Pathology Underrates Colon Cancer Extranodal and Nodal Metastases; Ex vivo Radioimmunodetection Helps Staging

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Abstract

Purpose: Colorectal carcinoma is frequently accompanied by small lymph nodes metastases that often escape pathologic examination. We evaluated whether ex vivo radioimmunodetection with the Affinity Enhancement System (AES) could improve detection of mesocolonic metastases.

Experimental Design: A bivalent 111In-labeled hapten was injected (16 patients) 4 days after a bispecific antibody (anticarcinoembryonic antigen, antihapten). Surgery was done 1 to 3 days later, and radioactive uptake in the mesocolon was recorded. Extensive pathologic examination of the mesocolon (reference method) was done after fat dissolution. This method visualizes all lymph nodes but is not in routine use.

Results: The reference method disclosed 705 nodes. There was no significant difference between the number of node metastases detected by AES or by the reference method (16 versus 17). Better detection would have been obtained by AES than by routine pathology (P < 0.01). In addition 12 extranodal metastases were found in this study of which eight were detected by AES. The prognostic importance of such extranodal metastases has been underlined in the literature. Routine pathology combined with AES would have disclosed all node metastases and 86% of total metastases versus 35% by routine pathology alone.

Conclusions: Ex vivo radioimmunodetection could improve nodal and extranodal metastases detection in patients with colorectal cancer. Its value for improving pathologic analysis, together with the effect of these small metastases on prognosis, should be further evaluated. The benefit of adjuvant chemotherapy for patients upstaged with radioimmunodetection should also be assessed because adjuvant chemotherapy improves the 5-year survival of stage III patients.

Five-year survival rate is about 60% to 80% in patients with tumor-node-metastasis stage II (no lymph node involvement) colorectal cancer and 30% to 60% in patients with tumor-node-metastasis stage III (node-positive) disease (1–4). Adjuvant chemotherapy improves the 5-year survival of stage III patients (5–8) but is not commonly used in stage II patients because of its side effects (3, 4, 9). However, some patients with stage II disease may be underscored and would benefit from this treatment if a more precise staging could be obtained. Routine pathology with node detection by palpation, followed by serial macroscopic sectioning of the mesocolon, may understage the disease (10–16). Indeed, studies using a fat clearance technique showed that most lymph node metastases involved nodes smaller than 5 mm that usually escape routine pathologic examination (10, 12, 17). Fat dissolution of surgical specimens thus improves the detection of small node metastases.

Radioimmunoguided surgery was proposed (21) to improve colorectal cancer staging, using a labeled anti–carcinoembryonic antigen (CEA) antibody Fab’-fragment (CEA-Scan). Ex vivo gamma-probe scanning of surgical specimens obtained after injection of a 111In-labeled antibody was also shown to be
useful for detecting occult metastases and involved lymph nodes of 5 mm or less (10). Besides, pretargeted immunoscintigraphy with the Affinity Enhancement System (AES; refs. 22, 23) has allowed us to preoperatively detect medullary thyroid carcinoma metastases smaller than 5 mm (24).

This study examined whether it was possible to improve the detection of metastases in mesocolon resection specimens by the AES radioimmunodetection method. This method was compared with the extensive pathologic examination of mesocolon specimens after fat dissolution.

Patients and Methods

Patients. The protocol was approved by our institutional review board in keeping with French bioethical regulations. All patients gave a written informed consent before participating in the study.

In the surgery department of Rothschild Hospital, Paris (Assistance Publique-Hôpitaux de Paris), 16 consecutive patients (nine women, seven men) were eligible for this study. Criteria for enrollment were primary colonic or upper rectal adenocarcinoma, serum CEA level of lower than 100 ng/mL, and no previous history of mouse antibody administration. Minors, pregnant or breast-feeding women, patients with metastases detected during the preoperative work-up, and patients having received chemotherapy or radiotherapy were not eligible.

Mean age was 67 years (range, 46-84 years). The adenocarcinoma was located in the right colon in seven cases, the left colon in eight cases, and the upper rectum in two cases (both left and right colons were involved in one case).

Radioimmunotargeting protocol. The bispecific anti-CEA/anti-diethyl-tylenediaminepentaacetic acid [DTPA(In)] mouse monoclonal antibody (25) was kindly supplied by Immunotech Pharma SA, and a dose of 0.1 mg/kg was given as a 30-min i.v. infusion. The bivalent hapten di-DTPA-TL (kindly supplied by Immunotech Pharma) labeled with 125 ± 43 MBq of 111InCl3 (Mallinkrodt) was injected 4 days later (24). High immunoreactivity (96.7 ± 2.7%) allowed i.v. injection of the labeled hapten without purification. No side effects were recorded after injections of the AES reagents.

Operative protocol. Patients underwent surgery 2 to 4 days after the radiotracer injection. After inspection of the abdominal cavity for carcinosis and hepatic metastases, the colonic lumen was excluded by a lace tied on each side of the tumor; vessels draining the tumor were tied, and then cut at their aortic origin (left colon and upper rectum) or at their superior mesenteric origin (right colon). The colon was excised, together with the mesocolon and its corresponding vessels. A fragment of ~4 cm2 of uninvolved large epiploon was sampled as a healthy tissue reference. The surgical specimen was sent immediately, unfixed, to the pathology laboratory.

Pathology and radioactive detection. Mesocolon specimens were macroscopically examined, radioactive uptake was detected with a manual probe, and the blocks of mesocolon showing radioactive uptake were excised for histologic examination. Thereafter, the specimens were submitted to fat dissolution and all nodes and masses present in the clarified tissue were examined as described below.

Each analysis was done by a different operator, unaware of the results of other analyses.

Macroscopic analysis. Photographs of the fresh specimen were taken to record the relationship between the tumor and the mesocolon, which was then separated from the colonic tube along the line of colonic insertion. Systematic pathologic analysis of zones of interest on the colonic tube, including the tumor, was done. The standard technique (5-mm slices) was not used to search for node involvement because anatomic integrity of the mesocolon resection specimen had to be preserved. Instead, to simulate routine pathologic examination, nodules found after fat dissolution were classified in two size groups: those equal or larger than 4 mm (5 mm before dehydration) and those smaller than 4 mm because most nodules of this size escape routine pathologic examination (12, 18).

The unfixed mesocolon, a fragment of the primary tumor and a fragment of uninvolved large epiploon, serving as reference, were placed between two grids, bearing centimeter scales to identify each square centimeter by a pair of coordinates (Fig. 1). A map of the specimen was drawn including the line of colonic insertion, surgical sections, the zone of tumor insertion, and the origin of the vascular pedicle(s). The support and specimen were then immersed in a bath of 4% buffered formalin.

Detection of isotope uptake on surgical specimens. The radioactivity of each square of the grid was counted with a Gammed II gamma probe (EURORAD). Uptake was considered significant when radioactivity...
counts were equal to or exceeded 2.5-fold that of the healthy reference tissue (mean of five counts).

To avoid pathology report delay, zones of mesocolon containing radioactive hotspots were excised and embedded in paraffin for immediate histologic examination, after their location on the grid was recorded.

Mesocolon fat clarification. The remains of the specimen, still held in the grid, was fixed in three consecutive formalin baths (first week), dehydrated in three baths of 5 L of absolute ethyl alcohol (second week), and cleared in three baths of 5 L of Histosol (Shandon; third week; refs. 14, 26). All mesocolon areas showing tissue densification, detected on an illuminated plate, were included in paraffin after their coordinates had been recorded, followed by further histopathologic examination.

Histology. A first analysis was done on a section of each inclusion block, stained with H&E-saffron. All blocks containing a nodal structure or an inflammatory focus in connective tissue, which had initially been considered metastasis-free, were subjected to further analysis with five staged sections in the block. One slide at each level was stained with H&E-saffron, and two were subjected to immunohistochemical detection of cytokeratin and CEA antigen. All slides were independently read by two pathologists. Their written results were then validated by consensus during a third reading with a dual-observer microscope.

Statistical analysis. Fisher’s exact test was used to evaluate the significance of the results using GraphPad Prism (GraphPad Software, Inc.).

Results

Fat clarification: the reference method. Overall, 7 of 16 patients had mesocolonic metastases. Full anatomic study of the areas exhibiting radioactive uptake and of the remaining mesocolon, subjected to fat clarification, detected 705 lymph nodes in the 16 surgical specimens (mean, 44 nodes per patient; range, 16-93 nodes; Table 1). Seventeen nodes were metastatic, of which four nodes revealed micrometastases (size, between 2 mm and 0.2 mm; Table 2). Immunohistologic analysis detected no additional metastasis or micrometastasis and no isolated tumor cells or clusters measuring 0.2 mm or less.

In addition, 12 metastatic lesions (41% of all metastases) found in the mesocolon lacked the structural features of lymph nodes (Table 3 and Fig. 2). Eleven (38% of all metastases) were tissue transplants of vascular embolic origin. Eight of these had visible venous vascular origin, and three corresponded to irregularly shaped tumor nodules, to which it is recommended to assign a vascular origin (35, 36). The other one, which had a histologic aspect of isolated metastatic nodule in fat tissue, with no visible residual nodal structure and with smooth contour, was classified as N+ as recommended (36).

Assuming that routine pathology misses lymph nodes smaller than 4 mm (5 mm before dehydration), only eight nodal and two extranodal metastases would have been detected by routine pathology.

Radioimmunodetection. The blocks of mesocolon with significant radioactivity contained 226 nodes (mean, 14 nodes per patient; range, 0-44 nodes) and 24 metastases.

Sixteen were node metastases, of which nine measured <4 mm (Table 2). The only metastatic node missed by the probe measured between 4 and 5 mm. This node was infiltrated with a few tumor cells on a mucoid cystic pattern. These tumor cells were moderately positive to immunohistochemical analysis with an anti-CEA antibody. For the detection of node metastases, sensitivity was 94.1%, specificity was 87.2%, and negative predictive value was 99.9%. AES immunodetection provided a significantly better detection than that would have been obtained by routine pathology (P < 0.01). There was no significant difference between AES detection and the reference method. Association of AES radioimmunodetection and routine pathology would have revealed all existing node metastases (Table 2).

Eight (67%) of the twelve metastases that did not correspond to lymph nodes were detected by AES. The four missed metastases measured <4 mm (Table 3).

Table 1. Size distribution of detected lymph nodes

<table>
<thead>
<tr>
<th>No. lymph nodes (mean per patient; range)</th>
<th>&lt;2 mm</th>
<th>2 to &lt;4 mm</th>
<th>≥4 mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>705 (44; 16-93)</td>
<td>308, 43.7% (19; 7-35)</td>
<td>256, 36.3% (16; 4-51)</td>
<td>141, 20.0% (9; 3-18)</td>
</tr>
</tbody>
</table>

Table 2. Distribution of metastatic lymph nodes per patient according to the detection method

<table>
<thead>
<tr>
<th>Patient identification no.</th>
<th>T category</th>
<th>Size (mm)</th>
<th>Simulation of routine pathology*</th>
<th>AES radiimmunodetection</th>
<th>Simulation of AES guided routine pathology</th>
<th>Reference: fat clearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>pT3</td>
<td>≥4</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>9</td>
<td>pT3</td>
<td>≥4</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>12</td>
<td>pT3</td>
<td>0.2 to &lt;2</td>
<td>4</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 to &lt;4</td>
<td>0</td>
<td>5</td>
<td>5</td>
<td>5</td>
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<tr>
<td></td>
<td></td>
<td>≥4</td>
<td>5</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>pT3</td>
<td></td>
<td>8</td>
<td>16</td>
<td>17</td>
<td>17</td>
</tr>
</tbody>
</table>

*To simulate routine pathologic examination, nodules were grouped into two size classes; those ≥4 mm (5 mm before dehydration) and those <4 mm because most of these small nodules escape routine pathologic examination (12, 18).

†Results of the full anatomic study of the areas with radioactive uptake and of the remaining mesocolon after fat clearance.
Examples of results of the mesocolon specimen of patient 9 are shown on Figs. 1 and 2.

Overall, pathology guided by AES would have identified 25 of 29 nodal and extranodal metastases, whereas only 10 would have been disclosed by routine pathology alone ($P < 0.001$).

**Discussion**

Metastatic status determines prognosis and indication for adjuvant chemotherapy in patients with colorectal cancer (3, 6). A recent population-based study in the United States concluded that, in 2001, most patients with colorectal cancer received inadequate lymph node evaluation (27). There is, thus, an important risk of understaging that may exclude patients from postoperative chemotherapy, a treatment with proved benefits in colon cancer with node metastases (7, 8).

Fat dissolution reveals all lymph nodes present in mesocolon specimens (10–15). In this study, histologic examination after fat dissolution revealed 16 to 93 nodes per patient (mean, 44 nodes) in agreement with previous results obtained with the fat dissolution method (10, 11, 13–16, 18, 28). It is generally accepted that this time-consuming method cannot be used routinely.

Abdel Nabi et al. proposed a considerably shorter procedure (10). Radioimmunodetection in excised specimens from patients with primary colorectal cancer, after preoperative injection of an indium 111–labeled anti-CEA antibody, detected a significantly higher number of metastases than standard pathologic examination. Radioimmunodetection has been improved by the AES method, which provides higher tumor to normal tissue radioactivity uptake ratios than does directly labeled antibodies (23, 25, 29). It is a pretargeting approach that uses unlabeled bispecific antibodies (directed to both the carcinoembryonic antigen and a hapten) and a labeled molecule bearing two haptons. We evaluated in the present study whether the AES method may improve ex vivo metastasis detection in the mesocolon.

AES immunodetection provided better detection of node metastases than that which would have been obtained by routine pathology, assuming that every lymph node larger than 4 mm is found by routine examination ($P < 0.01$). There was no significant difference between the detection of node metastases by AES and by the reference method. AES detection of lymph node involvement had a sensitivity of 94.1%, a negative predictive value of 99.9%, and a specificity of 87.2% as several probe-positive sites did not harbor metastases.

There is no consensus on the optimal number of mesocolon nodes to be analyzed during routine examination, numbers from 12 to 15 being recommended (16, 20, 30). However, these figures are based on controversial data and analyses, which lead some authors to consider that the number of nodes per specimen that can guarantee identification of all patients with metastases is not established (2, 31). The statistical study of Goldstein predicted that to find a single metastasis with 60% chance, 24 nodes would have to be examined (31). Our histologic analysis of the mesocolon areas with positive radioactive signals (14 nodes per patient in average) revealed 94% of the node metastases. In this study, AES pretargeting associated with routine pathology would have disclosed all node metastases (Table 2), suggesting that AES could guide the pathologist to improve node metastasis detection.

We were surprised by the frequency of metastases without detectable nodal structures (41% of all metastases). Pathologic examination after fat clearance disclosed 12 metastases of this type, among which eight were detected by the probe.

Eleven of the extranodal foci were tissue transplants of vascular embolic origin. The prognostic importance of these extramural tumor nodules of vascular origin, discontinuous from the primary tumor mass, has been underlined in the literature (3, 32–34), and they are now included in the T category as pt3 (35). These extramural metastases of vascular origin should be distinguished from intravascular spread via lymphatics or venous vessels, which are categorized as V or L (venous or lymphatic) substages of the T category (36–38).

The last extranodal metastasis, with a relatively smooth contour, resembled an isolated tumor nodule in connective tissue. It has been suggested that these isolated nodules should be categorized as metastatic nodes (N1 or N2) even if no residual lymphoid tissue is visible (36, 37).

The large number of metastases of vascular origin in this study suggests that their frequency may have been underestimated because they are difficult to detect. Tackling into account nodal metastases only, 3 of the 16 patients would have been wrongly diagnosed as metastasis-free in the mesocolon. Overall,

<table>
<thead>
<tr>
<th>Table 3. Distribution of extranodal metastases per patient according to the detection method</th>
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<tbody>
<tr>
<td><strong>Patient identification no.</strong></td>
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<tr>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>1</td>
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<tr>
<td>6</td>
</tr>
<tr>
<td>9</td>
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<tr>
<td>10</td>
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<tr>
<td>12</td>
</tr>
<tr>
<td>Total</td>
</tr>
</tbody>
</table>

*To simulate routine pathologic examination, nodules were grouped into two size classes: those ≥4 mm (5 mm before dehydration) and those <4 mm because most of these small nodules escape routine pathologic examination (12, 18).

†Results of the full anatomic study of the areas with radioactive uptake and of the remaining mesocolon after fat clearance. Abbreviations: V-v, vascular origin with venous vascular structures; V-i, vascular origin with no visible structure but irregular in shape; S, smooth contour (35, 36).
AES-guided pathology would have identified 86% of nodal and extranodal metastases, whereas only 35% would have been disclosed by routine pathology ($P < 0.001$).

**Conclusion**

These results suggest that standard pathologic examination underestimates the number of metastases in mesocolon specimens from colorectal cancer patients by failing to detect not only small involved nodes but also extramural extranodal metastases. AES immunodetection ex vivo identified a large proportion of these small metastases. Routine pathologic examination guided by immunodetection, using either commercially available anti-CEA antibodies (39) or the new reagents under development for the AES method (40, 41) in conjunction with a dedicated imaging device, could detect a larger proportion of metastatic sites, including extranodal tumor foci, and help to refine the staging of patients with colorectal cancer. Another interesting finding in this study is the large proportion of extranodal metastases in the mesocolon of these patients. The value of this technique for improving routine pathology, together with the effect of the detection of additional nodal and extra nodal small metastases on prognosis, should be further evaluated. The benefit of adjuvant chemotherapy for patients upstaged with radioimmunodetection should also be assessed.

**Acknowledgments**

We thank Dr. Alain Vandewalle for helpful discussions, Dr. S. Moore for correcting English grammar, Immunotech Pharma (Marseille, France) for the generous supply of AES reagents, Mecaserto (Saint Thibault des Vignes, France) for lending us an imaging device, and M. Elii Mulliez for invaluable help with radioisotopic detection. The AES technology is now under development by IBC Pharmaceuticals, Inc. (Morris Plains, NJ).
Detection of Colon Cancer Small Metastases

References

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