Molecular Detection of Neoplastic Cells in Lymph Nodes of Metastatic Colorectal Cancer Patients Predicts Recurrence

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ABSTRACT

Disseminated disease, especially to the liver, constitutes the major risk of recurrence for colorectal cancer patients. However, successful resection can still be achieved in 25–35% of colorectal cancer patients with isolated metastases. To evaluate the clinical value of occult micrometastatic disease detection in lymph nodes, we tested genetic (K-ras and p53 gene mutations) and epigenetic (p16 promoter hypermethylation) molecular markers in the perihepatic lymph nodes from colorectal cancer patients with isolated liver metastases. DNA was extracted from 21 paraffin-embedded liver metastases and 80 lymph nodes from 21 colorectal cancer patients. K-ras and p53 gene mutations were identified in DNA from liver metastases by PCR amplification followed by cycle sequencing. A sensitive oligonucleotide-mediated mismatch ligation assay was used to search for the presence of K-ras and p53 mutations to detect occult disease in 68 lymph nodes from tumors positive for these gene mutations. Promoter hypermethylation at the p16 tumor suppressor gene was examined in both liver lesions and lymph nodes by methylation-specific PCR. Sixteen of the 21 (76%) liver metastases harbored either gene point mutations or p16 promoter hypermethylation. Twelve of the 68 lymph nodes were positive for tumor cells by molecular evaluation and negative for tumor cells by histopathology and cyto-keratin immunohistochemistry, whereas none were positive for tumor cells by histopathology or negative for tumor cells by molecular analysis (P = 0.0005, McNemar’s test). Moreover, in three patients with lymph nodes that were histologically negative at all sites, molecular screening detected tumor DNA at one or more lymph nodes. Survival analysis showed a median survival of 1056 days for patients without evidence of lymph node involvement by molecular analysis and 165 days for patients with positive lymph nodes by this approach (P = 0.0005). These results indicate that lymph node metastasis screening in colorectal cancer patients by molecular-based techniques increases the sensitivity of tumor cell detection and can be a good predictor of recurrence in colorectal cancer patients with resectable liver metastases.

INTRODUCTION

Colorectal cancer constitutes a major public health problem. This disease affects about 1 in 20 people in Western countries, and more than 155,000 new cases are diagnosed in the United States each year. Overall, 25% of the patients with resectable disease at the time of the diagnosis will have recurrent disease, presumably from local, regional, and peritoneal seeding. Major improvements in surgical procedures have allowed tumor resection in patients with isolated liver, lung, or pelvic metastases. However, only 25–30% of patients achieve a 5-year disease-free survival. There is not much information on the prognosis factors of this subset of colorectal cancer patients. Perihepatic lymph node involvement has been associated with a poor prognosis in patients with isolated liver metastases (1), indicating the need for improved control of micrometastases.

Genetic-based techniques have been used to detect micrometastases in regional lymph nodes and were reported to be more sensitive than standard histopathology (2). Although several genetic techniques have been used, they are all based on the ability to perform PCR on tissue samples. Hayashi et al. (3) have detected a small number of cancer cells by screening for K-ras gene alterations with the mutant allele-specific amplification analysis in lymph nodes negative for tumor cells by morphological analysis. Other studies have used the reverse transcription-PCR technique to detect gene expression for the presence or absence of a specific tumor marker (4–6). Moreover, it has been reported that the detection of micrometastases by molecular-based techniques in morphologically negative lymph nodes is a prognostic tool in stage II colorectal cancer patients (5, 7), suggesting that it could serve as a selective marker for intensive postoperative adjuvant chemotherapy.

We have used a mismatch ligation assay to screen for K-ras and p53 gene mutations (8) and the MSP technique (9) to look for p16 promoter hypermethylation in the perihepatic lymph nodes from colorectal cancer patients who underwent...
were then separated on a 12% denaturing polyacrylamide gel. Subsequent incubation at 37°C for 30 min. The ligation products were removed by the addition of 1 unit of alkaline phosphatase and allowed to cool at room temperature for 15 min, at which time the [32P]phosphate on the unligated 3' oligomer was assayed. A section from a liver metastasis was deparaffinized using 100% xylene followed by 100% ethanol. The pellet was then resuspended in a buffer containing SDS-proteinase K, and DNA was extracted with phenol-chloroform followed by ethanol precipitation. Finally, precipitated DNA was resuspended in 100 µl of Tris-EDTA buffer as described previously (10). DNA was quantified spectrophotometrically, and 100 ng were used as a template for each PCR amplification.

**Mutation Detection.** Exon 1 of K-ras and four individual exons of p53 (exons 5–8) were amplified by PCR from liver metastasis DNA. All PCR products were purified and sequenced directly using the AmpliCycle sequencing kit (Perkin-Elmer, Foster City, CA). The primers for the PCR and the sequencing conditions used for each exon are described elsewhere (8). The lymph nodes from those tumors positive for any mutation were tested using a mismatch ligation assay. The mismatch ligation procedure has been described previously (8). Briefly, the exon containing the mutation was amplified by PCR, and the product was purified before the mismatch ligation assay. Each PCR product (50 ng) was mixed with 8 ng of a common 32P-labeled oligomer in a 20-µl reaction containing 150 mM NaCl, 10 mM MgCl2, 100 mM Tris-HCl (pH 7.5), 1 mM spermidine, 1 mM DTT, 1 mM ATP, and 3 µg of T4 gene 32 protein (Boehringer Mannheim). This mixture was denatured at 95°C for 5 min and allowed to cool at room temperature for 15 min, at which time 1 unit of T4 ligase was added. The ligation was carried out at 37°C for 1 h and terminated by heat inactivation at 68°C for 10 min. The [32P]phosphate on the unligated 3' oligomer was removed by the addition of 1 unit of alkaline phosphatase and subsequent incubation at 37°C for 30 min. The ligation products were then separated on a 12% denaturing polyacrylamide gel.

**RESULTS**

**Identification of Genetic Alterations at the Liver Metastasis Tissue.** We identified either K-ras and p53 gene mutations or p16 gene promoter hypermethylation at 16 of the 21 (76%) liver metastases analyzed. Ten of the 21 (48%) tumors showed K-ras mutations, 6 of 21 (29%) tumors harbored p53 mutations, and 3 of 21 (14%) tumors had p16 promoter hypermethylation (Table 1). The molecular alterations found for each tumor are summarized in Table 2. Primary screening for mutations in the tumor tissue allowed us to then search for the same specific mutation in the lymph nodes with a specific mismatch ligation or MSP assay, both of which are several times more sensitive (capable of detecting at least 1 tumor cell in 1000 normal cells) than the direct sequencing of PCR products.

**Molecular Alterations at the Perihepatic Lymph Nodes in Colorectal Cancer Patients with Liver Metastases.** A mismatch ligation assay was used to test for K-ras and p53 mutations in the 68 lymph nodes of the 16 patients whose liver metastases had a mutation, and the MSP assay was used for p16 promoter methylation detection (Fig. 1). Among the 68 lymph nodes, the examination by standard histopathological procedures diagnosed 17 nodes as a positive (28%), whereas molecular analysis increased this number to 29 (45%). Those lymph nodes, the examination by standard histopathological procedures diagnosed 17 nodes as a positive (28%), whereas molecular analysis increased this number to 29 (45%).

**Table 1** Molecular alterations detected in liver metastases from colorectal cancer patients

<table>
<thead>
<tr>
<th>Molecular alteration</th>
<th>No. of tumors (%)</th>
<th>Detection method</th>
</tr>
</thead>
<tbody>
<tr>
<td>K-ras mutations</td>
<td>10/21 (48%)</td>
<td>Cycle sequencing</td>
</tr>
<tr>
<td>p53 mutations</td>
<td>6/21 (29%)</td>
<td>Cycle sequencing</td>
</tr>
<tr>
<td>p16 methylation</td>
<td>3/21 (14%)</td>
<td>MSP</td>
</tr>
<tr>
<td>Total</td>
<td>16/21 (76%)</td>
<td>Any</td>
</tr>
</tbody>
</table>

The corresponding mutation in the tumor DNA was used as a positive control, and a negative tumor for the mutation was used as a negative control in each reaction. Oligonucleotide sequences for the K-ras ligation assay have been described previously (11). Specific oligomers were designed individually to identify p53 gene mutations (8). M.S.P. For p16 promoter hypermethylation analysis, 1 µg of DNA was denatured by NaOH and then modified by sodium bisulfite. DNA samples were then purified using Wizard DNA purification resin (Promega, Madison, WI), treated again with NaOH, precipitated with ethanol, and resuspended in water. PCR was performed separately with methylation-specific primers and nonmethylation primers for each tumor sample (9). Controls without DNA and positive controls for unmethylated and methylated reactions were performed for each set of PCR reactions. PCR products were analyzed on nondenaturing 6% polyacrylamide gels, stained with ethidium bromide, and visualized under UV illumination.

**Statistical Analysis.** The cumulative survival rates for patients groups were calculated using the Kaplan-Meier method (12) and compared using the log-rank test. To evaluate the difference in sensitivity between histopathological and molecular lymph node metastasis detection, we used McNemar’s test. All reported P values are two-sided.
Micrometastases Detection in Colorectal Cancer Patients

We calculated the survival rate in the group of 16 evaluable patients (patients with any molecular alteration in the liver tumor tissue) according to the presence or absence of liver tumor tissue) according to the presence or absence of molecular alterations in liver tumor DNA. The discordance is probably due to slight differences in the sensitivity of each assay.

**Table 2** Comparison of lymph nodes metastasis detection between histopathology and molecular approaches in patients with molecular alterations in liver tumor DNA

<table>
<thead>
<tr>
<th>Patient</th>
<th>Path. Dx</th>
<th>Molec. Dx</th>
<th>VS</th>
<th>FU (days)</th>
<th>Molecular alteration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0/2</td>
<td>0/2</td>
<td>D</td>
<td>1280</td>
<td>p53: substitution ATC(195)ACC</td>
</tr>
<tr>
<td>2</td>
<td>2/2</td>
<td>2/2</td>
<td>D</td>
<td>163</td>
<td>K-ras: Gly13Asp</td>
</tr>
<tr>
<td>3</td>
<td>7/12</td>
<td>8/12</td>
<td>D</td>
<td>6</td>
<td>K-ras: Gly12Asp</td>
</tr>
<tr>
<td>5</td>
<td>0/1</td>
<td>1/1</td>
<td>D</td>
<td>442</td>
<td>K-ras: Gly12Asp</td>
</tr>
<tr>
<td>7</td>
<td>0/6</td>
<td>0/6</td>
<td>D</td>
<td>368</td>
<td>K-ras: Gly12Val</td>
</tr>
<tr>
<td>9</td>
<td>2/8</td>
<td>4/8</td>
<td>D</td>
<td>265</td>
<td>K-ras: Gly12Val</td>
</tr>
<tr>
<td>11</td>
<td>0/1</td>
<td>1/1</td>
<td>LOF</td>
<td>445+</td>
<td>K-ras: Gly12Val</td>
</tr>
<tr>
<td>12</td>
<td>0/8</td>
<td>2/8</td>
<td>D</td>
<td>37</td>
<td>p53: substitution CAG(284)CAAG</td>
</tr>
<tr>
<td>14</td>
<td>0/4</td>
<td>0/4</td>
<td>A*</td>
<td>829+</td>
<td>p16 methylation</td>
</tr>
<tr>
<td>21</td>
<td>0/5</td>
<td>4/5</td>
<td>D</td>
<td>102</td>
<td>p53: substitution CGG(248)TGG</td>
</tr>
<tr>
<td>24</td>
<td>0/1</td>
<td>0/1</td>
<td>D</td>
<td>736</td>
<td>p16 methylation</td>
</tr>
<tr>
<td>27</td>
<td>0/3</td>
<td>0/3</td>
<td>NA</td>
<td>1081+</td>
<td>p53: substitution GGA(266)TGA</td>
</tr>
<tr>
<td>28</td>
<td>0/3</td>
<td>0/3</td>
<td>A</td>
<td>348</td>
<td>K-ras: Gly12Ala</td>
</tr>
<tr>
<td>29</td>
<td>1/6</td>
<td>3/6</td>
<td>D</td>
<td>125</td>
<td>p53: substitution CGG(248)CAG</td>
</tr>
<tr>
<td>31</td>
<td>5/5</td>
<td>5/5</td>
<td>D</td>
<td>423</td>
<td>K-ras: Gly12Val</td>
</tr>
<tr>
<td>35</td>
<td>0/1</td>
<td>0/1</td>
<td>A</td>
<td>1160+</td>
<td>K-ras: Gly12Val</td>
</tr>
<tr>
<td>Any positive</td>
<td>17/68</td>
<td>29/68</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Path. Dx, pathological diagnosis; Molec. Dx, molecular diagnosis; VS, vital status; FU, follow-up; D, dead; LOF, loss of follow-up; A, alive; NA, not available.

**Fig. 1** Evaluation of the presence (+) or absence (−) of neoplastic cells according to histopathology (H) or molecular analysis (M) in the perihepatic lymph nodes of colorectal cancer patients with liver metastases. T, tumor tissue for each patient. The patient number is indicated to the left of each panel. A, mismatch ligation assay for K-ras gene mutations. Example of a Val (valine) mutation in several lymph nodes from patient 29. A sensitivity of 1:100 and 1:1000 indicates 1 neoplastic cell in 100 or 1000 normal cells, respectively. B, mismatch ligation assay for p53 gene mutations. Point mutation in codon 248 resulting in a CGG (arginine) to TGG (serine) change in patient 21. C, MSP to detect promoter hypermethylation at the p16 gene. Lanes U and M indicate the unmethylated and methylated reaction for each sample. C+ and C− represent the positive and negative controls for methylation, respectively. At the far right, Lane − represents the negative control (without DNA) for the PCR reaction.
median survival for patients with positive lymph nodes was 165 days, whereas the median survival for patients with negative lymph nodes was 1056 days ($P = 0.0005$). The same analysis based on histopathological evaluation only indicated a median survival of 175 days for patients with tumor cells at the lymph nodes and 798 days for patients negative for the same test ($P = 0.011$; Fig. 2).

**DISCUSSION**

The identification of genetic changes associated with neoplastic development provides us with a variety of potentially powerful molecular markers for the clinical detection and follow-up of cancer patients. Tumor-specific molecular abnormalities in several bodily fluids have been investigated as a diagnostic tool in many tumor types. Different genetic alterations have been found in the sputum of lung cancer patients (13), the stool of colorectal cancer patients (14), the urine of bladder cancer patients (15), and oral rinses from head and neck cancer patients (16). In patients with head and neck cancer, molecularly positive margins provided a high rate of local tumor recurrence and were correlated with survival (17). Recently, several studies have been undertaken to evaluate the accuracy and sensitivity of occult micrometastatic disease detection in the lymph nodes by molecular-based approaches in many tumor types, including colorectal cancer (2, 18). Molecular screening of metastases represents a more sensitive approach compared to cytological examination and has an additional advantage in that it allows the examination of the whole lymph node, whereas standard histological diagnosis only analyzes a few slides. In agreement with previous reports, our assays for metastasis detection are more powerful than conventional histopathology ($P = 0.0005$, McNemar’s test). We recognize that the analysis based on the lymph nodes assumes that all nodes are independent of one another. This is not strictly true, because patients contribute more than one lymph node, making this analysis slightly anti-conservative. Perhaps most importantly, molecular-based analysis found positive lymph nodes in three patients classified as completely negative by histopathological evaluation and cytokeratin immunostaining, accounting for the significant difference in survival outcome based on molecular diagnosis.

The mismatch ligation assay used in the present work has been shown to have a sensitivity of about 1 tumor cell in 1000 normal cells, and the MSP assay has a similar sensitivity (9). MSP evaluation has the advantage of being a nonradioactive method and has been found to be reliable in detecting p16 promoter hypermethylation in bronchoalveolar lavages from lung cancer patients (19). Several approaches for lymph node micrometastasis detection have been described, and most of them are based on DNA or RNA isolation followed by PCR. The DNA-based approaches tended to be absolutely specific because they detect genetic abnormalities present only in the neoplastic DNA and not in the normal DNA. Furthermore, DNA-based techniques can be used robustly in paraffin-embedded tissue, allowing evaluation in retrospective studies. A major inconvenience for DNA-based tests is that only those tumors with identified genetic changes can be evaluated. In the present study, 76% of the patients could be analyzed because they had at least one molecular abnormality detected by either mutation analysis or promoter hypermethylation. However, our percentage of p16 promoter hypermethylation and p53 mutations was low compared to those of previous reports (20, 21), probably due to random sampling. Therefore, a good percentage of patients evaluable for genetic screening should generally be higher. Moreover, analysis of other common mutations such as the APC and β-catenin genes and further elucidation of genetic changes in colorectal cancer may allow the use of these approaches to study virtually every patient.

The high sensitivity of the molecular assays can have an important impact on clinic management. The identification of metastases in regional lymph nodes is routine practice for surgical pathologists and is one of the most significant prognostic factors derived from the staging process. In Dukes B colorectal cancer, the assessment of metastases by molecular techniques in pathologically negative lymph nodes has been correlated with a poor prognosis, indicating that molecular-based techniques may be a useful prognostic factor in colorectal cancer that could also serve as a selective marker for intensive postoperative adjuvant chemotherapy (5, 7). Although additional studies with a higher number of patients must be done to confirm the findings, our results indicate that the molecular screening of perihilar lymph nodes can be a good prognostic indicator of recurrence in colorectal cancer patients with resectable hepatic metastases.
is likely that the presence of tumor cells at the lymph nodes indicates secondary spread of the disease from the liver metastases or represents the presence of more widely disseminated disease from the initial primary tumor. There is little data about the determinants of prognosis in this specific group of patients. Some potential prognosis factors include the number of liver metastases (one to three versus four or more), carcinoembryonic antigen levels, ploidy, and hepatic lymph node involvement (22). In agreement with the last parameter, we found that patients affected with metastases at the perihepatic lymph nodes have a significantly worse survival than those without lymph node involvement. Moreover, molecular detection of occult tumor cells enhances the differences between both groups of patients. Although the data are still preliminary, the use of molecular approaches to detect perihepatic lymph node tumor involvement in colorectal cancer patients with liver metastases may be a tool in the selection of those patients that can benefit from liver metastasis resection.

We have used molecular-based techniques (specific mismatched ligation assay and p16 promoter hypermethylation) to increase the sensitivity of tumor cell detection in the perihepatic lymph nodes from colorectal cancer patients with recurrent disease. Moreover, molecular detection of occult node metastases predicts a poor prognosis in colorectal cancer patients with resectable liver metastases. Prospective studies should evaluate whether patients positive for tumor cells by molecular analysis should be subjected to the morbidity of aggressive surgery or instead entered into trials of intensive adjuvant therapies.

REFERENCES


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