Tumor cells are dislodged into the pulmonary vein during lobectomy.

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Tumor Cells are Dislodged into the Pulmonary Vein During Lobectomy

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Abstract

Objective—Intraoperative tumor shedding may facilitate tumor dissemination. Earlier studies defined shed tumor cells primarily by cytomorphological examination, and could not always distinguish normal epithelial cells from tumor cells. We sought to accurately identify tumor cells using single-cell sequencing and determine whether these cells were mobilized into the circulation during pulmonary lobectomy.

Methods—We analyzed 42 blood samples collected from the tumor-draining pulmonary vein at the end of lobectomy procedures. We used arrays of nanowells to enumerate and retrieve single EpCAM+ cells. Targeted sequencing of 10–15 cells and nested PCR of single cells detected somatic mutations in shed epithelial cells consistent with patient-matched tumor but not normal tissue.

Results—The mean of EpCAM+ cells in VATS lobectomy (no wedge) specimens (n=16) was 165 (median 115; range 0–509) but sampling cells from three patients indicated that only 0–38% of the EpCAM+ cells were tumor cells. The mean of VATS lobectomy (wedge) specimens (n=12) was 1128 (median 197; range 47–9406) and all of the EpCAM+ cells were normal epithelial cells.

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in two patients sampled. The mean of EpCAM$^+$ cells in thoracotomy specimens (n=14) was 238 (median 22; range 9–2920) and 0–50% of total EpCAM$^+$ cells were tumor cells based on four patients sampled.

**Conclusion**—Surgery mobilizes tumor cells into the pulmonary vein, along with many normal epithelial cells. EpCAM alone cannot differentiate between normal and tumor cells. On the other hand, single-cell genetic approaches with patient-matched normal and tumor tissue can accurately quantify the number of shed tumor cells.

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**Introduction**

Surgical resection of a primary tumor is the first line of treatment in early stage non-small cell lung cancer (NSCLC), but 30% of the patients relapse and succumb to distant metastases or local recurrence \(^1\). Intraoperative tumor shedding can potentially contribute to tumor recurrence \(^2\). A number of studies have reported incidences of tumor seeding during surgery \(^2\), \(^3\), or local recurrences as a result of surgery \(^4\). In particular, a study by Yamanaka et al. sampled blood through a catheter inserted into the mesenteric vein, and found clusters of tumor cells released into circulation in patients with colorectal cancers and portal invasion \(^3\). In addition, a no-touch isolation technique was developed to reduce intraoperative tumor shedding \(^5\), \(^6\). Therefore, it is of interest to quantify how many tumor cells are dislodged during the physical manipulation of the tumor during surgical resection.

These earlier studies identified shed tumor cells primarily by cytomorphological examination, immunohistochemical staining or indirect detection of epithelial cell markers such as cytokeratin and EpCAM using RT-PCR \(^2\), \(^7\)–\(^9\). Using cytokeratin staining, it was previously estimated that the number of tumor cells shed during surgery ranged from 10 to 7 × 10^6 \(^2\). Another study reported a high number of tumor cells found in the pulmonary vein (mean 1195, median 81) using EpCAM staining \(^8\). It remains to be determined, however, whether normal epithelial cells inflate the counts of tumor cells since none of the epithelial markers used—cytokeratin or EpCAM—are tumor-specific. The lack of single-cell isolation techniques when performing genetic analysis such as RT-PCR also limits the sensitivity of detection to about ten cells \(^8\), \(^10\). This sensitivity may be suboptimal when the amount of tumor cells shed is extremely rare.

We made use of recent advances in single-cell isolation techniques and genomic analysis \(^11\) to interrogate single epithelial cells shed intraoperatively. We obtained whole blood from ligated tumor-draining pulmonary vein, and isolated individual epithelial cells using arrays of subnanoliter wells (nanowells) previously developed \(^12\). The array comprises 84,762 cubic wells of 275 pL each. Because the shed cells are rare, loading biased the occupancy of the wells to single epithelial cells. We then used a robotic micromanipulator to retrieve individual cells for single-cell targeted or whole genome sequencing. Somatic mutations identified in this highly enriched sample of shed epithelial cells are compared against patient-matched tumor and adjacent normal tissue, allowing us to pinpoint whether the shed cells originate from the tumor.
Materials and methods

Patients and sample collection

Patients were recruited according to Institutional Review Board-approved protocol at the Lahey Hospital and Medical Center and Committee on the Use of Humans as Experimental Subjects approved study at MIT. Patients identified had biopsy validated lung cancer, or had tumors suspicious for lung cancer by CT scan characteristics and/or PET scan findings and had intraoperative diagnostic wedge resections at the time of their lobectomy. Lung cancer patients underwent lobectomy either via thoracotomy or video-assisted thoracoscopy (VATS). Once the lobe was removed, the remaining blood (1 – 8 ml) in the pulmonary vein specimen was placed in a separate EDTA tube. If the tumor was at least 1.5 cm in size then a 5 mm × 5 mm × 5 mm segment of tumor was removed and placed in saline and on ice. A 2 cm × 2 cm × 1 cm segment of the adjacent normal tissue was removed at least 8 cm outside the tumor margin. The tissue specimens were transported to MIT within 2 h. Table 1 shows the patient characteristics (Refer to Table S1 for individual patient data in the supplementary materials).

Enrichment of epithelial cells from blood samples

The blood sample was enriched for epithelial cells using RosetteSep CD45 depletion kits (StemCell Technologies) (Refer to additional methods in the supplementary materials). Following depletion of CD45+ cells, the residual cells from whole blood were stained with EpCAM, a cocktail of lineage markers (Lin) for leukocytes including CD3, CD16, CD20, CD38 and CD45 (all from Biolegend) in a dilution of 1:20 and 1 μM Calcein AM violet (Molecular Probes) at room temperature for 1 hr. Afterwards, the cells were rinsed with PBS and stained with Annexin V FITC (BD Pharmingen) in Annexin V binding buffer (BD Pharmingen) for 10 min at room temperature.

Cells were loaded onto the array of nanowells. The entire array was imaged with an epifluorescence microscope (Zeiss) (Refer to additional methods in the supplementary materials for imaging specifications). EpCAM+ cells were identified with Enumerator, a custom image analysis software developed in house. For each nanowell array, we generated a list of cell information including the well IDs, cell size and fluorescent intensities. The text file was converted into a FlowJo-readable text format. Gating and cell statistics were analyzed in Flowjo (Treestar) to identify the viable epithelial cells with their well IDs. See Figure 1A for workflow.

Single-cell retrieval

Enumerator generates a list of well positions containing epithelial cells of interest. Borosilicate capillary tubings (internal diameter 0.86 mm, outside diameter 1.5 mm, length 10 cm, Sutter Instrument) were purchased and shaped with a micropipette puller (Sutter Instrument P-97). The tip was manually scored with a ceramic tile (Sutter Instrument) to achieve an internal diameter of 50 to 60 μm, and mounted on a robotic micromanipulator (CellCelector, AVISO GmbH). The instrument was calibrated to the positions of each well of the array and instructed to retrieve the cells from the specified wells. The robot aspirated 1 μl of medium directly above each well and deposited the cells into a 96-well plate for
downstream assays. The capillary tube only aspirates the contents from the well of interest, but not the neighboring wells (Figure 1B).

**Whole genome amplification**

Whole genome amplification (WGA) was performed using the REPLI-g single cell kit (Qiagen). 10 – 15 single cells from blood samples were deposited into a single well containing 8 μl Ultrapure water (Life Technologies), 0.3 μl DLB buffer (reconstituted in 55 μl water) and 0.1 μl 1M DTT buffer by the robot manipulator. The volume of aspiration was reduced to 0.2 μl per cell. The cells were lysed at 65 °C for 10 min. Subsequently, 3 μl Stop solution was added to quench cell lysis, followed by 29 μl Reaction Buffer and 2 μl DNA polymerase. Isothermal amplification was carried out at 30 °C for 8 hr.

**Targeted sequencing**

Amplicon enrichment was performed using the Lung Cancer Panel within the GeneRead DNAseq Targeted Exon Enrichment Panels for NGS (Qiagen). Library preparation was done with the NEBNext DNA Library Prep Master Mix Set for Illumina (NEB) and barcoded with the NEBNext Multiplex Oligos for Illumina (NEB). Sequencing of barcoded pools was performed with paired-end 150 reads using the Illumina MiSeq and data were analyzed using a combination of the Cloud-Based DNAseq Sequence Variant Analysis (Qiagen).

**Copy number variation analysis**

WGA products were quantified by PicoGreen (Invitrogen), adjusted to 2.5 ng/μl for library preparation using the Nextera DNA Sample Prep Kit (Illumina), and barcoded with the Nextera Index Kit (Illumina). Sequencing of barcoded pools was performed with paired-end 150 reads using the Illumina MiSeq.

**Mutation analysis of single cells using nested PCR**

Single cells from blood samples were deposited into 0.8% triton X-100 in 15 μl Ultrapure water (Invitrogen) and 1 μl Proteinase K (600 mAU/ml, Qiagen) by the robot manipulator as described in the cell retrieval section. Cell lysis was achieved by one freeze-thaw cycle and 1 hr Proteinase K digestion at 55 °C. Two rounds of nested PCRs were performed to amplify the exons of interest with multiplex PCR kit (Qiagen). The first round of PCR amplification was performed for 15 cycles (each cycle consists of denaturation 95 °C 30 s, annealing 60 °C 4 min, ext ension 72 °C 90 s). The second round of PCR amplification was performed for 35 cycles (each cycle consists of denaturation 95 °C 30 s, annealing 60 °C 3 min, ext ension 72 °C 90 s). Refer to Table S2 in the supplementary materials for primers used for p53 and kras sequencing.

**Results**

**Surgery released EpCAM+ cells into the tumor-draining pulmonary vein**

Forty-two early stage NSCLC patients recruited in our study underwent either VATS lobectomy (with or without diagnostic wedge) or thoracotomy and lobectomy. In order to
investigate whether tumor cells were shed intraoperatively, we examined whole blood (1–8 ml) removed from the ligated tumor-draining pulmonary vein. Because tumor cells are rare in the blood, we developed a platform to enumerate and retrieve single cells (Figure 1, Methods). After the epithelial cells were enriched by depletion of erythrocytes and leukocytes, the remaining cells were loaded into an array of 84,672 cubic wells and imaged for surface marker expression. Viable epithelial cells in the blood were defined as Calcein AM+/Annexin V−/EpCAM+/Lin− staining. The nanowells partitioned the cells into units of single cells or preformed clusters such that the robotic micromanipulator retrieved only the viable epithelial cells from predetermined wells of interest (Figure 1B). Epithelial cells found in the normal and tumor tissue were isolated by flow cytometry for comparison with the epithelial cells found in the blood.

Enumerating the number of viable cells using the nanowells revealed a significant number of EpCAM+ cells in the pulmonary vein with the counts as follows: VATS lobectomy (no wedge) (mean 165, median 115, range 0 to 509), VATS lobectomy (wedge) (mean 1128, median 197, range 47 to 9406), thoracotomy/lobectomy (mean 238, median 22, range 9 to 2920) (Figure 2). The high number of EpCAM+ cells in VATS lobectomy (wedge) was surprising initially because the tumor tissue was already removed prior to the ligation of the pulmonary vein. This result implied that many EpCAM+ cells were normal epithelial cells. Therefore, we needed to differentiate between normal and malignant epithelial cells using methods other than EpCAM staining to accurately quantify the number of tumor cells shed intraoperatively.

**Targeted sequencing confirmed consistent mutations between primary tumor and pulmonary vein blood after surgery**

One accurate way to distinguish between normal and tumor epithelial cells is to look for somatic mutations unique to tumor cells. We retrieved 10–15 EpCAM+ cells from the pulmonary vein of each patient and pooled them together for targeted sequencing against a panel of 20 frequently mutated genes in NSCLCs. Targeted sequencing affords each gene a high coverage (typically 50x to 5000x), enabling sensitive detection of low-abundance somatic mutations from an admixture of normal and tumor cells. The fraction of the reads mapped to the alternate alleles is indicative of the abundance of tumor cells (whereas the reference allele refers to wildtype/normal phenotype).

Targeted sequencing found that only a proportion of EpCAM+ cells obtained from blood harbored the same somatic mutations as the patient-matched primary tumors (Table 2). For VATS lobectomy (no wedge), ~30% of the reads from both patients CW46 and CW48 contained point mutations in TP53 and KRAS respectively, whereas none of the reads from CW54 contained the point mutation present in the tumor. On the other hand, none of the VATS lobectomy (wedge) samples carried somatic mutations in the blood specimens even though such mutations were present in the primary tumors, implying that wedge removed most if not all of the tumor tissue (Table 2). Therefore, all of the EpCAM+ cells shed in the VATS lobectomy (wedge) procedures we examined were normal epithelial cells.

The thoracotomy/lobectomy specimens gave a spectrum of frequencies. CW56 had no detectable mutations in the blood specimen whereas CW51 and CW59 had approximately
10% of the reads containing the same TP53 mutation and STK11 mutation found in their respective primary tumors. Since CW51 and CW59 only had 31 and 14 EpCAM\(^+\) cells, 10% of the total EpCAM\(^+\) cells yield only 1–3 tumor cells, implying that the number of tumor cells shed in these two patients was almost negligible. On the other hand, the blood specimen of CW62 had a high proportion of the reads (81%) harboring an activating EGFR mutation at codon 719. Since CW62 had 2920 EpCAM\(^+\) cells, a significant number of tumor cells was shed intraoperatively in this patient.

**Copy number variation analysis confirmed the malignancy of shed EpCAM\(^+\) cells in the blood**

While targeted sequencing identified single point mutations, copy number variation allowed us to survey the entire genomic landscape and determine the malignancy base on chromosomal gains and losses. We performed a low-pass copy number variation analysis between normal tissue, tumor and blood.

In general, the copy number variation analysis agreed well with the targeted sequencing. In VATS lobectomy (no wedge) specimens that contained a high tumor content such as CW46, the profile of epithelial cells found in the blood resembled that of the tumor tissue (Figure 3A). On the other hand, the profile of VATS lobectomy (wedge) blood specimens such as CW47 was relatively uniform (Figure 3B), implying that no or few tumor cells were released into the pulmonary vein. For the thoracotomy/lobectomy patients with a low abundance of tumor cells in the blood such as CW51, the profile of the blood was mostly uniform except in regions corresponding to high chromosomal gains in the primary tumor (Figure 3C). On the other hand, CW62, with a high tumor content in the blood, had chromosomal aberrations in the blood specimen (Figure 3C).

**Mutation analysis of single cells identified tumor cells released into the pulmonary vein during thoracotomy**

While targeted sequencing and copy number variation analysis gave convincing evidence for the presence of shed tumor cells, this analysis was conducted with pools of 10–15 cells (mainly due to cost limitations). We developed a second method to detect somatic mutations applicable to single cells and estimate the abundance of tumor cells. Multiplex nested PCR followed by Sanger sequencing was cost effective and sensitive down to single cells. We designed multiplex primers against TP53 exons 4–8, KRAS exon 2 and EGFR exons 18–21 as these regions contain common mutations in NSCLC. For each patient, we first confirmed the mutations present in the bulk tumors, then screened for the same mutations in single cells of the matched blood specimens.

Consistent with targeted sequencing, we detected individual tumor cells and clusters of cells in VATS lobectomy (no wedge) procedures. Two out of eight EpCAM\(^+\) cells from patient CW46 were tumor cells, and three out of eight EpCAM\(^+\) cells from patient CW48 were tumor cells (Figure 4A). Morphologically, the tumor cells were larger and more irregularly shaped. No tumor cells were detected in the VATS lobectomy (wedge) procedures in CW47 (Figure 4B) and CW61. In the thoracotomy/lobectomy samples, we did not detect single tumor cells in CW50 (Figure 4C) and CW56, CW59. However, we found four tumor cells
out of eight EpCAM+ cells to contain the activating EGFR G719A mutation in CW62 (Figure 4C). Morphologically, the tumor cells were indistinguishable from normal cells, further supporting the inadequacy of using cytomorphological means to quantify the number of tumor cells.

**Discussion**

Although prior studies have reported an abundance of intraoperatively shed cells, we used single-cell genetic means to parse these cells into normal and malignant epithelial cells. We interrogated 421 EpCAM+ cells from 9 patients using three genetic approaches - single-cell nested PCR, targeted sequencing and copy number variation analysis. We found that lobectomy mobilizes viable tumor cells into the pulmonary vein. Other than tumor cells, a high number of normal epithelial cells were also dislodged during the surgery; these cells stained positive for EpCAM but did not contain the same driver mutations as the ones found in the primary tumors.

Many previous studies used EpCAM-based approaches to quantify the number of tumor cells shed during surgery. While nearly all EpCAM+ cells in the peripheral circulation (circulating tumor cells) are tumor cells, too many normal epithelial cells are dislodged during surgery to render EpCAM an equally good marker for intraoperatively shed tumor cells. We found no correlation between the number of EpCAM+ cells shed during surgery to either tumor size, lymph node metastases, blood vessel/lymphatic invasion or tumor grade (supplementary figure 1), further suggesting that EpCAM is limited as a marker of surgery-induced tumor shedding. Instead, genetic analysis is necessary to differentiate between tumor and normal cells.

By using genetic approaches to pinpoint true tumor cells, we have identified a group of patients who have significant intraoperative tumor shedding. In our limited sample size, we find that only 20% of the thoracotomy patients (1/5 patients) have significant tumor shedding. Patient CW62 had ~1000 tumor cells mobilized into the pulmonary vein. Other thoracotomy patients either had no detectable tumor cells (CW50, 56) or a very low number of tumor cells based on the results of targeted sequencing (CW51, 59). A more extensive, longitudinal study is necessary to establish whether the number of tumor cells shed directly affects patient outcome. The sample size of our current study is too small to address this hypothesis. It also remains to be determined whether the mobilized tumor cells are capable of forming metastases.

In addition to sampling the pulmonary vein after the surgery, we also sampled the peripheral blood and pulmonary vein prior to surgical manipulation. However, the baseline rate of tumor shedding is low (supplementary figure 2). Although we might not have captured all of the baseline shedding using EpCAM as a marker, the disparity between the number of cells in the pulmonary vein after surgery and peripheral blood is corroborated by a previous study. Because the number of circulating tumor cells in early stage lung cancer patients is low, we believe that sampling the pulmonary vein post surgery might provide an alternative, non-invasive method of identifying patients who may be at a higher risk of recurrence.

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Generally, we detected more tumor cells in the pulmonary vein blood of the specimen from VATS lobectomy (no wedge) procedures than thoracotomy/lobectomy procedures. We believe that only the thoracotomy cases, not the VATS cases, truly reflect the degree of tumor cell mobilization as the manipulation of specimen after thoracotomy is minimal. Once the lobe was extracted, we were careful not to manipulate the tumor or lobe and only drained the pulmonary vein by removing the staple line from the vein. On the other hand, compression of the tumor during its extraction by a VATS lobectomy may greatly inflate the number of tumor cells shed.

The presence of mobilized tumor cells raises the question as to whether the order and timing of pulmonary vein ligation is important in minimizing this phenomenon. A recent study compared the sequence of pulmonary vessel ligation in thoracotomy patients and its impact on tumor cell shedding using CD44v6 and CK19 as the tumor marker, but the results showed no appreciable differences whether their pulmonary vein was ligated before or after the ligation of the pulmonary artery. It is possible that our genetic approaches may give a different count of tumor cells and reveal a difference in the degree of tumor cell mobilization during manipulation of the lobe during lobectomy.

In this study, we confirmed the presence of intraoperative tumor cell shedding by identifying consistent mutations between EpCAM+ cells found in the pulmonary vein blood and patient-matched tumor. It remains to be determined whether the shed tumor cells are viable for any significant length of time in circulation or are potentially tumorigenic and whether these mobilized tumor cells influence local or systemic recurrence.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References


Figure 1. Isolation of intraoperatively shed tumor cells
A) Whole blood from the tumor draining pulmonary vein was enriched by depletion of red and white blood cells. The remaining cells were stained with fluorescent antibodies, loaded into nanowells and imaged for enumeration of epithelial cells. Viable epithelial cells were defined as Calcein AM⁺/Annexin V⁻/EpCAM⁺/Lin⁻. Tumor and normal tissue were disaggregated into single-cell suspensions and sorted by flow cytometry using the same markers. B) Image analysis software extracted the fluorescence intensities of all the cells on the array, which could then be plotted similar to a flow cytometry plot. EpCAM⁺ cells were gated out and a list of their corresponding well ID was generated. Single EpCAM⁺ cells were retrieved with a robotic manipulator according to the well IDs. The robot only removed cells from a defined well (red box) but not from the neighboring wells. EpCAM⁺ cells from normal and tumor tissue were harvested by flow cytometry.
Figure 2. Count of EpCAM$^+$ cells in the pulmonary vein
The number of EpCAM$^+$ cells in blood from the pulmonary vein was enumerated using nanowells and plotted according to the type of surgery performed. Each bar shows the median value. One zero value is excluded from the VATS (no wedge) category. ** p-value < 0.001.
Figure 3.
Copy number variation analysis. EpCAM+ cells from normal tissue, tumor and pulmonary vein were whole-genome amplified and subjected to next generation sequencing. The copy number variation analysis was performed with HMMCopy, a software that segments chromosomes using a Hidden Markov Model. Chromosomal gains are colored in red; losses are colored in green; neutral copies are colored in blue. Patients underwent either VATS (no wedge) in A), or VATS (wedge) in B) or thoracotomy in C).
Figure 4. Nested PCR of single cells or single preformed clusters

The bulk tumor, normal tissue and individual epithelial cells of four patients from blood were subjected to two rounds of PCR amplification reactions against specific somatic driver mutations in TP53, KRAS and EGFR. The sequences of mutated codons are indicated next to the patient ID with the normal tissue boxed in black and the tumor tissue boxed in red. The bright field and fluorescence images of single shed tumor cells are shown next to their sequences. Tumor cells are boxed in red. A) Patient CW48 who underwent VATS (no wedge) have three tumor cells. * A single tumor cell that has a mutation in KRAS F28S instead of the more prevalent G12D mutation as in the primary tumor. B) Patient CW47 who underwent VATS (wedge) have no tumor cells shed. C) Patients CW50 and CW62 both underwent thoracotomy. CW50 have no tumor cells but CW62 have four tumor cells out of the eight cells sampled.
Table 1

Patient Characteristics

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<td>I</td>
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<tr>
<td>Negative</td>
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<tr>
<td>Positive</td>
<td>8 (19)</td>
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<tr>
<td>Negative</td>
<td>34 (81)</td>
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Targeted sequencing

The whole-genome-amplification products of tumor, normal tissue or shed epithelial cells found in the blood were used in targeted sequencing of 20 commonly mutated genes in NSCLC. The table indicates the number of reads mapped to the reference alleles (indicating the presence of normal cells) or alternate alleles (indicating the presence of tumor cells). Percentages of total reads are indicated in parenthesis. Shown are three patients who have undergone VATS (no wedge), two patients who have undergone VATS (wedge) and four patients who have undergone thoracotomy.

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Procedure</th>
<th>Mutations</th>
<th>Tumor (%)</th>
<th>Normal (%)</th>
<th>Blood (%)</th>
<th>EpCAM+ cells</th>
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