Pulmonary venous blood sampling significantly increases the yield of circulating tumor cells in early-stage lung cancer

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ABSTRACT

Objective: To identify circulating tumor cells (CTCs) in the blood of patients with early-stage lung cancer and to show that sampling pulmonary vein (PV) blood using microfluidic chip technology will yield significantly more CTCs. Improving early detection of lung cancer is critical to improving lung cancer survival. Reproducible detection of CTCs is limited currently in early stage tumors.

Methods: Patients undergoing pulmonary resection had PV blood drawn before resection. Peripheral blood was sampled at preoperative, intraoperative, and postoperative times. Samples were analyzed on microfluidic chips using antibody-based capture.

Results: A total of 32 patients with primary lung cancer were evaluated. Twenty patients had 1 or more CTCs detected in at least 1 sample (62.5%). The mean number of CTCs from peripheral vein sources at the preoperative, intraoperative, and postoperative time points was 1.3, 1.9, and 0.6 respectively. The average number of CTCs in the PV was 340.0 (range, 0.0-5422.50; $P > .01$). When PV CTCs were present, the number of CTCs was correlated with pathological tumor size ($P = .0236$). The number of PV CTCs was not correlated with any other clinical feature (eg, smoking status, preoperative or postoperative stage). Furthermore, the number of PV CTCs was significantly higher when preoperative bronchoscopic biopsy was performed, compared with computed tomography-guided biopsy ($P = .0311$). Seven patients had evidence of CTC clusters, or microemboli.

Conclusions: With a single vein draining the entire tumor basin, lung cancers are unique, allowing the high-yield isolation of CTCs from the PV. This method may facilitate future studies to improve the detection and analysis of early-stage lung CTCs. (J Thorac Cardiovasc Surg 2016;151:852-8)

Cluster of lung cancer circulating tumor cells found in the pulmonary vein.

Central Message

Pulmonary vein sampling results in significantly higher yields of early-stage lung cancer circulating tumor cells.

Perspective

Identifying circulating tumor cells offers the opportunity to develop blood tests for early detection of lung cancer. Increasing the yield of cells will allow for improved characterization to find better markers to detect low concentrations of cells, creating an alternative to radiologic films by leading to a cost-effective and low-risk early detection method for lung cancer.

See Editorial Commentary page 859.

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Improving the early detection of lung cancer, a disease that causes 27% of all cancer-related deaths in the United States, is critical, given that patients with early-stage tumors have a 5-year survival rate of 52%, compared with <5% for patients with metastatic disease.¹ Almost three-quarters of lung cancers are diagnosed at a stage too advanced for surgical intervention. Recent computed tomography (CT) scan screening trials have shown that early detection results in improved long-term survival,² but increased radiation from CT scans has been implicated in increasing long-term cancer risk.³ Alternative methods to detect or rule out early-stage cancers would dramatically change the current paradigm of lung cancer diagnosis and

Abbreviations and Acronyms

CT	= computed tomography
CTC	= circulating tumor cell
DAPI	= 4',6-diamidino-2-phenylindole
EpCAM	= epithelial cell adhesion molecule
PET SUV	= positron emission tomography standardized uptake value
PV	= pulmonary vein

treatment, and result in significant improvement in survival by stage-shifting patients alone.

Identifying circulating tumor cells (CTCs) offers a unique strategy for detecting cancers through a blood sample. Circulating cells originating from solid tumors can be separated from normal hematopoietic cells based on size and other inherent physical and biological properties. Immunomagnetic separation using epithelial-based antibodies, such as epithelial cell adhesion molecule (EpCAM), has been used successfully to identify CTCs in patients with lung, prostate, colon, breast, and pancreatic cancers, but isolates only small numbers of CTCs (range, 1-11 CTCs/mL in solid organ tumors), with very low purity (0.01%-0.1%), and low yield (only ~20%-60% of patients).⁴ The level of “biological noise” associated with the low sensitivity, selectivity, and yield of magnetic bead-based technologies is prohibitive to their capacity for early cancer detection. Also limiting early cancer detection is the inability to further analyze the rare cells found using current methods. The development of strategies for increasing the yield of CTCs is needed to identify new CTC markers specific to early cancers that can be used to identify antibodies that will lead to increased sensitivity and specificity of CTC detection.

Microfluidic technology offers advantages over immunomagnetic separation and other strategies for isolating CTCs, including improved yield, lower blood volume required, and the potential to yield viable CTCs.⁵ Recent advances in microfabrication and microfluidics have led to the ability to detect CTCs at levels of 5 to 100 CTCs/mL in whole blood.^{6,7} Along with EpCAM, further labeling using cytokeratin (CK), 4',6-diamidino-2-phenylindole (DAPI), and CD45 antibodies allows the isolation of a subpopulation of circulating cells separate from normal hematopoietic cells that are likely CTCs. A complementary strategy for increasing the yield of CTCs, which may be unique to lung cancer among solid organ tumors owing to lung anatomy and operative technique, is to sample blood directly from a pulmonary vein (PV) within the lung itself, from the lobe that contains the tumor. Sampling PV blood from the lobectomy specimen after resection has been described using CellSearch (Janssen Diagnostics, LLC, Raritan, NJ), an immunomagnetic bead separation platform.⁸

Although antibody-based immunomagnetic and microfluidic techniques result in cells that are fixed and stained, future microfluidic antibody-free methods would allow for the capture of viable cells. By increasing the number of cells, fixed or otherwise, further analysis of CTC phenotype and genotype is possible. The aim of the present study was to evaluate the sampling of blood from the PV before resection of the affected lobe in early-stage lung cancers, using EpCAM-based microfluidic chips to increase the yield of CTCs compared with that achieved with peripheral blood sampling. As a secondary analysis, we hoped to evaluate clinical factors (eg, stage, histology, tumor manipulation) and their relationship to the number of CTCs found.

METHODS

Between January 2013 and January 2014, we prospectively enrolled 32 patients with surgically resectable lung cancer. Enrolled patients underwent lung resection via open thoracotomy or video-assisted thoroscopic surgery, with wedge resection, wedge resection followed by lobectomy, or lobectomy. Between 6 and 8 mL of blood was drawn from a peripheral vein before the start of surgery (preoperative peripheral). The PV draining the affected lobe was isolated first using minimal manipulation of the lung. Up to 5 mL of PV blood was removed using a 25-gauge needle attached to a 10-mL syringe (a 25-gauge spinal needle for video-assisted thoroscopic surgery) before removal of the lesion by wedge resection or lobectomy (intraoperative PV). Concurrently, 6 to 8 mL of peripheral blood was obtained via a peripheral arterial or venous line (intraoperative peripheral). The locations of blood sampling are shown in Figure 1, A. All specimens were collected in EDTA tubes and then submitted for immediate analysis. Pathological confirmation of lung cancer in patients with suspected malignancy was obtained by nonanatomic wedge resection biopsy, and the remainder of the pulmonary resection (lobectomy) procedure was completed using standard surgical techniques. A final 6 to 8 mL blood sample was drawn on postoperative day 1 or 2 after surgery (postoperative peripheral). The timing of blood draws is shown in Figure 1, B.

CTCs were isolated from the preoperative, intraoperative, and postoperative blood samples. Samples were processed using an EpCAM-based microfluidic chip. CK, DAPI, and CD45 labeling was used to score for CTCs and eliminate contaminating white blood cells. CK is positive in many CTCs, DAPI is positive in nucleated cells, eliminating red blood cells, and CD45 is positive in white blood cells, allowing for negative selection. Individual antibodies or combinations of EpCAM, EGFR, and CD133 antibodies were used to capture purported CTCs. For immunomagnetic bead strategies, the threshold of ≤ 5 CTCs/7.5 mL was considered “background noise”; that is, only the presence of >5 CTCs per 7.5 mL of blood was considered positive for CTCs. The data were analyzed as raw data and also as the number of CTCs above the threshold of 5. The concentration of CTCs was calculated from the number of CTCs identified histologically by volume of blood drawn and reported the number of CTCs/7.5 mL of blood, in concordance with previous CTC studies.

The Kruskal-Wallis test was used to compare the number of CTCs in PV samples and peripheral blood samples at preoperative, intraoperative, and postoperative time points. Clinical data for the enrolled patients were gathered prospectively, including staging information (eg, T stage, maximum positron emission tomography standardized uptake value, biopsy methods), perioperative data (eg minimally invasive vs open thoracotomy), and postoperative histology (eg, grade). Associations between perioperative factors and PV CTC numbers were evaluated using

FIGURE 1. A, Schematic showing sites of blood draws to identify CTCs. B, Timing of blood draws relative to surgery.

simple linear regression analysis for continuous factors or the Wilcoxon rank-sum test for binary factors. All analyses were performed using SAS 9.4 software (SAS Institute, Cary, NC). A P value $< .05$ was considered significant.

RESULTS

Clinical data from the 32 patients are presented in Table 1. The average patient age was 70 years, and 56% were male. Approximately two-thirds of the patients underwent preoperative biopsy, by either bronchoscopy or radiologic (CT scan) guidance. The average clinical and pathological tumor size was 3.2 cm, but notable upstaging was seen, with 53% of patients clinically stage IA but only 36% pathologically stage IA. A majority of patients had adenocarcinoma, with squamous cell cancer the second most common histology. Of note, 3 patients had small-cell lung cancer.

Using the EpCAM-based microfluidic chip capture system, 20 of 32 patients had detectable CTCs (62.5% detection rate) at any time point or sample. Seven patients had microemboli or CTC clusters (Figure 2). There were no clinical or pathological differences between the patients with and those without detectable CTCs. Table 2 displays the average number of CTCs (per 7.5 mL of blood) found at all sampling time points, giving raw data results, the using the 5 CTCs/7.5 mL of blood threshold, and the raw data when collating only the group of CTC-positive samples ($n = 20$). In all 3 groups, the PV samples contained a statistically significant higher number of CTCs, with an average of 544 CTCs/7.5 mL of blood in the patients with EpCAM-positive samples. When including the 12 patients who had no CTCs captured, the average from the PV samples was still 340 CTCs/7.5 mL of blood, using the 5-CTC threshold. No patients had more CTCs in the peripheral blood samples compared with the PV blood samples.

Using linear regression analysis to evaluate the number of PV CTCs in patients with CTCs present, we found that only postoperative tumor size was correlated with the

TABLE 1. Demographic data for 32 lung cancer patients

Variable	Value
Age, y, mean $\pm$ SD	70.0 $\pm$ 11.5
Male sex, n (%)	18 (56)
Pack years, mean $\pm$ SD	36.5 $\pm$ 29.8
Preoperative biopsy, n (%)	21 (66)
CT biopsy	12 (57)
Bronchoscopic biopsy	9 (43)
Preoperative stage, n (%)	
IA	17 (53)
IB	6 (19)
IIA	6 (19)
IIB	2 (6)
IIIA	1 (3)
cT size, cm, mean $\pm$ SD	3.2 $\pm$ 1.9
Postoperative stage, n (%)	
IA	12 (38)
IB	9 (28)
IIA	3 (9)
IIB	3 (9)
IIIA	4 (12)
IV	1 (3)
pT size, cm, mean $\pm$ SD	3.2 $\pm$ 2.0
Histology, n (%)	
Adenocarcinoma	17 (53)
Squamous cell	10 (31)
Small cell	3 (9)
Carcinoid	2 (6)
Lobectomy, n (%)	28 (88)
Lymph nodes, mean $\pm$ SD	10.2 $\pm$ 7.6
Lymph node stations, mean $\pm$ SD	3.5 $\pm$ 1.1

SD, Standard deviation; CT, computed tomography; cT, clinical T; pT, pathologic T.

FIGURE 2. Representative picture of a CTC cluster. EpCAM-positive CTCs are DAPI (blue) and cytokeratin (CK) 7/8 (red) positive, and CD45 (green) negative. The fluorescent images are combined in the lower right picture. DAPI, 4',6-diamidino-2-phenylindole.

number of PV CTCs using either the raw data or the standard threshold of >5 CTCs per 7.5 mL of blood (Table 3). Other factors, including preoperative tumor size, positron emission tomography standardized uptake value, and

positive lymph nodes, were not correlated. Figure 3, A shows a scatterplot of PV CTCs compared with pathological tumor size ($R^2 = 0.354$). In the CTC-positive patients, analysis of the associations between the number of CTCs and clinical factors revealed a correlation between the bronchoscopic biopsy method and the number of CTCs in the PV at both thresholds (Table 4). PV CTCs were detected in 8 of the 9 patients who underwent preoperative bronchoscopic biopsy, compared with only 4 of the 12 patients who underwent CT-guided biopsy. The use of bronchoscopic (vs CT-guided) biopsy did not correlate with peripheral versus central tumor location or with

TABLE 2. Number of CTCs

Variable	CTCs/7.5 mL of blood, mean ± SD
Raw data	
Preoperative peripheral	3.4 ± 5.6
Intraoperative peripheral	3.5 ± 6.9
Intraoperative PV	343 ± 1233
Postoperative peripheral	2.5 ± 3.4
5 CTC cutoff	
Preoperative peripheral	1.3 ± 4.4
Intraoperative peripheral	1.9 ± 5.6
Intraoperative PV	340 ± 1233
Postoperative peripheral	0.6 ± 1.6
+ CTCs only	
Preoperative peripheral	2 ± 5.4
Intraoperative peripheral	3.1 ± 6.9
Intraoperative PV	544 ± 1537
Postoperative peripheral	0.9 ± 1.9

Five CTCs/7.5 mL of blood is a common cutoff used for CTC detection systems. Raw data also are displayed as the number of CTCs/7.5 mL of blood. Only 20 of the 32 patients had detectable CTCs (62.5% rate of detection). The “+ CTCs only” rows presents the data from patients with only positive CTCs. There was a significant difference in the number of PV CTCs compared with other sample points ($P < .01$). CTC, Circulating tumor cell; SD, standard deviation; PV, pulmonary vein.

TABLE 3. Association of preoperative factors with the number of PV CTCs in patients with positive CTCs

Variable	5-CTC cutoff, Raw CTCs,	
	P value	P value
Age	.633	.7230
Positron emission tomography standardized uptake value	.5084	.6483
Postoperative tumor size	.0236	.0398
Number of lymph nodes	.5525	.8696
Pack years	.9299	.5587
Preoperative tumor size	.5138	.5539

The number of PV CTCs correlates with postoperative tumor size. P values < .05 are in red type. CTC, Circulating tumor cell.

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FIGURE 3. A, Scatterplot of PV CTCs on a log scale versus pathologic tumor size with all data points. B, Difference in pulmonary vein CTC numbers by preoperative biopsy analysis. Bronchoscopic biopsy specimens were associated with higher PV CTCs. PV, Pulmonary vein, CT, computed tomography.

tumor size; likewise, the number of CTCs did not correlate with central versus peripheral tumor location. The range of PV CTCs detected in the 2 biopsy methods is shown in Figure 3, B. When we used a threshold of 2 or 0 CTCs/7.5 mL of blood, the use of a lobectomy (vs wedge resection) was also correlated with the presence of CTCs. Only 4 patients underwent wedge resection secondary to limited pulmonary function and small tumor size; the remaining 28 patients underwent lobectomy (Table 4). Microemboli, or clusters, were present in both PV and peripheral blood samples in 7 patients. The presence of microemboli in the PV or peripheral vein samples was not correlated with any other clinical factor.

TABLE 4. Associations of preoperative factors and numbers of PV CTCs in patients with detectable CTCs

Variable	5-CTC cutoff, P value	Raw CTCs, P value
Biopsy method	.0311	.0498
Grade	.4995	.8955
Preoperative T	.3002	.3576
Preoperative stage	.269	.3019
Postoperative T	.6167	.4795
Postoperative M	.3544	.6232
Postoperative N	.745	.9242
Upstaged	.819	.5827
Postoperative stage	.6275	.8462
Histology	.396	.4644
Video-assisted thoracoscopic surgery	.6847	.8332
Lobectomy	.0513	.02
Wedge/lobectomy	.6253	.5834
Peripheral nodule	.4463	.9393
Pleural invasion	.6323	.48
Capture antibody number	.5923	.4156

P values < .05 are in red type. CTC, Circulating tumor cell.

DISCUSSION

Our data confirm that PV sampling combined with a sensitive microfluidic chip capture system can provide a much higher yield of CTCs in early-stage lung cancers than has been reported previously. As others have noted, not all tumors shed EpCAM-positive cells; in this study, only 20 of 32 subjects had CTCs captured. Even when using the additional capture antibodies of EGFR and CD133, we still had samples with no CTCs detected. We found no clinical differences between patients with CTCs detected and those without CTCs detected. Either the 12 patients without detectable CTCs truly had zero CTCs, or we may have missing alternative CTCs that possibly may have a mesenchymal phenotype and cannot be captured using an EpCAM-based strategy.^{9,10} Alternative capture antibodies specific to mesenchymal cells could be used in the future to detect potential CTCs in this group, which composed more than one-third of the dataset. Alternative approaches to increase the detection rate of CTCs include size-based capture systems, which also could minimize this limitation.^{11,12} Along with the CTCs detected in the PV samples, CTCs were present at low levels in the peripheral blood samples of these patients with early-stage cancer. Further work is needed to confirm that these CTCs are truly tumor cells and not just epithelial cells above the 5-CTC/7.5 mL threshold.

Analysis of clinical and histological factors related to the number and presence of CTCs, revealed that patients who underwent bronchoscopic biopsy were more likely to have detectable CTCs than those who underwent CT-guided biopsy. Other authors have reported that lung manipulation at the time of surgery results in increased shedding, or release, of CTCs,^{13,14} suggesting that our findings could be real. The clinical implications of these higher numbers of CTCs are unknown.

Related to the lung manipulation studies, our comparison of patients who underwent minimally invasive surgery and those who had open surgery revealed no difference in CTC numbers based on approach. The majority of our lung resections were performed with a minimally invasive approach, limiting the potential to analyze this variable. Even for the biopsy data, our sample size was small and not appropriately powered, and further work is needed to confirm these findings. If these results are validated, follow-up studies may be needed to evaluate the effect of higher numbers of PV CTCs on long-term survival and to investigate whether or not the type of biopsy actually contributes to worse outcomes.

The other novel finding of this study is that in patients with detectable CTCs, the number of PV CTCs was correlated with pathological tumor size. We found no correlation with nodal stage or with overall stage, but a larger series may be needed to show a relationship to overall stage.

This study is a preliminary foray in combining 2 novel strategies, PV sampling and microfluidic technology, to increase the yield of CTCs, and as such has several limitations. Twelve patients had no detectable CTCs using our current EpCAM-based capture system. The specific capture antibodies must be modified to improve CTC detection, looking specifically for mesenchymal markers. Despite statistically significant differences based on biopsy method, a greater number of subjects is needed to evaluate the biopsy findings. We are currently prospectively sampling peripheral blood from selected patients within this cohort to determine whether there is a correlation between CTCs and recurrence.

With a single vein draining the entire tumor basin, lung cancers are unique among solid organ tumors. Reproducible and high-yield isolation of CTCs from pulmonary venous drainage may facilitate future studies of CTCs by helping to identify better CTC markers, given our ability to analyze hundreds, if not thousands, of cells for more specific surface markers. This would allow the use of CTCs as a possible biomarker in the diagnosis and treatment of lung cancer patients. We envision the ability to use a peripheral blood sample to identify lung cancer in high-risk patients, or to differentiate lung cancer from benign nodules in patients with indeterminate lung nodules on radiologic scans.

Conflict of Interest Statement

Rishindra M. Reddy reports consulting and lecture fees from Covidien and as well consulting fees from Intuitive. All other authors have nothing to disclose with regard to commercial support.

You can watch a Webcast of this AATS meeting presentation by going to: http://webcast.aats.org/2015/Video/Tuesday/04-28-15_613_0720_Reddy.mp4.

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Key Words: lung cancer, circulating tumor cells, early cancer detection

Discussion

Dr J. Donington (New York, NY). Rishi, congratulations. This is a great presentation and really interesting technology. I also really congratulate you on having Dr Del Nido provide the basic science lecture that really gave us insight into how this kind of physiologic flow and shear stress helps in the detection of these circulating tumor cells.

CTCs have tremendous potential in lung cancer, especially in advanced disease where biopsy can be hard, and yet it is important to follow how people respond to therapy and how they may mutate their tumors in response to therapy. Clearly the role here is a little more ambiguous.

I have a couple of questions. One, you chose to do your peripheral samples in a venous draw rather than an arterial

draw. Is there any concept that a first pass through the capillaries in the periphery may decrease yield in a peripheral yield?

Dr Reddy. That is an excellent question. We have not investigated that. Actually, the peripheral draws were done through both, and in that first foray we actually didn't document that. Having said that, we are documenting this going forward.

Dr Donington. My next question really has to do with the concept of using this for diagnosis. It seems like a pretty long leap right now. I don't know of any studies right now that are using this for early detection, either as a stand-alone or as a companion biomarker. What about prognosis? Is it something we can use? Is there any evidence that finding CTCs, especially in a pulmonary vein, which you are not going to be accessing in a wide range of people, might help us to know who would require adjuvant therapy or different therapy postoperatively?

Dr Reddy. That is definitely a possibility. Originally when we were looking at this, I thought the benefit would be from getting a larger number of CTCs that can allow us to look for other markers that may help us find those rare CTCs in a peripheral blood draw in the future. Having said that, one of our highest CTC counts was from someone who clinically had stage IIIA but then later was found to have stage IV and didn't recognize it at the time, and that person had approximately 5000 CTCs in a pulmonary vein blood draw. We are tracking all patients prospectively who have had pulmonary vein sampling to determine this, and thus there may be a role for that also.

Dr Donington. Great. Excellent work. Thank you.

Dr D. Harpole (*Durham, NC*). I have just one quick comment. Did you look at the location of the tumor and the relationship between the numbers? I'm wondering if your bronchoscopic biopsy patients actually had more

central tumors, which may be the reason that the number of CTCs you saw was higher than the ones that were done by CT, which may have been more peripheral.

Dr Reddy. That is an excellent question. Actually, I discuss that in the manuscript. After finding the difference in biopsy method, I went back and actually reevaluated personally all of the scans to document location. Tumor location, central versus peripheral, did not correlate with CTC numbers, nor did it correlate with type of biopsy. Our pulmonologists do our bronchoscopic biopsies with Super-D if needed for even peripheral tumors.

Dr Harpole. This is excellent work. I think that one of the problems that people have who are investigating this is they are trying to get yields from peripheral blood that then they can look at molecular markers and so forth and their yield is low, and so they abandoned this for anyone but those with advanced disease. So I think this actually, as a proof of principle, allows you to collect enough CTCs to really do some investigative diagnostics, and then move that to the periphery. I applaud you for sort of reintroducing this field that had languished for a while, and you are using a better technology. Thank you.

Dr D. Nguyen (*Miami, Fla*). I just want to ask you one question. Are these cells viable when you collect them?

Dr Reddy. In the current platform, they are not. They are fixed and stained. But we do have a label-free or antibody-free chip that uses size-based detection that we are also investigating right now that will produce viable cells.

Dr Nguyen. Can you speculate if the cells that are collected peripherally and centrally are morphologically or genotypically different?

Dr Reddy. We don't know yet. We are doing that work currently.