Radioimmunoguided Surgery for Gastrointestinal Malignancies: An Analysis of 14 Years of Clinical Experience

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Radioimmunoguided surgery can assist with the intraoperative identification and staging of abdominal adenocarcinoma.

Background: The identification of all sites of intra-abdominal adenocarcinoma is key to optimal surgical resection and tumor staging. Conventional imaging methods and direct visualization and palpation have limited sensitivity and specificity. Radioimmunoguided surgery (RIGS) has a potential to improve these parameters.

Methods: The development of the RIGS concept is presented, and the studies of tumor localization, detection of disseminated disease, staging, and survival are correlated with the tumor biopsy of gastrointestinal carcinoma, particularly colorectal carcinoma.

Results: RIGS can detect clinically and histologically occult neoplasm. Also, by providing immediate intraoperative information, the RIGS approach improves surgical staging, impacts on surgical and medical care, and affects patient prognosis.

Conclusions: RIGS may become the standard of care for the surgical staging and treatment of colorectal cancer and other gastrointestinal malignancies.

The Concept of Radioimmunoguided Surgery

The original concept of radioimmunoguided surgery (RIGS, Neoprobe Corp, Dublin, Ohio) was to intraoperatively assess the malignant nature of tissue undergoing visual and manual evaluation by an operating surgeon and to make this information immediately available. RIGS technology is comprised of three interrelated but separate components - a radionuclide, a gamma-detecting probe (GDP), and a monoclonal antibody (MAb) - that work in unison toward the common objective of intraoperative detection of primary or recurrent tumor and disseminated disease. RIGS requires a conceptual and working knowledge of the system’s components and their function in the process of intraoperative tumor identification, and it depends on the affinity of the MAb for the neoplasm. It is not dependent on the light microscope for tumor detection, but it requires the interaction of its three components with the skill of the surgeon for the enhancement of intraoperative tumor localization. Today, RIGS uses a low-energy isotope (Iodine-125), a second-generation murine antitumor-associated glycoprotein-72 (TAG-72) MAb, CC49, with high affinity and specificity for antigens expressed by malignant human colorectal cells, and a gamma detector, the Neoprobe 1000 (Neoprobe Corporation, Dublin, Ohio), comprised of a cadmium telluride scintillation crystal.

The RIGS protocol has been previously described. Briefly, patients undergo preoperative laboratory studies to document serum tumor markers and liver function. Imaging studies are performed to exclude obvious unresectable disseminated disease. Saturated solution of potassium iodide (10 drops by mouth twice daily) is prescribed two days before injection of the radiolabeled MAb and continued for three weeks to minimize uptake of $^{125}$I by the thyroid. After the injection, precordial (blood pool) gamma probe counts are monitored weekly with the GDP until they are less than 20 counts per 2 seconds (usually three to four weeks later). At the time of surgery, the abdomen is explored through a midline incision from the xiphoid process to the pubis in a standard manner, and all sites of readily apparent tumor are documented. A second survey of the abdomen is then performed with the GDP in a systematic fashion. First, gamma probe counts are obtained from the blood pool (usually over the abdominal aorta) to determine the level of background radioactivity in the blood. The instrument is adjusted (squeched) so that the gamma detector’s audible signal is not emitted unless radioactivity 3 standard deviations above mean background counts is detected. The audible signal then detects focal uptake of radioactivity or a RIGS-positive tissue focus. In the absence of widely disseminated disease, the tissue is resected if this can be accomplished safely. The surgical specimens are then sent to pathology for histologic evaluation. Using this protocol, RIGS studies have yielded information in three major areas: tumor localization, disease dissemination, and staging and survival. Our experience in these three areas is reviewed and the significance of our findings is discussed.

Tumor Localization

The first clinical study of RIGS began in 1983 and used a baboon polyclonal ant carcinoembryonic antigen (anti-CEA) antibody radiolabeled with the high-energy isotope, Iodine-131. A total of 28 patients with primary and recurrent colorectal carcinoma were injected with $^{131}$I-anti-CEA. Whole-body external images were obtained preoperatively and were compared with intraoperative findings using a hand-held gamma detector. Using the criterion of a tumor-to-background (T:B) count ratio of 1.5:1, $^{131}$I-anti-CEA localized gross tumor in all but one patient with malignant primary colorectal disease (Table 1). An additional patient with a large bowel neoplasm was found to have an adenomatous polyp whose T:B count ratio was 1:1, indicating no focal uptake of radioactivity and therefore no binding of radiolabeled antibody to tumor antigen. Preoperative scintigraphy accurately localized tumor in 33% of patients with primary neoplasms and in 64% of those with recurrent disease.

Most importantly, this study reported the first intraoperative assessment of malignant tissue by RIGS technology with the information provided immediately to an operating surgeon during tumor extirpation. The ability of RIGS to localize to the majority of obvious tumor, to detect metastatic disease to the liver, and to distinguish...
between benign and malignant neoplasms of the colon was also demonstrated in this investigation. Additionally, the combined use of the $^{131}$I-anti-CEA and the GDP intraperitoneally was shown to be superior to the external scintillation camera in the assessment of tumor burden. These observations also illustrated the limitation of a gamma detector imposed by the inverse square law: $S = \frac{1}{D^2}$, where $S$ indicates sensitivity or specificity and $D$ indicates the distance of the detector from the source of radiation. Therefore, sensitivity or specificity will increase as the distance from the detector to the radiation source is decreased. The GDP comes into direct contact with the source of radiation (the tumor), while the external scintillation camera will never achieve this proximity to the neoplasm it attempts to image.

The second phase of RIGS clinical trials reflected not only the development of hybridoma technology and the production of large quantities of MAb with high affinity and specificity for tumor antigens, but also the further refinement of the hand-held gamma-detecting instrument. In a study by O’Dwyer et al.,$^4$ the MAb 17-1A or its fragment $\text{F(}ab’)_2$ radiolabeled with the low-energy isotope $^{125}$I, was injected preoperatively into 18 patients with primary and recurrent colorectal carcinoma. Only 16 patients were included in the final analysis of the data because the gamma detector malfunctioned at the time of surgery in two instances. As shown in Table 1, using a low-energy isotope, an MAb, and a T:B count ratio of 1.5:1 or greater, the radiolabeled antibodies were taken up by the tumor in all but one patient, by the majority of tumor sites, and by metastatic tumor to the liver. Of greater significance is the fact that RIGS technology detected clinically occult disease, ie, tumor not obvious to the surgeon.

To further assess the use of the MAb 17-1A in RIGS, Petty et al.$^5$ reported an experience with 13 patients in which the criterion for a positive finding was a T:B ratio of 2:1. Tumor localization was achieved in only 61% of patients. Occult disease was again localized, but it was found unexpectedly in lymphatics remote from the colon, including the peripancreatic area. These findings demonstrated occult colorectal carcinoma disseminated to sites that were not previously well described. Of the 12 tumor sites localized, hematoxylin and eosin (H&E) staining with standard microscopy detected adenocarcinoma in only eight sites. However, using immunohistochemistry (IHC) and autoradiography, malignant cells were identified in the remaining four specimens. These observations documented the ability of RIGS technology to identify H&E occult tumor and demonstrated for the first time tumor detection that was not dependent on light microscopy.

The third phase of RIGS investigations used the first-generation anti-TAG-72 murine MAb, B72.3, radiolabeled with $^{125}$I. Thirty patients with primary colorectal carcinoma participated in the study (Table 1).$^2$ Tumor localization occurred in 23 patients (77%). With B72.3 as a component of the RIGS system, benign and malignant lesions were again differentiated. However, in this study, the neoplasm in question was detected in the liver, a site common for metastatic colorectal carcinoma. RIGS findings altered adjuvant therapy in 4 (17%) of the 23 patients. Additionally, the identification of occult tumor in sites remote from the primary tumor that were not typically included as sites of distant disease for colorectal carcinoma further supported the observation that the RIGS system appeared to provide more accurate surgical staging than could be provided by even the most experienced surgeon.

In a second study of the MAb, B72.3, Sickle-Santanella et al.$^6$ showed a tumor localization rate of 89% in six patients with primary disease and 82% in 31 with recurrent malignancy. The authors reported identification of clinically occult tumor in 26% of patients. Additionally, metastases were detected in the liver and the lung (Table 1).

These two studies of the GDP and B72.3 reaffirmed the ability of RIGS technology to assess the malignant nature of tissue intraperoperatively and to provide this information immediately to the operating surgeon. Since these observations of the RIGS system took place in the institution where the technology was conceived and
developed, it was evident that assessment in a larger surgical community was required to determine its future use in the care of cancer patients.

Cohen et al later reported a multicenter trial of B72.3 and the GDP in 104 patients with both primary and recurrent colorectal carcinoma. Tumor localization was achieved in 78% of study participants (Table 1). In six patients with recurrent disease, no tumor could be identified by traditional exploration or the GDP. In 27% of patients whose tumor was localized, occult neoplasm was identified and subsequently confirmed histopathologically. Disseminated disease was again detected in the liver, periporal regions, retroperitoneum, and pelvis. Of the 72 patients with recurrent colorectal cancer, 37 were deemed unresectable. In 27% of these patients, data provided by RIGS led to the decision to abandon further tumor extirpation. In the remaining 35 patients, operative resection was extended in 23% based solely on the information provided by the RIGS cancer detection system.

The development of human antiguine antibodies (HAMA) was also investigated in this large patient cohort. Within five weeks of injection of the radiolabeled MAbs, 40% of individuals developed HAMA. By chi-square analysis, no significant correlation between the development of these antibodies and tumor localization could be found. Additionally, when serum TAG-72 levels were analyzed, no relationship between blood levels and tumor detection was demonstrated.

The data from this study confirmed that detection of occult tumor in 20% and 30% of patients with colorectal carcinoma could be achieved in multiple centers by numerous surgeons and that this information could affect the operative management of the patient. In this large study cohort, RIGS was shown to have a sensitivity of 77% and a positive predictive value of 78%.

The fourth phase of localization studies used the second-generation anti-TAG MAbs CC83 and CC49, radiolabeled with 125I. A comparison of the affinity constants (Ka) for these two MAbs of 27.7 x 10^9 M^-1 and 16.2 x 10^9 M^-1 respectively, with the Ka of 2.5 x 10^9 M^-1 for B72.3, would predict superior tumor binding using CC83 and CC49. For RIGS, this should be reflected in a better tumor localization rate than was obtained with B72.3. To test this hypothesis, a clinical trial was conducted with 125I-CC83 in patients with recurrent colorectal carcinoma. In this study cohort, tumor was localized in all 15 evaluable patients (100%). Of the 27 tumor sites confirmed histologically to be carcinoma, 100% were localized. Of these 27 sites, four were clinically occult and identified intraoperatively only by gamma-probe detection (Table 1). In this study, the sensitivity of the RIGS protocol was 100%, an improvement over the 77% for B72.3. However, the positive predictive value was only 69% (less than the 78% for B72.3). Operative therapy was altered in 3 (20%) of 15 patients, resulting in more extensive tumor resection.

The second generation anti-TAG-72 MAb, CC49 radiolabeled with 125I, was evaluated as a component of RIGS in a cohort of 54 patients with primary and recurrent colorectal adenocarcinoma. In this study group, 3 of 24 patients with primary disease had tumor resected either endoscopically or during previous surgery. In the 21 remaining patients, 125I-CC49 and the GDP localized the primary neoplasm in 18 (86%). Of the 30 patients with recurrent disease, tumor was detected in 29 (97%). From the entire group of 54 patients, 99 sites of carcinoma were identified; 88% were localized using RIGS, and 14% were clinically occult to the surgeon (Table 1). RIGS findings altered therapeutic intervention in 26 (44%) of 54 study participants. This additional information led to extended resection in most patients.

In the latter two studies, tumor localization using CC83 and CC49 was found to be better than using B72.3; however, the detection of other gastrointestinal malignancies using these second-generation MAbs remained to be demonstrated. LaValle et al, in a cohort of 10 patients with adenocarcinoma of the pancreas, showed that nine patients with primary disease and one patient with a hepatic recurrence had all sites of obvious neoplasm localized using 125I-CC49 and the GDP. Nine RIGS-positive lymph nodes that were histologically negative for tumor were further evaluated by IHC using a combination of anticytokeratin (AE1/AE6, CAM 5.2) and anti-MOC-31 (an epithelial membrane antigen) MAbs. Carcinoma was documented in six of these nine lymph nodes. These findings demonstrated again that RIGS technology unequivocally detects H&E occult tumor. Detection of pancreatic cancer using 125I-CC49 and the GDP established the applicability of the RIGS system to gastrointestinal malignancies other than colorectal adenocarcinoma.

In summary, the localization studies demonstrated that (1) RIGS technology was developed using the radiolabeled polyclonal antibody, 131I-anti-CEA and the MAbs 17-1A and B72.3 radiolabeled with 125I, but was enhanced using the second-generation anti-TAG-72 MAbs CC83 and CC49 radiolabeled with 125I, (2) it detected obvious gastrointestinal adenocarcinoma, clinically occult neoplasm, and H&E occult tumor, (3) it had a potential role in the surgical staging of gastrointestinal malignancies, and (4) it provided immediate intraoperative information to a surgeon that impacted on the management of the cancer patient.

**Disease Dissemination**

In the studies described above, four types of tissue defined by both RIGS status and histopathology were resected during RIGS for gastrointestinal adenocarcinoma. Type I was RIGS negative with no tumor cells by H&E staining, type II was RIGS negative with tumor cells by H&E staining, type III was RIGS positive with no tumor cells by H&E staining, and type IV was RIGS positive with tumor cells by H&E staining.

Type III RIGS-positive tissue was comprised most commonly of lymph nodes and was most frequently detected in the celiac, periporal, periaortic, and retroperitoneal areas. These findings were not observed in RIGS-negative nodes removed from the same patient. IHC of type III RIGS lymph nodes with MAbs to TAG-72 demonstrated staining concentrated in the germinal centers and in the radial sinuses of the lymph node (Fig 1A). Autoradiography of type III RIGS-positive nodal tissue showed silver grain deposition (Fig 1B) indicative of focal radioactivity from the radiolabeled MAb concentrated in the germinal centers and radial sinuses of the lymphoid follicles, a localization pattern identical to that seen in IHC.

To further assess the frequency of H&E occult tumor in type III RIGS nodal tissue, Cote et al analyzed lymph node specimens resected during RIGS with five additional sections and IHC consisting of a cocktail of MAbs to cytokeratin. A total of 57 nodes excised from 16 colorectal carcinoma patients (nine primary and seven recurrent) were included in the study. Of the 39 (68%) lymph nodes that were RIGS positive, 14 (36%) were H&E positive for colorectal carcinoma. In the remaining 25 RIGS-positive nodes, H&E-negative samples (140%) demonstrated the presence of occult metastases after serial section and IHC. Of the original 57 lymph nodes, 27 contained tumor. Of these, neoplasm was visualized by H&E staining in 17 (14 RIGS-positive nodes and 3 RIGS-negative nodes) and by IHC after H&E staining was negative for tumor in 10 (all RIGS positive). In this study, routine histologic evaluation identified adenocarcinoma in 17 (63%) of 27 specimens, while the RIGS system identified malignancy in 24 (89%) of 27 nodal samples (P < .05). Fifteen lymph nodes removed were RIGS negative and H&E negative. Occult disease was not detected in any of these tissues by cytokeratin staining.

Arnold et al described the sites of tumor dissemination detected by RIGS technology in a cohort of 86 patients with primary and recurrent colorectal carcinoma. In all patients, the GDP used with 125I-CC49 detected more foci of tumor than did traditional surgical exploration. In patients with primary disease, traditional surgery identified 45 sites of neoplasm, while RIGS detected 153 sites. In patients with recurrent disease, surgical exploration found 116 tumor foci, while RIGS detected 184. The liver was the
Carcinoma was found disseminated to regions not typically described for metastatic colorectal cancer, including the celiac axis, gastrohepatic ligament (GHL), retroperitoneum, and the root of the small bowel mesentry. The identification of tumor by RIGS in the GHL, around the celiac axis, and in the retroperitoneum around the aorta was associated with liver metastasis. Focal radioactivity was detected in GHL with equal frequency in patients with both primary (86%) and metastatic (67%) disease. This was also true of RIGS-positive tissue detected around the celiac axis, which was observed in 49% of both primary and recurrent tumor patients. Also of note, these authors found disease dissemination was not influenced by the location of the primary tumor. Metastatic disease, when present, was found in the same regions regardless of whether the original lesion was in the colon (right, transverse, or left) or the rectum. These findings suggested that unappreciated disseminated tumor is present at the time of primary resection. The sites of this unrecognized tumor are characteristic of metastatic disease found in patients with recurrent disease and therefore represent the early detection of disease progression. Lymphatic dissemination of colorectal carcinoma appears to be more extensive and more complex than previously believed.

To further investigate the clinical significance of disseminated disease detected by RIGS technology, Schneebaum et al. performed a retrospective study of periportal RIGS-positive lymph nodes in 124 patients with recurrent colorectal carcinoma. RIGS was performed using $^{125}$I-B72.3 in 87 patients and $^{125}$I-CC49 in 37 patients. Of the entire patient population, 47 (38%) were found to have RIGS-positive periportal lymph nodes. Thirteen (28%) of these 47 patients had tumor identified by routine H&E staining of lymphatic tissue. In the remaining 34 patients, lymph node tissue was studied for 31 patients (three specimens were not suitable for examination) by IHC using a polyclonal cocktail of anticytokeratin antibody (antikeratin AE1/AE3). From this group of patients, an additional eight were found to have tumor in their periportal lymph nodes by IHC. From the entire study group, 21 (48%) of 44 patients had tumor identified in their RIGS-positive periportal lymph nodes using a combination of H&E staining and IHC for cytokeratin. In this patient cohort, there was no significant difference in the incidence of periportal node involvement in those with liver metastasis (44%) and those without tumor in the liver (28%). A significantly higher incidence ($P<0.05$) of RIGS-positive periportal lymph nodes was detected with $^{125}$I-CC49 (44%) than with $^{125}$I-B72.3 (28%). Additionally, in this study, no correlation between RIGS-positive periportal lymph nodes and preoperative CEA level was found.

These authors also discussed nine patients who presented with recurrence in the periportal area following RIGS. In all nine, focal uptake of radioactivity was documented by GDP counts in this region using a T:B count ratio of 1.5:1 at the time of the original surgery. A biopsy was performed of the RIGS-positive lymph nodes in all but one patient. The microscopy on frozen section was reported as ‘reactive lymph node, no tumor seen.’ Despite these negative results, a biopsy was performed in one patient on all lymph nodes in the periportal region. After nonroutine serial section of these nodes and subsequent H&E staining, tumor was finally documented. In the remaining eight patients, cytokeratin staining was performed on their nodal tissue and in four of these patients, H&E occult neoplasm was identified by IHC. Therefore, microscopic tumor was found in five (56%) of the nine patients with RIGS-positive periportal lymph nodes.

In these nine patients, disease involving the periportal lymph nodes became clinically apparent at an average of 9.8 months later (range = 6 to 32). In six patients, gross tumor in the periportal area was an operative finding. In two patients, a mass consistent with recurrence was seen on abdominal computed tomography. In six patients who were re-explored, the periportal tumor was unresectable in four. On the basis of these observations, 38% of patients with recurrent colorectal carcinoma can have RIGS-positive periportal lymph nodes, and at least 48% of these nodes can be found to contain neoplasm. Even in the absence of tumor by routine microscopy, a clinically significant recurrence can develop in this site as early as six months after RIGS-positive activity is detected in this region.

More recent studies of type III RIGS lymph nodes have been performed for the purpose of investigating the biologic behavior of the cells found in the germinal centers and radial sinus of this nodal tissue. These two regions of a RIGS-positive lymph node are the areas where TAG-72 antigen expression is detected by IHC and where the radioactivity is documented by autoradiography. In these investigations, cells were isolated from a total of 24 lymph nodes -- 22 RIGS-positive lymph nodes from patients with colorectal carcinoma and two from patients undergoing laparotomy for benign processes. The cells were characterized for malignancy by an assessment of proliferative properties, antigen expression, and studies of tumorigenesis.

Under sterile conditions, the fresh tissue was cut into sections 400 to 500 μm in thickness. The sections were placed in 36-mm Petri dishes and immobilized with fibrin clot. A solution of 0.05% type I collagenase and 0.0003% DNAse in a balanced salt solution was added to each dish and incubated at 33 degrees Celsius for 15 to 30 minutes. Using an inverted phase contrast microscope and a micropipette, cells from nodal radial sinuses and germinal centers were aspirated from each lymph node section and cultured in vitro. The aspirated cells, in a concentration of 1 x 10⁷ to 2 x 10⁵, were initially placed in T-25 culture flasks containing Dulbecco’s minimal essential media (DMEM) with 10% fetal bovine serum (FBS). After 24 hours, the free-floating cells were removed. The remaining adherent cells were cultured in vitro for one week and expanded under similar conditions. After six days of expansion, the cells were transferred to fibroblast limiting media containing 1% HITES (100 μM of hydrocortisone, 5 ng/mL of insulin, 5 μg/mL of transferrin, 100 μM of estrogen, and 5 μg/mL of selenium) to avoid fibroblast dominant cultures. After six weeks of expansion under these conditions, the remaining viable cells were used in studies of proliferation and doubling time, used in tumorigenesis assays (described below), or stored in liquid nitrogen.

Studies of proliferation and doubling time (eternal growth) were conducted using nodal cells and human fibroblasts. Both cell populations were seeded into six-well plates in a concentration of 1 x 10⁴ in DMEM with 10% FBS and allowed to proliferate for 50 and 100 hours of culture. Cell counts and doubling times were calculated for both cell types and both time intervals and were compared. The potential tumor cells were found to double at a steady rate and to maintain a constant cell count, which are typical characteristics of malignant cells in vitro. The fibroblasts, however, showed prolonged doubling times and decreased cell numbers over time.

Tumorigenesis assays were then performed in vitro. The cells isolated and selected from the initial in vitro studies were then cultured in DMEM containing 10% FBS and 0.5% agarose (soft agar). After 12 days, the cells grew in discrete colonies under these anchorage-independent growth conditions (Fig 2). Cells from these tumor colonies were counted, isolated, then placed in 24-well plates and allowed to proliferate. They were subsequently used for in vivo tumorigenesis assays in immunocompromised animals (described below).

After the culture for adherence, inhibition of fibroblasts, and eternal growth, the viable potential tumor cells were then characterized for antigen expression using immunofluorescent staining of TAG-72 with a C49 MAb and by immunofluorescence using flow cytometry. Cells for immunofluorescent staining were removed from storage in liquid nitrogen, placed in DMEM containing 10% FBS, and allowed to proliferate. During adaption to in vitro culture conditions and during proliferation, cells were harvested and stained with an immunofluorescent CC49 MAb. Cells still adapting to culture and in a nonproliferating state were found to have positive staining indicating cell-surface expression of TAG-72 (Fig 3). This observation was not noted for cells in a proliferating state. This variable expression of TAG-72 requires further study of conditions peculiar to early culture following preservation and storage in liquid nitrogen. Antigen expression as a function of pH may also be a factor in the variable expression of TAG-72 seen in these potential tumor cells from RIGS-positive lymph nodes.
Cells incubated in saturating concentrations of conjugated MAb to collagen IV, CD11b, CD14, CD19, and CD34. Cells incubated with irrelevant conjugated antibodies served as controls and were used to establish specific binding. Normal human B cells, endothelial cells, fibroblasts, and bone marrow cells were used as positive controls. The cells were sorted using a flow cytometer (FACSort, Becton Dickinson & Co, Franklin Lakes, NJ), and data were analyzed using Lysis II software (Becton Dickinson). As shown in Table 2, potential tumor cells grew as adherent cells in culture, were not growth inhibited in HITES-containing medium, showed eternal growth properties in vitro, grew as colonies in soft agar, and lacked the other characteristics of cells found in normal lymph nodes.

In vivo tumorigenesis assays were developed in immunocompromised animals. Potential tumor cells were harvested from culture using 1 g of trypsin and 0.02 g of EDTA per 100 mL of phosphate-buffered saline. They were initially injected in a concentration of 1 x 10⁷ viable cells into the cecum of anesthetized nude mice through a small abdominal incision. The incision was closed with a stainless steel wound clip. At four weeks, the animals were sacrificed, and the tumors were removed and enzymatically dissociated with collagenase (type I, 200 U/mL) and DNASE (270 U/mL). The resulting cell suspensions were filtered and washed in serum-free media in preparation for injection into SCID mice. A small incision was made in the abdomen of anesthetized animals, and the spleen was injected with viable potential tumor cells in concentrations of 5 x 10⁵ or 2 x 10⁶ cells/mL. The wound was closed with stainless steel staples. After four weeks, the mice were sacrificed and the organs were harvested.

Evidence of disseminated human tumor cells originally isolated from type III RIGS nodal tissue was obtained by the extraction of human DNA from the murine organs, followed by polymerase chain reaction (PCR) amplification of human Alu sequences. After removal, the murine tissues were frozen in liquid nitrogen, homogenized, and lysed in buffer containing 75 mm of NaCl, 25 mm of EDTA (pH 8.0) and 1% sodium dodecyl sulfate. Proteins were digested by incubation with proteinase K at 55 degrees Celsius overnight. Proteins were then extracted with NaCl (6 M) in a final concentration of 1.5 M and chloroform in a volume of 1:1 for 30 to 60 minutes. The sample was centrifuged and DNA was extracted from the supernatant by adding propanol in a volume of 1:1. The DNA pellet was washed with 70% ethanol overnight, dried, and resuspended in TE buffer (10 mm of Tris-HCl, 1 mm of EDTA, pH 8.0). PCR amplification of human Alu sequences was performed using 100 ng of DNA from the various murine organs as template and the Human DNA Identification Kit (Boehringer Mannheim Diagnostics, Indianapolis, IN) according to the manufacturer’s instructions.
As shown in Fig 4, human Alu sequences were present in mouse lung, liver, spleen, and bone marrow. While this assay is semiquantitative, it is evident that murine bone marrow contained the greatest amount of human DNA. These sequences would be found in animal organs only if disseminated there in human cells. These findings provide evidence that RIGS-positive lymph nodes harbor cells not normally present in lymphatic tissue, are capable of tumorigenesis both in vitro and in vivo, and are disseminated in vivo.

Taken together, these studies of disseminated disease and RIGS technology indicated the following: (1) Analysis of type III nodal tissue revealed the germinal centers and radial sinuses are the sites of RIGS activity as these are the regions with silver grain deposition by autoradiography and the sites of antigen binding by IHC. (2) When tumor dissemination detected by RIGS was compared to histologic evaluation, RIGS identified significantly more foci of carcinoma (P<0.05). (3) Metastatic colorectal carcinoma was found in sites not previously well described and not routinely evaluated during traditional exploratory laparotomy. (4) Tumor dissemination detected by RIGS was indicative of disease progression with or without the histologic confirmation of tumor as demonstrated in studies comparing sites of RIGS-positive tissue in patients with primary and recurrent colorectal carcinoma and in studies of RIGS-positive peritumoral lymph nodes. (5) RIGS-positive lymph nodes harbored cells in the regions of RIGS activity (germinal centers and radial sinuses) with malignant characteristics.

### Staging and Survival

The impact of RIGS on surgical staging was evaluated by Arnold et al\(^1\) in 36 patients with primary colorectal cancer. Tumor localization using \(^{125}\)I-CC49 occurred in 30 (83\%) patients. In 24 (68\%) of these individuals, the RIGS system detected additional sites of neoplasm in the liver, GHL, celiac axis, regional lymph nodes, retroperitoneum, omentum, small bowel mesentry, abdominal wall, around the iliac vessels, in the pelvis, and in gynecologic organs that were not identified by traditional exploration. As a result of these RIGS findings, disease was upstaged in 11 (37\%) of 30 patients, and the operative strategy was altered in nine patients. From these data, it is evident that the RIGS system provided immediate intraoperative information that led to more accurate surgical staging than traditional exploratory laparotomy and therefore provided the surgeon with a better tool with which to assess both regional and systemic spread of tumor.

A study of survival based on RIGS findings was first reported by Martin and Carey\(^7\) in 1991 for a cohort of 86 patients with recurrent colorectal carcinoma. When tumor dissemination procedures were performed using RIGS technology to determine three groups of patients. The groups were comprised of 53 patients (62\%) with resectable tumor by standard visual and manual inspection, 33 patients with nonresectable disease by traditional determinations, and 13 patients from the traditionally nonresectable group. The traditional resectable group found by RIGS assessment to have unresectable neoplasm. Therefore, the RIGS survey for resectability concurred with the traditional assessment in 40 patients (75\%). Of greater significance, 25\% of patients assessed to have operable tumor were found by RIGS to have disease too widely disseminated for surgical cure. The two-, three-, and five-year survival rates were evaluated for each patient group (Table 3). The median survival was 60+ months for the 40 patients in the traditional/RIGS resectable group, 29.4 months for the RIGS nonresectable group, and 18 months for the traditional nonresectable patients. Probability values comparing survival by Lee-Desu statistical analysis were \(P<0.0001\) for traditional/RIGS resectable vs traditional nonresectable, \(P=0.0008\) for traditional/RIGS resectable vs RIGS nonresectable, and \(P=0.24\) for traditional nonresectable vs RIGS nonresectable.

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<thead>
<tr>
<th>Carcinoma</th>
<th>Antibody</th>
<th>Tumor Status</th>
<th>Survival</th>
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<tbody>
<tr>
<td>Recurrent colorectal n=86</td>
<td>mB72.3 ref 17</td>
<td>Positive Tumor RIGS</td>
<td>2 yrs: 13/28 (46%) P&lt;0.05; 3 yrs: 12/28 (43%) P&lt;0.019</td>
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<tr>
<td>Primary colorectal n=31</td>
<td>mCC49 ref 18</td>
<td>Positive Tumor RIGS</td>
<td>2 yrs: 13/19 (68%) P=0.0001; 3 yrs: 12/19 (63%) P=0.0001</td>
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<tr>
<td>Recurrent colorectal n=69</td>
<td>mCC49 ref 18</td>
<td>Positive Tumor RIGS</td>
<td>2 yrs: 13/19 (68%) P=0.0001; 3 yrs: 12/19 (63%) P=0.0001</td>
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These data indicate that significant differences occurred in the survival of patients found to have resectable tumor by RIGS (traditional/RIGS resectable) and both groups of patients with nonresectable disease (traditional nonresectable and RIGS nonresectable). At five years, RIGS nonresectable tumor impacted on survival in a manner identical to traditionally nonresectable malignancy. While the survival rates for the two nonresectable groups of patients were different at two and three years, they reflect a similar tumor biology that is even more evident at five years.

In a study of RIGS and survival, Arnold et al\(^1\) reported an experience of 31 patients with primary colorectal carcinoma who underwent tumor extirpation using RIGS. At the conclusion of the procedure, 14 patients (45\%) had no detectable residual RIGS-positive tissue, while 17 patients (55\%) had unresectable disease. Final RIGS status was compared to TNM staging, and both were analyzed with regard to outcome (Table 3). When survival was examined with respect to stage, patients without evidence of disseminated disease (stage I/II) lived longer than those with regional or widespread disease (stage III/IV) \((P<0.019)\). Patients with no residual RIGS-positive foci (RIGS-negative) following primary resection had a survival advantage over those who had residual RIGS-positive tissue \((P<0.0001)\). This study demonstrated unresectable RIGS tissue to be associated with poor survival. This difference did not appear to be solely dependent on stage, as TNM stage I, II, and III patients were in the RIGS-negative group.

Bertsch et al\(^9\) and survival in 45 patients with recurrent colorectal carcinoma following resection using RIGS and \(^{125}\)I-CC49. Ten patients by RIGS criteria were found to have resectable tumor, five had RIGS nonresectable tumor, 20 had traditionally nonresectable disease, and 10 patients had tumor classified as addressed disease. This last group included patients with RIGS-positive macroscopic tumor treated by other intraoperative and perioperative protocols as well as patients with extensive peritoneal carcinomatosis treated by tumor cyoreduction and intraperitoneal hyperthermic perfusion. In this group of patients with recurrent disease, the RIGS-negative individuals again had a survival advantage.

In a more comprehensive analysis of 212 patients with both primary and recurrent colorectal carcinoma who underwent RIGS with either \(^{125}\)I-B72.3 or \(^{125}\)I-CC49,
patients were divided into three groups based on final RIGS status. As shown in Fig 5, the 74 RIGS-negative patients had a significantly better outcome at five years than either of the RIGS-positive groups.

In summary, studies of RIGS technology and staging and survival have demonstrated consistently that (1) surgical staging with RIGS was superior to traditional exploratory laparotomy resulting in stage change (upstaging) in more than one third of patients with primary colorectal carcinoma, and (2) the identification of unresectable RIGS-positive tissue in patients with both primary and recurrent disease was a significant predictor of poor outcome and at five years had the same clinical relevance as unresectable tumor confirmed by histopathology.

Conclusions

On the basis of this review, we conclude that RIGS technology detects obvious gastrointestinal adenocarcinoma, clinically occult neoplasm, and H&E occult tumor. It provides immediate intraoperative information to a surgeon that impacts on the management of the cancer patient, and it detects tumor dissemination indicative of disease progression with or without the histologic confirmation of tumor. RIGS technology provides surgical staging superior to traditional exploratory laparotomy, and it identifies unresectable RIGS-positive tissue, a significant predictor of poor outcome, in patients with both primary and recurrent disease. RIGS may become the standard of care for the surgical staging and treatment of colorectal carcinoma as well as other gastrointestinal malignancies.

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References


Fig 5. -- Survival curves based on final RIGS status for patients with primary and recurrent colorectal carcinoma following RIGS using (125)I-B72.3 and (25)I-CC49. Three patient cohorts are represented totaling 212 patients.
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