluoro-
uracil adjuvant therapy in patients with MSI+ tumors, but benefit was shown for those with MSI– tumors in stage II/III disease. This benefit apparently was seen even if stages II and III were separately analyzed. Unlike in our study, older patients made up a significant proportion of those who received chemotherapy. This finding of similar outcomes in patients with stage III disease is likely a product of small numbers combined with using only fluorouracil therapy, but it leads to the speculation of whether risk stratification within stage III would allow better decisions for chemotherapy.

The findings herein raise more questions and certainly lend credence to the idea of stratifying patients on the basis of molecular findings to help us interpret outcomes in a more defined fashion. Although the Eastern Cooperative Oncology Group (ECOG) 5202 trial is randomizing patients on the basis of allelic loss of 18q and MSI, our data suggest that lymphocytic infiltration should be added in this study to more accurately evaluate outcomes. Patients who were MSI+ and LI– have outcomes similar to those who were MSI+ but LI+, and this fact may confound the picture.

Interpretation of our data are tempered by the limited sample size, but what seems apparent is that these 2 patient groups, MSI+/LI+ and MSI–/LI–, are distinctly different, in terms of both prognosis and response to adjuvant fluorouracil-based chemotherapy. Current adjuvant regimens have evolved to include oxaliplatin18 or biological agents.19 However, on analysis specifically of patients with stage II disease in the MOSAIC trial, the addition of oxaliplatin did not improve outcome.20

We conclude that the role for adjuvant therapy is still questionable, particularly in patients with stage II disease. We propose that future trials to answer that question should use MSI and LI status as part of risk stratification.

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REFERENCES

DISCUSSION
Clifford Ko, MD, Los Angeles, California: This study exemplifies the innovative and important work by surgeons in advancing our understanding of colon cancer. Recently, the TNM system has been modified. For stage II, as an example, T3s are now separated from T4s because of the different survival rates in these subsets. Currently, we seem to be perseverating as a group on nodal status and number of positive and negative nodes.
There is probably an upcoming paradigm shift in cancer staging. This work nicely illustrates the direction of this shift by using architectural pathology and biomarkers to advance staging and prognostication.

The authors have investigated 2 of the potential markers, MSI and LI. Microsatellite instability is found in HNPCC, which makes up a minority of the cancers seen clinically but also may be found in up to one-quarter of sporadic cancers. The LI is also very timely. An important recent article by Lynch showing that patients with MSI probably have an increased cytotoxic response by these lymphocytes fits nicely with the work that was just presented.

There are 2 main findings of this study. The first is that the MSI+ and LI+ tumors had better survival. This is very consistent with the literature because we know that the patients with HNPCC tend to do better. The second finding is noteworthy because it is not exactly what we would expect. The finding is that the MSI− and LI− patients have worse outcomes, particularly with chemotherapy. The chemotherapy that was given in the study is fluorouracil and leucovorin. Regarding this finding, I have a few questions.

One question is whether perforation and obstruction are the reason for the worse outcomes of the chemotherapy patients, or whether there are other clinical factors that are likely equivalent to an issue of patient selection. Are we giving chemotherapy to the worst actors in stage II, and that is why you have this big discrepancy in outcomes?

The second question is that fluorouracil and leucovorin is probably not the standard regimen anymore, and do you have any further information regarding the addition of oxaliplatin?

The third question is about the use of biomarkers. Specifically, for the practicing colon surgeons caring for patients with colon cancer, how should we use these biomarkers now? Are they ready yet or in a few years from now?

Dr Frankhouse: This paper is an attempt to help us move beyond the TNM system that we have today. Similar to the situation with breast cancer, we who treat colorectal cancer are ready to move toward molecular markers, or personalized medicine as it is called by some. What drives me is that only 25% of patients with stage II colorectal cancer develop recurrence, and current chemotherapy helps a small number of these patients.

We are excited about the MOSAIC trial comparing the FOLFOX regimen (oxaliplatin, leucovorin, and fluorouracil) vs fluorouracil for stage II colorectal cancer but bothered by the lack of a control arm of observation only. Furthermore, if we look at stage III disease, where we all accept that chemotherapy is warranted, historical controls suggest that almost half of patients do not develop recurrence when forgoing chemotherapy. So considering patients within these 2 groups as homogeneous is a mistake. We need to risk stratify within groups.

Since patients with HNPCC nearly uniformly show MSI, and they have a better prognosis, we looked at MSI in our sporadic colorectal cancer population. We found about a 30% prevalence and that about a third of our MSI+ tumors had LI compared with about 8% of tumors without MSI.

I am a bit surprised, too. I thought that we might find that this is indeed a subgroup in stage II that would benefit from chemotherapy, but we can all look at our data and say, “At best it provided no value. At worst, it was actually a detriment.” I’m hoping this will be a springboard for further studies. Currently, the ECOG 5202 trial will look prospectively at MSI and 18q deletions in stage II patients to stratify high and low risk. We can do more than just those 2 markers and add LI. We clearly showed that LI adds much to the better prognosis seen in MSI tumors, and its absence detracts from benefits of MSI. In this age of cost containment, we do it in our hospital on a routine basis, and it does not cost anything.

The cost of MSI is a consideration. True genetic studies are probably about $1000, but City of Hope helped with discounting. Currently, immunohistochemical testing can be done for about $250 and is easily employed in most hospital systems. My hope for the future is to look at LI and identify what stimulates immunogenicity of those tumors. The MSI might be one factor, but there is probably more to it. Whether gene array analysis will show a molecular pattern, or whether analysis of protein expression is the key, I do not know yet. But that is the basic direction in which we have to move.

Dr Ko asked about the poor prognosis of MSI−/LI− patients and failure of chemotherapy to improve on it. I concede that perforation, obstruction, and T4 tumors were more common in patients who received chemotherapy and might have influenced our outcomes. However, typically what I expect to see in a nonrandomized cohort of patients is that those who do not receive chemotherapy are the ill and elderly. So there is usually a negative bias to those who do not receive treatment. We showed just the opposite.

We add oxaliplatin only for stage III and stage IIB. We add oxaliplatin only when we prove with an observation control arm that it is truly a benefit for average-risk stage II cancer. Whether in ECOG 5202 or other upcoming studies, we must identify stage II patients at high risk and perhaps stage III patients who are at low risk by using molecular markers. Then those prognostic markers may predict response to chemotherapy. A number of biomarkers have been proposed, and these 2 are a start. Microsatellite instability and LI are ready to be used at present for study purposes at least. They are inexpensive and easy to do in almost any hospital setting. So I encourage you all to think about using them in your own institutions.

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