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ORIGINAL ARTICLE – CANCER RESEARCH

Prevalence and number of circulating tumour cells and microemboli at diagnosis of advanced NSCLC

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Abstract
Purpose Timing and magnitude of blood release of circulating tumour cells (CTC) and circulating tumour microemboli (CTM) from primary solid cancers are uncertain. We investigated prevalence and number of CTC and CTM at diagnosis of advanced non-small cell lung cancer (NSCLC).
Methods Twenty-eight consecutive patients with suspected stage III–IV lung cancer gave consent to provide 15 mL of peripheral blood soon before diagnostic CT-guided fine-needle aspiration biopsy (FNAB). CTC and CTM (clusters of ≥3 CTC) were isolated by cell size filtration (ScreenCell), identified and counted by cytopathologists using morphometric criteria and (in 6 cases) immunostained for vimentin.
Results FNAB demonstrated NSCLC in 26 cases. At least one CTC/3 mL blood (mean 6.8 ± 3.7) was detected in 17 (65 %) and one CTM (mean 4.5 ± 3.3) in 15 (58 %) of 26 NSCLC cases. No correlation between number of CTC or CTM and tumour type or stage was observed. Neoplastic cells from both FNA and CTC/CTM were positive for vimentin but heterogeneously.
Conclusions CTC can be detected in two-thirds and CTM in more than half of patients with advanced NSCLC at diagnosis. Reasons underlying lack of CTC and CTM in some advanced lung cancers deserve further investigations.

Keywords Circulating tumour cells · Circulating tumour microemboli · Non-small cell lung cancer

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Introduction

Detection, enumeration and molecular characterization of circulating tumour cells (CTC) and circulating tumour microemboli (CTM) in the peripheral blood featuring a type of «liquid biopsy» represent an emerging field in lung cancer research (Huang et al. 2013; Krebs et al. 2012; Wendel et al. 2012; Carlsson et al. 2014). In particular, it has been hypothesized that CTM might be more capable of metastasizing as compared with single CTC (Bottos and Hynes 2014).

Better understanding of metastatic haematogenous dissemination is one of the aims of research on CTC (Hou et al. 2011; Ilie et al. 2014a). Usually CTC and CTM are searched in samples of peripheral blood taken at variable time interval after diagnosis of the primary solid lesion (Huang et al. 2013). As a matter of fact, timing and magnitude of CTC and CTM release from the malignant solid tumour are not yet established. We considered diagnosis a

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reasonable time point in the disease course in an attempt to better outline temporal dynamics and entity of the haematogenous delivery of CTC and CTM.

We investigated prevalence and number of CTC and CTM of advanced non-small cell lung cancer (NSCLC) in blood samples taken just before diagnosis with percutaneous fine-needle aspiration biopsy under computed tomography guidance (CT-FNAB).

Patients and methods

Case selection

From October to December 2014, we prospectively enrolled 28 consecutive patients with suspected and previously untreated stage III or IV lung cancer scheduled for diagnostic CT-FNAB at the Careggi Hospital of Florence, Italy. Due to the unsettled possible therapeutic and prognostic implications of CTC detection in early stages of lung cancer and the consequent ethical issues, we decided to skip stage I and II lung cancer lesions in the present investigation. All patients signed a written consent for CT-FNAB, blood collection aimed to evaluation of CTC and anonymously participating to the study. The clinical stage of the presumed lung cancer was determined before FNAB according to the American Joint Committee on Cancer staging system (7th edition) (Detterbeck et al. 2009) with joint review by two radiologists [M.Mas. and E.B.] and one nuclear medicine physician [A.V.] of the patient's contrast-enhanced CT and 2-[18F]flu-2-deoxy-D-glucose positron emission tomography examinations.

CT-FNAB of the lung lesion was performed by one of the two experienced interventional radiologists [M.N. and M.F.] on a 40-detector CT scanner with CT fluoroscopy capability (Sensation Open, Siemens, Erlangen, Germany). The lesion aspirates were processed and assessed by one of the two expert cytopathologists [C.M. and M.Mat.] with rapid on-site examination (ROSE) (Mazza et al. 2005). In each case, definitive cytological diagnosis was made according to the current classification of lung cancer (Travis et al. 2013).

Blood sample collection

Manipulation of tumour in animal models (Juratli et al. 2014) and in human lung cancer (Sawabata et al. 2007; Yao et al. 2014) is associated with delivery of CTC in the bloodstream. Since the same phenomenon might occur in lung tumours undergoing FNAB, in each patient 15 mL of blood was collected in two EDTA tubes (7.5 mL each) soon before CT-FNAB through the antecubital vein access routinely prepared before CT-FNAB. Blood samples were processed within 3 h after collection.

CTC analyses

CTC were isolated from blood by filtration on ScreenCell Cyto devices (ScreenCell, France) using isolation-by-size method, which does not involve any biological characterization of the putative CTC.

In an in vitro study, the ScreenCell Cyto device showed sensitivity of 74 and 91 % for the detection of two and five CTC, respectively (cultured NCI-H2030 cells derived from NSCLC), spiked in 1 mL of peripheral blood from a healthy donor (Desitter et al. 2011).

CTC were identified using morphometric criteria (Hofman et al. 2012). In six patients, CTC were characterized by immunocytochemistry. In particular, since CTC in lung cancer undergo epithelial–mesenchymal transition (EMT) and tend to lose positivity for epithelial surface markers, such as cytokeratin and EpCaM (Krebs et al. 2012), while developing mesenchymal markers, such as vimentin (Lecharpentier et al. 2011; Hou et al. 2011), we used the latter to test potential positivity of FNAB and CTC.

Isolation

Blood was filtered by the ScreenCell Cyto filtration devices according to the procedure previously described (De Giorgi et al. 2010).

Briefly, before filtration and in order to lyse red blood cells, 3 mL blood samples were diluted in 4 mL of the provided dilution buffer, according to the manufacturer's instructions (ScreenCell FC dilution buffer; ScreenCell). After blood filtration, filter was rinsed with an additional 1.6 mL phosphate-buffered solution to remove red blood cell debris. Filtration was usually completed within approximately 3 min. The filter was then disassembled from the filtration module and allowed to air-dry. For each patient's blood sample, filtration was performed in duplicate.

Identification

Identification and characterization of CTC were carried out in consensus by the same two experienced cytopathologists who performed FNAB with ROSE and were aware of the results of latter.

Cytological studies were conducted directly on the filter. The track-etched filters were stained with haematoxylin solution S (Merck KGaA, Germany), applied to the membrane for 2 min, and with Shandon eosin Y aqueous (Thermo Electron Corporation, Thermo Fisher Scientific Inc., USA) for 30 s. For microscopic observation, the ScreenCell Cyto filter was

placed on a standard microscopy glass slide, and a 7-mm circular cover slip (Menzel-Glaser, Germany) was laid on the filter with the appropriate mounting medium. CTC were identified among the entrapped cells according to the presence of at least four of the following cytomorphological criteria: nucleus size ≥16 µm, anisonucleosis (ratio > 0.5), high (>2:1) nuclear/cytoplasmic ratio, irregular nuclei and presence of three-dimensional sheets (Vona et al. 2000; De Giorgi et al. 2010; Hofman et al. 2012; Pinzani et al. 2013). CTM were defined as a group of contiguous CTC containing three or more nuclei and/or three-dimensional sheets of cells (Krebs et al. 2012).

Cytological characterization with vimentin immunostaining

Immunostaining with mouse anti-vimentin antibody (V9, Santa Cruz Biotechnology Inc., USA) was performed using "S.A. ScreenCell" protocol on ScreenCell filters. Accordingly, the ScreenCell Cyto filters were hydrated with Tris-Buffered Saline (TBS; pH 7.4). The excess TBS was removed with absorbent paper, and the filters were put on filter support in a humid chamber. The antibody required heat-induced epitope retrieval, so the circle filter spots were treated in a bath containing the retrieval solution (pH 6.0) at 95 °C for 20 min.

Filters were removed from the water bath and cooled at room temperature for 20 min. The circle filters were rinsed with TBS for 1 min, and the excess of TBS was removed with absorbing paper. Each filter was incubated for 5 min at room temperature with 70 mL of permeabilizing buffer.

The filters were washed once with TBS for 1 min and immersed in a bath containing distilled water, and then, the circle filter was incubated in a humid chamber with peroxidase-blocking solution for 30 min at room temperature.

The filters were washed once with TBS for 1 min and incubated with mouse anti-vimentin antibody (1:50 dilution, sc 6270, Santa Cruz Biotechnology Inc.) for 1 h at room temperature in a humid chamber. We used EnVision Detection System, Peroxidase DAB, Rabbit/Mouse (K 5007, Dako, Italy) for 40 min at room temperature, followed by the chromogen AEC (3-amino-9-ethyl-carbazole, Dako) for 10 min at room temperature. Each filter was counterstained with Harris's haematoxylin for 2 min. Finally, the filters were rinsed with tap water and covered with a coverslip.

Positive and negative controls were available for each vimentin immunostaining.

Statistical analysis

Data are expressed as mean ± SD and were analysed by SPSS 18.0 for Windows (Statistical Package for the Social Sciences). Category variable was assessed with contingency table and Chi-square test. Univariate correlation was carried out using Spearman's correlation test. P values of less than 0.05 were considered statistically significant.

Results

CT-FNAB demonstrated NSCLC in 26 cases and single-lung metastasis (from intestinal adenocarcinoma and breast cancer) in 2 cases. Table 1 details the demographic data, cytological diagnosis and clinical stage of the 26 NSCLCs. Diagnoses included adenocarcinomas in 15 cases, squamous cell carcinomas in 10 cases and not otherwise specified NSCLC in 1 case. The clinical stage of NSCLCs was IIIA in 6, IIIB in 8 and IV in 12 patients.

Figure 1 displays examples of CTC and CTM. The presence and number of CTC, the presence and number of CTM and the number of cells forming each CTM are reported in Table 1. At least one CTC in 3 mL was detected in 17 of 26 (65 %) NSCLCs. The mean number of CTC was 6.8 ± 3.7 per 3.0 mL. CTM were detected in 15 of 17 (88 %) patients with CTC corresponding to 58 % of NSCLC patients. The number of CTM ranged between 1 and 12 (mean 4.5 ± 3.3), and the number of cells comprised in the aggregate between 3 and 30 (mean 9.3 ± 7.2). All six cases (number 21–26) tested for vimentin staining displayed some but not all positive cells in both the FNA and filter with a substantial concordance in the cytoplasmatic staining pattern.

There was no significant correlation between cytological type or clinical stage, on the one hand, and the presence and number of CTC/CTM, and the number of CTM forming cells, on the other hand.

Discussion

Prevalence of CTC and CTM in previous studies conducted on patients with lung cancer ranged between 18 and 100 % (Huang et al. 2013; Maheswaran et al. 2008).Several factors might account for this wide range, including methods to isolate and identify CTC, type of lung cancer (NSCLC vs. small cell lung cancer), tumour stage, effects of treatments and, presumably, the time interval between tumour development and CTC release and blood sampling.

In our study, we focused on untreated advanced NSCLC and performed blood sampling just before FNAB diagnosis. Moreover, we used a sensitive method for isolation of CTC and CTM (Krebs et al. 2012) and applied cytomorphometric criteria proposed to identify them (Hofman et al. 2012).

The 65 % CTC prevalence we found is higher than in most prior studies of NSCLC and substantially in line with the 80 % prevalence reported in one study of advanced

Table 1 Cytopathological diagnosis, clinical stage, number of CTC and of CTM in 26 cases of advanced non-small cell lung cancer

Patient number/sex/age	Cytopathological diagnosis	Clinical stage	CTC Number[a]	CTM[b] Number: number of CTM forming cells
1/M/67	Squamous cell carcinoma	IIIA	3	1:3 cells
2/M/64	NSCLC not otherwise specified	IV	0	0
3/M/74	Squamous cell carcinoma	IIIB	3	4:6, 7, 10, 20 cells
4/M/83	Squamous cell carcinoma	IIIA	12	0
5/M/70	Squamous cell carcinoma	IV	4	8:4, 5, 5, 6, 7, 10, 10, 15 cells
6/M/78	Squamous cell carcinoma	IIIB	0	0
7/M/54	Adenocarcinoma	IV	0	0
8/M/78	Squamous cell carcinoma	IIIB	0	0
9/M/76	Adenocarcinoma	IV	5	0
10/M/68	Squamous cell carcinoma	IV	5	1:3 cells
11/M/84	Adenocarcinoma	IIIB	9	7:5, 5, 12, 19, 22, 26, 27 cells
12/F/75	Adenocarcinoma	IV	15	5:3, 4, 4, 5, 30 cells
13/F/51	Adenocarcinoma	IV	0	0
14/M/71	Squamous cell carcinoma	IIIB	0	0
15/M/79	Squamous cell carcinoma	IIIB	5	2:5, 14 cells
16/M/48	Adenocarcinoma	IIIB	0	0
17/F/77	Adenocarcinoma	IIIB	0	0
18/F/80	Adenocarcinoma	IIIA	12	5:4, 7, 10, 15, 22 cells
19/M/78	Adenocarcinoma	IV	4	2:3, 3 cells
20/F/83	Adenocarcinoma	IIIA	0	0
21/M/78	Adenocarcinoma	IV	8	6:9, 10, 10, 14, 16, 16 cells
22/F/66	Adenocarcinoma	IV	6	4:3, 5, 5, 7 cells
23/M/79	Adenocarcinoma	IIIA	4	8:4, 4, 4, 5, 5, 6, 7, 8 cells
24/M/68	Adenocarcinoma	IV	4	1:5 cells
25/M/81	Adenocarcinoma	IV	11	12:3, 4, 5, 5, 5, 8, 10, 10, 13, 16, 21, 22 cells
26/M/72	Squamous cell carcinoma	IIIA	5	1:30 cells

[a] Per 3 mL of peripheral blood

[b] CTM are defined as clusters of ≥3 CTC

NSCLC (Krebs et al. 2012). On the other hand, the 58 % CTM prevalence we found is slightly in excess compared with prior results in NSCLC (Krebs et al. 2012; Wendel et al. 2012; Carlsson et al. 2014). As a matter of fact, available data indicate that CTC and CTM are detectable in most but not all patients with untreated advanced NSCLC (Krebs et al. 2012; Wendel et al. 2012; Carlsson et al. 2014). The reasons for lack of CTC and CTM in some advanced NSCLC are unclear. They include intermittent blood release of CTC from primary tumour, lymphatic rather than haematogenous tumour cell release and suboptimal sensitivity of the technical approach to isolate and detect them (Ilie et al. 2014a). All these possibilities deserve further investigations.

In our study, in line with some recent reports (Hofman et al. 2012; Krebs et al. 2012; Wendel et al. 2012; Carlsson et al. 2014), we did not find correlation between presence and number of CTC/CTM and the stage of advanced NSCLC, indicating that CTC/CTM number may not distinguish between the most advanced stages of the disease. However, a correlation between CTC and prognosis was reported in a meta-analysis that considered presence of CTC but not their numbers or phenotype characterization (Huang et al. 2013). In particular, it has been argued that, especially when the prognostic role of CTC is considered, it is important to address the biological specificity of CTC. This concerns the investigation of the capability of the CTC to invade, proliferate and cause metastasis (Wicha and Hayes 2011). In line with this pivotal point, in our samples we used vimentin staining that is a feature of EMT. Its heterogeneous positivity in some neoplastic cells from the tumour aspirate and in some CTC and CTM confirms previous evidence (Lecharpentier et al. 2011; Hou et al. 2011). A similar comparative evaluation between circulating and primary tumour neoplastic cells could be performed on

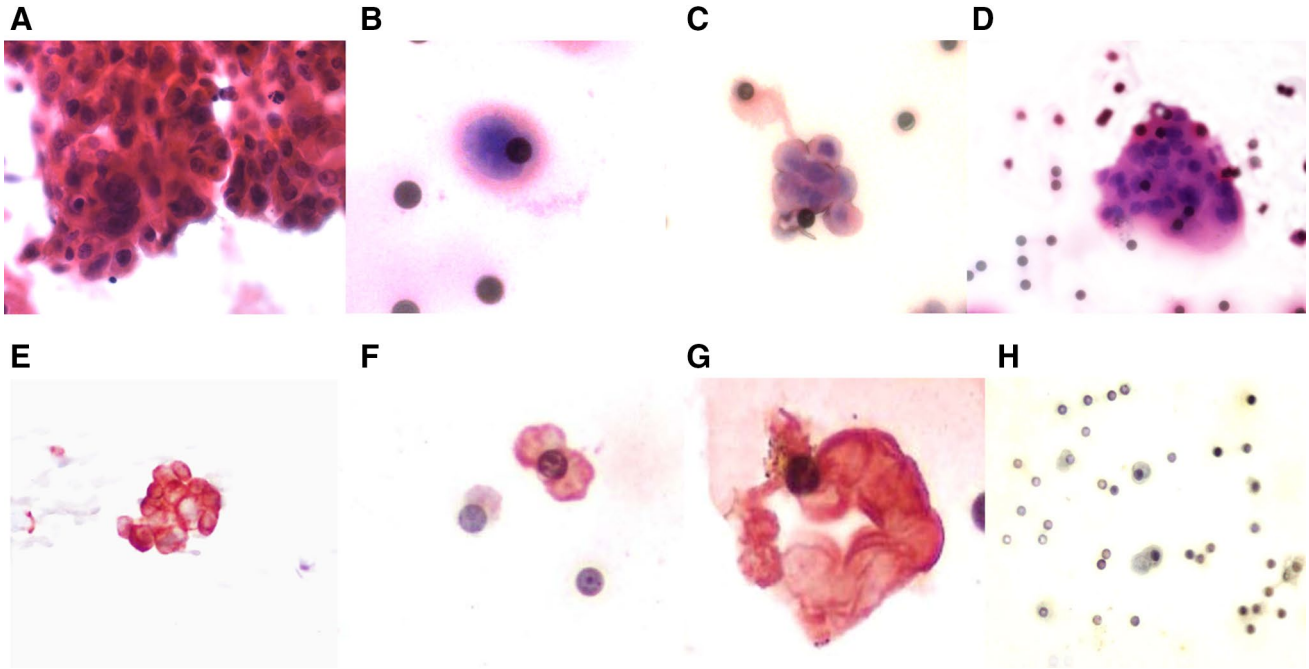

Fig. 1 Examples of neoplastic cells in fine-needle aspirates and of CTC and CTM in ScreenCell filters. **a**–**c** Case n.5. Squamous cell carcinoma stage IV. **a** Fine-needle aspirate (Papanicolaou staining, 40*x* magnification) showing a three-dimensional aggregate of cells with irregular and large nuclei. **b** Filter (haematoxylin and eosin staining, 40*x* magnification) showing a CTC with a large nucleus. **c** Filter (haematoxylin and eosin staining, 40*x* magnification) showing a CTM formed by 7 CTC. **d** Case n. 12. Adenocarcinoma stage IV. Filter (haematoxylin and eosin staining, 20*x* magnification) showing a CTM formed by 30 CTC. **e**–**g** Case n. 24. Adenocarcinoma stage IV. **e** Fine-needle aspirate (vimentin staining, 40*x* magnification) showing a cluster of tumour cells positive to vimentin. **f** Filter (vimentin staining, 40*x* magnification) showing CTC (two larger and more intensely stained nearby a pore and one smaller isolated nearby another pore) positive to vimentin. **g** Filter (vimentin staining, 40*x* magnification) showing a CTM positive to vimentin. **h** Case n. 22. Filter (vimentin staining, 20*x* magnification) showing CTC negative to vimentin. The *black dots* observed in **b**, **c**, **d**, **f**, **g** and **h** correspond to the 8 µm pores of the filters

their genotype (Maheswaran et al. 2008), but was not performed yet.

We recognize four limitations of our study. First, we investigated a relatively small sample of patients with NSCLC. However, the setting of CTC search was controlled and restricted to the time point of diagnosis of untreated advanced NSCLC. Second, we lacked serial follow-up "liquid biopsy" which may have relevant implications for the management and therapy in the single patient (Ilie et al. 2014a). This was due to the short follow-up available since diagnosis in most of the patients. Third, the cytopathologists who evaluated filters for presence of CTC and CTM were not blind to the results of FNAB. Finally, although the potential of CTC and CTM for diagnosis of lung cancer is actively investigated (Carlsson et al. 2014; Ilie et al. 2014b), this objective was beyond the realm of the present study.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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