Published in final edited form as:

Nanomedicine. 2014 April ; 10(3): 661–668. doi:10.1016/j.nano.2013.10.008.

Molecular characterization of scant lung tumor cells using iron-oxide nanoparticles and micro-nuclear magnetic resonance

Arezou Ghazani^{1,*}, Melina Pectasides^{1,2,*}, Amita Sharma^{2,*}, Cesar M. Castro^{1,5}, Mari Mino-Kenudson³, Hakho Lee¹, Jo-Anne O. Shepard^{2,#}, and Ralph Weissleder^{1,2,4,5,#}

¹Center for Systems Biology, Massachusetts General Hospital, 185 Cambridge St, CPZN 5206, Boston, MA 02114

²Department of Imaging, Massachusetts General Hospital, Fruit St, Boston, MA 02114

³Department of Pathology, Massachusetts General Hospital, Fruit St, Boston, MA 02114

⁴Department of Systems Biology, Harvard Medical School, 200 Longwood Ave, Boston, MA 02115

⁵Massachusetts General Hospital Cancer Center, 55 Fruit St, Boston, MA 02114

Abstract

Advances in nanotechnology and microfluidics are enabling the analysis of small amounts of human cells. We tested whether recently developed micro-nuclear magnetic resonance (μ NMR) technology could be leveraged for diagnosing pulmonary malignancy using fine needle aspirate (FNA) of primary lesions and/or peripheral blood samples. We enrolled a cohort of 35 patients referred for CT biopsy of primary pulmonary nodules, liver or adrenal masses and concurrently obtained FNA and peripheral blood samples. FNA sampling yielded sufficient material for μ NMR analysis in 91% of cases and had a sensitivity and specificity of 91.6 and 100% respectively. Interestingly, among blood samples with positive circulating tumor cells (CTC), μ NMR analysis of each patient's peripheral blood led to similar diagnosis (malignant vs benign) and differential diagnosis (lung malignancy subtype) in 100% and 90% (18/20) of samples, respectively. μ NMR appears to be valuable, non-invasive adjunct in the diagnosis of lung cancer.

Keywords

Bioorthogonal click chemistry; Circulating Tumor Cell; μ NMR; Iron-oxide nanoparticles; Point of care diagnosis

Introduction

Lung cancer is among the most common and fatal cancers worldwide. In 2013, over 225,000 new cases and 159,000 deaths are estimated to occur in the US alone¹. While most patients

© 2013 Elsevier Inc. All rights reserved.

[#]R. Weissleder, MD, PhD, Center for Systems Biology, Massachusetts General Hospital, 185 Cambridge St, CPZN 5206, Boston, MA, 02114, 617-726-8226, rweissleder@mgh.harvard.edu; Jo-Anne Shepard, MD, Division of Chest Radiology, Massachusetts General Hospital, 32 Fruit St, Boston, MA, 02114, 617-724-4256, JSHEPARD@PARTNERS.ORG.

*Equal contributions

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

are currently treated with a combination of surgery, radiation and chemotherapy, novel therapies are emerging for specific lung cancer subtypes²⁻⁴. Both treatment initiation and confirmation of recurrence commonly rely on primary tissue sampling occurring through bronchoscopy or CT guided lung biopsy. Either approach yields small cores of tissue which are embedded, sectioned and then stained for immunohistochemistry. Percutaneous core biopsies with 17-19G coaxial needles, however, confer morbidity and throughput is generally low^{5,6}.

Alternative sources of malignant cells for diagnostic and molecular testing include peripheral blood (circulating tumor cells or CTC)⁷⁻⁹, pleural fluid (thoracentesis) and fine needle aspirates (FNA). While these cells are attainable through less invasive measures, their challenges include their scant nature¹⁰⁻¹², limited viability, epithelial-to-mesenchymal transition (EMT)¹³ and heterogeneous protein expression levels. CTC are rare (~1-100 cells/mL of blood)^{11,14}, while a single FNA pass often yields < 200 cells depending on technique¹². Irrespective of the source and method, malignant cells are outnumbered by co-existing host cells from blood and tissue samples (immune cells, mesothelial cells, fibroblast).

Advances in nanotechnology and microfluidics have expanded the feasibility of molecular analyses using limited clinical specimens. A spectrum of new methods have appeared^{15,16} varying in throughput, accuracy and ease of use. We have recently developed a μ NMR approach that allows profiling of fine needle aspirates^{12,17}, exosomes¹⁸, and potentially individual cells¹⁹ across the spectrum of solid tumors. The method is fast, accurate and applicable in a point of care setting. To inaugurate testing of our technology for pulmonary malignancies, we enrolled 35 patients referred for biopsies of primary lung lesions or their potential liver or adrenal metastases. We then compared our FNA and CTC analyses to conventional pathology interpretation of each patient's core biopsy (Figure. 1). We show that our method is accurate and when combined with CTC analysis, could potentially avoid the need for core biopsies.

Methods

Patient population and analysis

The study was approved by the Institutional Review Board, and the procedures followed were in accordance with institutional guidelines. Informed consent was obtained from all subjects. Thirty-five subjects requiring biopsy were enrolled in this study. Thirty-two of them had pulmonary nodules that required tissue diagnosis, while 3 had known primary lung cancer with suspected adrenal (2) or liver (1) metastases. On the day of enrollment, both peripheral blood (7 mL) and fine needle aspirates from intraparenchymal masses were collected from each subject. Five clinicians (M.P., A.S., C.M.C, J.A.S. and R.W.), reviewed the documented clinical, imaging, and pathology data for each subject with cancer. Tables 1 and 2 summarize the different cohorts used for analyses.

Selection of biomarkers

Detection of malignancy—We used a previously identified cocktail of four (quad) markers (EGFR, EpCAM, HER-2, MUC-1) for CTC detection, as the combined application of these markers allows more accurate CTC counting than a single marker (EpCAM) based detection²⁰. **Subclassification of lung cancer**: we selected clinically relevant markers used in differentiating subtypes of lung cancer: adenocarcinoma (TTF1, Napsin A), squamous cell carcinoma (p40), small cell carcinoma (a cocktail of Synaptophysin + Chromogranin). Table 3, Figure S1 and Figure S2 show the markers, antibodies, and diagnostic algorithm used for the detection of cancer and differentiation of lung cancer subtypes.²¹⁻²⁴.

Sample processing and labeling

Each peripheral blood sample (7 ml) was lysed and the cell pellet resuspended in buffer (100 μ l of 1X PBS/1% FBS). FNA samples were fixed as described previously¹². For molecular profiling experiments of both blood and FNA specimens, primary antibodies were separately added to aliquots of parent samples. Cell pellets were incubated for 20 minutes. TCO-modified secondary IgG antibodies (10 μ g/ml) were then added to the pellet and incubated for 20 minutes. Cell pellets were then washed twice with 1 $\times$ phosphate buffered saline (PBS) and incubated with magnetic nanoparticles (100 nM Tz-CLIO) for 10 minutes. Excess Tz-CLIO was removed by washing the pellet twice with 1XPB, before being resuspended in 20 μ l of 1XPBS for μ NMR measurements. Fluorescent images were taken for visualization of CTCs using EasySep™ enrichment method. CTCs were isolated using the FITC Positive Selection kit (STEMCELL Technologies Inc). Cells were labeled with antibodies against EPCAM, EFGR, Her-2 and Muc and targeted with FITC-conjugated antibodies and dextran-coated magnetic nanoparticles. Labeled CTCs are separated using an EasySep™ magnet. All labeling experiments were performed at room temperature.

Preparation of transyclooctene (TCO)-modified antibodies

Monoclonal antibodies against targets were used for the primary labeling of antigens (Table S1). To enhance the labeling efficiency, TCO modification of antibodies were performed on secondary IgG antibodies instead of primary antibodies, (i.e. primary antibodies were incubated with cells per manufacturer's protocol). Secondary IgG antibodies were conjugated with (E)-cyclooct-4-enyl 2,5-dioxopyrrolidin-1-yl carbonate (TCO-NHS), as previously reported^{20,25,26,26,27}. TCO-conjugation was performed with 0.5 mg of mouse or rabbit IgG antibody in the presence of 1000 equivalents of TCO-NHS in phosphate-buffered saline (PBS) and 10% dimethylformamide at room temperature for 3 hours. Unreacted TCO-NHS was subsequently washed using 2 ml of Zeba desalting columns (Thermo Fisher, Rockford, IL), and antibody concentrations were determined by absorbance measurement.

Preparation of tetrazine (Tz)-modified nanoparticles

Cross-linked iron oxide (CLIO) nanoparticles were prepared as previously discussed²⁶. Briefly, Tz-modified nanoparticles were synthesized by reacting NH₂-MNPs with 500 equivalents of 2,5-dioxopyrrolidin-1-yl 5-(4-(1,2,4,5-tetrazin-3-yl)benzylamino)-5-oxopentanoate (Tz-NHS). The reaction was carried out in PBS/5% dimethylformamide at room temperature for 3 hours, as previously described^{20,26,27}. Excess Tz-NHS was removed by gel filtration using Sephadex G-50 (GE Healthcare, Pittsburgh, PA).

μ NMR measurements

The μ NMR technology and magnetic nanoparticle labeling have been extensively tested in cell lines^{17,20,26}, mouse models of cancer¹⁷ and in clinical samples^{12,20,28,29}. Moreover, the performance μ NMR for cancer detection was compared to gold standard method (CellSearch)²⁰. In the present study, sample labeling and μ NMR measurements (Figure 1) were performed as previously optimized^{20,26}. The transverse relaxation rates (R₂) were determined in an effective sample volume of 1 μ L. μ NMR measurements and analysis of cellular expression were performed as previously described²⁰. Briefly, the following Carr-Purcell-Meiboom-Gill pulse sequences were used: echo time, 4 ms; repetition time, 6 s; number of 180° pulses per scan, 500; number of scans, 8. R₂ values from samples were subtracted from those of PBS buffer to calculate Δ R₂. The Δ R₂ of samples were normalized to Δ R₂ obtained from negative control to account for background signal. Negative controls for each sample were prepared similarly to the test samples without inclusion of the antibody incubation step. For each sample, measurement was done three times and the average value was obtained for analysis.

Results

FNA yield sufficient numbers of cells for analysis

Table 1 summarizes the enrolled patient cohort consisting of consecutive patients referred for CT guided biopsy of lung lesions or suspected lung cancer metastases in the adrenal glands or liver, and who consented to participate in the study. Table 2 summarizes the different cohorts and final pathologic diagnosis. Just before each procedure and on the same day peripheral blood was drawn for CTC analysis.

Following placement of the co-axial needle by CT guidance, 3-5 FNA passes were placed in saline from each lesion (Figure 1). Samples were incubated with TCO-modified antibodies for key marker proteins (Table 3) revealed by bioorthogonal reactions using magnetic nanoparticles and μ NMR analysis. Figure 2 summarizes the yields of total and malignant cell number for each specimen. In two of the 35 patients, the biopsy procedure was aborted due to medical reasons, leaving 33 patients for analysis. Overall, the attained specimens were sufficient for μ NMR analysis in 30 of the 33 patients (91 %) yielding an average of 1900 cancer cells per patient. In 3 patients, insufficient numbers of FNA cells were noted after the procedure.

DMR analyses are accurate in establishing subtypes

To establish the diagnosis of malignancy, we used a cocktail of 4 antibodies (EGFR, MUC, Her2, EpCAM, collectively known as the “Quad marker”). Indeed, this signature is more sensitive than EpCAM alone for diagnosing epithelial malignancies^{20,2512}. Differentiating between different subtypes of primary lung cancer and lung metastases was based on the expression levels of key and validated proteins (TTF-1, napsin A, synaptophysin, chromogranin, p40; Table 3)²¹⁻²⁴.

Figure 3 summarizes the FNA profiles across the 30 patients with sufficient FNA material available for analyses. The quad marker was positive (i.e. μ NMR⁺ diagnosis = malignant) in all 27 cancer patients, including in lung cancer patients with stage 1 disease with a primary tumor as small as 0.7 cm. μ NMR finding was negative in 3 patients with benign nodules and in 12 peripheral blood samples obtained from healthy individuals. TTF-1 and/or napsin A were positive in all adenocarcinomas, while p40 was positive in the four squamous cell cancers. Altogether, FNA analysis by μ NMR showed excellent concordance with conventional histopathology, both for establishing overall diagnoses and differential diagnoses (Figure 3). Table 4 summarizes the sensitivity, specificity and predictive values of μ -NMR analyses.

CTC and FNA analyses are concordant

We next explored whether CTC analyses could be performed in the same patient cohort described above. Peripheral blood analyses from 35 patients demonstrated, on average, 38 CTC per patient (7ml blood) with proven malignancy (Figure 4). There were 11 true negative (i.e. diagnosis of negative for lung malignancy based on gold standard and μ NMR-finding for CTC), four false negative (i.e. diagnosis of positive for lung malignancy based on gold standard but μ NMR- finding for CTC) and zero false positive samples (Table 4). The overall sensitivity for detecting lung cancer in blood was 83.3% with the specificity of 100% (Table 4). We noted good correlation between lung cancer biomarkers across CTC and FNA ($r^2=0.83$). Using patient by patient analyses, we then asked whether peripheral blood analysis could have obviated a lung biopsy. In 70% of the patient population, CTC were positive while 20% displayed FNA positive / CTC negative profiles, and 10% were negative for both CTC and FNA (Figure 4).

Differential diagnosis of CTC samples

Since the same differential diagnostic markers can be applied to CTC, we next determined whether such analyses could establish the primary tumor type based on blood sample alone (Figure 5). Our data indicate that CTC analyses correctly identified histologic subtypes in 18 of 20 patients (90%) with available matched samples.

Discussion

Advances in nanotechnology, microfabrication and chemistries have catalyzed miniaturized diagnostic platforms to potentially analyze scant clinical specimens. Success here would potentially reduce the morbidity associated with more invasive procedures, improve cost effectiveness through efficient use of reagents and time, allow for serial testing to tailor treatments, and promote point of care testing. There are two generic approaches: I) methods to isolate and purify malignant cells for subsequent analysis^{7,30} and II) methods that allow direct measurements without the need for purification^{12,19}. Each analytical approach has its unique advantages and applications in the clinical workflow. For any method to succeed it must be accurate, reproducible, fast and cost-effective. In particular, methods that allow real-time feedback would be highly advantageous to guide decisions during interventional procedures.

Here we leveraged the benefits of μ NMR to analyze clinically accessible samples. The approach has a number of advantages including fast turn around, high sensitivity and readouts without need for lengthy purification. The technology has previously been optimized for FNA analyses of abdominal malignancies¹², CTC²⁰ and exosome analyses¹⁸. The current study capitalized on some of the previously established malignancy markers (notably the quad signature of EpCAM, EGFR, Muc1 and Her2)¹² but also added clinically relevant differential diagnostic markers of lung cancers (napsin A, p40, TTF-1, synaptophysin and chromogranin; Table 3)²¹⁻²⁴.

Our results show that point-of-care, operator-independent μ NMR protein analysis of patient-derived FNA and peripheral blood samples is highly accurate in identifying the presence and establishing the type of lung malignancy. Achieving this in peripheral blood and/or limited biopsies can lead to a shift in the current standard of practice, with peripheral blood analysis preceding any invasive procedure, and FNA acquisition replacing the need of a more invasive core biopsy.

While the current results are very encouraging, we did not explore all potential aspects in this feasibility study. For example did not test for specific mutations to inform eligibility for molecular drug treatments (e.g. EGFR or Alk translocations) but such assays could be easily established through use of TCO labeled antibodies²⁷ (or other affinity ligands) with sufficient specificity. Second, the μ NMR method used here is based on read-outs of bulk samples (requiring ~ 50 cells depending on target and antibodies) and is not a single cell profiling technology. For the latter, alternative approaches such as μ Hall sensors¹⁹ or others could be used³¹. While not tested here, the μ NMR method is capable of broader genetic analyses as recently demonstrated for bacterial profiling^{32,33}. It is conceivable to integrate such testing with antibody approaches on the same chip platform. Finally, we did not test the method with bronchoscopy specimens although we would expect similar results given the typical cell numbers that are harvested³⁴. Similarly, we expect that the method could be used for analyses of other clinically relevant samples such as pleural fluid³⁵.

The current data justifies future lung cancer focused studies to further develop the technology and inform preclinical and early phase drug testing. We anticipate the use of additional markers to accompany diagnostic panels by leveraging the advanced multiplexing

capabilities of digital NMR technology. Attaining lung cancer subtype information along with any actionable molecular alterations rapidly (~1 hour) and at the point-of-care can help streamline the clinical research process. Same visit readouts can help initiate treatment strategies (e.g. selecting targeted therapies if adenocarcinoma versus chemotherapy if squamous cell) and promptly identify resistance markers to alter treatment course. Coupled with CTC as surrogates to invasive core biopsies, as demonstrated here, there is also strong potential for cost effective and safety advantages. Point-of-care μ NMR lung cancer cell analyses could thus function as a peripheral window into each individual's underlying biology and help meet the goals of precision medicine in this prevalent and often lethal disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank N. Sergeyev for synthesizing cross-linked dextran iron oxide nanoparticles, J. Klubnick for preparing TCO, S. Hilderbrand for synthesizing the tetrazine precursor, D. Issadore for μ NMR developments, M. Sebas, and B. Tricot for reading NMR samples.

This work was funded in part by K12CA087723 and DOD W81XWH-11-1-0706

References

1. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. *CA Cancer J Clin*. 2013; 63(1):11–30. [PubMed: 23335087]
2. Alamgeer M, Ganju V, Neil Watkins D. Novel therapeutic targets in non-small cell lung cancer. *Curr Opin Pharmacol*. 2013
3. Kwak EL, Bang YJ, Camidge DR, Shaw AT, Solomon B, Maki RG, et al. Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. *N Engl J Med*. 2010; 363(18):1693–1703. [PubMed: 20979469]
4. Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med*. 2004; 350(21):2129–2139. [PubMed: 15118073]
5. Tam AL, Kim ES, Lee JJ, Ensor JE, Hicks ME, Tang X, et al. Feasibility of Image-Guided Transthoracic Core-Needle Biopsy in the BATTLE Lung Trial. *J Thorac Oncol*. 2013; 8(4):436–442. [PubMed: 23442309]
6. Wu CC, Maher MM, Shepard JA. Complications of CT-guided percutaneous needle biopsy of the chest: prevention and management. *AJR Am J Roentgenol*. 2011; 196(6):W678–82. [PubMed: 21606253]
7. Nagrath S, Sequist LV, Maheswaran S, Bell DW, Irimia D, Ulkus L, et al. Isolation of rare circulating tumour cells in cancer patients by microchip technology. *Nature*. 2007; 450(7173):1235–1239. [PubMed: 18097410]
8. Sequist LV, Nagrath S, Toner M, Haber DA, Lynch TJ. The CTC-chip: an exciting new tool to detect circulating tumor cells in lung cancer patients. *J Thorac Oncol*. 2009; 4(3):281–283. [PubMed: 19247082]
9. Yu M, Stott S, Toner M, Maheswaran S, Haber DA. Circulating tumor cells: approaches to isolation and characterization. *J Cell Biol*. 2011; 192(3):373–382. [PubMed: 21300848]
10. Abouzgheib W, Bartter T, Dagher H, Pratter M, Klump W. A prospective study of the volume of pleural fluid required for accurate diagnosis of malignant pleural effusion. *Chest*. 2009; 135(4):999–1001. [PubMed: 19017891]
11. Alix-Panabieres C, Pantel K. Circulating tumor cells: liquid biopsy of cancer. *Clin Chem*. 2013; 59(1):110–118. [PubMed: 23014601]

12. Haun JB, Castro CM, Wang R, Peterson VM, Marinelli BS, Lee H, et al. Micro-NMR for rapid molecular analysis of human tumor samples. *Sci Transl Med*. 2011; 3(71):71ra16.
13. Nurwidya F, Takahashi F, Murakami A, Takahashi K. Epithelial mesenchymal transition in drug resistance and metastasis of lung cancer. *Cancer Res Treat*. 2012; 44(3):151–156. [PubMed: 23091440]
14. Maheswaran S, Haber DA. Circulating tumor cells: a window into cancer biology and metastasis. *Curr Opin Genet Dev*. 2010; 20(1):96–99. [PubMed: 20071161]
15. Ozkumur E, Shah AM, Ciciliano JC, Emmink BL, Miyamoto DT, Brachtel E, et al. Inertial focusing for tumor antigen-dependent and -independent sorting of rare circulating tumor cells. *Sci Transl Med*. 2013; 5(179):179ra47.
16. Shah AM, Yu M, Nakamura Z, Ciciliano J, Ulman M, Kotz K, et al. Biopolymer system for cell recovery from microfluidic cell capture devices. *Anal Chem*. 2012; 84(8):3682–3688. [PubMed: 22414137]
17. Lee H, Sun E, Ham D, Weissleder R. Chip-NMR biosensor for detection and molecular analysis of cells. *Nat Med*. 2008; 14(8):869–874. [PubMed: 18607350]
18. Shao H, Chung J, Balaj L, Charest A, Bigner DD, Carter BS, et al. Protein typing of circulating microvesicles allows real-time monitoring of glioblastoma therapy. *Nat Med*. 2012; 18(12):1835–1840. [PubMed: 23142818]
19. Issadore D, Chung J, Shao H, Liong M, Ghazani AA, Castro CM, et al. Ultrasensitive clinical enumeration of rare cells ex vivo using a micro-hall detector. *Sci Transl Med*. 2012; 4(141):141ra92.
20. Ghazani AA, Castro CM, Gorbato R, Lee H, Weissleder R. Sensitive and direct detection of circulating tumor cells by multimarker micro-nuclear magnetic resonance. *Neoplasia*. 2012; 14(5):388–395. [PubMed: 22745585]
21. Bishop JA, Teruya-Feldstein J, Westra WH, Pelosi G, Travis WD, Rekhtman N. p40 (DeltaNp63) is superior to p63 for the diagnosis of pulmonary squamous cell carcinoma. *Mod Pathol*. 2012; 25(3):405–415. [PubMed: 22056955]
22. Mukhopadhyay S, Katzenstein AL. Subclassification of non-small cell lung carcinomas lacking morphologic differentiation on biopsy specimens: Utility of an immunohistochemical panel containing TTF-1, napsin A, p63, and CK5/6. *Am J Surg Pathol*. 2011; 35(1):15–25. [PubMed: 21164283]
23. Tan D, Zander DS. Immunohistochemistry for assessment of pulmonary and pleural neoplasms: a review and update. *Int J Clin Exp Pathol*. 2008; 1(1):19–31. [PubMed: 18784820]
24. Turner BM, Cagle PT, Sainz IM, Fukuoka J, Shen SS, Jagirdar J. Napsin A, a new marker for lung adenocarcinoma, is complementary and more sensitive and specific than thyroid transcription factor 1 in the differential diagnosis of primary pulmonary carcinoma: evaluation of 1674 cases by tissue microarray. *Arch Pathol Lab Med*. 2012; 136(2):163–171. [PubMed: 22288963]
25. Ghazani AA, McDermott S, Pectasides M, Sebas M, Mino-Kenudson M, Lee H, et al. Comparison of select cancer biomarkers in human circulating and bulk tumor cells using magnetic nanoparticles and miniaturized micro-NMR system. *Nanomedicine*. 2013
26. Haun JB, Yoon TJ, Lee H, Weissleder R. Molecular detection of biomarkers and cells using magnetic nanoparticles and diagnostic magnetic resonance. *Methods Mol Biol*. 2011:72633–49.
27. Haun JB, Devaraj NK, Hilderbrand SA, Lee H, Weissleder R. Bioorthogonal chemistry amplifies nanoparticle binding and enhances the sensitivity of cell detection. *Nat Nanotechnol*. 2010; 5(9):660–665. [PubMed: 20676091]
28. Lee H, Yoon TJ, Figueiredo JL, Swirski FK, Weissleder R. Rapid detection and profiling of cancer cells in fine-needle aspirates. *Proc Natl Acad Sci U S A*. 2009; 106(30):12459–12464. [PubMed: 19620715]
29. Shao H, Min C, Issadore D, Liong M, Yoon TJ, Weissleder R, et al. Magnetic Nanoparticles and microNMR for Diagnostic Applications. *Theranostics*. 2012; 2(1):55–65. [PubMed: 22272219]
30. Talasaz AH, Powell AA, Huber DE, Berbee JG, Roh KH, Yu W, et al. Isolating highly enriched populations of circulating epithelial cells and other rare cells from blood using a magnetic sweeper device. *Proc Natl Acad Sci U S A*. 2009; 106(10):3970–3975. [PubMed: 19234122]

31. Zong C, Lu S, Chapman AR, Xie XS. Genome-wide detection of single-nucleotide and copy-number variations of a single human cell. *Science*. 2012; 338(6114):1622–1626. [PubMed: 23258894]
32. Chung HJ, Castro CM, Im H, Lee H, Weissleder R. A magneto-DNA nanoparticle system for rapid detection and phenotyping of bacteria. *Nat Nanotechnol*. 2013; 8(5):369–375. [PubMed: 23644570]
33. Liong M, Hoang AN, Chung J, Gural N, Ford CB, Min C, et al. Magnetic barcode assay for genetic detection of pathogens. *Nat Commun*. 2013; 4:1752. [PubMed: 23612293]
34. Iyoda A, Baba M, Shibuya K, Moriya Y, Yasufuku K, Sekine Y, et al. Transbronchial fine needle aspiration cytological examination: a useful tool for diagnosing primary lung cancer. *Thorac Cardiovasc Surg*. 2006; 54(2):117–119. [PubMed: 16541353]
35. Light RW. Clinical practice. Pleural effusion. *N Engl J Med*. 2002; 346(25):1971–1977. [PubMed: 12075059]

We tested our recently developed point-of-care, operator-independent μ NMR device to perform protein analysis of tumor cells for diagnosing pulmonary malignancy. In this clinical study, analysis of both fine needle aspirate (FNA) of primary tumors and blood circulating tumor cells (CTC) was shown to be highly accurate in identifying the presence and establishing the type of lung malignancy.

Figure 1. Study outline

Patients were consented for a peripheral blood sample prior to the procedure as well as an FNA sample during a routine CT guided lung biopsy. Aliquots of samples were incubated with either TCO-modified antibody cocktails (quad, cocktail) or single, specific antibodies. After washing, antibodies were revealed with magnetic nanoparticles detected by μ NMR.

Figure 2. Obtaining cells from fine needle aspirates (FNA)
Left: Representative example showing CT guided placement of a 20G coaxial needle within a nodule in the middle lobe of the right lung. Right, top: Total number of cells harvested via FNA. Right bottom: Number of cancer cells (quad positive) harvested by FNA sampling.

Figure 3. μ NMR analysis of FNA samples

Left: Raw μ NMR data for the different biomarkers tested. Analysis of the different biomarkers into histologic types shows good agreement between μ NMR and conventional histology (from surgical samples or core biopsies). All malignant and benign masses were correctly classified. Right: Histology images of lung cancer subtypes. Top: Lung adenocarcinoma exhibiting gland-forming malignant cells. Middle: Moderately differentiated squamous cell carcinoma demonstrating a nest of polygonal cells with eosinophilic cytoplasm and intercellular bridges. Bottom: Small cell carcinoma characterized by nests of malignant cells with a high nuclear cytoplasmic ratio, prominent apoptosis and mitotic activity. All images were obtained at 400X magnification.

Figure 4. Analysis of circulating cancer cells

Quad-NMR analysis of peripheral blood samples showed variable numbers of CTC. In 71% of all cases with positive FNA, there were also CTC present. CTC were negative in 19% of positive FNA samples. There was a correlation between circulating cancer cells and number of cancer cells harvested by FNA analysis ($r^2 = 0.83$).

Figure 5. Differential diagnosis of CTC

Left: raw μ NMR data for the different biomarkers tested in CTC. Analysis of the different biomarkers into histologic types shows reasonable agreement in over 85% of cases. Right: fluorescent images of quad marker labeling of CTC (top) and CTC and DAPI staining (bottom). Fluorescent images were obtained at 40X magnification.

Table 1

Overview of patient population

Characteristic	Number	Percentage
Number of patients	35	
Age		
Median	70.5	
Range	21-87	
Gender		
Male	21	60
Female	14	40
Primary Lung Cancer	24	68.6
Tumor Subtypes		
Adenocarcinoma	17	48.6
Squamous Cell Carcinoma	4	11.4
Large Cell Carcinoma	1	2.9
Small Cell Carcinoma (SCLC)	2	5.7
Stage		
IA	4	11.4
IB	3	8.6
IIA	1	2.9
IIB	4	11.4
IIIA	2	5.7
IIIB	0	0
IV	8	22.9
Limited Stage SCLC	2	5.7
Advanced Stage SCLC	0	0
Lung Metastases	7	20
Melanoma	4	11.4
Renal Cell Carcinoma	1	2.9
Ewing's Sarcoma	1	2.9
Unknown Primary	1	2.9
Benign Lesions	4	11.4

Detailed patient information

The table shows patients' clinical information and whether FNA and blood were obtained from each patient (last 2 columns). FNA samples from three patients (pts 4, 6 and 12) were acellular; with not enough material to proceed with DMR. All patient had pulmonary nodules or masses ranging from 0.7 -9.0 cm in diameter. There were 17 adenocarcinomas, 4 squamous cell carcinoma, 2 small cell carcinomas, 1 large cell carcinoma, 7 metastases and 4 benign nodules.

Table 2

#	Pathology	Sex	Age	Diameter	Stage	FNA	Blood
1	Adeno	Female	69	2.6 cm	IIB	yes	yes
2	Adeno	Female	71	3.0 cm	IIB	yes	yes
3	Adeno	Female	71	1.2 cm	IA	yes	yes
4	Adeno	Female	75	0.7 cm	IV	acellular	yes
5	Adeno	Female	70	4.7 cm	IV	yes	yes
6	Adeno	Female	67	2.8 cm	IV	acellular	yes
7	Adeno	Male	56	2.3 cm	IA	yes	yes
8	Adeno	Male	71	8.2 cm	IIIA	yes	yes
9	Adeno	Male	74	1.9 cm	IV	yes	yes
10	Adeno	Male	73	6.4 cm	IV	yes	yes
11	Adeno	Female	77	1.0 cm	IA	yes	yes
12	Adeno	Male	78	3.7 cm	IIB	acellular	yes
13	Adeno	Male	87	2.4 cm	IIIA	yes	yes
14	Adeno	Male	58	2.4 cm	IA	yes	yes
15	Adeno	Female	47	4.2 cm	IIA	yes	yes
16	Adeno	Male	57	3.5 cm	IB	yes	yes
17	Adeno	Male	65	2.0 cm	IV	yes	yes
18	Squamous	Male	75	5.0 cm	IV	yes	yes
19	Squamous	Male	64	2.2 cm	IB	yes	yes
20	Squamous	Male	75	3.2 cm	IB	yes	yes
21	Squamous	Female	81	2.0 cm	IV	yes	yes
22	Small	Female	81	3.2 cm	Limited Stage	yes	yes
23	Small	Female	73	2.0 cm	Limited Stage	yes	yes
24	Large	Male	54	6.5 cm	IIB	yes	yes
25	Met	Male	73	1.7 cm	IV	yes	yes

#	Pathology	Sex	Age	Diameter	Stage	FNA	Blood
26	Met	Male	78	3.1 cm	IV	yes	yes
27	Met	Female	43	2.0 cm	IV	yes	yes
28	Met	Male	65	1.9 cm	IV	no	yes
29	Met	Male	70	4.3 cm	IV	yes	yes
30	Met	Male	21	1.2 cm	Metastatic	yes	yes
31	Met	Female	78	2.3 cm	IV	yes	yes
32	Benign	Male	68	4.9 cm		yes	yes
33	Benign	Male	78	2.3 cm		no	yes
34	Benign	Female	59	1.4 cm		yes	yes
35	Benign	Male	66	2.2 cm		yes	yes

Molecular markers used for diagnosis and sub-typing

Table 3

The quad marker test was used to determine if a malignancy was present. The quad marker contains four antibodies against common tumor markers (EGFR, EpCAM, MUC-1 and Her2). Adenocarcinoma was identified by positivity for TTF-1 and/or Napsin A. Squamous cell carcinoma was identified by positivity of p40. Small cell lung cancer was diagnosed by a positive signature for synaptophysin and/or chromogranin. Large cell carcinoma and metastasis were negative for all lung cancer specific panel of markers (TTF-1, Napsin A, p40, synaptophysin, chromogranin).

Category	Question	Quad EGFR + EpCAM + Muc + Her2	TTF1	Napsin A	p40	Cocktail Synaptophysin + Chromogranin
Malignancy	Malignant	+				
	Benign	-				
Primary	Lung	+	+			
	Met	+	-			
Type	Adenocarcinoma	+	+	+	+/-	-
	Squamous cell	+	-	-	+	-
	Small cell carcinoma	+	+/-	-	-	+
	Large cell carcinoma or metastasis	+	-	-	-	-

Table 4
Diagnostic accuracy of μ NMR

Data are shown separately for FNA analysis of primary lung lesions and for CTC analysis. Note that calculations are based on all samples, including those with insufficient material for DMR processing (thus the overall reported sensitivity is reduced).

	FNA (n = 33)	CTC (n = 35)
Sensitivity	22/24 (91.6%)	20/24 (83.3%)
Specificity	8/8 (100%)	11/11 (100%)
PPV	22/22 (100%)	20/20 (100%)
NPV	8/10 (80%)	11/15 (73.3%)
Total samples for analysis	33	35
Not enough cells	3	
True neg	8	11
False neg	2	4
True pos ("lung" cancer cases)	22	20
False pos	0	0