Molecular Lymphatic Mapping of the Sentinel Lymph Node

Bret Taback,* Kahoko Hashimoto,*
Christine T. Kuo,* Arden Chan,*
Armando E. Giuliano,[†] and Dave S. B. Hoon*

From the Department of Molecular Oncology* and the Joyce Eisenberg Keefer Breast Center,[†] John Wayne Cancer Institute, Saint John's Health Center, Santa Monica, California

Intraoperative lymphatic mapping to identify the sentinel lymph node (SLN) has significantly changed the management of regional lymph node basin of patients with various types of solid tumors such as melanoma and breast cancer. The procedure has improved the diagnosis of micrometastasis in the regional tumor-draining lymph nodes by providing a focused histopathological assessment of select lymph nodes most likely to harbor occult disease. Blue dye and/or radioisotopes are efficient mapping agents but the lack of accurate methods to quantify their presence and the potential for dissolution and decay, respectively, throughout time limit their role as reliable markers for identifying a sentinel node from additional secondary lymph nodes that may be either blue and/or radioactive to some degree. A consistently durable marker is needed that can be introduced during surgery and successfully quantitated among tumor-draining lymph nodes to permit a more accurate assessment of hierarchical organization. This may be of particular importance in retrospective analysis of archival tissues as there are no inherent markers to denote the SLN from successive echelon nodes. A procedure of molecular lymphatic mapping (MLM) was developed in a rat model to label the SLN preoperatively with rice gene DNA containing plasmid or linear rice DNA fragment (rDNA). The MLM efficiency was demonstrated by polymerase chain reaction (PCR) detection of the molecular marker in both frozen and paraffin-embedded SLN; 1.25 µg of rDNA injected with blue dye could be reproducibly detected by PCR. The MLM procedure was validated in a rat breast tumor model with lymph node metastasis. The procedure was successful in permanently labeling and identifying by PCR both frozen and paraffin-embedded SLN. MLM in conjunction with a conventional mapping agent can be used as a valuable asset for molecular assessment of the SLN and retrospective analysis of paraffin-embedded specimens. (*Am J Pathol* 2002, 161:1153–1161)

The technique of intraoperative lymphatic mapping and sentinel lymph node dissection (SLND) has revolutionized the management of regional tumor-draining lymph nodes in melanoma and other solid cancers that metastasize via the lymphatics. The procedure is minimally invasive and reduces morbidity. Furthermore, this approach provides for a more focused assessment of occult metastasis in a cost-effective manner, which may improve clinicopathological staging. Since Morton and colleagues¹ introduced the technique in melanoma, it has been successfully applied to breast cancer,^{2,3} colon cancer,^{4,5} thyroid cancer,⁶ and gastric cancer.⁷ Because the SLN is the first lymph node to harbor metastatic cancer cells from the primary tumor, its tumor status is highly predictive of the histopathology of the associated lymphatic drainage basin. It has been shown that patients who have tumor-free SLN do not need a complete lymph node dissection, thus avoiding the cost and potential morbidity associated with a formal elective lymph node dissection.^{1,7–10} The clinical benefits of SLND will be assessed on the completion of several large ongoing multicenter randomized trials in melanoma, breast, and colon cancer.^{11–16}

Concurrently to the initial phases of developing the SLN procedure at the John Wayne Cancer Institute in the 1990s, our laboratory has been developing molecular detection approaches to improve identification of occult metastatic tumor cells in the SLN(s) of melanoma, breast cancer, and colon cancer patients.^{16–21} We have shown in frozen sections of SLN using multimarker reverse transcriptase-polymerase chain reaction (RT-PCR) assays that melanoma, colon, and breast cancer patients can be upstaged compared to hematoxylin and eosin (H&E) and immunohistochemistry analysis.^{16,18,20} More recently, through extensive studies, it has become known that immunohistochemistry detection for micrometastasis and occult tumor cells in the SLN is more efficient if performed on paraffin-embedded sections compared to frozen sections. This approach also minimizes the variability of analysis among institutes. Studies in our laboratory have now evolved to assess for metastatic tumor cells in paraffin-

Supported in part by a grant from QVC and the Fashion Footwear Charitable Foundation (New York, NY) (FANY), the Gonda (Goldschmied) Research Laboratories at the John Wayne Cancer Institute, and the Ben B. and Joyce E. Eisenberg Foundation (Los Angeles, CA).

Accepted for publication June 17, 2002.

Address reprint requests to Dr. Dave S. B. Hoon, Department of Molecular Oncology, John Wayne Cancer Institute, 2200 Santa Monica Blvd., Santa Monica, CA 90404. E-mail: hoon@jwci.org.

embedded SLN. As methods for identification and analysis of SLN become more molecularly orientated, new approaches are needed to accurately and inherently label the SLN for additional studies.

Although the SLN is highly predictive of the histological status of the tumor-draining lymph node basin when using the technique of blue dye and/or radioisotopes for detection, their ability to accurately categorize draining lymph node hierarchy is limited. This is particularly true when multiple lymph nodes may stain blue and/or demonstrate radioactivity. Additionally, because these substances dissolve and decay, respectively, throughout time they lack utility as an enduring label of the sentinel node as well as additional lymph nodes for subsequent analysis of paraffin-embedded tissues. Introduction of a durable substrate during surgical lymphatic mapping, which can be readily quantitated among the tumor-draining lymph nodes, may provide a unique method to accurately label the sentinel and secondary lymph nodes during their evaluation.

In this report, we introduce the molecular lymphatic mapping (MLM) technique that can be used in conjunction with the blue dye to label the SLN for archiving, histopathology, and molecular studies. We established a rat model system to demonstrate the utility of the MLM technique. A xenogeneic DNA marker with no significant homology to known rat or human genomic sequences was used to provide a highly specific marker. We successfully demonstrated the effectiveness of MLM using a plasmid containing rice DNA as well as a linear rice DNA fragment in both frozen and paraffin-embedded SLN. A rat metastatic breast tumor model was used to demonstrate the potential application to human studies. The study also demonstrated the successfulness of MLM in targeting the first tumor-draining lymph node in the regional lymphatic basin.

Materials and Methods

Rice Gene Construction

Rice genomic DNA of *Olyza sativa* encoding a cytoplasmic male sterility peptide in the mitochondria was chosen as the molecular identification marker. The rice gene library cloned in EMBL3 SP6/T7 (host strain K802) was purchased from Clontech (Palo Alto, CA). A 1050-bp fragment from the rice gene genomic library was amplified by PCR primers: sense-5'-CATAAGCCATCCGAA-ACCAGTA-3' and antisense-5'-AATAGCATAGTCCAA-GCGAACC-3'. The PCR product was isolated by gel electrophoresis and then cloned into the pcDNA3 plasmid (Invitrogen, La Jolla, CA) (Figure 1). The plasmid was subsequently transfected into *Escherichia coli* and expanded as previously described.²² A large amount of plasmid was isolated and purified by QiaAmp (Qiagen Inc., Valencia, CA). The purified 5.6-kb plasmid DNA was used for MLM. The linear 1050-sbp rice gene fragment was obtained either by digestion of the rice gene fragment cloned in the pcDNA3 plasmid with specific restriction enzymes or by PCR amplification and cDNA product

Figure 1. Rice DNA plasmid construct. The rice DNA fragment of 1050 bp was inserted into plasmid pcDNA3. PCR amplification using RA (176-bp product) and RD (134-bp product) primers were used to detect the rice DNA fragment.

isolation. The purified linear rice gene fragment will be referred to as rDNA from this point on.

Animals and Treatment

All animal studies were performed on male Sprague-Dawley or Fisher 344 rats. All studies were approved and conducted under the guidelines of the Animal Use Committee at Harbor-University of California at Los Angeles Research and Education Institute. Rats were anesthetized with ketamine (75 mg/kg) and xylazine (7.5 mg/kg) mixture during MLM and SLND. MLM was performed by subcutaneously injecting 100 μ l of 1% isosulfan blue (Lymphazurin; U.S. Surgical Corp, Norwalk, CT) solution mixed with different dosages of the rice DNA into the rat footpad. After ~5 minutes, dissection of the rat was performed. The blue-stained lymph nodes (SLN) and adjacent downstream nodes were excised under the anesthetized condition. The dissected lymph nodes were frozen or formalin (10%)-fixed for 48 hours and then paraffin-embedded before DNA extraction.

DNA Extraction and PCR Analysis

DNA was extracted using DNAzol (Molecular Research Center Inc., Cincinnati, OH) from frozen and paraffin-embedded lymph nodes. Briefly, DNA extraction was performed on frozen lymph nodes using the DNAzol isolation protocol. The paraffin-embedded nodes were bisected and serially sectioned into several 10- μ m sections, deparaffinized with xylene, and DNA was extracted using a QIAamp kit (Qiagen Inc.). At optimal established

conditions of PCR approximately three sections were needed; for plasmid DNA analysis a lesser number of sections were required. To evaluate the amount of DNA extracted, the DNA concentration was quantitated using the Picogreen assay kit (Molecular Probes Inc., Eugene, OR). Purified total DNA was dissolved in molecular grade water, quantified, and then PCR-amplified for the rice gene. The PCR conditions were set up as previously described with 1.5 mmol/L $MgCl_2$, 0.8 mmol/L dNTP, 0.25 U AmpliTaq polymerase (Applied Systems, Foster City, CA).^{13,17} Five sets of primers (RA, RB, RC, RD, RE) were initially designed targeting different sites within the cloned rice gene fragment and optimal PCR conditions were established for each primer set. RA (sense, CCATGTGATCGCTACTAAAG; anti-sense, CATTGAGGAGTTTCCAGAT) and RD (sense, CCTTGTCTATGGCGGTA-ACT; anti-sense, CAGGTTTCAGCACGAAATC) primer sets proved most reliable after serial assessment under the following conditions: 95°C for 10 minutes, followed by 40 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, and a final extension of 72°C for 10 minutes. After PCR amplification, cDNA products were assessed by 2% agarose gel electrophoresis and ethidium bromide staining. Gel electrophoresis data were visualized and recorded using an Alphamager (Alpha-Innotech, San Leandro, CA). The housekeeping gene β -actin was used in all PCR assays to verify the integrity of the DNA in each sample.

Histochemistry Analysis

A 962-bp segment of the rice gene was PCR amplified using a biotin-labeled sense primer 5'-CCATGTGATCGCTACTAAAG-3' and unlabeled anti-sense primer 5'-GCTCTAGAAATAGCATAGTCCAAGCGAACC-3'. The biotinylated rice linear DNA fragment was purified from agarose gel with the QIAquick DNA purification kit (Qia-gen Inc.) and prepared for injection into the animals. To confirm the presence of rDNA, lymph nodes of rats injected with biotin-labeled rice gene DNA (biotin-rDNA) were formalin-fixed, paraffin-embedded, sectioned, and stained. Slides of paraffin-embedded tissue sections were deparaffinized with xylene 3× for 3 minutes, treated with ethanol 3× for 3 minutes, and then hydrated with decreasing concentrations of absolute ethanol. To block endogenous peroxidase activity, 0.3% hydrogen peroxide in methanol was overlaid on the sections for 20 minutes. To reduce nonspecific binding, the sections were incubated with 20% normal human serum in phosphate-buffered saline (PBS), pH 7.2, for 20 minutes. Horseradish peroxidase-conjugated Neutroavidin (Pierce, Rockford, IL) in PBS was added to sections and incubated for 30 minutes. After several washes with PBS, the sections were then developed with diaminobenzidine substrate solution (Vector Laboratories, Burlingame, CA). To remove the excess substrate, the sections were washed in deionized water for 3 minutes. Counterstaining with hematoxylin (Mayer's hematoxylin solution; Sigma, St. Louis, MO) was then performed on sections. Multiple sections from different SLN were evaluated by light microscopy and confirmed by at least two readers.

Rat Tumor Metastasis Model

The rat MAT13672 III breast adenocarcinoma cell line (American Type Culture Collection, Rockville, MD) was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (heat-inactivated). Cells in exponential growth phase were harvested, washed several times in RPMI 1640 medium, and prepared for injection. One million cells were injected subcutaneously into the footpad of syngeneic female Fisher 344 rats to establish primary tumors as previously described.²³ Approximately 14 days after injection, tumor growth (swelling) in the footpad was physically detectable. For MLM, the rDNA and 1% isosulfan blue dye mixture was injected into the primary tumor site. After ~5 minutes, blue dye containing SLN was dissected for assessment.

Real-Time PCR

To quantify the amount of rice gene DNA in lymph nodes of injected rats, quantitative PCR was performed using iCycler iQ Real-Time Detection System (Bio-Rad, San Diego, CA). Tenfold dilutions of 10^7 copies of the rice gene containing plasmids were PCR-amplified along with DNA samples extracted from lymph nodes of injected rats. A dual-labeled fluorescent resonance energy transfer probe 5'-CCGAGGCCAGTTGAGATCAGTC-3' (Genset, La Jolla, CA) was designed and used in conjunction with the RGA primer sets for the PCR reaction. PCR conditions were kept the same except for the addition of the dual-labeled probe and the increase of $MgCl_2$ concentration from 1.5 mmol/L to 2 mmol/L. DNA samples were extracted from lymph nodes of rats injected with 2.5 or 1.25 μ g of rDNA. DNA extracted from lymph nodes of rats injected with solution not containing rDNA was used as negative controls. Five hundred, 100, and 10 ng of DNA were used as templates for real-time PCR in the analysis of rice gene copy number. After 35 cycles of PCR, results were analyzed using iCycler iQ software. A standard curve of the threshold cycle (C_t) of PCR was constructed using the data collected of dilutions of rice plasmid DNA with known copy number. Sample starting rice gene copy number was determined by its threshold cycle relative to the standard curve. Negative controls were included in all experiments: reagents alone and SLN without rDNA.

Results

Rice Gene Marker

In designing a marker for MLM, the following criteria are desired of the DNA marker: sufficient size to withstand degradation, ability to efficiently flow into the lymph node during MLM, and a unique sequence with no significant homology to known human or rat gene sequences. The GenBank data base search (Internet) verified that there was no significant homology between *O. sativa* (rice) genomic DNA to known human or rat genes. A 1050-bp DNA fragment was selected from the mitochondrial mem-

Figure 2. Representative MLM and SLN identification. **A:** Photograph shows when blue dye was injected into the rat footpad (**yellow arrow**) as traced to the first draining popliteal lymph node (SLN) (**blue arrow**). **B:** Blue dye injected in the colon (**yellow arrow**) and traced to the first draining mesenteric lymph node (SLN) (**blue arrow**).

brane glycoprotein from exons II to V in the rice gene and cloned into pCDNA3 plasmid (Figure 1).

The PCR primers were designed to obtain the highest efficacy in detection of the rice gene marker in frozen or paraffin-embedded tissue sections. Five different primer sets were initially developed for PCR detection of the rice DNA in the SLN after MLM. By having more than one set of primers covering different regions of the DNA, we were able to circumvent the potential problem of DNA subjected to *in vivo* DNase activity and also increase the detection sensitivity. The initial primer sets studied were RA, RB, RC, RD, and RE that produced DNA products of 176, 198, 177, 134, and 198 bp, respectively. The primer sets covered both the 5' site and 3' site of the rice gene fragment (Figure 1). Based on our assessment of efficacy of the primer sets and detection sensitivity when assessing rice DNA serially diluted in unrelated DNA, we determined that the two primer sets, RA and RD were the most efficient.

MLM with Plasmid DNA

In the initial MLM studies, the purified 5.6-kb rice plasmid DNA was mixed with 1% isosulfan blue. Serial dilutions of rice plasmid DNA were assessed from 0.625 to 5 $\mu\text{g}/100\ \mu\text{l}$. Because the 1% isosulfan blue (Lympazurin) is used for lymphatic mapping in melanoma, breast, and colon cancer patients at our institute, we also used this blue dye for the animal studies. For MLM the plasmid DNA in blue dye was injected into the footpad of rats under anesthetized condition. Approximately 5 minutes after injection of the plasmid DNA and dye solution, an oblique incision was made in the popliteal fossa to identify the first draining lymph node (SLN). The blue dye-stained

lymph node (Figure 2) was excised for PCR analysis. To confirm whether injected DNA had reached the popliteal node (SLN), PCR analysis was performed on the frozen SLN. The SLN was then thawed, DNA extracted, and PCR amplified with the two optimal rice gene primer sets RA and RD. Representative examples of DNA marker detection using different dosages of plasmid DNA in MLM are shown in Figure 3. The DNA marker could be detected using three serial sections from lymph nodes when 1.25 and 2.5 μg were used for MLM. In Table 1, a summary of the detection efficacy of plasmid DNA of 2.5 μg after MLM is shown. In a representative experiment, only one of four SLN was negative for the rice DNA. However, using both primer sets RA and RD, the rice plasmid DNA was always detected in the SLN after MLM. All SLN

Figure 3. Rice plasmid DNA detection by PCR in frozen SLN after MLM. Representative examples of PCR amplification with RA, RD, and β -actin on SLN. MLM performed with plasmid and blue dye in the rat footpad. **Lane A** is the negative control, muscle tissue. **Lanes B–E** are SLN (popliteal): MLM on rats was performed with 0, 0.625, 1.25, or 2.5 μg of rice plasmid DNA, respectively. **Lanes F–I** are mesenteric lymph nodes: MLM was performed with 0, 0.625, 1.25, or 2.5 μg of rice plasmid DNA, respectively.

Table 1. MLM with Rice Plasmid DNA: PCR Detection in the SLN

Specimen	RA primer	RD primer	RA or RD primer
Frozen popliteal SLN	4/4 (100%)	4/4 (100%)	4/4 (100%)
Paraffin-embedded popliteal SLN	3/4 (75%)	3/4 (75%)	3/4 (75%)
Frozen mesenteric SLN	5/5 (100%)	5/5 (100%)	5/5 (100%)
Paraffin-embedded mesenteric SLN	4/4 (100%)	4/4 (100%)	4/4 (100%)

MLM was performed with 2.5 μ g of rice plasmid DNA. Results shown indicate number of rats in which molecular marker was detected in SLN by PCR after MLM. RA or RD primer results indicate positive PCR for either marker.

isolated from control animals in which LM was performed with isosulfan blue only were negative for the rice DNA marker by PCR analysis.

In human SLND, both frozen and paraffin sections are used to assess for metastases. MLM studies were performed at various dosages of plasmid DNA and assessed in both frozen and paraffin-embedded SLN. The extracted DNA from multiple sections was analyzed by PCR using RA and RD primer sets. In Figure 4 representative PCR detection of different dosages of plasmid DNA used in MLM is shown. The studies demonstrated that plasmid DNA used in MLM could be detected efficiently in both frozen and paraffin-embedded SLN.

MLM with rDNA

To further simplify the MLM procedure, we produced a smaller DNA marker using a linear rice DNA fragment. The advantage of linear DNA (nonvector) is the elimination of potential plasmid-promoter element insertion into the host genome. An additional advantage with this approach is the avoidance of pooling at the injection site and inadequate transit to the SLN, which may occur with the larger plasmid DNA. Using rDNA, the same MLM procedures were applied to identify the SLN. First, the rDNA was used in MLM, and the efficiency of SLN targeting was assessed at various dosages ranging from 0.625 to 1.25 μ g of rDNA. Frozen SLNs were isolated and assessed by PCR (Figure 5). The rDNA was detected in SLN when as low as 0.625 μ g was used in MLM however, at higher dosages it was more consistent. The detection

of rDNA by PCR primers RA and RD was highly efficient at 1.25 μ g (Table 2). There was 100% detection of the rDNA using the RD primer set alone in frozen SLN. In all control animals in which blue dye alone was used in MLM the PCR with RA and RD primers were negative. The rDNA was not degraded in frozen SLN kept for more than a month at -30°C as detected by PCR.

We further investigated MLM using rDNA in paraffin-embedded SLN. The PCR assay was performed as described above on excised SLN that were formalin-fixed and paraffin-embedded. Initially serial sections of the paraffin-embedded tissue were extracted for DNA and the PCR assay was performed to assess multiple primer sets and conditions. Representative examples of rDNA detection by PCR in SLN when different dosages of rDNA in MLM were used are shown in Figure 6. In assessment of multiple animals, the rDNA detection in the SLN was highly efficient and could be detected by both RA and RD PCR primers in 100% of the specimens, by evaluating one to three tissue sections, when 1.25 μ g of DNA was used for MLM (Table 2). These MLM studies with rDNA indicate that low copies of the DNA marker can be detected from DNA isolated from a few paraffin-embedded sections.

Mesenteric SLN Analysis

Clinically, we have successfully performed lymphatic mapping in patients with early-stage colorectal cancer to identify SLN(s) that may harbor micrometastasis.^{8,16} These studies have been very successful in identifying occult metastasis in the SLN from these patients. As a

Figure 4. Rice plasmid DNA detection by PCR in paraffin-embedded SLN after MLM. Representative examples of PCR amplification with RA and RD and β -actin PCR on paraffin-embedded SLN performed with rice plasmid DNA and blue dye in the footpad. **Lane A** is the negative control, muscle tissue. **Lanes B–F** are SLN (popliteal): MLM performed with 0, 0.625, 1.25, 2.5, or 5 μ g of rice plasmid DNA, respectively. **Lanes G–I** are mesenteric lymph nodes: MLM performed with 0, 0.625, or 5 μ g of rice plasmid DNA, respectively.

Figure 5. Detection of rDNA by PCR in frozen SLN after MLM. Representative examples of PCR amplification with RA, RD, and β -actin primers on SLN. MLM performed with rDNA and blue dye in the rat footpad. **Lane A** is the negative control, muscle tissue. **Lanes B–G** are SLN (popliteal): MLM performed with 0, 0.625, 1.25, 2.5, 5, or 10 μ g of rDNA, respectively. **Lanes H–M** are mesenteric lymph nodes: MLM performed with 0, 0.625, 1.25, 2.5, 5, or 10 μ g of rDNA, respectively.

Table 2. MLM with rDNA: PCR Detection in the SLN

Specimen	RA primer	RD primer	RA or RD primer
Frozen popliteal SLN	11/12 (92%)	12/12 (100%)	12/12 (100%)
Paraffin-embedded popliteal SLN	26/26 (100%)	26/26 (100%)	26/26 (100%)
Frozen mesenteric SLN	9/9 (100%)	9/9 (100%)	9/9 (100%)
Paraffin-embedded mesenteric SLN	2/4 (50%)	2/4 (50%)	2/4 (50%)

MLM was performed with 1.25 μ g of rDNA. Results shown indicate number of rats in which molecular marker was detected in SLN by PCR after MLM. RA or RD primer results indicate positive PCR for either marker.

model for MLM of colorectal cancer we developed a protocol targeting the mesenteric lymph nodes in the rat. Representative PCR detection with primer sets RA and RD for plasmid DNA and rDNA in frozen mesenteric SLN is shown in Figures 3 and 5, and in paraffin-embedded mesenteric SLN in Figures 4 and 6. If blue dye alone was used for lymphatic mapping, the SLN were negative when assessed by PCR with either primer sets RA or RD. The detection of rDNA in frozen mesenteric SLN was 100%, whereas in the paraffin-embedded SLN the detection was 50%.

Quantitation of Gene Copies in the SLN

Quantitation of DNA in the SLN after MLM allows us to determine the level of the DNA marker that reached the SLN. To quantify the copies of the DNA marker in the SLN after MLM, we performed quantitative real-time PCR (Figure 7). Animals were injected in the footpad with two different dosages of rDNA, 2.5 and 1.25 μ g. Control animals were those in which lymphatic mapping was performed with the blue dye only. Using 500 ng of DNA in real-time PCR, 5.29×10^6 copies of rice gene was found in the SLN when MLM was performed with 2.5 μ g of DNA marker whereas, 2.00×10^6 copies were found in the SLN when MLM was performed with 1.25 μ g of DNA marker (Table 3). Decreasing copy numbers of rDNA were found when lesser amounts of DNA templates were assessed.

Figure 6. Detection of rDNA by PCR in paraffin-embedded SLN after MLM. Representative examples of PCR amplification with RA, RD, and β -actin primers on paraffin-embedded SLN. MLM performed with rDNA and blue dye in the rat footpad. **Lane A** is the negative control, muscle tissue. **Lanes B-F** are SLN (popliteal): MLM performed with 0, 0.625, 1.25, 2.5, or 5 μ g of rDNA, respectively. **Lanes G-K** are mesenteric SLN: MLM performed with 0, 0.625, 1.25, 2.5, or 5 μ g of rDNA, respectively.

Histochemical Verification of the DNA Marker

To verify the presence of the DNA marker in the SLN, we performed *in situ* hybridization. MLM was performed using a 5' biotin-labeled rDNA mixed with blue dye by injection into the footpad. The SLN was dissected, fixed, and paraffin-embedded as previously described. Tissue sections were cut and stained with avidin-conjugated peroxidase, counterstained, and developed for light microscopy assessment. Analysis of all 10 to 12 sections demonstrated that the majority of the labeled marker was in the subcapsular and paratrabeular sinus of the SLN (Figure 8). The DNA marker was detected in all of the sections stained demonstrating the utility of using a limited number of sections for DNA analysis. These studies indicate that the marker can be used to direct the viewer to potential sites in the SLN where metastatic tumor cells may distribute. The studies also validate by visual detection the presence of the DNA marker in the SLN after MLM.

Rat Tumor Metastasis Model

There are very few animal models in which tumor progression occurs sequentially from the primary tumor to the draining lymph nodes via the lymphatics and then to systemic sites as in human breast cancer. The mammary rat tumor model MAT13762 progresses in a similar man-

Figure 7. Representative example of real-time PCR quantitation. **Top:** Quantitation of rDNA in SLN is shown as log reporter fluorescence units (RFU). Samples of unknown copies of rDNA are amplified and the RFU are determined. **Horizontal line** is the threshold cycle. **Bottom:** Standard curve for determining rDNA copy number using known amounts of rice plasmid DNA in real-time PCR.

Table 3. Quantitation of DNA Marker Copies in SLN after MLM

DNA (μ g) used in MLM	DNA in PCR (ng)	Threshold cycle	DNA copy number in SLN
2.5	500	16.3	5,290,000
	100	17.6	2,720,000
	10	20.5	590,000
1.25	500	18.2	2,000,000
	100	20.2	694,000
	10	23.9	93,400
0	500	>35	0
	100	>35	0
	10	>35	0

Representative of individual rat SLN after MLM with 2.5 or 1.25 μ g rDNA. Real-time PCR performed on DNA isolated from SLN after MLM with rDNA.

ner as human breast cancer from the primary tumor to the draining lymph node. This mammary tumor metastasizes to the popliteal lymph node 100% of the time when injected into the hind footpad. One million exponentially growing MAT13762 cells were inoculated into the rat footpad ($n = 12$ animals). Within ~ 14 days the tumor was easily identifiable in the footpad. MLM was performed using rDNA in blue dye by injection around the tumor area in a manner similar to human breast cancer SLN mapping. The popliteal SLN was identified, dissected, and processed for DNA or formalin-fixed and paraffin-embedded for histopathological analysis. Serial sections were cut and H&E stained for analysis of tumor metastasis. Histochemical analysis of the SLN indicated micro-metastasis presence in the SLN identified by MLM in all animals. PCR analysis of adjacent serial sections from SLN containing tumor cells confirmed by H&E demonstrated the presence of rDNA in all cases.

Discussion

In this preclinical study, we demonstrated a novel approach referred to as MLM for identifying, detecting, and permanently labeling the SLN. The concept of MLM using the DNA marker in conjunction with blue dye should provide a more accurate approach in identifying and assessing SLN; particularly with molecular based detection methods. Initially, rice plasmid DNA was assessed to determine whether a DNA vector could be effectively delivered to the SLN. We demonstrated that plasmid DNA could be efficiently delivered to the SLN through the lymphatics using MLM. However, a smaller DNA fragment with no potential of integration into the host genome will be more desirable for human studies. These preliminary rDNA studies demonstrate that a smaller DNA fragment was as efficient as the plasmid for MLM. The design of the two primer sets for the assay provides a fail-safe should potential degradation of the DNA marker occur in the SLN. Furthermore, this approach improves the assay sensitivity and enhances its accuracy for marker detection. A DNA sequence ~ 1 to 5 kb is efficient in MLM for identification and labeling the SLN.

The studies performed on frozen SLN were subsequently adapted to paraffin-embedded SLN using both rice plasmid DNA and rDNA. No significant loss in de-

tection of the markers was observed in paraffin-embedded SLN as compared to frozen SLN for rice plasmid DNA. The PCR detection of low dosages of rDNA from a limited number of paraffin-embedded sections demonstrates the assay sensitivity. There was some loss when assessing rDNA from paraffin-embedded mesenteric SLN as compared to frozen mesenteric SLN. These differences, which did not occur with popliteal SLN, may be a result of less rapid diffusion of the linear DNA through the SLN because of its proximity to the injection site at the bowel wall, and/or technical problems with inadvertent injection into the lumen of the very thin bowel wall. Furthermore, mesenteric SLN were notably smaller and more difficult surgically to identify in comparison to the popliteal node dissection. Formalin fixation and paraffin embedding for permanent sections are the standard procedures in hospitals throughout the world for archiving lymph nodes dissected from surgical operations. The stability of marker DNA (rDNA, rice plasmid DNA) in long-term archival tissue remains to be determined. In general, DNA of paraffin-embedded tissue does not significantly degrade within 10 years. The permanent labeling of SLN will be very important in retrospective molecular analysis. The inherent molecular labeling provides an added assurance that the SLN is truly the SLN during molecular analysis. As DNA and RNA molecular assays for tumor cell detection in paraffin-embedded tissue continue to show prominence, a molecularly labeled SLN will become very important. This will allow simultaneous analysis of tumor markers and SLN verification.

Analysis of rDNA copies in the SLN was assessed using quantitative real-time PCR. The objective was to determine whether the DNA marker could be quantitated in the SLN. The quantitation of the rDNA was very successful and provided an accurate measure to delineate the SLN by MLM. The distribution level of rDNA among SLN and downstream nodes can be assessed rapidly by this approach. In human melanoma and breast cancer SLN studies, there can be more than one SLN identified during SLND.^{9,11,13} Also, depending on the method used (blue dye or radiocolloids) and the surgeon's technical expertise, multiple potential SLN may be identified during the operation.^{8,11,24-28} This can cause difficulties for pathologists analyzing and determining the true first draining SLN. The ability to quantify an inherent marker will

Figure 8. Representative photographs of *in situ* staining with biotinylated rDNA in the SLN. **A:** Control SLN after MLM with blue dye only. **B:** Biotinylated rDNA detection (brown) in SLN after MLM. **C:** Biotinylated rDNA detection (brown) in SLN after MLM. Sections of SLN were hematoxylin-counterstained. Original magnifications: $\times 200$ (**A**, **C**); $\times 100$ (**B**).

provide an additional parameter to more accurately assess lymphatic distribution and appropriately label the SLNs as well as secondary lymph nodes for correlation. This may be especially important in characterizing complex lymphatic drainage patterns, ie, those that may occur with colorectal cancers.

As molecular approaches become more frequent for evaluating occult metastasis, MLM will provide a valuable tool. The presence of an inherent molecular marker in the

SLN will improve the integration of molecular techniques for accurately assessing high-risk tumor-draining lymph nodes. Lymphatic mapping and SLN identification significantly helps the pathologist to focus on the node(s) most likely to contain metastatic tumor cells thus improving accuracy of detecting occult metastasis.^{24,29,30} This significantly saves time and reduces the associated costs of traditional approaches for detecting and analyzing multiple lymph nodes from standard lymphadenectomy specimens. This can be further enhanced with real-time PCR techniques. The distribution of the DNA marker determined by *in situ* histochemistry can orient the pathology reviewer to the potential area likely to contain occult metastatic tumor cells in the SLN. The biotinylated rDNA was detected in the subcapsular and paratrabeular sinus of the SLN. This compares with similar observational studies using carbon dye as a lymphatic mapping agent.³¹

The studies also provide a novel approach of delivering gene vectors to draining lymph nodes. One of the major problems in gene therapy for vaccination or activating immune responses is targeting specific lymphoid organs such as lymph nodes. Utilization of MLM with plasmids for specific genes such as antigens or cytokines can be used to target draining lymph nodes and activate regional immunity. This may be very useful in activating tumor-draining lymph nodes to specific antigens and induce immunity or reverse immune suppression. The studies on the frozen tissue with the plasmid DNA showed that the plasmid could effectively reach the SLN within minutes. This approach demonstrates delivery of a vector to lymphoid tissue sites that can be transfected *in vivo*. Secondary lymphoid organs can be very useful sites to directly augment regional and systemic immunity through lymphatic directed vaccination. To date, most gene vaccination approaches have been administered through intramuscular, subcutaneous, or intradermal routes.³² Better approaches are needed to activate anti-tumor-specific immunity in tumor-draining lymph nodes, such as by intralymphatic immunization, which may act to control local, in-transit, and regional recurrence.

Future studies will involve assessing MLM in patients undergoing SLND to determine its efficacy. Studies will be needed to determine whether the injection site or the draining lymph node distance will influence labeling with a DNA marker. In the rat model, the distance from the injection to the lymph node is relatively short and constant. Pilot studies need to be conducted in humans to assess marker distribution with varying distances between the injection site and SLN during lymphatic mapping. Although these studies were performed with isosulfan blue dye, it should be similarly compatible with radiocolloids used for lymphatic mapping. We believe if MLM is used to inherently label the SLN this technique will facilitate the clinical integration of molecular studies to detect occult metastasis and provide a reliably valuable tool for retrospective reviews of archival paraffin-embedded tissues.

Acknowledgments

We thank Dr. R. Turner and V. Vu for their advice and technical assistance.

References

- Morton DL, Wen DR, Wong JH, Economou JS, Cagle LA, Storm FK, Foshag LJ, Cochran AJ: Technical details of intraoperative lymphatic mapping for early stage melanoma. *Arch Surg* 1992; 127:392-399
- Giuliano AE, Kirgan DM, Guenther JM, Morton DL: Lymphatic mapping and sentinel lymphadenectomy for breast cancer. *Ann Surg* 1994; 220:391-398
- Giuliano AE, Dale PS, Turner RR, Morton DL, Evans SW, Krasne DL: Improved axillary staging of breast cancer with sentinel lymphadenectomy. *Ann Surg* 1995; 222:394-399
- Saha S, Wiese D, Badin J, Beutler T, Nora D, Ganatra BK, Desai D, Kaushal S, Nagaraju M, Arora M, Singh T: Technical details of sentinel lymph node mapping in colorectal cancer and its impact on staging. *Ann Surg Oncol* 2000; 7:120-124
- Bilchik AJ, Giuliano A, Essner R, Bostick P, Kelemen P, Foshag LJ, Sostrin S, Turner RR, Morton DL: Universal application of intraoperative lymphatic mapping and sentinel lymphadenectomy in solid neoplasms. *Cancer J Sci Am* 1998; 4:351-358
- Kelemen PR, Van Herle AJ, Giuliano AE: Sentinel lymphadenectomy in thyroid malignant neoplasms. *Arch Surg* 1998; 133:288-292
- Kitagawa Y, Fujii H, Mukai M, Kubota T, Ando N, Watanabe M, Ohgami M, Otani Y, Ozawa S, Hasegawa H, Furukawa T, Kumai K, Ikeda T, Nakahara T, Kubo A, Kitajima M: The role of sentinel lymph node in gastrointestinal cancer. *Surg Clin North Am* 2000; 80:1799-1809
- Tsioulis GJ, Wood TF, Morton DL, Bilchik AJ: Lymphatic mapping and focused analysis of sentinel lymph nodes upstage gastrointestinal neoplasms. *Arch Surg* 2000; 135:926-932
- Giuliano AE, Jones RC, Brennan M, Statman R: Sentinel lymphadenectomy in breast cancer. *J Clin Oncol* 1997; 15:2345-2350
- Brobeil A, Cruse W, Messina JL, Glass LF, Haddad FF, Berman CG, Marshburn J, Reintgen DS: Cost analysis of sentinel lymph node biopsy as an alternative to elective lymph node dissection in patients with malignant melanoma. *Surg Oncol Clin North Am* 1999; 8:435-445
- Veronesi U, Paganelli G, Galimberti V, Viale G, Zurrida S, Bedoni M, Costa A, de Cicco Geraghty JG, Luini A, Sacchini V, Veronesi P: Sentinel-node biopsy to avoid axillary dissection in breast cancer with clinically negative lymph-nodes. *Lancet* 1997; 349:1864-1867
- Giuliano AE, Haigh PI, Brennan MB, Hansen NM, Kelley MC, Ye W, Glass EC, Turner RR: Prospective observational study of sentinel lymphadenectomy without further axillary dissection in patients with sentinel node-negative breast cancer. *J Clin Oncol* 2000; 18:2553-2559
- Morton DL, Thompson JF, Essner R, Elashoff R, Stern SL, Nieweg OE, Roses DF, Karakousis CP, Mozzillo N, Reintgen D, Wang HJ, Glass EC, Cochran AJ: Validation of the accuracy of intraoperative lymphatic mapping and sentinel lymphadenectomy for early-stage melanoma: a multicenter trial. Multicenter Selective Lymphadenectomy Trial Group. *Ann Surg* 1999; 230:453-463
- Haigh PI, Giuliano A: A critical evaluation of sentinel lymph node dissection in malignancy. *Cancer: Principles and Practice of Oncology*, vol 14. Edited by JR Devita, VT Hellman, SA Rosenberg. Philadelphia, Lippincott-Raven, 2000, pp 1-11
- Harlow SP, Krag DN: Sentinel lymph node—why study it: implications of the B-32 study. *Semin Surg Oncol* 2001; 20:224-229
- Bilchik AJ, Saha S, Wiese D, Stonecypher JA, Wood TF, Sostrin S, Turner RR, Wang HJ, Morton DL, Hoon DS: Molecular staging of early colon cancer on the basis of sentinel node analysis: a multicenter phase II trial. *J Clin Oncol* 2001; 19:1128-1136
- Hoon DS, Sarantou T, Doi F, Chi DD, Kuo C, Conrad AJ, Schmid P, Turner R, Giuliano A: Detection of metastatic breast cancer by beta-HCG polymerase chain reaction. *Int J Cancer* 1996; 69:369-374
- Bostick PJ, Huynh KT, Sarantou T, Turner RR, Qi K, Giuliano AE, Hoon DS: Detection of metastases in sentinel lymph nodes of breast cancer patients by multiple-marker RT-PCR. *Int J Cancer* 1998; 79:645-651
- Bostick PJ, Hoon DS, Cote RJ: Detection of carcinoembryonic antigen messenger RNA in lymph nodes from patients with colorectal cancer. *N Engl J Med* 1998; 339:1643-1644
- Bostick PJ, Morton DL, Turner RR, Huynh KT, Wang HJ, Elashoff R, Essner R, Hoon DS: Prognostic significance of occult metastases detected by sentinel lymphadenectomy and reverse transcriptase-polymerase chain reaction in early-stage melanoma patients. *J Clin Oncol* 1999; 17:3238-3244
- Wascher RA, Bostick P, Huynh KT, Turner R, Qi K, Giuliano A, Hoon DB: Detection of MAGE-A3 in breast cancer patients' sentinel lymph nodes. *Br J Cancer* (in press)
- Okamoto T, Kaneda Y, Yuzuki D, Huang SK, Chi DD, Hoon DS: Induction of antibody response to human tumor antigens by gene therapy using a fusogenic viral liposome vaccine. *Gene Ther* 1997; 4:969-976
- Hoon DB, Ziola B, Ramshaw I: Circulating immune complexes in rats bearing 6-thioguanine-resistant variants of the 13762 mammary adenocarcinoma. *Cancer Res* 1984; 44:2406-2409
- Cochran AJ, Balda BR, Starz H, Bachter D, Krag DN, Cruse CW, Pipers R, Morton DL: The Augsburg Consensus. Techniques of lymphatic mapping, sentinel lymphadenectomy, and completion lymphadenectomy in cutaneous malignancies. *Cancer* 2000; 89:236-241
- Turner RR, Ollila DW, Krasne DL, Giuliano AE: Histopathologic validation of the sentinel lymph node hypothesis for breast carcinoma. *Ann Surg* 1997; 226:271-276
- Bostick P, Essner R, Glass E, Kelley M, Sarantou T, Foshag LJ, Qi K, Morton D: Comparison of blue dye and probe-assisted intraoperative lymphatic mapping in melanoma to identify sentinel nodes in 100 lymphatic basins. *Arch Surg* 1999; 134:43-49
- Cox CE, Pendas S, Cox JM, Joseph E, Shons AR, Yeatman T, Ku NN, Lyman GH, Berman C, Haddad F, Reintgen DS: Guidelines for sentinel node biopsy and lymphatic mapping of patients with breast cancer. *Ann Surg* 1998; 227:645-651
- Hill AD, Tran KN, Akhurst T, Yeung H, Yeh SD, Rosen PP, Borgen PI, Cody III HS: Lessons learned from 500 cases of lymphatic mapping for breast cancer. *Ann Surg* 1999; 229:528-535
- Turner RR, Ollila DW, Stern S, Giuliano AE: Optimal histopathologic examination of the sentinel lymph node for breast carcinoma staging. *Am J Surg Pathol* 1999; 23:263-267
- Turner RR, Giuliano AE, Hoon DS, Glass EC, Krasne DL: Pathologic examination of sentinel lymph node for breast carcinoma. *World J Surg* 2001; 25:798-805
- Haigh PI, Lucci A, Turner RR, Bostick PJ, Krasne DL, Stern SL, Morton DL: Carbon dye histologically confirms the identity of sentinel lymph nodes in cutaneous melanoma. *Cancer* 2001; 92:535-441
- Kaneda Y, Hoon DSB: DNA vaccines for cancer treatment. *Drugs*. London, PharmaPress Ltd., 2001, pp 301-311