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Fluid biopsy for Circulating Tumor Cell identification in Patients with early and late stage Non-Small Cell Lung Cancer; a glimpse into lung cancer biology

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Abstract

Circulating tumor cell (CTC) counts are an established prognostic marker in metastatic prostate, breast, and colorectal cancer, and recent data suggests a similar role in late stage non-small cell lung cancer (NSCLC). However, due to sensitivity constraints in current enrichment-based CTC detection technologies, there is little published data about CTC prevalence rates and morphologic heterogeneity in early stage NSCLC, or the correlation of CTCs with disease progression and their usability for clinical staging. We investigated CTC counts, morphology, and aggregation in early stage, locally advanced, and metastatic NSCLC patients by using a fluid phase biopsy approach that identifies CTCs without relying on surface receptor-based enrichment and presents them in sufficiently high definition (HD) to satisfy diagnostic pathology image quality requirements. HD-CTCs were analyzed in blood samples from 78 chemotherapy-naïve NSCLC patients. 73% of the total population had a positive HD-CTC count (> 0 CTC in 1 mL of blood) with a median of 4.4 HD-CTCs/mL (range 0–515.6) and a mean of 44.7 (±95.2) HD-CTCs/mL. No significant difference in the medians of HD-CTC counts was detected between stage IV (n=31, range 0–178.2), stage III (n=34, range 0–515.6) and stages I/II (n=13, range 0–442.3). Furthermore, HD-CTCs exhibited a uniformity in terms of molecular and physical characteristics such as fluorescent

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cytokeratin intensity, nuclear size, frequency of apoptosis and aggregate formation across the spectrum of staging.

Our results demonstrate that, despite stringent morphologic inclusion criteria for the definition of HD-CTCs, the HD-CTC assay shows high sensitivity in the detection and characterization of both early and late stage lung cancer CTCs. Larger studies are warranted to investigate the prognostic value of CTC profiling in early stage lung cancer. This finding has implications for the design of larger studies examining screening, therapy, and surveillance in lung cancer patients.

INTRODUCTION

Circulating tumor cell (CTC) research has attracted the attention of both cancer biologists and oncologists over the past decade. Cancer biologists view CTCs as a tool to potentially unravel mechanisms of metastases in order to help clinicians with new drug targets and treatment decisions. Several methods for CTC detection have been developed and multiple studies on CTC enumeration and molecular characterization have been published in a variety of different cancers including breast, prostate, colorectal, lung, and mesothelioma, to name a few [1–6]. Increasing evidence suggests that CTC numbers are prognostic in both early and late stage cancers and are predictive of radiographic response in some settings [7–10]. Despite that, CTCs are not widely used by clinicians to make treatment decisions nor have they become a routine research tool to study cancer. There are several reasons for the lack of adoption of CTC quantification in clinical practice. First is the lack of randomized data showing improvement in outcomes based on modulation of therapy guided by CTC numbers. Second is the low prevalence or inability to detect CTCs in some cancers, exemplified by NSCLC. The current gold-standard of CTC enumeration, the FDA-approved CellSearch® system (Veridex, Raritan NJ), is based on the immunomagnetic enrichment of EpCAM-positive cells. One major limitation of CellSearch® is its inability to detect low EpCAM expressing tumor cells such as those derived from NSCLC [11]. Using CellSearch® assay and the widely accepted 2 CTC in 7.5 mL of blood cutoff, only 0%, 7% and 32% of patient with chemotherapy naïve stage IIIA, IIIB and IV NSCLC, respectively, have detectable CTCs [12]. Even after lowering the threshold to 1 CTC in 7.5 mL of blood, overall positivity remains low at 36%, 45%, 40% and 37% for patients with stages I, II, III, and IV NSCLC, respectively [13]. Such a low yield of detected CTCs in lung cancer patients reduces the utility of the CellSearch® assay as a widely usable biomarker. Isolation by size of epithelial tumor cells (ISET platform), being independent of EpCAM expression, performs slightly better with a combined rate of CTC detection of up to 50% across all stages. The stage specific performance of the ISET method in NSCLC is 48%, 60%, 45%, and 47% for the same respective stages [13].

High definition CTC technology is a new modality for detecting circulating tumor cells based on morphological characterization and high throughput counting. This technology uses a non-enrichment method, and thus potentially increases the sensitivity of the assay. We report here prevalence rates and characteristics of CTCs in patients with different stages of NSCLC using the high definition CTC (HD-CTC) technology, with the goal of understanding how these cells ultimately affect malignant progression and metastasis.

PATIENTS & METHODS

Study design

This was an international multicenter study of CTC prevalence and characteristics in patients with histologically proven NSCLC. Patients were enrolled in 4 prospective studies on characterization, enumeration and biomarker profiling of CTCs conducted at University

of California San Diego, Moores Cancer Center (San Diego, CA, USA), Billings Clinic (Billings, MO, USA) and The Netherlands Cancer Institute - Antoni van Leeuwenhoek Hospital (Amsterdam, The Netherlands).

The first study recruited patients newly diagnosed or with progressive metastatic lung cancer. This study enrolled 150 patients at University of California, San Diego and Billings Clinic. The second study recruited 24 chemotherapy naïve patients with stage III NSCLC at The Netherlands Cancer Institute - Antoni van Leeuwenhoek Hospital. The third study enrolled 30 treatment naïve patients with stages I–III lung cancer at University of California, San Diego. The fourth study collected 26 chemotherapy naïve patients of all stages from University of California, San Diego and Billings Clinic. All studies were approved by respective institutional review boards (IRB), and informed consent was obtained from each patient. Each study had identical collection and shipment requirements pertaining to blood sample handling. Peripheral blood samples were collected in Cyto-Chex® tubes (Streck Innovations, Omaha, NE) according to institution specific IRB-approved protocols, Good Clinical Practice and standard operating procedures. Prior adjuvant chemotherapy was not exclusionary for patients with metastatic lung cancer recurrences. Some patients received prior palliative radiation. Data was collected regarding age of the patient, smoking status, performance status, histology, TNM staging according to AJCC 7.0 and sites of metastatic disease [14]. We will report on a chemotherapy naïve cohort of patients with different stages of lung cancer.

HD-CTC enumeration and characterization

Blood samples collected in preservative tubes were shipped in temperature-controlled containers and processed at Scripps Physical Sciences Oncology Center within 24 hours after phlebotomy. The technical specifics, sensitivity, accuracy, linearity, and reproducibility of the HD-CTC assay as well as the cytomorphological inclusion criteria for HD-CTCs are described by Marrinucci et al. in this issue of Physical Biology including both the sensitivity and specificity of the overall assay. In brief, following red blood cell lysis nucleated cells were attached as a monolayer to custom-made glass slides. For HD-CTC identification, slides were subsequently incubated with antibodies against pan-cytokeratin (CK) and CD45 and nuclei were counterstained with DAPI. An automated digital microscopy technique was used for imaging. Potential CTCs were located and identified by computational analysis of the resulting data. Images of CTC candidates were then presented to a hematopathologist-trained technical analyst for analysis and interpretation. Cells were classified as HD-CTCs if they were CK-positive, CD45-negative, contained an intact DAPI nucleus without identifiable apoptotic changes or a disrupted appearance, and were morphologically distinct from surrounding white blood cells (WBCs). Importantly, this platform avoided discarding any images that did not meet HD-CTC inclusion criteria, but digitally cataloged all cells and associated analysis in databases for subsequent re-analysis of HD-CTCs and otherwise defined cell categories. Ancillary HD-CTCs characteristics investigated in this study included relative nuclear size and frequency of homotypic aggregation.

Relative nuclear sizes of HD-CTCs were calculated as the ratio of individual HD-CTC nuclear sizes (in pixels) and the mean nuclear size of surrounding WBCs. For HD-CTC cluster analysis, two or more HD-CTCs that were separated by one pixel or less were counted as a homotypic HD-CTC aggregate.

HD-CTC and HD-CTC cluster counts were enumerated on a per slide basis. WBC counts of whole blood were determined automatically (WBC system, HemoCue, Cypress, CA) and the number of leukocytes detected by the assay per slide was used to calculate the actual amount of blood analyzed per slide. For this reason, fractional values of HD-CTCs/mL and clusters/mL are possible.

To confirm non-biased retention of various cell types by the CTC slide, paired samples of EDTA and cytochex fixed peripheral blood were collected from a single healthy donor. Automated differentials were performed on each sample using a CELL-DYN Sapphire (Abbott Diagnostics, Abbott Park, IL) instrument calibrated to clinical laboratory standards. An aliquot of each sample was then processed using the standard HD-CTC process, through all steps including imaging. A random series of ten fields of view from each slide was imaged in high resolution in the DAPI channel. Images were reviewed by a hematopathologist and a three part manual differential conducted based on the nuclear morphology of the cells. Results demonstrate essential congruence for pre- and post-process white blood cell differential, demonstrating non-biased retention of various white blood cell types.

Statistical analysis

The D'Agostino & Pearson omnibus normality test was used to determine whether parametric or non-parametric tests were used for comparisons. Means between groups were compared using parametric tests (unpaired t test for comparison between two groups and one-way ANOVA for comparison among three groups). Multiple comparisons after one-way ANOVA were performed with Tukey's test. As CTC levels were not normally distributed, non parametric tests were used for group comparisons. Medians between groups were compared using non-parametric tests (Mann-Whitney U test for comparison between two groups and Kruskal-Wallis test for comparison among three or more groups). Multiple comparisons after non-parametric ANOVA were performed with Dunn's post test. Correlation of HD-CTC counts with clinical variables was assessed by contingency table analysis using Fisher's exact test for comparison between two groups or the Freeman-Halton extension of the Fisher's exact test for comparison among three or more groups. Statistical dependence was analyzed by calculating Spearman's rank correlation coefficient. Statistical analyses were performed using GraphPad Prism 5.0. Two-tailed *p* values of ≤ 0.05 were considered significant.

RESULTS

Patient population

A total of 230 patients with NSCLC were enrolled in all studies between January 2008 and January 2011. 78 of these patients were chemotherapy-naïve at the time of first blood sampling and were selected for this subgroup analysis: 10 patients with stage I, 3 patients with stage II, 34 patients with stage III and 31 patients with stage IV disease. Due to the small number of available stage II patients, stage I and II patients were pooled into an early stage group for all subsequent analyses. Patient demographics and stage distribution are presented in Table 1.

Morphology and prevalence of HD-CTCs in chemotherapy-naïve patients

HD-CTCs presenting with heterogeneous morphological features were identified in NSCLC patients of all tumor stages. HD-CTC shapes and sizes varied within and across patients and no obvious morphological characteristics unique to each stage were identified by qualitative assessment. (Fig. 1)

We detected HD-CTCs in 73% of the total population with a median of 4.4 HD-CTCs/mL (range 0–515.6) and a mean of 44.1 (± 95.2) HD-CTCs/mL. No significant differences were detected in median HD-CTC count between tumor stages ($p=0.32$). (Fig. 2) Of the 31 stage IV patients 61% had quantifiable HD-CTCs/mL (median 2.9, mean 28.4, range 0–178.2). 79% of stage III patients had detectable HD-CTC (median 6.9, mean 51.1, range 0–515.6). 85% of stage I and II patients had detectable HD-CTCs (median 2.5, mean 67, range 0–

442.3). The prevalence of HD-CTCs in correlation with clinical characteristics is listed in Table 2. An HD-CTC count of $\geq 1/\text{mL}$ was significantly associated with pleural metastases but no other metastatic site. Tumor histology and the number of metastatic sites did not correlate with HD-CTC numbers.

Molecular and physical characteristics of HD-CTCs

To gain insights into the biology and heterogeneity of CTCs, we investigated molecular and physical characteristics of HD-CTCs. Recent studies have identified a process termed epithelial to mesenchymal transition (EMT) as a prerequisite for carcinoma invasion and metastasis. One of the hallmarks of EMT in tumor cells is the progressive loss of epithelial antigens, including cytokeratins, and it is postulated that CTCs are characterized by a shift towards a mesenchymal phenotype [13, 15]. We examined the levels of CK expression on HD-CTCs from the blood of patients with different clinical stages of the disease. In addition, the nuclear size of early and late stage HD-CTCs relative to surrounding WBCs was assessed as this might reflect overall cell size and thus could influence the extent to which CTCs are filtered out of the circulation by capillary beds in distant organs. Recently, it was reported that CTCs isolated from central venous blood of metastatic breast cancer patients were significantly larger than those from peripheral venous blood in 50% of investigated patients [16].

We detected a median relative CK intensity of 19 on HD-CTCs of stage I/II patients. Median relative CK intensities for stages III and IV HD-CTCs were 15.7 and 31.1, respectively (Fig. 3A). CK levels on stage IV HD-CTCs were significantly higher compared to their stage I/II and III counterparts ($p=0.01$). On average, HD-CTC nuclei measured to be $1.6 (\pm 0.25)$, $1.8 (\pm 0.66)$, and $1.7 (\pm 0.61)$ times the size of WBC nuclei in stages I/II, III, and IV, respectively (Fig. 3B). No significant difference was detected in nuclear sizes across stages, but sizes of the nuclei of patients with stages I/II disease were more tightly grouped. There was no correlation between HD-CTC counts and median CK intensity and HD-CTC counts and mean nuclear size for all tumor stages. Of note, our previous results demonstrated that, after correction for fixation, the mean nuclear size of tumor cells in histological lymph node sections was 35% larger than the mean nuclear size of CTCs (see Torrey et al. in this issue of *Physical Biology*).

Other cell categories

In addition to enumerating cells falling into the HD-CTCs category, stringently defined to identify a population of intact and potentially metastasizing epithelial cells, we tracked additional cell categories with different inclusion criteria. These cells, some of which are oftentimes included in the CTC category when using alternative CTC detection platforms, might very well contribute to overall tumor burden and tumor dissemination. We defined a subset of CD45-negative cells with positive CK staining whose nuclear size was too small (less than 1.3 times the mean size of surrounding WBCs) to meet HD-CTC criteria as “CTC-small”. CD45-negative and CK-dim or negative cells (less than 5 SDOM brighter than the mean CK intensity of surrounding WBCs) otherwise meeting the morphological inclusion criteria for HD-CTCs were defined as “CTC-CK low” and, finally, CD45-negative CK-positive cells exhibiting cytoplasmic and/or nuclear signs of apoptosis were defined as “CTC-Ap”.

Analysis of other cellular events was performed for a subset of the study population. The cell count distribution for the above defined cellular events is depicted in Fig. 4. Median prevalence rates were consistently lower than those observed for HD-CTCs and no statistical differences in prevalence rates were detected between tumor stages for any of the three cell categories.

HD-CTC aggregation

In addition to EMT as one of the mechanisms behind cancer cell invasion and metastasis it is believed that cohesive or collective cell migration may also play a major role in the formation of distant metastasis [17]. It is postulated that aggregated cancer cells get trapped in the capillaries easier and/or evade immune defenses more efficiently. A recent study in patients with renal cell carcinoma detected the presence of tumor clumps in renal venous blood that correlated with the presence, or development, of pulmonary metastases [18]. We determined the percentage of HD-CTC associated in homotypic aggregates and number of HD-CTC clusters per mL of blood. Homotypic aggregates are defined as aggregates containing at least 2 HD-CTCs with or without interspersing WBC. No significant difference was observed between stages in the percentage of HD-CTCs associated in aggregates, with an average of 32.4% of stage I/II, 46.8% of stage III and 32.2% of stage IV HD-CTCs being observed to form homotypic aggregates (Fig. 5A). The mean HD-CTC clusters per mL of blood were 11.6, 8.1, and 5.8 for stages I/II, III, and IV, respectively, with no statistical difference ($p=0.6$) (Fig. 5B). There was a significant correlation between HD-CTC counts and % of HD-CTCs in clusters ($r=0.7$, $p<0.0001$) as well as between HD-CTC counts and HD-CT clusters/mL ($r=0.92$, $p<0.0001$).

Patients with stage I–II disease and > 10 HD-CTC/mL had a higher median number of CTC clusters/mL of 20. The median was smaller for patients with stage III and IV disease with 5.8 and 10.7 respectively but did not reach statistical significance. (Fig. 6)

DISCUSSION

Technology in the field of circulating tumor cells has advanced to allow detection of these cells to better understand tumor biology and metastases. In the recent past, there has been an increase in enrichment-based rare cell detection technologies with several platforms already developed and even more in planning stages. CTCs have already been shown to act as prognostic biomarkers in multiple cancers. Counts > 0.67 CTCs/mL (5 CTCs/7.5 mL) in patients with metastatic breast cancer at weeks 3 and 5 after initiation of chemotherapy correlate with a shorter overall and progression free survival [9]. Similar cutoffs using the CellSearch® assay have been shown to predict outcomes in locally advanced and metastatic NSCLC [12]. Although the current platforms have shown promise with respect to other cancers, they are inadequate for NSCLC due to low discovery rate of CTCs in those patients, especially in early stages. For example, using the CellSearch® assay and a threshold of ≥ 2 CTC per 7.5 mL only 21% of treatment naïve patients were reported to have detectable CTCs, leaving nearly 80% of lung cancer patients without access to a prognostic marker. This is likely due to an inherent limitation of an EpCAM based enrichment strategy as lung cancer cells do not commonly express EpCAM [11]. The HD-CTC assay applied in NSCLC identifies CTCs in 73% of all patients, including 85% of patients with early stage disease and 79% of patients with locally advanced disease, making this assay more promising in NSCLC. No differences were seen based on histologic type, which is congruent with other studies [12, 13]. In turn, as shown by Marrinucci in this issue of *Physical Biology* the high sensitivity of the assay does not impact its specificity. One or less HD-CTC/mL is seen in healthy donors.

Using EpCAM based enrichment approaches, it has been shown in lung and other cancers that patients with early stage disease present with fewer CTCs [12, 19]. This is contrary to non-EpCAM enrichment assays where no such relationship is observed [13]. This is most likely due to different subpopulations of CTCs being selected by each platform, ranging from inclusion of EpCAM low, perhaps EMT-like cells by non-EpCAM based platforms to more differentiated EpCAM positive cells for EpCAM based assays. The non-enrichment based method used in this study is not only more sensitive in lung cancer, but adds an ability

to examine multiparametric characteristics of CTCs such as nuclear size, CK expression, clustering and other cell categories. Qualities such as cell size, nuclear size, and cohesiveness are commonly evaluated by pathologists when making diagnosis of cancer and estimating cancer behavior. Therefore, the HD-CTC platform may be viewed as a fluid biopsy in a cancer patient.

CTC are known to be heterogeneous. Others have reported different sizes of CTC retrieved from central vs. peripheral circulation in patients with metastatic breast cancer [16]. We did not examine the absolute size of CTCs in this trial, but investigated differences in relative nuclear sizes among stages. We did not see a statistically significant difference in nuclear sizes among stages. However, a tighter distribution of nuclear size in patients with early stage cancer was noted. (Fig. 3) This might indicate the existence of a more homogeneous population of escaped cells in patients with early stage cancer. In our data set only one of thirteen patients had regional (N1) lymph node metastasis. Consequently, all HD-CTC in this subset of patients were presumably shed from the primary tumor. Contrary to that, one might imagine that in patients with bulky lymph node metastases and distant metastases cells can be shed from any combination of those sites which might account for greater variability in nuclear size.

CK intensity was selected as a potential marker due to its known loss during EMT transformation. It has been reported that 11% of cells extracted by the ISET method do not express CK. We saw significant differences in CK intensity in patients with non-metastatic lung cancer compared to their metastatic counterparts. A potential explanation would be that circulating cells from patients with early stage lung cancer are biologically different from cells in widely metastatic disease. This is only a hypothesis and needs to be further supported by future studies.

One potentially promising feature of our HD-CTC assay is the ability to observe CTCs in clusters. Animal data suggest that tumor cells injected in clumps have a greater propensity to form metastases than single cell injected in similar total numbers [20, 21]. Animal models also showed that cluster size increases with longer duration of tumor growth and distribution of metastatic sites differ if animals are injected with clusters vs. single cells [22]. In humans clusters released from clear cell renal cell carcinoma correlated with pulmonary metastases [18]. On average, we observed 32 to 47% of cells traveling in clusters. We did not examine cluster size in this study; however, physical properties of CTC clusters were analyzed by Cho et al. in this issue of Physical Biology. As with nuclear size, we did not observe a statistically significant difference in the percentage of CTCs in clusters between stages, again pointing to uniformity of inherent cell characteristics for those cells that are able to make it to the circulation.

In summary, our study shows high prevalence rates of CTC positive patients in all stages of NSCLC, thus increasing the potential applicability of this technology for prognostication of lung cancer patients. We believe that the HD-CTC assay has a lot of potential that could not be fully tapped in this study due to small patient groups and the lack of outcome correlations. It is interesting that cells shed in the circulation from patients with different stages of lung cancer are surprisingly uniform with regards to nuclear size, CK expression and their ability to aggregate. We postulate that this may reflect the specific qualities a tumor cell must possess to be able to make it into the circulation and survive there. Our observation that patients with non-metastatic NSCLC circulate cells with lower CK intensities is interesting. Perhaps the larger, more CK intense CTCs represent a function of overall body tumor burden and consist of dead or dying cells that 'fall off' into the bloodstream in high numbers in high stage patients but do not truly represent 'metastasizable' cells. However, this observation needs to be confirmed in other cancers and

on a larger numbers of patients. Our future direction is to correlate multiparametric characteristics of HD-CTC with patient outcomes.

NSCLC is a most common cancer in the United States and a leading cause of cancer related deaths [23]. Although progress has been made in 5 year survival, the degree of the improvement is dismal compared to other common cancers such as colorectal, prostate and breast [24]. It is not known if such differences in outcome result from the lack of a clinically practical screening program or from an innately aggressive biology of the disease. Poor survival even for patients with the earliest stages of lung cancer points to the latter. An ability to perform serial fluid biopsies and to compare those results across cancer types, within and between individual patients with the same diagnosis could carry a great potential in CTCs helping treat and understand the aggressive nature of NSCLC.

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ABBREVIATIONS

CTC	circulating tumor cell
NSCLC	non-small cell lung cancer
HD	high definition
HD-CTC	high definition CTC
WBCs	white blood cells
CK	pan-cytokeratin

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Figure 1. Representative HD-CTCs detected in the blood of (A) a stage II NSCLC patient and (B) a stage IV NSCLC patient (red, CK; blue, DAPI; green, CD45; original magnifications $\times 40$)

Figure 2. Distribution of HD-CTC counts in NSCLC patients according to tumor stage. Red lines depict the median.

Figure 3. Distribution of relative CK levels (**A**) and nuclear sizes of HD-CTCs (**B**) according to tumor stage. Each dot represents the mean CK level and mean relative nuclear size, respectively, of all HD-CTCs detected in one individual patient. Red lines depict the median.

Figure 4. Distribution of CTC-Small (A), CTC-Ap (B), and CTC-CK low counts (C) according to tumor stage. Red lines depict the median.

Figure 5. Percentage of HD-CTCs associated in clusters (A) and prevalence of HD-CTC clusters (B) according to tumor stage in NSCLC patients with a positive HD-CTC count. Red lines depict the median.

Figure 6.
HD-CTC clusters per mL, high vs. low HD-CTC concentrations, differentiated by stage.
Bars indicate SEM.

Table 1

Patient demographics

Characteristic	No.	%
Age		
Median	64	
Range	37–83	
Sex		
Female	36	46.2
Male	42	53.8
Histology		
Adenocarcinoma	44	56.4
Squamous Cell Carcinoma	20	25.6
Poorly differentiated	1	1.3
Large Cell	2	2.6
NSCLC, NOS	8	10.3
Mixed AdCa/BAC	3	3.8
Tumor Stage		
I	10	12.8
II	3	3.8
III	34	43.6
IV	31	39.7

Table 2

HD-CTC prevalence in association with clinical characteristics

		HD-CTCs/ml					
		> 0	> 1	> 2	> 5	> 10	
All patients (n=78)		57 (73.1%)	54 (69.2%)	51 (65.4%)	38 (48.7%)	30 (38.5%)	
Stage I/II (n=13)		11 (84.6%)	10 (76.9%)	9 (69.2%)	6 (46.2%)	5 (38.5%)	
Stage III (n=34)		27 (79.4%)	27 (79.4%)	26 (76.5%)	19 (55.9%)	15 (44.1%)	
Stage IV (n=31)		19 (61.3%)	17 (54.9%)	16 (51.6%)	13 (41.9%)	10 (32.3%)	
<i>p</i>		0.178	0.1011	0.1089	0.5440	0.6734	
Adenocarcinoma (n=44)		33 (75%)	31 (70.5%)	29 (65.9%)	21 (47.7%)	15 (34.1%)	
Squamous cell carcinoma (n=20)		12 (60%)	11 (55%)	10 (50%)	8 (40%)	8 (40%)	
Other (n=14)		11 (78.6%)	11 (78.6%)	11 (78.6%)	9 (64.3%)	7 (50%)	
<i>p</i>		0.4054	0.3421	0.256	0.3907	0.5345	
Number of metastatic sites							
1 (n=11)		7 (63.6%)	6 (54.5%)	5 (45.5%)	4 (36.4%)	4 (36.4%)	
2 (n=11)		8 (72.7%)	8 (72.7%)	8 (72.7%)	6 (54.5%)	5 (45.5%)	
3+ (n=9)		4 (44.4%)	3 (33.3%)	3 (33.3%)	3 (33.3%)	1 (11.1%)	
<i>p</i>		0.5309	0.217	0.2366	0.6603	0.3049	
Site of metastasis							
Brain							
Yes (n=6)		3 (50%)	3 (50%)	3 (50%)	2 (33.3%)	1 (16.7%)	
No (n=25)		16 (64%)	14 (56%)	13 (52%)	11 (44%)	9 (36%)	
<i>p</i>		0.6526	1	1	1	0.0671	
Bone							
Yes (n=14)		7 (50%)	5 (35.7%)	5 (35.7%)	3 (21.4%)	2 (4.3%)	
No (n=17)		12 (70.6%)	12 (70.6%)	11 (64.7%)	10 (58.8%)	8 (47.1%)	
<i>p</i>		0.2883	0.0759	0.1556	0.0669	0.068	
Lung							
Yes (n=12)		6 (50%)	5 (41.7%)	5 (41.7%)	4 (33.3%)	3 (25%)	
No (n=19)		13 (68.4%)	12 (63.2%)	11 (57.9%)	9 (47.4%)	7 (36.8%)	
<i>p</i>		0.4521	0.2883	0.4725	0.484	0.6972	
Liver							

		HD-CTCs/ml				
		> 0	> 1	> 2	> 5	> 10
	Yes (n=3)	1 (33.3%)	1 (33.3%)	0 (0%)	0 (0%)	0 (0%)
	No (n=28)	18 (64.3%)	16 (57.1%)	16 (57.1%)	13 (46.4%)	10 (35.7%)
<i>p</i>		0.5435	0.5764	0.1012	0.2452	0.5328
Lymph nodes						
	Yes (n=17)	12 (70.6%)	11 (64.7%)	11 (64.7%)	10 (58.8%)	7 (41.2%)
	No (n=14)	7 (50%)	6 (42.9%)	5 (35.7%)	3 (21.4%)	3 (21.4%)
<i>p</i>		0.2883	0.2895	0.1556	0.0669	0.2802
Adrenal						
	Yes (n=3)	1 (33.3%)	1 (33.3%)	1 (33.3%)	1 (33.3%)	1 (33.3%)
	No (n=28)	18 (64.3%)	16 (57.1%)	15 (53.6%)	12 (42.9%)	9 (32.1%)
<i>p</i>		0.5435	0.5764	0.5996	1	1
Pleura						
	Yes (n=5)	5 (100%)	5 (100%)	5 (100%)	5 (100%)	3 (60%)
	No (n=26)	14 (53.8%)	12 (46.2%)	11 (42.3%)	8 (30.8%)	7 (26.9%)
<i>p</i>		0.1284	0.0482	0.0434	0.0076	0.2955