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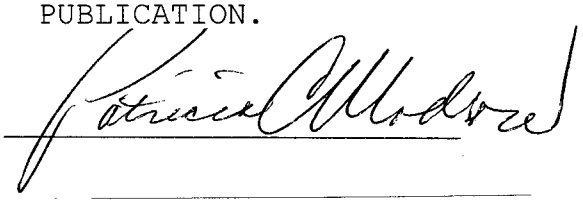

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13. ABSTRACT *(Maximum 200 Words)*

The purpose of this study is to use sentinel node technology to better predict prognosis for node-negative breast cancer patients.

Patients with a new diagnosis of unilateral, unifocal breast cancer and a clinically negative axilla were offered enrollment in this protocol. Sentinel nodes were localized and resected along with a standard staging axillary dissection. The sentinel nodes were processed in standard fashion and were then retained for specialized studies including microsectioning and telomerase studies. Short term goals are to determine the proportion of patients considered to be node-negative who harbor micrometastasis or telomerase in the sentinel node and to determine the association of nuclear grade in Ki67 with the finding of micrometastasis in the sentinel node. Long term goals include evaluation of the association of sentinel node micrometastasis and telomerase with disease free survival and overall survival.

In the time interval covered by this report, a total of 150 patients ranging in age from 35-87. All enrolled patients have been female. Short term technical outcomes and some pathologic outcomes are presented in this report. Long term outcomes are pending as required by protocol.

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INTRODUCTION

While the majority of node-negative breast cancer patients will be cured by local therapy alone, approximately 25-30% will go on to recur and die of their disease within 10 years of the time of the diagnosis.[1,2,3] At present our ability to predict which of the node negative group of patients will recur is limited. It is likely that a significant proportion of node negative patients who would not have recurred currently receive adjuvant therapy from which they cannot benefit. It is currently very difficult to predict which node negative patients are likely to recur and therefore stand to benefit from adjuvant therapy, and which patients can safely forgo adjuvant therapy. Sentinel lymph node biopsy is an emerging technique for the evaluation of regional lymph nodes in patients with newly diagnosed cancers. One possible advantage of this technique is that it provides one or two lymph nodes which can be subjected to more extensive pathologic review than is typically done with nodes from a standard staging dissection. For those patients considered to be node negative, the presence of unidentified micrometastasis within lymph nodes may be one feature which predicts a poorer outcome. Micrometastases are known to be missed on routine histologic sectioning in approximately 10-30% of cases.[4,5,6] Additionally, there has been a great deal of interest in the potential usefulness of molecular markers as a means of identifying malignant cells. Telomerase is a ribonucleoprotein which is telomere specific and adds nucleotide repeats to chromosome ends. Telomerase is known to be active only in cells with proliferative capacity such as germ line cells, stem cells and malignancies [7,8,9] and is under investigation as a way of identifying malignant cells in breast cancer. This protocol uses sentinel node technology to identify and selectively remove the sentinel node as a separate specimen from the remainder of the axillary dissection. The sentinel node is processed as a standard lymph node for pathologic review, and paraffin blocks are saved and are serially sectioned at a delayed interval. A tiny portion of the sentinel node is saved for telomerase studies. Short-term outcomes include association of characteristics of the primary tumor with the finding of micrometastasis and telomerase in the sentinel node. The long-term outcomes of this study are the association between micrometastasis or telomerase in the sentinel node and disease free and overall survival.

BODY

Experimental Methods

Protocol Design. The design is a prospective cohort study. Patients with newly diagnosed early stage breast cancer were offered enrollment and underwent a sentinel lymph node biopsy as part of their surgical staging as well as a standard level I and II axillary dissection. The sentinel lymph nodes were evaluated both with routine histopathology as currently practiced and also with specialized techniques to look for evidence of microscopic disease that may not be appreciated on routine sectioning. The decision to offer a patient adjuvant therapy was based on the pathologic findings of the primary tumor and the standard evaluation of the lymph nodes. The findings from the specialized studies were not introduced into the clinical chart. Patients are then to be followed to determine their disease free survival and overall survival.

Patient Population. The Johns Hopkins Breast Center and the Johns Hopkins Greenspring Station Breast Center are the sites for recruitment of patients. Inclusion criteria include 1) Newly diagnosed operable invasive breast cancer. 2) Clinically negative ipsilateral axilla. 3) Patients for whom a staging axillary lymphadenectomy is considered the current standard of care. 4) Primary tumor size estimated by clinical and radiographic criteria to be less than or equal to three centimeters. 5) Willing participation following informed consent. Exclusion criteria are: 1) Clinically positive lymph nodes. 2) Pregnancy. 3) Previous axillary lymphadenectomy, 4) Multifocal or bilateral disease. Patients were registered at the time of their informed consent and were assigned consecutive identification numbers.

Statistical Methods. The sample size needed was calculated based on the survival endpoint, and is estimated to be approximately two hundred and sixty patients assuming an alpha error of 0.05, beta error of 0.2. This assumes that seventy percent of patients enrolled will be node negative, that twenty five percent of those who are node negative will be micrometastasis positive and that there will be a twenty percent difference in disease free survival at five years between the groups that are micrometastasis positive and micrometastasis negative. This sample size provides eighty percent power to determine the difference between seventy percent and ninety percent disease-free survival at five years.

Study Procedure. Technetium - 99m Sulfur colloid is prepared by the radiopharmacy at Johns Hopkins for each case. The material is used unfiltered. A total of 0.95 mCi of technetium is diluted into a final volume of 4.0 ml. A total of 4.0 ml of the radiotracer is injected into the

breast of each patient enrolled; 1.0 ml per injection site is injected into normal tissue superior, inferior, medial and lateral to the primary cancer or biopsy cavity. At each injection site the 1.0 ml volume is injected from the deepest to the most superficial level of the tumor or biopsy cavity within the breast. The injection is guided by ultrasound or stereotactic mammography whenever possible. The injection is performed by personnel certified in the handling of radioactive substances. Precautions are maintained according to radiation safety guidelines of handling open containers of radiopharmaceutical. All materials used for injection (syringes, gloves, gauze used to wipe the skin or spills) are handled and disposed by the personnel of the nuclear medicine department. A total of 0.95 mCi of technetium is the total amount injected per patient. The time interval between injection and surgery is from one to six hours. The gamma detector used intraoperatively is the C-Trak gamma detector (Carewise Medical, Morgan Hill, CA.) . The settings used are a threshold of 130 keV and a window of 40 keV. All counts are accumulated over 10 seconds intraoperatively.

The sentinel lymph node biopsy is performed at the same time the patient is scheduled for a staging axillary lymphadenectomy. The patient is brought to the operating room approximately one to six hours following the injection of the radioisotope. The procedure is performed as it would be in the absence of a sentinel node biopsy. In the course of the procedure when the axilla is first entered either during the course of a modified radical mastectomy or an axillary dissection, the hand held gamma probe is used intraoperatively to localize the sentinel lymph node. This lymph node is dissected from surrounding tissues and removed as a separate specimen.

In the operating room the sentinel lymph node is dissected by the surgeon, two touch preps or tiny silvers of tissue are prepared on glass slides and the glass slides dropped into liquid nitrogen for telomerase studies. The sentinel lymph node is then labeled and sent to surgical pathology.

The remainder of the patient's primary surgical therapy for breast cancer is completed at the same setting, including a standard level one and level two axillary lymph node dissection.

Telomerase. The slides of the touch preps made in the operating room from the sentinel lymph node are briefly thawed and overlaid with thirty-five microliters of CHAPS lysis buffer. Cells are scraped off with the pipette tip and the lysate collected. The five microliter aliquot is used for assay of total protein and the remainder of the sample is allocated and snap frozen in liquid nitrogen. The PCR-based telomeric repeat amplification protocol (TRAP) is performed as described [10,11] with the following exceptions: 10 ag of a PCR control (ITAS) is added to the

reaction mixture, the reactions will be incubated for 45 minutes, and the annealing temperature used is 56°. Each cell extract is accompanied by the same cell extract 1) inactivated by heating to 94°C for ten minutes and assayed as a negative control and 2) assayed in a concurrent PCR using primer for a housekeeping gene, RPA, as a positive control for a cellular aspirate, since the amount of protein in the lysate is often too low to be assayed by standard techniques. The RPA primers amplify under the same reaction conditions as the telomerase primers. The TRAP assay is scored in a binary fashion with a positive result defined as any banding pattern (laddering) beyond background.

Processing of the Sentinel Node in Surgical Pathology. Upon receipt by surgical pathology the sentinel node(s) is processed and embedded in a routine fashion after appropriate gross inspection of the specimen. One section will be made per block. Routine hematoxylin stains is done and the remainder of the paraffin block will be saved for future review. At an interval of four to six months recently accumulated sentinel node blocks are processed in the surgical pathology research laboratory in a batch fashion. The blocks are serially sectioned. All paraffin ribbons are collected, mounted on slides and examined for evidence of micrometastasis using standard H&E stain.

Surgical Pathology of the Primary Breast Cancer and Axillary Contents. The breast specimen (whether it be mastectomy or partial mastectomy) is processed in the routine fashion for definition of the exact pathologic size of the tumor and histologic evaluation. The primary tumor is evaluated in the standard fashion and analyzed for histologic grade according to the Elston grading system. The tumor is analyzed for Ki-67, as is currently routine. The axillary contents are processed in a routine fashion with dissection of individual lymph nodes and processing of each node with one or two sections with H&E staining.

Follow Up. Patients are followed for a minimum of five years and for longer whenever possible to determine disease free survival and overall survival. Disease free survival is defined as ending at the first diagnosis of distant (not locoregional) disease. Patients enrolled in this protocol are screened according to the Johns Hopkins breast follow up protocols which include at least every six-month evaluation by a physician.

Pathologic Outcomes. Sentinel lymph nodes that are negative for metastases by routine histology are assessed for micrometastases and telomerase. The proportion of these nodes found to be positive for micrometastases or telomerase is calculated. The nuclear grade (Elston grading) or a primary tumor and Ki-67 in the primary tumor is tested for association with the

finding of micrometastasis in the sentinel lymph as an indirect measure of outcome in two by two tables using the Chi square test.

Survival Outcomes. Patients who prove to be node negative by routine histologic criteria will be followed for a minimum of five years to determine disease free survival. Disease free survival is then analyzed according to the presence or absence of micrometastases and according to the presence or absence of telomerase in the sentinel lymph node. Factors controlled for include menopausal status, the size of the primary tumor and whether or not the patient received adjuvant therapy. Statistical modeling is done according to the Cox proportional hazards model to estimate the risk ratio attributable to micrometastases and telomerase on disease-free survival and overall survival.

Results

Protocol Entry. Between January 1998 and October 1999, 150 patients were entered in this protocol. All patients had a unilateral, unifocal lesion estimated preoperatively to be less than 3 cm in greatest dimension and had a clinically negative axilla. Each gave voluntary, written informed consent. Protocol procedures were followed for each patient. Each patient underwent a peritumoral injection of approximately 0.95 mCi of Technetium sulfur colloid. In the operating room the gamma probe was used to localize and assist with the resection of the sentinel node as a separate specimen and the specimens were processed as outlined above. By design of the protocol the study outcomes are processed in a delayed fashion. Long-term outcomes of disease free survival and overall survival will be accrued over the next few years.

Patient Characteristics. Data for the first enrolled 150 patients are presented in Table 1 in appendix B. Patients range in age from 35-87 with a median age of 51. For 84 (56%) their initial presentation was with a mammographic abnormality, and for 63 (42%), their initial presentation was with a palpable abnormality; three had other presentations. Of these 150 patients, 112 (74.7%) had breast conserving surgery. Pathologic tumor size ranged from 0.1 to 3.9 cm with a median of 1.3 cm. The majority (69.3%) of the primary tumors were of a ductal histology. Overall 28% of enrolled patients had a positive axillary node, either sentinel or non-sentinel.

Technical Data. At the inception of this protocol the technique of sentinel node biopsy for breast cancer was new to this institution. The first 10 cases for each participating surgeon were

monitored by the principal investigator. Data was collected on the identification rate to assess each surgeon's ability to find a sentinel node and the number of sentinel nodes retrieved per case. Each sentinel node excision was followed by a completion axillary dissection. False negatives were identified and a false negative rate was calculated. Overall in the first 150 patients, the identification rate was 85.3%. The number of sentinel nodes per patient averaged 1.8 with a range from 0-5 per patient. There were three false negatives in this series for an overall false negative rate of 7.9% using as a denominator the number of patients who had a sentinel node identified and had any positive axillary node. Data for the entire group as well as for each surgeon is presented in Table 2 in appendix B. As is consistent with the published literature there is some variability between surgeons. The ability to identify a sentinel node, the number of sentinel nodes identified per patient and the false negative rate varied among the three surgeons participating in this protocol. In the published literature the number of sentinel nodes retrieved per patient is 2 - 2.2. In our series overall the number of sentinel nodes per patient was approximately 1.8, varying from 1.5 to 2.2 for different surgeons. Of some interest but uncertain significance, the surgeon with the lowest number of sentinel nodes identified per patient also had the highest false negative rate. It is well documented in the literature that there exists a shallow learning curve for sentinel node biopsy in breast cancer. Most groups report lower identification rates and higher false negative rates early in their experience. Technical outcomes for this protocol were reevaluated for the most recent 30 cases of each surgeon and are presented in Table 3 in appendix B. In this overall group there was still some variability from surgeon to surgeon regarding the ability to identify a sentinel node and the number of sentinel nodes identified per patient. There was only one false negative within these ninety cases. These data are in some ways comparable to that in the described literature. Our overall identification rate of 85.3% is somewhat lower than in most of the published literature, but is not dissimilar to that reported in a large multi-center study.[12] The false negative rate among these ninety cases is 4.2%, lower than in the overall series of 150 patients.

Telomerase. Telomerase testing was done according to protocol on a total of 50 sentinel nodes retrieved from 28 patients early in the experience of this trial. All sentinel nodes that were positive by routine histology for metastatic disease were also positive for telomerase. Of 42 histologically negative nodes 32 (76%) were positive for telomerase. These data are reported in detail in the accompanying document in Appendix A. The rate of telomerase positivity among histologically negative sentinel nodes was felt to be too high to have clinical

relevance therefore telomerase studies were ended. Tissue continues to be collected for testing with other molecular markers.

Pathology and Survival Outcomes. Pathologic endpoints are processed in a delayed fashion per protocol requirement and are not reportable at this writing. The outcomes of greatest interest are the survival data. Data on local and distant recurrences are being collected and will be analyzed with five year disease-free survival as the major endpoints.

Statement of Work

The following lists accomplishments related to each Task outlined in the Statement of Work.

1. The initial implementation of this protocol was overseen by the principal investigator for the first 10 cases of each participating surgeon. The protocol was adhered to.

2. Data was collected for each surgeon regarding accuracy of technique and the false negative rate. This data is presented in the preceding section.

3. Telomerase specimens were processed in Dr. Sukumar's lab. The data was maintained separately from the clinical record and was not used for clinical care. This data is included as appendix A in the form of a draft of an article for potential publication. This data was also presented at the Era of Hope meeting in Atlanta in June 2000.

4. Microsectioning of the sentinel lymph node in surgical pathology is still ongoing and this information is not ready for reporting.

5. Patient accrual continues.

6. The proportion of patients who are telomerase positive is reported in appendix A.

7. This task is still underway as the microsectioning has not yet been completed.

8. Patients are being followed. Disease-free survival and overall survival data is being gathered. This is not ready for reporting.

Summary

A clinical trial evaluating the use of sentinel node biopsy in patients with early breast cancer is ongoing at Johns Hopkins. All enrolled patients gave voluntary, informed consent on a JCCI approved protocol. Enrolled patients are typical of general populations of early stage breast cancer patients in their age distribution, presentation and histology. Technical outcomes are similar to other reported series of sentinel node biopsy in the identification rate, the false

negative rate and the presence of inter-operator variability. Pathologic outcomes are pending at this writing and survival data continues to be collected.

KEY RESEARCH ACCOMPLISHMENTS

- Sentinel node biopsy has been successfully implemented at Johns Hopkins with satisfactory technical outcomes.
- Specimens have been collected according to protocol and processed in the designated manner.
- The telomerase studies have been completed and are presented as appendix A.
- Patient accrual to the overall study is continuing.
- The pathologic specimens have been processed according to protocol.
- Patients are being followed and disease free survival and overall survival is being recorded.

REPORTABLE OUTCOMES

1. The telomerase data was presented as a poster and as a platform presentation at the Era of Hope meeting in Atlanta in June 2000.

2. The telomerase data is assembled in a draft of a paper which is included here as appendix A.

CONCLUSIONS

We have completed the first portion of a large ongoing study of sentinel node biopsy and survival outcomes at Johns Hopkins. Data for the first enrolled 150 patients are reported in this document. Technical outcomes are satisfactory and are comparable to those reported at other institutions. Evaluation of the sentinel nodes for the presence or absence of telomerase is presented here in the form of a draft of an article for future publication. The rate of telomerase positivity in the sentinel nodes from patients in this series was strikingly high. We found in 76% of histologically negative sentinel nodes. The significance of this finding is unknown. Clinical followup continues to be accrued on these patients. Survival outcomes for the overall project will not be ready for reporting for another 3-5 years.

These data support the concept of sentinel node biopsy as a staging procedure for breast cancer. After adequate experience with the technique the false negative rate is less than 5%. With regard to telomerase studies on the sentinel node, at this point we believe that telomerase is not an appropriate or clinically relevant marker of disease in the sentinel node. The proportion of histologically node negative sentinel nodes which were positive for telomerase was 76%, which greatly exceeds the expected number of clinical events in this population. Therefore the telomerase studies have been halted. Other potential molecular markers are under investigation for potential use in microstaging of the sentinel node.

There are many reasons why the use of sentinel node biopsy in patients with early stage breast cancer may contribute to improved staging. First, it is a minimally invasive procedure and offers the possibility of accurate staging with a very low morbidity. The risk of lymphedema following excision of a sentinel node(s) is expected to be minimal. The concept of the sentinel node in breast cancer appears to be valid. Although there are minor variances in technique from one reported study to another, the ability to find a sentinel node and the reported node positivity rate among different groups of patients appears to be comparable and appropriate.

Second, another important advantage of using sentinel node biopsy in breast cancer patients is that the most important lymph node is not likely be overlooked. The traditional staging dissection yields a random sampling of lymph nodes from anatomic levels I and II with no attempt to remove every lymph node. One lesson learned from sentinel node studies is that

lymphatic drainage can be unpredictable. While the majority of sentinel nodes are found within the area of a standard staging dissection, a significant minority may be located in other areas such as the internal mammary chain, the supraclavicular area and sites at the periphery of the axillary nodes. In one large series, the sentinel node was found outside the usual limits of an axillary dissection in 8% of cases.[12] Thus, in a small percentage of cases the use of the sentinel node biopsy will yield a lymph node that otherwise would have been left behind. Even in the majority of cases where the sentinel node is within the area of a standard axillary dissection, it allows the opportunity for specialized processing of that node.

Third and perhaps most importantly, the sentinel node allows a limited amount of tissue to be submitted for specialized analysis. Microsectioning of nodes from standard axillary dissection has shown the presence of micrometastases in a significant minority of patients who would otherwise have been classified as node negative. Based on numerous retrospective studies it seems likely that these patients have a somewhat worse prognosis than those who lack micrometastases.[6,13,14,15] Early studies in sentinel nodes using serial sectioning and immunohistochemical staining have demonstrated upstaging of otherwise negative sentinel nodes in 9.4-23% of cases.[16,17] The use of molecular markers to detect even smaller amounts of disease might provide additional stratification. Early reports using PCR for CEA and mammaglobin to evaluate sentinel nodes demonstrate upstaging of significant numbers of nodes.[18,19] The clinical implications of these studies are unknown.

Specialized processing of the sentinel node is likely to result in more specific stratification of which "node negative" patients are more likely to relapse and therefore are more likely to benefit from systemic therapy. In some patients the finding of micrometastasis or trace amounts of disease in the sentinel node may have no implications for systemic therapy. For example a patient with a 2 cm primary tumor would most likely receive systemic adjuvent even if the node were entirely negative. In a patient with a primary tumor less than 1 cm in greatest dimension, however, the finding of disease in the lymph node found only by specialized technique might result in a recommendation for systemic treatment which would not otherwise have been made. Perhaps ultimately more importantly it may be possible to identify a subgroup of patients (perhaps those whose sentinel nodes are negative by a panel of examinations) who are at extremely low risk of relapse and could therefore safely forgo systemic treatment.

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Appendix A
Unpublished

AN EVALUATION OF TELOMERASE AS A TUMOR DETECTION MARKER IN SENTINEL NODES FROM PATIENTS WITH EARLY BREAST CANCER

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ABSTRACT

Objectives. Node-negative breast cancer patients have an approximately 30% risk of systemic recurrence. Telomerase is a telomere-specific ribonucleoprotein and is active in cells with proliferative capacity. The purpose of this study was to evaluate telomerase as a tumor marker in sentinel nodes by comparing the standard pathology with the finding of telomerase activity in sentinel nodes.

Methods. Patients with newly diagnosed unifocal, unilateral breast cancer less than 3 cm in size were offered entry. After peritumoral injection of 0.95 mCi technetium sulfur colloid, the sentinel node was identified using a hand-held gamma probe at the time of the primary breast surgery. The sentinel nodes were processed with one or two levels for hematoxylin and eosin staining, retaining a tiny portion of the node for telomerase studies. Each sentinel node was evaluated using a PCR-based telomeric repeat amplification protocol (TRAP) and scored in a binary fashion with positive defined as banding beyond background. Telomerase results were compared with the results of routine histologic processing.

Results. Twenty-eight patients ranging in age from 36 to 87 were included. Of a total of 50 sentinel nodes (1-4 per patient) 8 were positive for metastasis by routine histology; all 8 were telomerase positive. Of the 42 histologically negative nodes, 32 (76%) were positive for telomerase. Nodes that had significant reactive changes were no more likely to be telomerase positive than nodes that lacked reactive changes. Clinical follow-up continues to be accrued.

Conclusion. Telomerase is not likely to be clinically useful as a method of evaluating sentinel lymph nodes in patients with early stage breast cancer.

INTRODUCTION

The majority of newly diagnosed breast cancer patients are node negative.[1] However they are a group with a heterogeneous prognosis. Although the majority will be essentially cured by local therapy alone, approximately 25-30% of node-negative patients will ultimately recur and die of their disease within 10 years of their diagnosis.[2,3,4] While many features of the primary tumor have been demonstrated to be associated with prognosis, there is still no reliable way to predict which of the node negative group of patients will recur and which will be cured by local therapy alone. It is therefore difficult to identify the patients who are most likely to benefit from adjuvant therapy and those who may safely forego adjuvant therapy.

Whether or not the presence of micrometastases or tumor markers in axillary lymph nodes imply a poorer prognosis remains controversial. A few retrospective studies show a diminished survival associated with the presence of nodal micrometastasis.[5,6,7] In recent years the development of sentinel node biopsy for breast cancer patients has allowed the opportunity to look at the primary draining lymph node(s) in great detail. These sentinel lymph nodes can be examined with multiple histologic sections, specialized staining or molecular markers. Telomerase is a ribonucleoprotein which is telomere-specific and adds nucleotide repeats to chromosomal ends. It is known to be active only in cells with proliferative capacity such as germ line cells, stem cells and malignant cells. Telomerase is active in the majority of primary breast cancers [8,9] and has been studied in numerous other malignancies including lymphoma, [10] non-small cell lung cancer, [11] colon cancer [12] and thyroid cancer.[13]

The purpose of this study was to evaluate telomerase as a sensitive marker for tumor cells in sentinel nodes from patients with newly diagnosed breast cancer. Telomerase may be able to identify microscopic or submicroscopic amounts of metastatic disease in sentinel nodes. The presence or absence of telomerase in the sentinel node(s) may have prognostic implications. In this study, the results of routine histologic staining of sentinel nodes were compared with the results of telomerase testing.

METHODS

Design. This study was part of a larger, ongoing prospective cohort trial to evaluate intensive analysis of sentinel nodes (SN) with serial sectioning, immunohistochemical staining, and molecular markers in predicting prognosis for patients with newly diagnosed breast cancer. Telomerase testing of sentinel nodes was performed on a consecutive series of patients within this ongoing trial. Patients with newly diagnosed breast cancer who were evaluated at Johns Hopkins Medical Institutions beginning in January 1998 were considered for entry into this trial. Entry criteria included 1) newly diagnosed primary invasive breast cancer which is unilateral and unifocal and less than or equal to 3 cm, 2) clinically negative ipsilateral axilla, 3) patients for whom a staging axillary lymphadenectomy was considered the standard of care, and 4) willing participation following informed consent. Excluded were patients who 1) had a previous axillary lymphadenectomy, 2) were pregnant or 3) had multifocal, bilateral, metastatic or recurrent disease. Patients were registered at the time of their informed consent and were assigned consecutive identification numbers. Formal protocol approval was obtained from the Johns Hopkins Joint Committee on Clinical Investigation.

Study Procedure. Technetium-99m sulfur colloid was used as the tracer for the sentinel node procedure. Unfiltered technetium-99m sulfur colloid was prepared by the radiopharmacy immediately prior to dosing. A total of 0.95 mCi of technetium was injected peritumorally to a total volume of 4.0 ml with 1.0 ml per injection site. The tracer was injected superior, inferior, medial and lateral to the primary tumor or biopsy cavity with ultrasound or mammographic guidance. The time interval from tracer

injection to surgery was from one to six hours. The injection site was massaged briefly to stimulate lymphatic flow. Intraoperatively the C-Trak (Carewise Medical Products, Morgan Hill, CA) gamma detector was used with settings of a threshold of 130 keV and a window of 40 keV. Counts were accumulated over 10 seconds. A sentinel node was defined as a node containing counts $\geq$10x background. The sentinel lymph node biopsy was performed at the same time the patient had definitive surgical therapy. The sentinel node was identified, removed and labeled as a separate specimen. The surrounding nodes were interrogated with the gamma probe to search for other nodes meeting the criterion for a sentinel node. Each node meeting the definition of a sentinel node was labeled separately.

Specimen Handling. The sentinel node(s) obtained were handled in a standardized fashion. A tiny fragment (<10% of the nodal volume) was snap frozen at –80°C, labeled with the study number and sent to the research laboratory for telomerase studies described below. The bulk of the node was sent to surgical pathology as a separate specimen along with the remainder of the patient's specimens. Upon receipt in surgical pathology the sentinel node(s) was (were) processed and embedded in paraffin in a routine fashion after appropriate gross inspection of the specimen. One or two levels were made per node. Routine hematoxylin and eosin (H&E) stains were done and the remainder of the tissue block was retained for future study. The primary breast cancer and the remainder of the axillary contents were processed in a routine fashion with measurement of pathologic size and standard evaluation for histology.

Telomerase. The nodal tissue obtained from the sentinel node at the time of the surgery was immediately snap frozen at –80°C with a coded identifier. Tissue blocks were embedded in OCT and two consecutive 10μ cryostat sections were obtained and immediately frozen in liquid nitrogen and stored at –80°C. One slide was stained with H&E. The second cryostat tissue section was suspended in 50 μl of telomerase lysis buffer (TLB) and extracts were prepared as described.[14] Protein concentration was determined by Bradford assay.[15] Aliquots of tissue extract containing 0.5μg of protein with and without inactivation with RNAse,[14] were used for the telomerase assays. Standard telomere repeat amplification protocol (TRAP) assays were performed as described.[14] All assays contained 10 ag of the 150 bp internal telomerase assay standard (ITAS) for detection of Taq polymerase inhibitors.[16] When ITAS inhibition was detected the assay was modified to include an extraction with phenol/chloroform/isoamyl alcohol at 25:24:1 followed by ethanol precipitation[17] before the PCR amplification step. PCR products were electrophoresed in a 10% non-denaturing polyacrylamide gel. The DNA ladders were visualized using a Molecular Dynamics Phosphorimager after a 12-hour exposure. Detectable telomerase activity was defined as a hexanucleotide ladder of 3 or more bands not present in matched RNAse-treated controls. The assays were scored in a binary fashion and were performed without knowledge of sample diagnosis.

Histologic Evaluation of Sentinel Nodes. The H&E sections from the routine surgical pathology of sentinel node were reviewed and scored for the presence or absence of histologic evidence of metastatic disease. Additionally, the lymph nodes were evaluated

and scored for the presence or absence of significant reactive changes by the study pathologist without knowledge of the telomerase status.

Analysis. Telomerase assay scoring results were compared with the results of the routine histology of the sentinel nodes. Among the sentinel nodes which were histologically negative, the presence or absence of reactive changes was compared with the telomerase results. Fisher's exact test was used to compare results.

RESULTS

Clinical Data. Sentinel nodes from 28 patients who fit the entry criteria for this protocol were used in this study. The patients ranged in age from 36 to 87 with a median age of 50. All were female. The majority of patients in this study (82%) had a breast preserving procedure. The majority of patients (89.3%) had a primary infiltrating ductal carcinoma. The median size of the primary was 1.2 cm with a range of 0.6 to 2.5 cm. Eight of the enrolled patients had at least one histologically positive axillary node. There were a mean of 1.79 sentinel nodes per patient with a range of one to four sentinel nodes per patient. There was one false negative sentinel node in this series. Table 1 lists the characteristics of the patients entered.

Sentinel Nodes. A total of 50 sentinel nodes were evaluated from the 28 patients. Each sentinel node was evaluated at one or two levels by routine H&E staining and also by telomerase assay as described above. Table 2 shows the results of routine pathology and telomerase studies. Of the 8 nodes that were positive by routine histology, all were telomerase positive. Of the 42 nodes that were negative by routine histology, 32 (76%) were positive for telomerase ($p = 0.18$, Fisher's exact test).

The presence or absence of reactive changes in sentinel nodes that were negative by routine histology was evaluated. In thirty-six nodes available for review, thirteen had moderate to marked reactive changes, and eight (61.5%) of these were telomerase positive. However, in the twenty-three nodes that lacked significant reactive changes,

nineteen (82.6%) were telomerase positive ($p = 0.23$, Fisher's exact test). Table 3 summarizes the comparison of significant reactive changes and telomerase results.

DISCUSSION

We have evaluated telomerase as a marker of metastatic disease in sentinel nodes from patients with recently diagnosed early stage breast cancer. As expected, all lymph nodes that were histologically positive were also positive for telomerase. Among nodes that were histologically negative, a surprisingly high proportion (76%) were telomerase positive. Of the nodes that were histologically negative, but telomerase positive, only 30% showed significant reactive changes. Thus, the presence of reactive changes in histologically negative sentinel nodes does not explain the high rate of telomerase positivity in this series. The clinical implication of a lymph node which is histologically negative but telomerase positive is unknown.

Most molecular markers that have been evaluated in breast cancer samples have been found to be of limited utility because of the lack of uniform positivity in primary breast cancer. Telomerase is a ribonucleoprotein polymerase which is active in the majority of malignancies, immortal cell lines and in germ cells. It has been studied extensively in breast cancer and is of interest as a possible tumor marker because of its high rate of positivity in primary breast cancers.

A high percentage of primary breast cancers are positive for telomerase. Reported positivity rates for invasive breast cancers range from 79% to 100%.[8,9,18,19,20] Additionally, the majority of in situ breast carcinomas are positive for telomerase.[20] Telomerase therefore is consistently positive in the majority of primary breast cancers and may be useful as a marker of disease. Some early studies reported that advanced breast cancers are more likely to be telomerase positive than are early breast cancers.[9,21] More recent studies have not confirmed this. Nawaz reported no difference in telomerase

positivity rates between node positive and node negative breast cancers.[19] One large study showed that a quantitative assessment of telomerase levels in primary breast cancers could be associated with survival among patients who had positive lymph nodes.[22] Another study, however, did not find any association of telomerase activity with outcome or with most prognostic variables.[23]

Telomerase has also been studied in other malignancies, most notably in colon cancer,[12] where increasing stage was associated with increased telomerase positivity as were other prognostic features such as the differentiation status, mitotic index and Ki-67. Telomerase has also been studied in non-small cell lung cancer with an 82.5 – 100% rate of telomerase positivity in primary tumors.[11,24] Eighty percent of non-Hodgkin's lymphomas had evidence of telomerase activity but only 7% of samples of Hodgkin's disease expressed telomerase.[10]

There is currently great interest in molecular staging of the regional lymph nodes from patients with newly diagnosed breast cancer. Molecular markers may be able to detect smaller amounts of cancer than can be detected by conventional histologic examination. Examination of regional lymph nodes for molecular markers is likely to detect disease in a greater percentage of patients than any form of serial sectioning or immunohistochemistry. The sentinel node is an ideal tissue in which to search for molecular markers for staging. The sentinel node is anatomically the first lymph node that receives lymphatic drainage from the primary tumor and has been demonstrated to be the node that is most likely to contain metastatic disease if any has occurred.[25,26] Evidence of metastatic disease in the draining regional nodes is of tremendous prognostic significance and can affect treatment recommendations.

Evaluating lymph nodes for telomerase, however, presents some difficulties. It has been demonstrated that the TRAP assay can be positive in activated lymphoid tissues such as the tonsils, hyperplastic lymph nodes, as well as in lymphocytic infiltrates in thyroid and breast tissues.[10,13,21] Other studies using quantitative assessments show low, but detectable, levels of telomerase activity in activated lymphocytes.[27] Thus lymph nodes may sometimes test positive for telomerase activity even in the absence of malignancy.

Our results demonstrate that telomerase by the simple TRAP assay is positive in all histologically positive lymph nodes tested and in a high percentage of histologically negative sentinel nodes. In this study histologically identifiable reactive changes in the sentinel nodes cannot explain the high frequency of telomerase positivity. The clinical significance of the finding of positive telomerase in a node that is histologically negative is unknown. Clinical follow-up on this group of patients continues to be accumulated. At present, because of the high percentage of sentinel nodes which were histologically negative but telomerase positive, we feel that telomerase is not likely to be a clinically useful method of examining sentinel nodes.

Table 1. Patient Characteristics (N=28)

Age, y (median, range)	50, 36 – 87
Type of Surgery (% BCS)	82%
Tumor Size, cm (median, range)	1.2, 0.6 – 2.5
Histology (% ductal)	89.3%
Number of Sentinel Nodes per Patient (mean, range)	1.79 (1 – 4)

Table 2. Comparison of the presence or absence of telomerase activity with the presence or absence of histologic evidence of metastasis in 50 sentinel nodes

		Telomerase	
		Positive	Negative
Histology	Positive	8	0
	Negative	32	10

Table 3. Comparison of the presence or absence of telomerase with the presence or absence of significant reactive changes in 36 histologically negative sentinel nodes

		Telomerase	
		Positive	Negative
Reactive Changes	Present	8	5
	Absent	19	4

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Table 1. Patient Characteristics (N=150) in the Johns Hopkins Sentinel Node Trial for Breast Cancer

Age, y: median, range		50, 35-87
Type of Surgery (% BCT)		74.7%
Tumor size, cm: median, range		1.3, 0.1-3.9
Histology (% of total):	ductal	69.3%
	lobular	8.7%
	mixed	16.0%
	other	6.0%
Presentation (% of total):	mammographic	56%
	palpable	42%

Table 2. Technical Outcomes for the Johns Hopkins Sentinel Node Trial for Breast Cancer. Patients #1-150

Patient Group	N	Identification Rate	#SN/ Patient	False Negative	FN Rate
All	150	85.3%	1.8 (0-5)	3	3/38 (7.9%)
Surgeon 1	42	88.1%	2.2 (0-5)	0	0/11 (0%)
Surgeon 2	50	78.0%	1.9 (0-4)	1	1/13 (7.7%)
Surgeon 3	58	89.7%	1.5 (0-4)	2	2/15(13.3%)

Table 3. Technical Outcomes for the Johns Hopkins Sentinel Node Trial for Breast Cancer, last 30 cases for each surgeon

Patient Group	N	Identification Rate	#SN/ Patient	False Negative	FN Rate
All	90	83.3%	2.0	1	(1/24) 4.2%
Surgeon 1	30	86.7%	2.4	0	(0.8) 0%
Surgeon 2	30	80.0%	1.9	0	(0/7) 0%
Surgeon 3	30	83.3%	1.6	1	(1/9) 11.1%

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 23 Aug 01

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FOR THE COMMANDER:

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