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Morphological analysis of circulating tumour cells in patients undergoing surgery for non-small cell lung carcinoma using the isolation by size of epithelial tumour cell (ISET) method

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Background and objective: Recurrence rates after surgery for non-small cell lung cancer (NSCLC) range from 25 to 50% and 5-year survival is only 60–70%. Because no biomarkers are predictive of recurrence or the onset of metastasis, pathological TNM (pTNM) staging is currently the best prognostic factor. Consequently, the preoperative detection of circulating tumour cells (CTCs) might be useful in tailoring therapy. The aim of this study was to characterize morphologically any circulating non-haematological cells (CNHCs) in patients undergoing surgery for NSCLC using the isolation by size of epithelial tumour cell (ISET) method.
Methods: Of 299 blood samples tested, 250 were from patients with resectable NSCLC and 59 from healthy controls. The presence of CNHCs was assessed blindly and independently by 10 cytopathologists on May-Grünwald–Giemsa stained filters and the cells classified into three groups: (i) malignant cells, (ii) uncertain malignant cells, and (iii) benign cells. We assessed interobserver agreement using Kappa (κ) analysis as the measure of agreement.
Results: A total of 123 out of 250 (49%) patients showed CNHCs corresponding to malignant, uncertain malignant and benign cells, in 102/250 (41%), 15/250 (6%) and 6/250 (2%) cases, respectively. No CNHCs were detected in the blood of healthy subjects. Interobserver diagnostic variability was absent for CNHCs, low for malignant cells and limited for uncertain malignant and benign cells.

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Conclusion: Identification of CTCs in resectable NSCLC patients, using ISET technology and according to cytopathological criteria of malignancy, appears to be a new and promising field of cytopathology with potential relevance to lung oncology.

Keywords: circulating tumour cells, isolation by size of epithelial tumour cells, non-small cell lung carcinoma, diagnosis, cytopathology, interobserver agreement.

Introduction

Detection of circulating tumour cells (CTCs) in the bloodstream of cancer patients can be prognostic and may have a large impact on the development of new strategies for cancer treatment.[1,2] CTCs can be detected during follow-up after tumour resection and in the metastatic phase.[3] Moreover, these cells can be isolated in patients undergoing surgery for completely resectable tumours.[4] CTCs can be detected using indirect or direct methodological approaches.[2,5] Among the indirect techniques, the Cell Search method is probably the one most frequently employed, and has been authorized by the USA Food Drug Administration for the follow-up of patients with breast, colonic and prostate metastases.[2] Currently, direct methods for detecting CTCs, although less developed, have the advantage of allowing cytomorphological analysis, in a similar way to that performed in exfoliative cytology and in fine needle aspiration cytology.[6,7] However, very few studies have been carried out to describe and define the cytomorphological criteria of CTCs and to examine whether the classical cytological criteria of malignancy can be applied to CTCs.[8–11] Among the direct methods, the fibre-optical array scanning (FAST) technology has been used recently to identify CTCs using cytomorphology, in a small number of lung, colonic and prostatic cancer patients.[9,12,13] Another direct method, called isolated by size of epithelial tumour cells (ISET) technology, first described in the field of oncology by Vona *et al.*[7,10,11] has been used in patients with liver or breast carcinomas. This latter technique is sensitive, simple, has a low cost and allows cytomorphological analysis and characterization of CTCs.

Circulating non-haematological cells (CNHCs) can be detected in the blood of patients with non-malignant diseases, and more exceptionally in healthy individuals.[2] The origin of these epithelial cells is uncertain and their morphological characterization is unknown. Therefore in practice, even in cancer patients, the detection of CNHCs could not always be considered as malignant cells, particularly if these CNHCs do not show cytological criteria of malignancy. In this regard, the cytomorphological analysis of CNHCs should be of great value in characterizing CTCs.

Lung cancer is the most prevalent neoplasm and the major cause of tumour-related mortality worldwide.[14–16] Despite recent advances in the management of resected lung cancers and more effective treatment of metastatic tumours, the cure rate of patients with lung cancer remains low.[14–16] In this regard, sensitive and specific detection of CTCs in the blood might be considered a potentially relevant prognostic biomarker for patients with NSCLC.[17] The detection of CTCs in patients with lung carcinoma has mainly been performed with indirect methods.[18–28] One study reported the cytomorphological details of CTCs isolated in one patient with lung adenocarcinoma using an immunofluorescent staining protocol.[9] Moreover, we recently demonstrated an interest in prognostic biomarkers of CTCs detected by the ISET method in NSCLC patients.[29] However, stringent cytomorphological criteria are now needed in order to better identify the different population of CNHCs, in particular those corresponding to malignant cells. No study has been performed prior to our study for morphological characterization of CTCs by a direct method in patients undergoing surgery for resectable NSCLC. This may be of interest, since in a subpopulation of patients with complete surgical tumor resection, loco-regional and distant metastases can occur rapidly, and the preoperative detection of CTCs could allow selection of these patients with a higher risk. [30–32]

This study was conducted in order to evaluate the usefulness and efficacy of the ISET method for preoperative detection and characterization of CTCs in NSCLC patients. For this purpose, CTCs were examined in a group of 250 patients with resectable NSCLC by a panel of 10 cytopathologists. Cytomorphological criteria had been established by this panel for classifying CNHCs into the three following categories: (i)

CNHCs with malignant features, (ii) CNHC with uncertain malignant features, and (iii) CNHC with benign features. In this paper, and for the purpose of simplicity, these categories of CNHCs were further called circulating malignant, uncertain malignant and benign cells, respectively. Interobserver agreement between these 10 cytopathologists was then studied for the three categories of CNHCs.

Methods

Patients and samples

Two hundred and fifty patients with NSCLC undergoing surgery from December 2006 to March 2010 gave their informed consent to participate in this study. Biopsies had not been performed for at least 15 days before surgery. The clinicopathological parameters of these patients are summarized in Table 1. Among 150 adenocarcinomas, 95 expressed the TTF1 antigen, as determined by immunohistochemical (anti-TTF1 antibody, diluted 1 : 100, Dako; Glostrup, Denmark) staining. Blood samples from 59 healthy volunteers were used as negative controls. There were 39 men (median age, 39 years; range, 25–65 years) and 20 women (median age, 45 years; range, 22–53 years), who were smokers (average, 14 pack years; range, 10–47 pack years), without evidence of neoplastic disease.

Table 1. Epidemiological and clinico-pathological data of non-small cell lung carcinoma patients

Variables	Overall*
Patient cohort	250
Age (years)	
Mean	65
Range	33–82
Gender	
Male	172 (69)
Female	78 (31)
Smoking status	
Never smoked	30 (12)
Former or current smokers	220 (88)
Mean (range) pack years	46 (1–85)
Tumour size (cm)	
Mean	4
Range	0.4–18
Histological cell type	
Adenocarcinoma	150 (60)
Squamous cell carcinoma	67 (27)
Neuroendocrine carcinoma	7 (3)
Large cell carcinoma	9 (3)
Adenosquamous carcinoma	2 (1)
Sarcomatoid carcinoma	10 (4)
NSCLC (NOS)	5 (2)
pTNM stage	
I	111 (44)
II	70 (28)
III	50 (20)
IV	19 (8)

*Values expressed as *n* (%).
pTNM, pathological tumour node metastasis; NSCLC, non-small cell lung carcinoma; NOS, not otherwise specified.

Methods

Ten millilitres of peripheral blood were collected in buffered EDTA before anaesthesia, maintained at 4 °C, and processed within 1 hour. Surgical lung specimens were taken for pTNM staging and histological evaluation, according to international classifications.[33,34] ISET was carried out as described previously.[7] After blood filtration, the membrane was gently washed with PBS, disassembled from the filtration module and allowed to air-dry. The spots were stained using a modified May-Grünwald–Giemsa (MGG) staining method with the following steps: May-Grünwald (undiluted, 5 minute), May-Grünwald (diluted 50% in PBS, 5 minute) and Giemsa (diluted 10% in PBS, 40 minute), followed by rinsing with PBS for 1 minute. Stained spots were examined by light microscopy using different steps: (i) observation at 100× and 200× original magnification to look for non-haematological cells and to count sheets of these cells, and (ii) observation at 630× and 1000× original magnification with oil immersion for detailed cytomorphological analysis. Different criteria were taken into account to characterize the detected non-haematological cells: nucleo-cytoplasmic ratio, anisonucleosis, irregularity of the nuclear membrane and size of the nucleus, nuclear hyperchromasia, and presence of three-dimensional sheets. Cells without visible cytoplasm were not included in this study. When features corresponding to early or late apoptotic cells (nuclear shrinkage and fragmented nuclei) were noted, the corresponding cells were not counted. Circulating malignant cells were defined by the presence of at least four of the following criteria: anisonucleosis (ratio > 0.5), nuclei larger than three calibrated pore sizes of the membrane (8 μm) (i.e. >24 μm), irregular nuclei, high nucleo-cytoplasmic ratio, and presence of three-dimensional sheets (Figure 1). CNHCs were defined as circulating uncertain malignant cells when they had fewer than two criteria, but at least one was

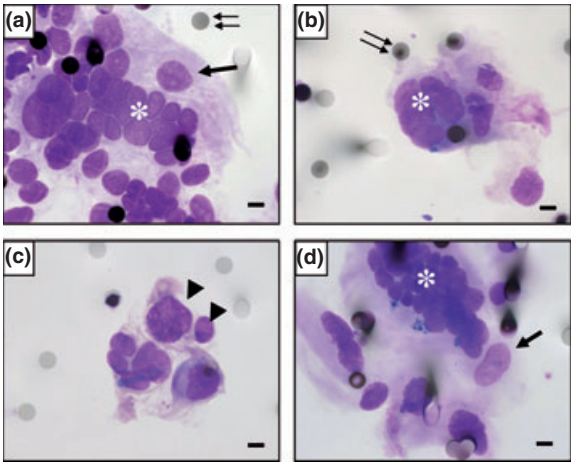

Figure 1. Cytomorphological criteria of circulating malignant cells isolated by the ISET method in patients with NSCLC. (a) Patient with T1N0 adenocarcinoma. (b) Patient with T2N1 adenocarcinoma. (c) Patient with T3N0 large cell carcinoma. (d) Patient with T2N1 large cell carcinoma. (a–d, original magnification ×1000, MGG staining; bars, 8 μm; arrows, anisonucleosis; arrow heads, irregularity and large nuclei; asterisks, three dimensional sheets; double arrows, pores of filters.)

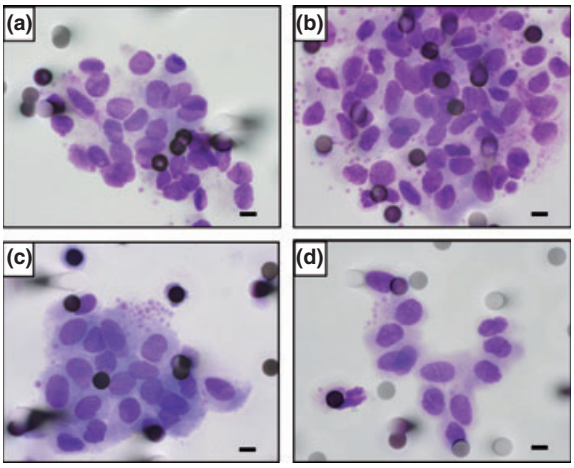

Figure 2. Cytomorphological criteria of circulating uncertain malignant cells isolated by the ISET method in patients with NSCLC. (a) Patient with T2N0 adenocarcinoma. (b) Patient with T3N1 large cell carcinoma. (c) Patient with T2N0 large cell carcinoma. (d) Patient with T1N1 squamous cell carcinoma. (a–d, original magnification ×1000, MGG staining; bars, 8 μm; double arrows, pores of filters.)

present (Figure 2). CNHCs defined as circulating benign cells were characterized by the absence of these criteria (Figure 3). A total of 917 pictures (average mean, seven pictures per filter; range, 1–

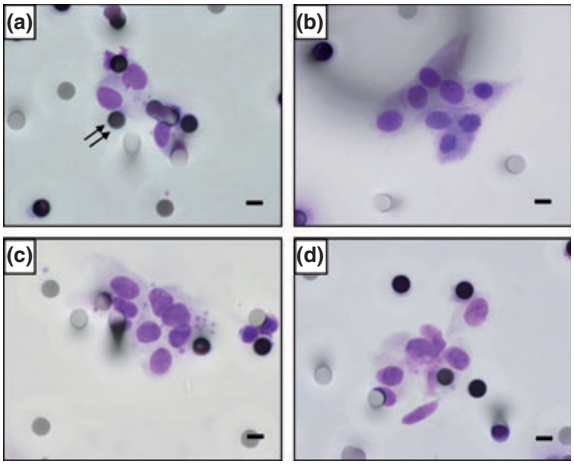

Figure 3. Cytomorphological criteria of circulating benign cells isolated by the ISET method in patients with NSCLC. (a) Patient with T2N0 adenocarcinoma. (b) Patient with T2N1 squamous cell carcinoma. (c) Patient with T2N1 large cell carcinoma. (d) Patient with T2N0 adenocarcinoma. (a–d, original magnification ×1000; bars, 8 μm; double arrows, pores of filters.)

32) were recorded and images were digitized at a ×1000 magnification and collected by three of the authors (VH, CB and PH). All images were then reviewed independently by the members of the panel of cytopathologists from eight different institutions (VH, CB, PV, SL, NM, JFF, TJM, JMV, EP and PH) without knowledge of the diagnosis. There were single observers in each institution, apart from at the Pasteur Hospital, Nice, where there were three observers. Images were scored independently by the cytopathologists and classified as circulating malignant, uncertain malignant or benign cells for each patient. Malignancy was confirmed if in at least one image a CTC (i.e. a circulating malignant cell) was identified and if at least five cytopathologists were in agreement. Similarly, a patient was classified in the category of patients with circulating uncertain malignant or benign cells when at least five cytopathologists agreed with this evaluation.

Statistical analysis

All calculations were performed with the statistical software R, a free language and environment for statistical analysis and graphics (version 2.9.0, Oregon State University, Corvallis, OR, USA). We used Kappa statistics, which reflect agreement between two measurements after removing chance agreement, as a

measure of reliability.[35,36] A value close to one represents almost perfect agreement whereas values close to or below zero represent poor agreement. A useful scale for the interpretation of the Kappa estimate was developed by Landis and Koch: 0.81–1.00, 'almost perfect'; 0.61–0.80, 'substantial'; 0.41–0.60, 'moderate'; 0.21–0.40, 'fair'; 0.00–0.20, 'slight'; and <0.0, 'poor' agreement.[36] We computed the weighted Kappa when the classification scheme had more than two categories; greater weight was given to differences in non-adjacent categories than to differences in adjacent categories.[35] Conditional agreement is reported using percentages. In addition to these analyses, we examined whether the magnitude of agreement for a given pathological characteristic differed at the level of another pathological characteristic. The following criteria were evaluated and compared using the chi-square statistical test; presence or absence of CNHCs: presence or absence of CNHCs according to the pTNM stages and the histological subtype; presence or absence of circulating malignant, uncertain malignant and benign cells. The Mann–Whitney test was used to compare quantitative and qualitative variables. A *P*-value of 0.05 or less was considered to be significant.

Results

Interobserver agreement between the three cytopathologists working in the same institution (VH, CB and PH) (Pasteur Hospital, Nice, France) was total ($\kappa = 1$) for detecting CNHCs on filters. CNHCs were present preoperatively in 123/250 (49%) patients undergoing surgery for NSCLC. In all cases (100%), five out the 10 cytopathologists agreed with the final diagnosis for each patient (circulating malignant, uncertain malignant or benign cells) ($\kappa = 1$; Table 2). Circulating malignant cells (CTC) were characterized morphologically in 102/250 (41%) of cases. It is noteworthy that interobserver variation was low for the diagnosis of malignant cells (Table 2). Patients showed circulating uncertain malignant and benign cells in only 15/250 (6%) and 6/250 (2%) cases, respectively. Among all the CNHC, circulating malignant, uncertain malignant and benign cells, represented 83% (102/123), 12% (15/123) and 5% (6/123) of cases, respectively. CNHCs were not found in the blood of the 59 healthy volunteers. Furthermore, the '5/10 cytopathologists' group was defined as a standard in order to calculate the inter-institution level of agreement (Table 3). It is noteworthy that inter-institution variability was similar, with or without stratification for the malignant features. As expected, there was higher variability between several institutions.

The frequency of detection of CNHCs in different histological subtypes was similar ($P > 0.05$) (Table 4). The cytopathological features of CNHCs from patients with lung adenocarcinoma were not distinguishable from those of CNHCs derived from the other histological subtypes (Figure 4). No correlation was

Table 2. Interobserver agreement between cytopathologists for identification of circulating malignant, uncertain malignant and benign cells

Agreement/cytopathologist	10/10	9/10	8/10	7/10	6/10	5/10
Circulating malignant cells						
Cumulative agreement/cases (%)	11 (9)	17 (14)	42 (34)	66 (54)	90 (73)	102 (83)
Circulating uncertain malignant cells						
Cumulative agreement/cases (%)	2 (1)	3 (2)	4 (3)	7 (6)	9 (7)	15 (12)
Circulating benign cells						
Cumulative agreement/cases (%)	1 (1)	2 (1)	3 (2)	4 (3)	5 (4)	6 (5)
Total						
Cumulative agreement/cases (%)	14 (11)	22 (17)	49 (39)	77 (63)	104 (84)	123 (100)

Results from the five out of 10 institutions were used as a standard for calculating Kappa level of agreement between the different institutions with or without stratification for malignant features. The percentages represent the number of positive responses made by the panel of cytopathologists for the evaluation of the different categories of circulating non-haematological cells. Numbers in brackets are the number of cases.

Table 3. Kappa values of agreement between the different institutions, with or without stratification for malignant features of cells

κ values/institution	8/8	7/8	6/8	5/8	4/8	3/8*
With stratification for malignant cells	0.12	0.19	0.45	0.68	0.89	1
Without stratification for malignant cells	0.11	0.18	0.40	0.69	0.84	1

*The 3/8-institution group was defined as a standard (κ = 1).
The results from the five out of 10 cytopathologists were defined as a standard (κ = 1) for calculating the inter-institution variability.

Table 4. Number of circulating non-haematological cells detected by isolation by the size of epithelial tumour cell method, according to histological classification of non-small cell lung carcinoma and pTNM staging

	ISET (+)	ISET (−)	Total	
Variables	*n* (%)	*n* (%)	*n* (%)	*P*-value
Histological subtype				
Adenocarcinoma	79 (53)	71 (47)	150 (100)	0.74
Squamous cell carcinoma	27 (40)	40 (60)	67 (100)	
Large cell carcinoma	5 (56)	4 (44)	9 (100)	
Neuroendocrine carcinoma	4 (57)	3 (43)	7 (100)	
Adenosquamous carcinoma	1 (50)	1 (50)	2 (100)	
Sarcomatoid carcinoma	4 (40)	6 (60)	10 (100)	
NSCLC (NOS)	3 (60)	2 (40)	5 (100)	
pTNM stage				
I	55 (49)	56 (55)	111 (100)	0.50
II	34 (48)	36 (52)	70 (100)	
III	24 (48)	26 (52)	50 (100)	
IV	10 (52)	9 (48)	19 (100)	
Overall	123 (49)	127 (51)	250 (100)	

ISET, isolation by size of epithelial tumour cell; pTNM, pathological tumor node metastasis; NSCLC, non-small cell lung carcinoma; NOS, not otherwise specified.
**P*-value significant at the 0.05 level.

observed between the detection of CNHCs and the pTNM stage ($P > 0.05$) (Table 4).

Discussion

The present study shows that CNHCs can be detected using ISET, a cytomorphological technology, in around 50% of patients undergoing surgery for resectable NSCLC. The presence of these CNHCs is not correlated with pTNM or the histological subtype of the primary tumour. In most cases, these cells were identified by a large majority of the cytopathologists on the panel as having malignant cytological criteria, and hence have been classified as CTCs. However, the invasive potential of these cells, as well as their metastatic potential, cannot be correlated, to date, with their cytological features. It would therefore be of interest to correlate the presence and the number of detected CTCs with patient follow-up. The different cytological criteria of malignancy adopted to classify the CNHCs as CTCs were those usually used in clinical cytopathology.[37] However, in the present study, certain criteria were more frequently observed than others. For example, anisonucleosis, increased size of nuclei and irregular nuclei were more often observed in CTCs than high nucleo-cytoplasmic ratio and three-dimensional sheets of cells. This can be explained, at least partially, by the fact that certain cytopathologists on the panel did not consider these cells as true CTCs (circulating malignant cells) and classified them as circulating uncertain malignant cells. Interestingly, isolated CNHCs in our population of patients were not diagnostic for a histological subtype of the corresponding primary NSCLC. In particular, no features of keratinization or intracytoplasmic secretory vacuoles, which could correspond to mucus secretion, were noted in these cells. This may be explained by the fact that these cells could correspond to cells pre-selected as poorly differentiated cells, which could be the only population able to cross the endothelial barrier and

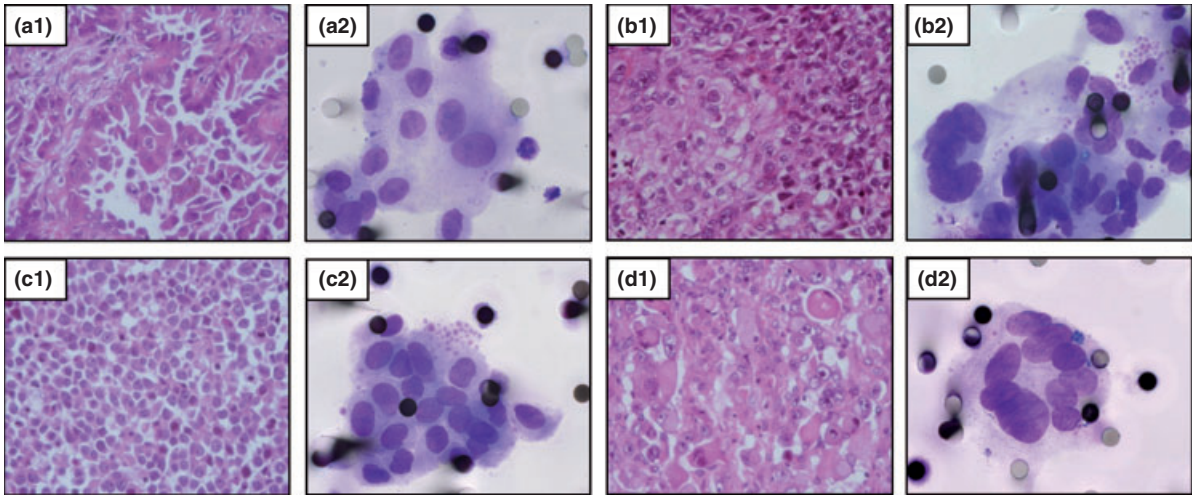

Figure 4. Comparison of the cytopathological features of circulating malignant cells and histological features of corresponding primary lung tumours. (a) Tubulo-papillary adenocarcinoma. (a1) H&E, original magnification ×400. (a2) Corresponding circulating malignant cells, original magnification ×1000. (b) Squamous cell carcinoma. (b1) H&E, original magnification ×400. (b2) Corresponding circulating malignant cells, original magnification ×1000. (c) Large cell carcinoma. (c1) H&E, original magnification ×400. (c2) Corresponding circulating malignant cells, original magnification ×1000. (d) Sarcomatoid carcinoma. (d1) H&E, original magnification ×400. (d2) Corresponding circulating malignant cells, original magnification ×1000.

migrate into the bloodstream.[1,2,38] The presence of CNHCs without any cytological criteria of malignancy, called circulating benign cells, is questionable. They may correspond to epithelial cells detached from peritumoural areas or from other sites subjected to tissue microtrauma leading to vascular barrier disruption. However, we cannot totally exclude the possibility that they could be tumour cells without cytological atypia. This situation is rarely observed in 'conventional' cytology obtained from exfoliative or fine needle aspiration specimens, but might be potentially more frequent in the morphological analysis of CTCs. Finally, it is possible that a few of these cells could be circulating endothelial cells, as these are frequently detected in the blood of cancer patients.[39,40] We did not consider cells with apoptotic features or naked nuclei for the diagnosis of CNHCs, although in some patients they were numerous, representing up to 30% of the isolated cells.

Some of the results of our study are not completely in keeping with some of the data on cytomorphogical observations described in a recent publication.[9] In this latter work, the authors showed a strong correlation between the cytopathological features of CTCs and the histological and cytopathological data observed in the corresponding primary lung adenocarcinoma.[9] Moreover, the majority of CTCs detected showed a low nucleo-cytoplasmic ratio.[9] The authors concluded that these CTCs were not de-differentiated.[9] This contradicts the current concept claiming that only poorly differentiated tumour cells can cross the endothelial barrier and then migrate into the blood stream.[1,2,38] However, the results obtained by Marinucci *et al.*[9] were observed in only one patient and used the FAST method.

Despite the importance of conventional cytopathological analysis based on MGG staining, it would certainly be of great interest to confirm these morphological criteria by an immunocytochemical study. However, immunocytochemistry of CTCs has a number of limitations because the phenotype of CTCs is usually different from that of the cells of the primary tumour.[1,2,38] Hence, and also as a consequence of the epithelio-mesenchymal transition phenomenon, some CTCs have a low or absent level of cytokeratins.[1,2,38] Moreover, no antibody is currently available for detecting the malignant potential of a given cell by immunocytochemistry. Recently, the detection of genomic aberrations in CTCs using an antigen-independent FISH-based assay has been suggested as a diagnostic and prognostic feature in patients with NSCLC.[41] Thus different molecular biological approaches applied to CNHCs detected by ISET could be very useful in the future to improve characterization of the malignant potential of these cells.[1,2,7,41]

In conclusion, the ISET method is a powerful approach not only for detection of CNHCs but also

for characterizing some or all of these CNHCs as CTCs. Our study shows that these CTCs are isolated in around half of patients undergoing surgery for resectable NSCLC. Whether or not their presence is related to prognosis remains to be evaluated. Quantification of these CTCs using automatic imaging devices would also be useful for looking for the prognostic impact according to the exact number of CTCs detected. Moreover, only the follow-up of our cohort of patients, with or without CTCs detected by the ISET approach, will tell us if these cells correspond to passively shed cells with little or no malignant potential, or actively migrating cells that are viable and may form further metastatic foci. The combination of 'classical' reference cytomorphological approaches with newly developed molecular markers derived from proteomic, molecular profiling and genetic analyses of the cells detected, should allow better classification and treatment of patients according to prognosis. Moreover, this might identify these CTCs as a pertinent molecular target for the development of new antitumour agents and raises the possibility that the presence of CTCs detected by ISET may guide clinicians in tailoring future therapy.

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