

## Preoperative Circulating Tumor Cell Detection Using the Isolation by Size of Epithelial Tumor Cell Method for Patients with Lung Cancer Is a New Prognostic Biomarker

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### Abstract

**Purpose:** Pathologic TNM staging is currently the best prognostic factor for non-small cell lung carcinoma (NSCLC). However, even in early-stage NSCLC, the recurrence rates after surgery range from 25% to 50%. The preoperative detection of circulating tumor cells (CTC) could be useful to tailor new therapeutic strategies in NSCLC. We assessed the presence of CTC in NSCLC patients undergoing surgery, using cytologic analyses, after their isolation by size of epithelial tumor cells (ISET method). The presence and the number of CTCs were considered and correlated with clinicopathologic parameters including patient follow-up.

**Experimental design:** Of the 247 blood samples tested, 208 samples were from patients with resectable NSCLC and 39 from healthy subjects. The mean follow-up was 24 months. An image of detected cells with presumably nonhematologic features [initially defined as "circulating nonhematologic cells" (CNHC)] was recorded. The presence of CNHC was assessed blindly and independently by 10 cytopathologists, using cytologic criteria of malignancy on stained filters. The count of detected CNHCs was made for each filter.

**Results:** One hundred two of 208 (49%) patients showed CNHCs corresponding to CNHC with malignant cytopathologic features in 76 of 208 (36%) cases. CNHCs were not detected in the control group. A level of 50 or more CNHCs corresponding to the third quartile was associated with shorter overall and disease-free-survival, independently of disease staging, and with a high risk of recurrence and death in early-stage I + II-resectable NSCLC.

**Conclusion:** A high percentage of NSCLC patients show preoperative detection of CNHC by the ISET method. The presence and level of 50 or more CNHCs are associated with worse survival of patients with resectable NSCLC. *Clin Cancer Res*; 17(4); 827-35. ©2010 AACR.

### Introduction

Lung cancer is the most prevalent neoplasm and the major cause of tumor-related mortality in the United States (1). Despite recent advances in the management of resected lung cancers and more effective treatments of metastatic tumors, the cure rate of patients with lung cancer remains low (2-6). Histologic classification of lung tumors distinguishes small and non-small cell lung carcinomas (NSCLC). Most NSCLCs display 3 histologic subtypes: adenocarcinoma, squamous cell carcinoma, and large cell carcinoma (7, 8). The prognosis of these NSCLC subtypes is quite similar (2-5).

Although pTNM staging is currently the only validated prognostic factor used in NSCLC patient follow-up and treatment, 25% to 50% of patients with early-stage NSCLC show tumor recurrence, even following extensive tumor resection, indicating the urgent need for more sensitive prognostic and predictive markers (9-11).

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### Translational Relevance

Besides pathologic tumor staging, a few prognostic biomarkers currently exist that correlate with the outcome of patients undergoing surgery for resectable non-small cell lung carcinoma (NSCLC). Therefore, improvement of relevant prognostic biomarkers, in particular for predicting recurrence, is urgently needed in lung clinical oncology. Local recurrence and metastatic dissemination of the primary tumor may arise from dissemination of circulating tumor cells (CTC) in the patient's blood prior to surgery for radical tumor resection. In this regard, early detection of CTC in patients having resectable NSCLC might be considered as a potentially relevant prognostic biomarker, which could also tailor new therapeutic strategies. We show in this study that the presence and number of CTCs detected according to their size (ISET, isolation by size of epithelial tumor cells) and then characterized by a panel of 10 cytopathologists, using a cytomorphologic analysis, are significantly correlated with shorter overall and disease-free survival in patients undergoing surgery for resectable NSCLC. We conclude that CTC detection using the ISET method in this population has a strong clinical impact.

A sizable body of evidence indicates that metastases may develop from circulating tumor cells (CTC) that spread into blood vessels before, during, and/or after surgery (12). Moreover, it has been reported that the presence of occult metastatic disease correlates with disease recurrence in stage I NSCLC patients (13). Thus, sensitive and specific detection of CTC in the blood might be considered as a potentially relevant prognostic biomarker for patients with resectable NSCLC. Indeed, the main goal for preoperative detection of CTC was to identify NSCLC patients with a high risk of recurrence after surgery in order to perform the best follow-up and therapeutic strategy.

Despite the report of a large number of studies on CTC detection, methodologic aspects concerning sensitivity, specificity, and reproducibility have prevented a clear appraisal of the clinical impact (12). While reverse transcriptase PCR (RT-PCR) and immune-mediated methods can be very sensitive, specificity remains a critical issue for these approaches, as no transcript or antigen specifically characterizing tumor cells from solid tumors is known at present (12). In this setting, cytopathologic analysis of CTC, isolated according to their size (ISET, isolation by size of epithelial tumor cells), is considered a promising approach, as CTC enrichment is very sensitive and cell morphology is not damaged (12, 14). This methodology allows "classical" cytopathologic criteria of malignancy, already used in exfoliative cytology, to be used to identify malignant tumor cells (14). Currently, ISET technology has been reported previously to allow identification of CTC in patients with liver or breast cancers (15, 16). However, the

ISET method has never been used to detect CTC in patients with NSCLC.

The aim of this study was (i) to determine the diagnostic potential of the ISET method for preoperative detection of CTC in resectable NSCLC patients and (ii) to correlate the presence and number of CTCs with different clinicopathologic parameters, in particular pathologic stages, and patient outcome. For this purpose, cytomorphologic criteria have been established by a panel of 10 cytopathologists for classification of detected circulating nonhematologic cells (CNHC) into 3 groups: (i) circulating nonhematologic cells with malignant features (CNHC-MF), (ii) CNHC with uncertain malignant features (CNHC-UMF), and (iii) CNHC with benign features (CNHC-BF).

### Materials and Methods

#### Patients and samples

Two hundred eight consecutive patients with NSCLC undergoing surgery from September 2006 to January 2009 at the Pasteur Hospital (Department of Thoracic Surgery) University of Nice Sophia Antipolis, Nice, France, were entered into this study. All patients gave their informed consent to participate in this study. Follow-up of these patients was from 12 to 41 months (mean = 24 months). Preoperative diagnosis was made, in 50% of cases, on bronchial biopsies and/or bronchial aspirates, transbronchial or transparietal chest biopsies, or mediastinoscopy with biopsy. Biopsies were not performed for at least 15 days before surgery. Others patients underwent surgery without previous biopsy, after diagnosis based on cancer imaging and confirmation with frozen sections. The main clinicopathologic parameters of the 208 patients are summarized in Table 1. Morphologic classification was assigned according to WHO criteria (8). The tumors were staged according to the 7th edition of the international tumor-node-metastasis (TNM) system (17). Twenty-nine patients with stage IIIA disease had neoadjuvant chemotherapy. Patients with stage IV disease had solitary brain metastases and were considered as operable for their lung carcinoma (18). Among the adenocarcinomas, 90 expressed the TTF1 antigen, as determined by immunohistochemical (anti-TTF1 antibody, diluted 1:100; Dako) staining. The percentage of epithelial tumor cells in the formalin-fixed, paraffin-embedded tissue sections of the primary tumors was less than 30% (45/208 cases: 22%), between 30% and 80% (145/208 cases: 69%), and more than 80% (18/208 cases: 9%) and was defined by counting the relative proportion of tumor cells in 20 different fields of each tumor at a 200× magnification.

Blood samples from 39 healthy volunteers were used as negative controls. There were 29 men (median age = 39 years; range = 25–45 years) and 10 women (median age = 35 years; range = 22–43 years), smokers (average 11 pack-years; range = 10–17 pack-years), without knowledge of neoplastic disease.

**Table 1.** Main epidemiologic, clinical, and pathologic characteristics in NSCLC patients included in the study

Clinical and pathologic parameters	No of patients (%)
Overall	208 (100)
<i>Age, y</i>	
Mean	63
Range	37–84
<i>Gender</i>	
Male	141 (68)
Female	67 (32)
<i>Tobacco exposure, pack-years</i>	
Number	189 (91)
Average	41.3
Range	1–150
<i>Tumor size, cm</i>	
Mean	3.8
Range	0.4–17
<i>Histology</i>	
ADC	115 (55)
Mixed ADC	95 (46)
Acinar ADC	9 (4)
Mucinous carcinoma	5 (2)
Clear cell ADC	2 (1)
Solid ADC with mucin production	2 (1)
Papillary ADC	1 (0.5)
Bronchioloalveolar ADC	1 (0.5)
Squamous cell carcinoma	54 (26)
Large cell carcinoma	19 (9)
Sarcomatoid carcinoma	10 (5)
Adenosquamous carcinoma	5 (2.5)
Non-small cell carcinoma	5 (2.5)
<i>pTNM staging</i>	
I	86 (44)
IA	36
IB	50
II	51 (25)
IIA	26
IIB	25
III	58 (28)
IIIA	56
IIIB	2
IV	13 (6)
TTF1 antigen expression	120 (58)
Neoadjuvant therapy	29 (14)

Abbreviation: ADC, adenocarcinoma.

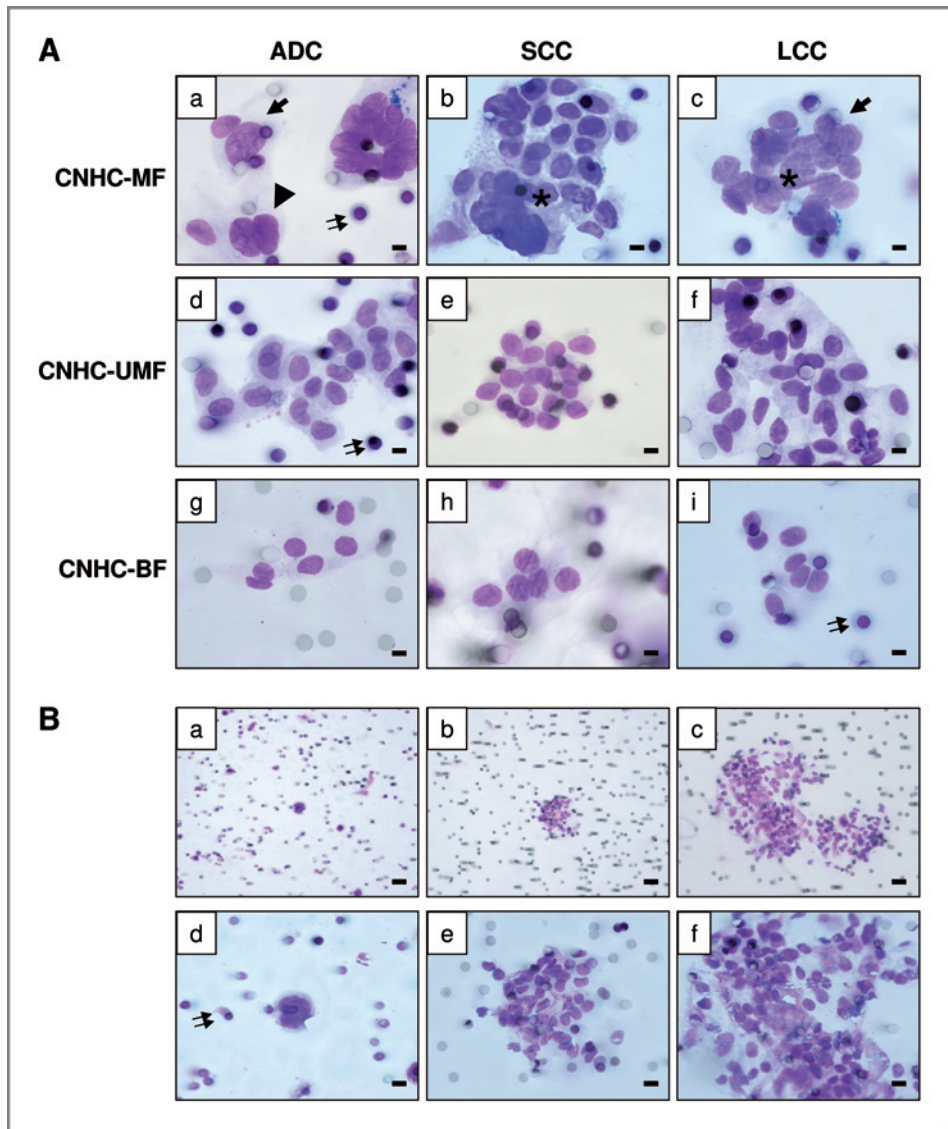
## Methods

Ten milliliters of peripheral blood was collected in buffered EDTA before anesthesia, maintained at 4°C, and processed within 1 hour. Surgical lung specimens were taken for pTNM staging and histologic evaluation. ISET was carried out as previously described (15). The module

of filtration has 10 wells, making it possible to load and filter 10 individual samples in parallel. Briefly, after blood filtration, the membrane was then gently washed with PBS, disassembled from the filtration module, and allowed to air-dry. The membrane was cut into 2 parts, containing respectively 6 spots for staining and 4 spots stored for further potential studies. The 6 spots were stained using a modified May-Grünwald-Giemsa (MGG) staining method with the following steps: May-Grünwald (undiluted, 5 minutes), May-Grünwald (diluted 50%, 5 minutes), and Giemsa (diluted 10%, 40 minutes), followed by rinsing with PBS for 1 minute. Membranes were then air-dried and kept in the dark at room temperature. Stained spots were examined by light microscopy, using different steps: (i) observation at 100× and 200× original magnification to look for CNHC and to count these cells, and (ii) observation at 630× and 1,000× original magnification with oil immersion for detailed cytomorphologic analysis. The following criteria were taken into account to characterize the detected nonhematologic cells: irregularity and size of the nucleus, anisonucleosis, nuclear hyperchromatism, nucleocytoplasmic ratio, size and number of nucleoli, and presence of tridimensional sheets. CNHC-MFs were characterized by the presence of at least 3 of the following criteria: anisonucleosis (ratio >0.5), nuclei larger than 3 calibrated pore size of the membrane (8 μm; >24 μm), irregular nuclear borders, and presence of 3-dimensional sheets (Fig. 1A, a–c). CNHCs were defined as uncertain malignant features (CNHC-UMF) when less than 3 of these criteria were present (Fig. 1A, d–f). CNHCs with benign features (CNHC-BF) were characterized in the absence of these criteria (Fig. 1A, g–i). A semi-quantitative analysis was performed on each filter, and patients were categorized into different groups according to the number of detected CNHC: group 1, less than 10 CNHCs (Fig. 1B, a and d); group 2, between 10 and 100 CNHCs (Fig. 1B, b and e); and group 3, more than 100 CNHCs (Fig. 1B, c and f). Moreover, CNHC was expressed as the median and as the interquartile difference, when considered as a continuous variable. The third quartile of the CNHC distribution function was used as the cutoff value. Moreover, this agrees with the cytomorphologic analysis because when more than 50 CNHCs were present, they were more easily diagnosed as malignant. Both semiquantitative stratification and the cutoff point of 50 or more CNHCs were used for statistical analyses. Eight hundred sixteen photographs (average mean 8 photographs per filter, range = 1–29) were recorded, and images were digitized and collected by 2 cytopathologists (V.H. and P.H.). All images were then reviewed independently by the panel of cytopathologists (V.H., C.B., P.V., S.L., N.M., J.F.F., T.J.M., J.M.V., E.P., and P.H.) in a blind way without knowledge of the diagnosis and clinical status. Images were scored independently by the cytopathologists as CNHC-MF, CNHC-UMF, or CNHC-BF for each individual.

## Statistical analysis

All calculations were performed with the statistical software R, a free language and environment for statistical



**Figure 1.** A, cytomorphologic criteria of CNHCs (a–c) with malignant features (CNHC-MF), (d–f) with uncertain malignant features (CNHC-UMF), and (g–i) with benign features (CNHC-BF), preoperatively isolated by the ISET method in patients with a resectable NSCLC. a–i, original magnification  $\times 1,000$ ; MGG staining; bars, 8  $\mu\text{m}$  (arrows, anisonucleosis; arrowheads, irregularity and large nuclei; asterisks, tridimensional sheets; double arrows, pores of the filter). B, a and d, isolated CNHCs (group 1, number of cells  $<10$ ). b and e, small sheet of CNHCs (group 2, number of cells between 10 and 100). c and f, large sheet of CNHCs (group 3, number of cells  $>100$ ; MGG; a–c, original magnification  $\times 100$ ; d–f, original magnification  $\times 630$ ). ADC, adenocarcinoma; SCC, squamous cell carcinoma; LCC, large cell carcinoma.

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analysis and graphics (version 2.9.0). The presence or absence of CNHCs, analyzed as a binarized qualitative variable, was compared with the following clinicopathologic variables: age, gender, smoker status, neoadjuvant therapy, pTNM stages, tumor size, histologic grade, histologic subtype, percentage of tumor cells in primary tumors, and the TTF1 immunostaining status in both patients with NSCLC and healthy volunteers by the  $\chi^2$  analysis or the Mann–Whitney test when applicable. We used  $\kappa$  statistics, which reflect agreement between 2 measurements after removing chance agreement, as a measure of reliability (19). A value close to 1 represents almost perfect agreement, whereas values close to or below zero represent poor agreement. The ANOVA was used to explore the association between the number of CNHCs, analyzed as a continuous variable, and the pTNM stage.

Patient outcome, including overall survival (OS) and disease-free survival (DFS) comparing the presence or absence of CNHC and according to the number of CNHCs, was assessed by the Kaplan–Meier analysis with a log-rank score for determining statistical significance. OS was defined as the interval between surgery and the date of death from any cause or the last follow-up. DFS was defined as the interval between the date of surgery and the date of relapse of the disease or the date of death from any cause. Patients who did not relapse or who died (for DFS) or remained alive (for OS) at the final follow-up were censored at that time. Multivariate Cox analyses were carried out to examine whether the presence of CNHC, according to the cutoff point of 50 or more CNHCs, is an independent prognostic factor for survival with adjustment for relevant clinicopathologic covariates. Multivariate

**Table 2.** Correlation of the levels of CNHCs stratified by the cutoff point detected by the ISET method with disease staging in resectable NSCLC patients

pTNM stage <sup>a</sup>	No. of patients	Levels of CNHC, n (%)	
		<50 CNHC	≥50 CNHC
Stage I	86	65 (76)	21 (24)
IA	36	29 (81)	7 (19)
IB	50	36 (72)	14 (28)
Stage II	51	35 (67)	16 (33)
IIA	26	17 (65)	9 (35)
IIB	25	18 (72)	7 (28)
Stage III	58	38 (66)	20 (34)
IIIA	56	37 (66)	19 (34)
IIIB	2	1 (50)	1 (50)
Stage IV	13	6 (46)	7 (54)
Overall	208 (100%)	144 (69)	64 (31)

NOTE: The cutoff point for grouping was the third quartile, 50 or more CNHCs, as described in the Materials and Methods section.  $P > 0.05$  for all groups. Values in parentheses are line percentages.

<sup>a</sup> $\chi^2$  analysis. Coding of variables: stages IA and IB were coded as 1, stages IIA and IIB were coded as 2, stages IIIA and IIIB were coded as 3, and stage IV was coded as 4. The  $P$  value for overall comparison was 0.15.

analyses using Cox regression models included all potential prognostic factors for survival with a  $P < 0.2$  value in univariate analysis. The variables included in the model for OS and DFS were pTNM stage, tumor size, and histology. A  $P \leq 0.05$  value was considered significant for all analyses.

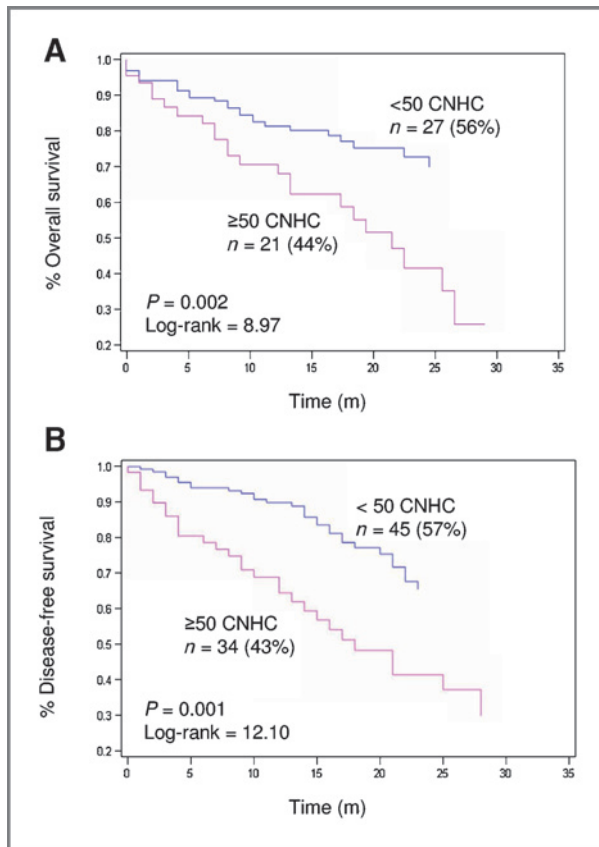
## Results

CNHCs were present preoperatively in 102 of 208 (49%) patients undergoing surgery for NSCLC (Supplementary Table 1). Interobserver agreement between the 2 initial cytopathologists was total ( $\kappa = 1$ ) for detection of CNHC on filters. The mean number of CNHCs was 42 (median = 0; range = 0–500; first quartile: 0, third quartile: 50) in NSCLC patients. CNHCs were present in 88 of 179 (49%) and 14 of 29 (48%) in patients without and with neoadjuvant chemotherapy ( $P = 0.86$ ), respectively. CNHC-MFs were characterized morphologically in 76 of 208 (37%) of cases (Fig. 1A) [in 65/179 (36%) and in 11/29 (38%) in patients without and with neoadjuvant chemotherapy ( $P = 0.96$ ), respectively]. In all cases (100%), at least 5 of the 10 cytopathologists agreed with the final diagnosis (CNHC-MF, CNHC-UMF, or CNHC-BF) for each patient ( $\kappa = 1$ ). CNHC-UMFs were diagnosed in 23 of 208 (11%) cases (Fig. 1A), whereas CNHC-BFs were observed in 3 of 208 (1%) of cases (Fig. 1A). The cytopathologic features of CNHC from patients with lung adenocarcinoma were not distinguishable from those of CNHCs derived from squamous cell carcinoma and large cell lung carcinoma (Fig. 1A) and the other histologic subtypes. CNHCs were not found in the blood of the 39 healthy volunteers.

No correlation was observed between the levels of CNHC ( $P = 0.15$ ; Table 2) or according to their presence by semiquantitative grouping ( $P = 0.35$ ; Supplementary Table 1) and the disease stage. Moreover, no correlation existed between the detection of CNHC and other clinicopathologic parameters (age, gender, tobacco exposure, tumor size, histologic subtype, histologic grade, percentage of epithelial tumor cells in the primary tumor, pleural invasion, presence of intratumoral emboli, and TTF1 staining;  $P > 0.05$ ; data not shown). Finally, no correlation was found between the number of CNHCs and the disease stage ( $P = 0.92$ ; Supplementary Fig. 1). Moreover, no significant relationship was noted between the number of CNHCs and the other different clinicopathologic parameters cited previously ( $P > 0.05$ ; data not shown).

The number of CNHCs (at the level  $\geq 50$  cells) was significantly associated with shorter OS and DFS ( $P = 0.002$ , and  $P = 0.001$ , respectively; Fig. 2). In addition, the level of 50 or more CNHCs was significantly associated with worse DFS for both early-stage I + II- and later-stage III + IV-resectable NSCLCs ( $P = 0.05$ , and  $P < 0.0001$ , respectively; Fig. 3). Finally, the presence of CNHCs, according to semiquantitative stratification, was associated with a shorter OS and DFS (Supplementary Fig. 2).

Subsequently, multivariate survival analyses using the Cox proportional hazard model were performed to examine the importance of the level 50 or more CNHCs in patient outcome when other prognostic factors were included. Both a level of 50 or more CNHCs and the pTNM stage were significantly independent prognostic factors for OS (Table 3). In addition, a level of 50 or more CNHCs, pTNM stage, histology cell subtype, and tumor size were



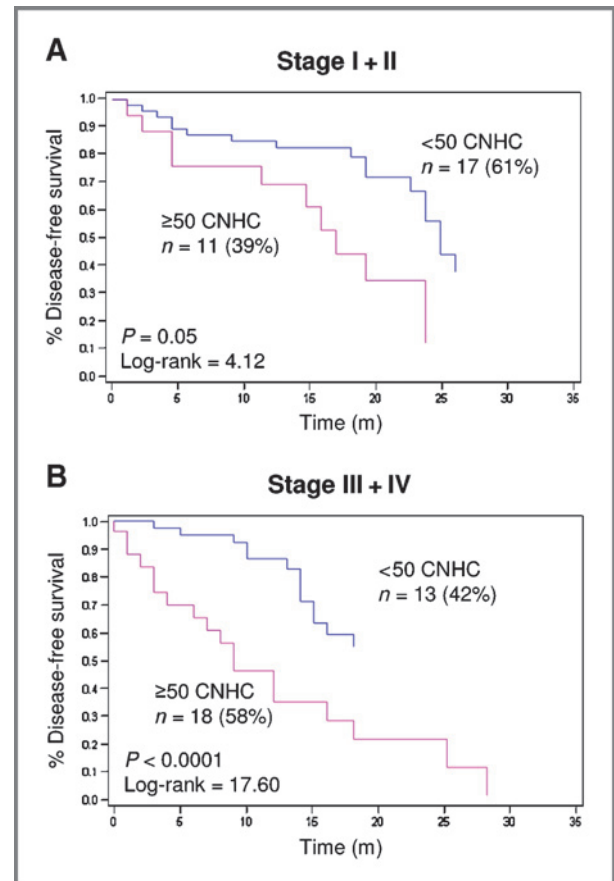
**Figure 2.** Kaplan–Meier curves of OS (A) and DFS (B) duration stratified according to the cutoff point of 50 or more CNHCs detected by the ISET method. The cutoff point of stratification was the third quartile, as described in the Materials and Methods section. Numbers in the legends are the number of events for each group.

significantly independent prognostic factors for DFS (Table 3).

## Discussion

This study shows the feasibility of the ISET method for preoperative isolation and identification of CTC from peripheral blood samples taken from patients with resectable NSCLC. We found that around half of these patients showed detected CNHC in their blood, mainly corresponding to CNHC-MF according to cytomorphologic criteria. Moreover, in these cases, interobserver variation was low for the diagnoses of these latter cells. It is noteworthy that detection of CNHCs by ISET had a strong clinical impact, as the presence and number of CNHCs correlated with a pejorative outcome (low OS and DFS). Other clinicopathologic parameters, including pTNM staging, did not correlate with the presence and number of CNHCs. These latter results show that preoperative CNHC detection in patients with resectable NSCLC is an independent new prognostic biomarker.

Different methods have been applied in the past to detect occult CTC in patients with NSCLC (20–30). Indeed, most



**Figure 3.** Kaplan–Meier curves of DFS for early-stage I + II NSCLC (A) and later-stage III + IV NSCLC (B). The cutoff value was defined as superior or equal to 50 CNHCs, as described in the Materials and Methods section, to define the presence of CNHC. Numbers in the legends are the number of events for each group.

of the previous studies have used indirect methods based on RT-PCR or quantitative RT-PCR before, during, and after surgery (21, 23, 24, 27–29) or nested RT-PCR (25) or used BErEP4-coated beads (22), magnetic bead enrichment, and laser scanning cytometric (26) and immunocytologic methods (CellSearch System; ref. 30). Different biomarkers such as the telomerase activity (22), the cytokeratin 19/carcinoembryonic antigen and c-met mRNAs (25, 27, 31), epidermal growth factor receptor (21, 32), and other different molecules of interest (23) have been used in these studies to specifically identify CTC. However, contrasting results have been obtained in all these previous studies because of technical characteristics such as marker- and method-related specificity and sensitivity and limitations such as the high cost and labor needed, which are not adapted for use in large cohorts of patients

The ISET method has been previously used in patients with liver and breast cancers (15, 16). We show here that this method can be applied to NSCLC patients. Interestingly, 49% of these patients showed preoperative CNHC in their blood. Among these CNHCs, diagnosis of CNHC-MF

**Table 3.** Multivariate Cox proportional hazard regression analysis of predicting factors for OS and DFS in 208 resectable NSCLC patients

Prognostic factor	HR	95% CI	P <sup>a</sup>
<b>OS</b>			
Histology			
Squamous cell carcinoma	1.610	0.909–2.850	0.10
Other subtypes	1		
pTNM stage			
I + II	0.190	0.080–0.455	<0.0001
III + IV	1		
Tumor size, cm			
≤3	0.664	0.413–1.068	0.09
>3	1		
Presence of CNHC			
≥50	2.096	1.331–3.300	0.001
<50	1		
<b>DFS</b>			
Histology			
Squamous cell carcinoma	2.346	1.129–4.876	0.022
Other subtypes	1		
pTNM stage			
I + II	0.225	0.604–0.840	0.0009
III + IV	1		
Tumor size, cm			
≤3	0.574	0.332–0.993	0.047
>3	1		
Presence of CNHC			
≥50	2.631	1.557–4.651	0.003
<50	1		

<sup>a</sup>P value significant at the 0.05 level.

was made by morphologic assessment in the majority of cases. The malignant characteristics of the detected CNHCs were assessed only according to cytopathologic criteria, which are classically used by cytopathologists in exfoliative and fine-needle aspiration cytopathology.

Interestingly, a group of CNHCs in this series has been classified as having uncertain malignant features (CNHC-UMF) by the cytopathologists. A few CNHCs were also classified as benign cells only by a couple of cytopathologists, as none of malignant criteria were morphologically present. However, certain cytopathologists of the panel hypothesized that CNHCs detected in peripheral blood cannot be benign cells in NSCLC patients. Interobserver variation was low for the different subgroups of CNHCs. However, given the new domain of CTC, we cannot rule out the possibility that some specific morphologic criteria could be required in the future to precisely identify and classify CNHCs. In this regard, it is clear that markers specific for CTC should be developed in the future and combined with cytomorphologic analysis. In particular, the different CNHC groups need to be better characterized phenotypically by immunocytochemical and/or by molecular biological approaches in further studies. In this

regard, when using a morphologic method, we cannot rule out the possibility that a couple of circulating endothelial cells can be isolated or associated with CTC (33). Thus, endothelial cells could also be further identified on filters by immunocytochemistry, using different markers (34). Finally, in our study, cytomorphologic criteria did not correlate with the histologic subtype of the primary lung tumor, underlying that no features of CNHCs were representative of the histologic subtype.

No correlation was found between CNHC, as quantitative or qualitative variables, and pTNM staging. In particular, our study on the correlation between the pTNM staging and the presence of CNHC showed the presence of CNHC in a group of patients with early-stage NSCLC before surgery. It will be of great interest in the future to follow-up this group of patients and compare them with early-stage carcinoma patients without preoperative detection of CNHC by looking for relapse and/or metastasis onset. Our study shows insight into this behavior, as the level of 50 or more CNHCs was significantly associated with a higher risk of recurrence in early-stage NSCLC. Tumor cell behavior depends on interactions between nuclear genetic changes in the malignant cells and a stroma

that is favorable for growth, invasion, and metastasis, in association with angiogenesis. Thus, an increase in different stromal biomarkers has been indicative of poor outcome in patients with NSCLC (35, 36). In this regard, we thought that it would be of interest to see whether the importance of the stroma, which is considerably variable from one NSCLC to another, even at the same TNM staging, was linked to the presence of CNHC. However, we found that the percentage of tumor cells in the primary tumor did not correlate with the number of CNHCs, indicating that a substantial stroma reaction associated with carcinoma could not influence the effraction of vessels by tumor cells and then their blood dissemination. Using univariate analysis, no significant difference was noted between CNHC, as quantitative and qualitative variables, and the pathologic stage, the size of the primary tumor, the histologic subtype, and the percentage of tumor cells in the primary tumor. This finding supports the idea that the presence of CNHC is independent of the well-established prognostic factors.

We then evaluated the prognostic significance of CNHC when preoperatively detected by ISET in patients with resectable NSCLC. It is noteworthy that a level of 50 or more CNHCs detected by ISET was associated with significantly decreased OS and DFS, independently of disease staging, as determined by univariate and multivariate survival analyses. Similar results were observed for univariate survival analyses when considering the presence of detected CNHCs, according to semiquantitative group stratification.

Currently, in most of the cases, patients with early-stage NSCLC with surgically resected tumors do not have adjuvant therapy. However, among these latter patients, a group will undergo relapse and/or develop metastases. In this regard, it is of great interest to look for a biomarker that is predictive of such disease evolution. Thus, the preoperative detection of CTC by ISET in this group can allow selection

of a population with a higher risk of relapse and poorer prognosis. Although the cytomorphologic approach alone cannot assess the malignant potential of CNHC, we strongly believe that it ensures a far higher specificity than other approaches based on epithelial-related antigens or transcripts and allows the study of cells with tumor like features by complementary approaches. Thus, by combining the reference "old" cytomorphologic approach with newly developed molecular markers derived from proteomics, molecular profiling, and genetic analyses, a promising path toward better classification and treatment of patients according to their prognosis should be obtained.

In conclusion, the presence of CTCs identified by ISET in patients undergoing surgery for resectable NSCLC is associated with poor prognosis, independently of disease staging. This finding identifies CTCs detected by ISET as a very strong, independent prognostic indicator in patients with resectable NSCLC. Moreover, it might identify these CTCs as a pertinent molecular target for the development of new antitumor agents.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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