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Tumor Acquisition for Biomarker Research in Lung Cancer

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Abstract

The biopsy collection data from two lung cancer trials that required fresh tumor samples be obtained for microarray analysis were reviewed. In the trial for advanced disease, microarray data were obtained on 50 patient samples, giving an overall success rate of 60.2%. The majority of the specimens were obtained through CT-guided lung biopsies ($N=30$). In the trial for early-stage patients, 28 tissue specimens were collected from excess tumor after surgical resection with a success rate of 85.7%. This tissue procurement program documents the feasibility in obtaining fresh tumor specimens prospectively that could be used for molecular testing.

Keywords

Lung cancer; Gene profiling; Bioinformatics

INTRODUCTION

A cytology specimen collected by fine-needle aspiration or bronchoscopic brushings/washings no longer provides adequate information to treat non-small cell lung cancer (NSCLC). Core needle biopsy or surgical specimen is now needed in the standard diagnostic workup of lung cancer to provide accurate histologic and molecular diagnosis (1–3). Furthermore, high quality, fresh tumor specimens that are adequate to assess proteomics, genomics, tumor immunology, and other molecular markers are necessary for biomarker discovery and validation. Much of what we know about the mutations present in NSCLC comes from important work by the Lung Cancer Mutation Consortium (4). However, most of those tumor specimens were from primary tumors for which there is no annotated clinical outcome data. It is important to collect and analyze adequate tumor samples to characterize the molecular characteristics of advanced stage NSCLC.

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DECLARATION OF INTEREST

The authors alone are responsible for the content and writing of the paper.

Tissue sampling in lung cancer presents different issues compared to other cancers. Breast and colon cancer patients often undergo surgery that will remove the bulk of their tumor, or in the case of breast cancer, have core biopsies of the primary tumor at initial diagnosis. Lung tumors are often central within the thorax and core needle biopsies require a more focused approach with specialized expertise on the part of the interventional radiologist or pulmonologist. This report describes our experience and challenges in obtaining fresh frozen lung tumor specimens for RNA extraction to utilize in biomarker discovery.

METHODS

The biopsy collection data from two IRB-approved lung cancer trials conducted at Duke University Medical Center were reviewed. As enrollment criteria, both protocols required that fresh tumor samples be obtained for microarray analysis. In the trial for advanced disease ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT00509366) Identifier NCT00509366), biopsies were obtained prior to first-line palliative chemotherapy using the following techniques: superficial percutaneous biopsies, image-guided biopsies, and excess tumor samples from standard surgery, bronchoscopy or mediastinoscopy. The trial for early-stage lung cancer ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT00545948) Identifier NCT00545948) enrolled patients with stage IB – IIIA NSCLC whose cancer was surgically resected and appropriate for platinum-based adjuvant chemotherapy. Research samples for this trial were collected from surgical specimens after standard resection. These trials were conducted at five clinical sites, in addition to Duke. An outline for specimen collection and processing is in Figure 1.

Patient consent

The consent process for patients in these clinical trials involved consent for the clinical trial itself, consent to have the tissue stored in a biorepository and sometimes consent for the procedure to obtain a research biopsy. Any tissue specimens that remained after appropriate diagnostic and microarray analyses were then stored in a biorepository for possible future research use if the patient signed the biorepository consent form.

Tissue collection

For patients with advanced disease, core needle biopsies (preferably with 18 gauge needles) were preferentially pursued over fine-needle aspirates in order to produce an adequate tumor specimen for histologic diagnosis and standard molecular evaluations, such as epidermal growth factor receptor mutation analysis. Extra core biopsies were obtained for research purposes. In some cases, dedicated research-only biopsies were performed as per protocol. Past-irradiated sites were avoided for biopsy due to the influence radiotherapy has on genetic material. When choosing where to get a tissue sample, the most accessible site was pursued. Peripheral thoracic lesions and metastases in the liver, bones, or adrenal glands were often targeted with CT-guided biopsies. Superficial lesions such as supraclavicular lymph nodes and cutaneous metastases were biopsied in the clinic. A maximum of up to five needle biopsy passes were allowed to obtain a tumor specimen. Patients undergoing surgery or bronchoscopy for standard clinical indications had excess tumor collected for study participation. For early-stage disease, tissue samples were obtained from excess surgically resected specimens after adequate tissue was reserved for all standard pathologic testing.

Specimen processing

At the time of specimen collection, a pathologist reviewed the core specimens to ensure that tumor was present with the use of touch prep analysis. If tumor was present, specimens proceeded through processing. If the biopsy was needed to confirm a patient's diagnosis as part of routine medical care, the diagnostic specimen was collected first. The remaining specimen intended for research was sequestered until it was determined that the pathologist had adequate tissue for all standard diagnostic tests. If it was later determined that a diagnostic specimen was inadequate, research specimens were released to pathology to be used for standard diagnostic workup, even if this used up the entire research-designated specimen. This occurrence was uncommon, but in several instances release of the research specimen benefited the patient by avoiding another biopsy procedure.

Research specimens were frozen using Tissue-Tek®OCT (optimal cutting temperature) compound (Sakura Finetek, Torrance, CA). Each biopsy specimen underwent further individual pathologic evaluation to determine the presence of tumor cells (percentage of cells present), inflammation, necrosis, and fibrosis. If the specimen had an acceptable histologic appearance, microdissection was performed in order to isolate tumor cells from tissue specimens to help increase nucleic acid yield by ensuring that tumor, and not necrotic or fibrotic tissue, was used for molecular analysis. RNA was extracted using a standard kit and quality of the RNA was evaluated with an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA), which indicates excessive RNA degradation or impurities if present. Metrics used to evaluate RNA quality were the UV absorbance 260/280 ratio and the RNA Integrity Number (RIN) with no impurities reflected by scores of 2 and 10 in each metric, respectively (5). If the RNA was of good quality (with 260/280 ratio of at least 1.8 and RIN of at least 5), microarray analysis was then performed in a CLIA-certified lab using the Affymetrix U133 plus 2.0 GeneChip platform (Affymetrix, Santa Clara, CA). Quality of the microarray results was assessed using standard metrics for Affymetrix platforms (6, 7), that included "percent present" and scale factors to measure overall intensity, and 3'/5' ratios of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β -actin to measure RNA degradation. The goal was for the GAPDH3'/5' ratio to be close to one and the β -actin 3'/5' ratio to be less than 3. The turn-around-time from biopsy to microarray analysis was planned to be 10 days per protocol. Specimens were frozen and stored at -70°C or below labeled with the patient's unique, coded number. Residual tumor specimens or RNA were stored in a biorepository, if the patient had consented to participation in the biorepository. The MAW3® program was used for sample tracking, inventory management, and chain of custody. MAW3® is a Duke-developed, HIPAA compliant software system and database that is used at Duke for sample and data banking (8).

Operational procedures for tissue processing

Tumor biopsies had to pass quality control (QC) criteria to qualify as successful: tumor present, high quality RNA, and successful microarray analysis. Inadequate biopsies were categorized by two main classifications: histological failures and RNA/microarray failures. Histological failures occurred when not enough tumor tissue was identified in the specimen or there was an extensive amount of necrotic material or fibrosis. Histological failures could be due to sampling error of the tumor, excessive necrotic tissue, or too small a biopsy

specimen. In these trials, RNA was extracted from biospecimens for use in microarrays, so failures could be due to extraction of too small an amount of RNA or poor quality of the RNA extracted (secondary to RNA breakdown from inflammation, improper specimen processing, necrotic tumor, and/or contamination). Microarray failures could occur due to technical problems with the microarray platform or poor quality of the reverse transcribed and biotin-labeled cRNA.

RESULTS

Advanced stage non-small cell lung cancer research biopsies

A total of 102 patients were consented to NCT00509366, of which 20 were determined to be ineligible by trial eligibility criteria, one withdrew consent, one died prior to tissue acquisition, and one had a tissue processing error occur. This resulted in 79 patients with advanced NSCLC being enrolled for tissue analysis (Figure 2). For five patients, initial tissue specimen processing failed, and a second attempt at tissue analysis was made. For one patient's single bronchoscopy procedure, the first tissue core failed QC, and the second passed. The other four patients required second biopsy procedures. This leads to a total of 83 procedures and 84 biospecimens being analyzed for the 79 trial patients.

Array data meeting all prespecified criteria were obtained on 50 patient samples, giving an overall success rate of 60.2%. Table 1 shows the types of procedures performed to obtain biopsies and success rates. Table 2 summarizes the reasons for biopsy failure per procedure type. Tissue specimen analysis failures were attributed to histological screening for viable tumor ($N = 17$), RNA failures ($N = 11$), or microarray failures ($N = 5$). The average turn-around-time for RNA processing for the specimens in this trial was 6 days with a range of 5 to 9 days. On average, 17.28 μg (range: 0.48–160.20 μg) of RNA was isolated with an average RIN of 7 (range: 2.2–9.8). Table 3 shows the average RIN and amount of RNA isolated by procedure type.

The majority of the specimens for this trial were obtained through CT-guided lung biopsies ($N = 30$), which were successful with a rate of 56.7%. Eighteen gauge needles were used in 26 of the 30 CT-guided lung biopsies. Twenty gauge needles were used in four of the procedures and produced an adequate specimen in only one patient. Bronchoscopy ($N = 13$) had a 30.8% success rate and was most likely to be successful when forceps were used to collect samples from endobronchial lesions when compared to the use of fine-needle aspiration or other techniques.

The single CT-guided biopsy of the liver ($N = 1$) was successful; whereas CT-guided biopsies of the adrenal gland ($N = 2$) and bone ($N = 1$) were not. Superficial lymph nodes ($N = 3$) and soft tissue masses ($N = 8$) were also biopsied as part of this study with adequate success rates (66.7% and 70.0%, respectively). Tru-cut biopsy needles were used for these superficial biopsies since they helped ensure retention of the collected specimen.

Tumor specimens obtained at surgery in the setting of advanced disease had a high rate of success (19 of 23, 82.6%). The most common anatomic site for surgical resection was the lung (11 cases) with a 90.9% success rate. Mediastinoscopy (six cases) had a 83.3% success

rate. Other sites in which tumor samples were obtained by surgical resection included brain and bone. Two sample failures were due to RNA poor quality and one was due to a microarray failure. One sample failure was due to lack of tumor being present on the research biopsy of a bone metastasis resection, where the diagnostic specimen contained histological confirmation of metastatic disease but there was too little tumor in the research specimen to allow microarray analysis.

Early-stage non-small cell lung cancer research biopsies

A total of 31 patients with resectable early-stage NSCLC were consented to NCT00545948, of which two were determined to be ineligible, and one withdrew consent, leaving 28 evaluable tissue specimens being collected from excess tumor after surgical resection (Figure 3). Four patients were collection failures due to histological failures ($N = 2$), RNA failures ($N = 1$) or microarray failures ($N = 1$), giving a success rate of 85.7%. The average turn-around-time for specimen RNA processing in this trial was 10 days with a range of 8 to 14 days. On average, 52.43 μg (range: 4.6–148.2 μg) of RNA was extracted with an average RIN of 7.6 (range: 5.4–9.5).

Combining all patients that underwent lung surgical resections in both trials, a total of 39 patients received thoracic surgery with 34 successful specimen collections with a rate of 87.2% success. Failures primarily occurred in tumors with extensive inflammation or necrosis.

Adverse events

Adverse events from tissue collection on NCT00509366 were collected according to protocol using NCI CTCAE version 3.0. There were no unanticipated adverse events during tissue acquisition. Among enrolled patients, CT-guided lung tumor biopsies resulted in seven pneumothoraces out of 30 (23.3%) with one grade 1, and six grade 2 events reported. There was no increase in pneumothorax for biopsies with an 18-gauge needle versus 20-gauge needle in our small sample size, with two out of four procedures using a 20-gauge needle resulting in a pneumothorax. Two of the seven patients with pneumothoraces required temporary small bore chest tube placement for approximately 24 hr. The other five patients were observed overnight. Surgical patients had post-procedure discomfort as expected for their surgery. No patient died as a result of tissue collection by surgery, bronchoscopy, or needle biopsy.

DISCUSSION

The lung cancer tissue procurement program described here documents our experience in obtaining fresh NSCLC tumor specimens from both advanced and early-stage patients prospectively that could be used for molecular testing and biomarker development. This process included explaining genomic medicine to patients, consenting to a tissue repository, developing collaborative relationships with interventionalists, utilizing a computerized tissue tracking system, quality control parameters for tumor/RNA quality, transfer of RNA to an independent outside gene array analysis company, transfer of genomic expression data to a secure data system, and report of outcome through a locked down model of gene expression

controlled by the study biostatistician. This process created an unbroken chain of custody for both biological specimens and genomic data.

Our experience demonstrated that core needle biopsies can provide adequate tissue for high-quality RNA analysis. CT-guided biopsies of the lung were the most common procedure used to obtain tissue in advanced lung cancer patients. The biggest concern when pursuing CT-guided lung biopsies is the risk of pneumothorax, especially in a population of patients who likely have poor pulmonary reserve due to smoking histories. Patients who are at higher risks for complications from CT-guided lung biopsies include patients age 60–69 years, smokers, and patients with COPD—all common findings in lung cancer patients (9). Other risk factors for pneumothorax include the presence of small lesions/nodules, long needle path (>4 cm), and repeated pleural puncture (10). The rate of pneumothorax for CT-guided lung biopsies averages from 20 to 25% with 2–5% requiring treatment such as chest tube placement (11–14). The pneumothorax rate for CT-guided biopsies in our program was within this range of expected incidence. Our biopsy success rate was optimal when we had a thoracic radiologist dedicated to the research program who screened patients prior to biopsy. Our limited experience showed no apparent increase in pneumothorax and hemorrhage rates for 18-gauge needles compared to 20-gauge needles, and that result is consistent with what has been reported in the literature (15).

In our limited experience, bone and adrenal biopsies were uniformly unsuccessful. Although bone biopsies can confirm metastatic disease upon histological examination, these biopsies may not consistently yield an appropriate tumor specimen for biomarker analysis. Non-diagnostic bone biopsies can range from 0 to 44%, with the rate increasing if the biopsy was performed without imaging guidance or without immediate onsite pathology review (16, 17). Additionally, decalcification techniques often affect nucleic acid yield. With the adrenal lesions, one of the two patients undergoing adrenal biopsies had a PET scan to assist in evaluating the malignant potential of the adrenal lesion. It has been shown that less than 50% of adrenal masses are malignant in lung cancer patients (18, 19). The number of attempted bone and adrenal biopsies in this series was small, so we cannot make definitive conclusions regarding these sites as biopsy sites for biomarker analysis. Overcoming the challenge of obtaining adequate specimens for biomarker analysis from bone and adrenal metastatic sites would be important for making progress in biomarker research.

When considering specimens collected from surgical procedures, even samples with adequate volume of tumor sometimes had RNA failure. Our experience with resected tumors showed that these specimens often had inflammation, fibrosis, and necrosis rather than faulty specimen handling. These poorly differentiated tumors have a poor prognosis natural history and are less likely to benefit from therapy. In validation studies of prognostic and predictive genomic biomarkers, we advocate that all specimens with adequate tumor volume should be included in an intent to analyze methodology. Otherwise the most poorly differentiated tumors will be excluded from the analysis and the outcome will be biased in favor of well differentiated, better prognosis cancers.

The benefits of obtaining lung cancer core biopsy specimens for histological and molecular analyses are validated for guiding therapy that improves outcome and should be standard of

care (2, 20, 21). Collecting appropriate specimens for biomarker discovery and validation can be difficult, since larger tissue samples are required for molecular analysis compared to those used for disease diagnosis. The results from the BATTLE Trial and the Lung Cancer Mutation Consortium demonstrate both the benefit of using tissue specimens in guiding the therapy of lung cancer patients and the feasibility of collecting the specimens (4, 22, 23). Many of the lung cancer specimens collected for repositories were from primary tumors. The samples collected in our program were from patients with advanced disease, which may have a different pattern of oncogenic driver mutations and mechanisms of resistance to therapy.

These trials collected tissue specimens and analyzed DNA in a time frame consistent with making decisions regarding cancer therapy. Other lung cancer trials in which tumor specimens were collected demonstrated that approximately 50% of the tumor samples were adequate for molecular analysis (24, 25). In comparison, a breast cancer study collecting image-guided fresh frozen core needle biopsies was able to obtain adequate tissue specimens for microarray analysis in 90% of patients (26). Core needle biopsies, typically 18 gauge, were used rather than small gauge needle biopsies. It is important to process each individual biopsy specimen for tumor content and this requires that a percentage of the specimen is lost. There is a much higher percentage of sample wastage to assess tumor content with multiple small core needle biopsies compared to one or two large core needle biopsies. Also there is degradation of genetic material around the surface of the biopsy specimen during the preservation process, and small core needle biopsies or aspirates have a high surface to volume ratio. There is a greater percentage of specimen loss for both of the above reasons in a small core biopsy compared to a larger diameter biopsy specimen, making small core needle biopsies more vulnerable to inadequate RNA yield. Thus, an 18-gauge needle biopsy allows for higher nucleic acid yield even, if the total volume of tissue obtained from smaller gauge biopsies is the same. Although an RNA preservative, such as RNALater®, was not used in this study due to concerns over its effects on histological evaluations and gene expression, several studies have shown that it can potentially improve the yield and quality of RNA extractions, especially for small tissue samples, when compared to snap freezing (15, 27–29). RNA preservatives can be beneficial when smaller samples cannot be avoided or if snap-freezing technology is not readily available. Snap freezing with OCT compound was used for these trials and has demonstrated it can be used with adequate RNA extraction results (30–33). Improper specimen processing after biopsy collection or lab processing errors can contribute to problems with adequate RNA extraction or microarray analysis. Studies that aimed to perform RT-PCR/FISH analyses had higher success rates compared to studies performing microarray analyses from extracted RNA, but the amplification of RT-PCR may be less accurate. Studies that incorporated microdissection to isolate tumor cells prior to RNA extraction, and studies that used larger gauge needles (16–19 gauge) for tissue collection were also more successful in having high rates of successful genomic analysis (25, 28).

When planning tissue biorepository programs or clinical trials requiring fresh tissue collection, the effect of inadequate tissue samples and number of failed biopsies should be considered when estimating how many patients need to be screened to complete enrollment. Given variable failure rates for biopsies and nucleic acid extraction, the experience of the

sites conducting the trial and processing the specimens, along with the expertise of the performers of the procedures used to obtain the tissue samples, should also be considered. The expertise of the professional obtaining the biopsies affects the success rate of the biopsies and the complication rates. In our experience, the success rate for tumor sample collection increased significantly when we identified an interventional radiologist who was proficient at assessing cases for biopsy attempts, and conducting the procedure. Multiple strategies for obtaining lung cancer biopsies have been evaluated, but one of the most successful and practical strategies incorporated performing additional research biopsies at the same time as diagnostic procedures, such as bronchoscopy. This allowed patients to undergo one procedure for diagnostic and research biopsy collection, in addition to ensuring tissue collection for advanced stage patients who typically are not surgical candidates (28, 34).

It is imperative to ensure that the tissue procurement process is safe for patients and appropriate consent is obtained. More detailed consent forms/processes are required for biospecimen collection trials, and an ECOG study showed that a more detailed consent form increased trial participation (35). Patients appreciate being given detailed information on how their biological specimens will be stored and potentially used in the future for additional research. Detailed or tiered consents enable patients to choose what type of future research would be allowed, if any, and whether they want to be contacted before their samples are used in the future (36). While the ability to use information obtained from tissue specimens can significantly improve the treatment plan for a patient, this must be balanced with the risks of the additional procedure.

In the future, collecting additional samples for research purposes at the time of standard diagnostic procedures will assist in making molecular analyses part of routine clinical care for lung cancer patients of all stages; and clinicians performing biopsies—surgeons, pulmonologists, interventional radiologists—should recognize the importance of obtaining adequate tumor tissue for molecular analyses. In addition, many clinical cancer trials testing new targeted therapies require that a tumor specimen is available for biomarker testing as part of eligibility. Collecting high-quality tumor specimens at multiple points before and after therapy will allow us to understand and develop strategies to overcome tumor-resistance mechanisms. Ultimately, the information obtained from these tissue samples for biomarker discovery and validation can allow us to pick the best possible therapy for an individual patient and maximize the therapeutic index throughout the course of a patient's treatment program.

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Figure 1.
Specimen collection and processing flow chart.

Figure 2.
REMARK diagram for [ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT00509366) Identifier NCT00509366.

Figure 3.
REMARK diagram for [ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT00545948) Identifier NCT00545948.

Table 1

Rates of Success in Processing Biospecimens in Advanced Stage NSCLC

	Total Procedures	Successes	
		Number	%
Bronchoscopy	13*	4	30.8
Mediastinoscopy	6	5	83.3
Soft tissue masses	3	2	66.7
Lymph node (tru-cut biopsies)	10**	7	70.0
Lung resection (including VATS)	11	10	90.9
Other surgical procedures	6	4	66.7
CT-guided biopsies—lung	30	17	56.7
CT-guided biopsies—adrenal	2	0	0
CT-guided biopsies—bone	1	0	0
CT-guided biopsies—liver	1	1	100
Total	83	50	60.2

* For one patient's bronchoscopy, the first tissue core failed QC, and the second passed.

** Eight patients underwent lymph node biopsies with two patients having a repeat biopsy due to failure of the first.

Table 2

Reasons for Biopsy Failures in Advanced Stage NSCLC

	Total Failures	Histologic Failures (Number (%))	RNA Failures (Number (%))	Microarray Failures (Number (%))
Bronchoscopy	9	6 (67%)	2 (22%)	1 (11%)
Mediastinoscopy	1	0	1 (100%)	0
Soft Tissue Masses	1	1 (100%)	0	0
Lymph Node (tru-cut biopsies)	3	2 (67%)	0	1 (33%)
Other Surgical Procedures	2	1 (50%)	0	1 (50%)
Lung Resection (including VATS)	1	0	1 (100)	0
CT-guided biopsies - lung	13	5 (38%)	6 (46%)	2 (15%)
CT-guided biopsies - adrenal	2	1 (50%)	1 (50%)	0
CT-guided biopsies - bone	1	1 (100%)	0	0
CT-guided biopsies - liver	0	0	0	0
Total	33	17 (52%)	11 (33%)	5 (15%)

Table 3

Mean RNA Isolated and RNA Integrity Number for Biospecimens in Advanced Stage NSCLC

	Mean RNA Isolated (μ g)	Mean RNA Integrity Number
Bronchoscopy	5.97	6.4
Mediastinoscopy	37.63	6.1
Soft tissue masses	7.30	6.3
Lymph node (tru-cut biopsies)	15.63	7.2
Lung resection (including VATS)	38.55	7.4
Other surgical procedures	47.85	9.3
CT-guided biopsies—lung	2.72	6.8
CT-guided biopsies—liver	1.3	9.4

* Data for CT-guided adrenal and bone biopsies are not available since these biopsies were not successful.