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Detection of Circulating Tumour Cells and Survival of Patients with Non-small Cell Lung Cancer

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Abstract. *Background: Detection of circulating tumour cells (CTCs) in the peripheral blood of lung cancer patients may predict survival. Various platforms exist that allow capture of these cells for further analysis; little work however, has been done with the ScreenCell device, an antibody-independent CTC platform. The aim of our study was to evaluate the ScreenCell device for detection of CTCs in lung cancer patients and to establish correlations of these findings with survival. Materials and Methods: Twenty-three patients, nine males, and fourteen females, underwent surgical treatment from February to May 2014 for non-small cell lung cancer. Thirteen patients had adenocarcinoma and ten squamous cell carcinoma, while eight were at an early stage (I-II) and five at a later stage (III-IV). Blood samples were obtained prior to surgery and following filtration through the ScreenCell device, were independently reviewed by 2 consultant pathologists. Results: The pathologists were able to independently identify CTCs in 78.3% (N=18) and 73.9% (N=17) of the cases examined, with overall 80.6% in early stages compared to 60.0% in late stages. The median survival times of positive vs. negative for CTC patients were 1011 and 711 days respectively, with a survival percentage rate of 77.8% and 60% in positive and negative CTC cohorts respectively. Conclusion: The results of this study suggest that the presence of CTCs analyzed by ScreenCell did not necessarily lead to a poorer prognosis in patients with lung cancer after curative surgery.*

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Mortality from cancer remains high and accounts for 29% of all deaths (1). The development of metastatic disease is responsible for 90% of these deaths (2).

Circulating tumour cells (CTCs) are cancer cells of epithelial origin that are present in the peripheral blood samples of cancer patients to form a subpopulation of tumour cells which intravasate to allow lymph haematogenous dissemination in other areas of the body (3).

CTC counts in peripheral blood prior treatment can independently predict the early recurrence in patients with cancer (3, 4). Overall survival in patients with stage III and IV non-small cell lung cancer (NSCLC) was significantly longer (8.1 months *vs.* 4.3 months) in patients with fewer than 5 CTCs compared to those with five or more CTCs before chemotherapy (5). Clusters of CTCs, also known as circulating tumour microemboli (CTM), involve a process of collective cell migration which is important in tumour cell invasion (6, 7). CTMs have fewer apoptotic markers than CTCs, and their ability to survive is enhanced. As a result detection of CTMs is associated with a poorer prognosis (8, 9). More advanced and/or metastatic cancers express higher levels of CTCs and CTMs (10).

Multiple platforms for CTC isolation exist with varying functionalities. In this study, we used ScreenCell® (Paris, France), which is an antibody-independent device to avoid bias introduced by antibody-dependent techniques such as the CellSearch (Paris, France). The aim of our study was to examine the relationship between the number of CTCs detected by ScreenCell® and survival percentage rate in a NSCLC cohort of patients with surgically resectable lung cancer. Based on our knowledge there are no previous studies that have examined such relationship.

Patients and Methods

Patients. Twenty-three patients undergoing surgical treatment for suspected or confirmed NSCLC were recruited from February to May 2014. Patients had a confirmed diagnosis of NSCLC either pre-operatively or on an intra-operative frozen section. Ethical approval was sought prior (10/H0504/9) and consent was obtained pre-

operatively. Surgery was performed under the care of four thoracic surgeons at a tertiary thoracic centre. All operations were performed *via* a thoracotomy beside one, which was done *via* a video-assisted thoracotomy approach.

The cohort consisted of nine males and fourteen females, with a median age of 66±11 years. All patients had primary lung cancer, with a subtype of adenocarcinoma in thirteen (56.5%) and squamous cell carcinoma in ten (43.5%) patients. Early stage (I-II) was diagnosed in eighteen and late stage (III-IV) in five patients (Table I). Patients were followed up for a median time period of 31.8 months post-operatively. Survival information and cause of death was obtained by contacting the patient's General Practitioner.

CTC detection using the ScreenCell device. The detailed technique of CTC detection using the ScreenCell device is described elsewhere (11). In brief, three millilitres of blood were collected from the peripheral vein of patients immediately prior to surgery in EDTA-containing tubes. Samples were incubated with 7 ml of a lysis and formaldehyde fixation buffer for 5 min, provided by ScreenCell. Samples were then filtered through the ScreenCell device as per the manufacturer protocol.

Post-filtration filters were removed and stained with Haematoxylin and Eosin (H&E, Sigma Aldrich, St Louis, MO, USA) staining. Stained filters were then mounted on to slides and viewed by two independent consultant pathologists to assess the presence or not of CTCs. Filters were scored as either positive or negative for atypical cells suspicious for cancer, based on cell morphology and size, typically much larger cells, with nucleus to cytoplasmic ratio larger than normally were deemed as positive (Figure 1).

Statistical analysis was performed using GraphPad Prism v5 (GraphPad, San Diego, CA, USA), survival rate was analysed by Kaplan-Meier curves, statistical significance was calculated using a Chi-square test, and a Hazards Ratio (HR) calculated with a 95% confidence interval (CI).

Results

There was no perioperative mortality and all patients were alive at discharge. At follow-up, 17 of the 23 (73.9%) patients were alive and 6 (26.1%) were dead. Two pathologists detected CTCs, one in 18 (78.3%) and the other in 17 (73.9%) of 23 cases. At early-stage cancer patients, CTCs were detected in 80.6% (N=29) of cases compared to 60.0% (N=6) at late-stage patients (Table II).

In the group of six patients (26.1%) who tested negative for CTCs, three (50%) died during follow-up, two from metastatic lung cancer and one as a result of bowel obstruction. There were three deaths (18%) in the group of seventeen patients who tested positive for CTCs, two patients died as a result of lung cancer progression and one patient died as a result of bronchopneumonia.

Out of total six patients that died, four were early-stage I-II and two were late-stage III-IV. In terms of CTC status in the above patients, there was concordance in five of the cases (83.3%) and disagreement in only one case (16.7%). The median survival time in patients who died in the CTC-negative and in the CTC-positive group was 650 days and 836 days, respectively. Median follow-up time of the survivors was 1,008 days (Table III).

Table I. *Details patient demographics and diagnosis*.

Variable	Value	Percent (%)
Total patients	23	-
Mean age (±SD) years	66±11	-
Males/Females	9/14	39.1%/60.9%
Pathology		
Primary lung cancer	23	100.0%
Adenocarcinoma	13	56.5%
Squamous Cell Carcinoma	10	43.5%
TNM (Tumour, node, metastasis) staging		
Early stage (IA-IIB)	18	78.3%
Late stage (III-IV)	5	21.7%

The Kaplan-Meier survival curves, showed a poorer survival proportion in the CTC negative group compared to the CTC positive group, with a chi-square value of 11.08, shown as being statistically significant ($p<0.0009$) and a HR of 0.04453 (95% CI; range, 0.007131 to 0.2781) (Figure 2).

Discussion

Detection of CTCs can be difficult as only one CTC may be found in a background of 1×10^5 to 1×10^6 peripheral blood mononuclear cells (12). Another challenge lies in the biological characteristics of CTCs that can differ from those of the primary tumour. CTCs acquire genetic mutations equipping them to respond to local growth factors and stimulate neovascularisation. Several studies in breast cancer patients have found that HER2 expression differed between CTCs and the primary tumour. It has thus been postulated that CTCs expressing different biomarkers from the primary tumour may respond differently to treatments (13-16).

Several studies have compared the performance of different technologies to find varying results, in particular between antibody-dependent and antibody-independent devices. It is becoming increasingly evident that some epithelial properties are lost from cancer cells, in some cancers more than others (17, 18). Hence, epithelial expression-based methods such as the CellSearch device, may have a disadvantage (despite being the only FDA approved CTC isolation device) over expression-independant, filtration-type platforms such as ISET (RareCells, Paris, France) or ScreenCell which rely on cell size and other physical cellular properties. There are a multitude devices available on the market, with varying functionality (19-25).

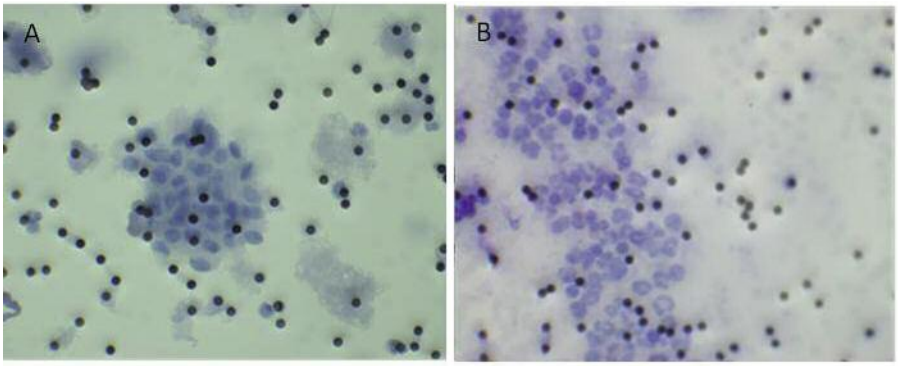

Figure 1. *Representative results of a positive slide of captured suspicious cancer cells on ScreenCell filter and a negative slide (haemotoxylin and eosin staining). (A) A large cluster of atypical cancer cells (40× magnification). Individual cell features can be seen, such as large irregular nucleus and small cytoplasmic layer. Overall size of cells is much larger (>8 μm, comparable to circular pores on the filter), compared to normal blood cells seen in the surrounding. (B) Negative slide showing contaminating leukocytes (40× magnification).*

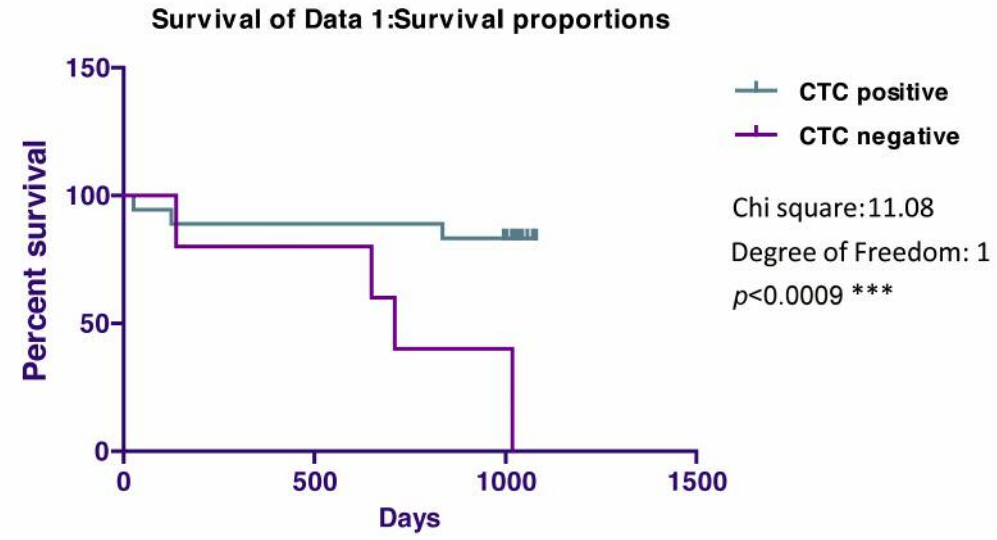

Figure 2. *Kaplan-Meier plot of survival, showing higher survival proportion to patients in the CTC negative group compared to those in the CTC positive group. A chi-square value of 11.08 was calculated as statistically significant with a p-value <0.0009 and HR of 0.04453 (95% CI; range, 0.007131 to 0.2781).*

Table II. *Results of ScreenCell filter viewing by 2 independent pathologists.*

Group	Pathologist A	Pathologist B	Overall Average
Cancer samples	18/23 (78.3%)	17/23 (73.9%)	35/46 (76.1%))
Early stage cancer (I-II)	15/18 (83.3%)	14/18 (78.0%)	29/36 (80.6%)
Advanced stage cancer (III-IV)	3/5 (60.0%)	3/5 (60.0%)	6/10 (60.0%)

The CTC detection rate by ScreenCell in our study, was higher compared to similar studies looking at patients with early-stage NSCLC. Freidin *et al*. (2014) reported CTC detection rates of 56% and 65% by two separate pathologists using ScreenCell in patients undergoing surgery for early-stage lung cancer in comparison to 78.3% (N=15) and 73.9% (N=14) detection rates in our study (Table II) (26). This could partly be explained by the subjective nature of the method of identification, particularly in the absence of any special or specific antibody stains. However, our

Table III. *Patient follow-up and survival data based on CTC presence or absence.*

	CTC positive	CTC negative
Number of patients	18	5
Alive at follow up	14	3
Median survival (days)	1011	711

detection rates were similar in comparison to studies looking at patients with more advanced NSCLC with 60% for both Pathologists, although it must be noted the advanced cancer sample numbers comprised a very small proportion of the cohort, Mascalchi *et al.* (2015) reported detection rates of CTCs using ScreenCell of 65% in a cohort of 26 patients with stage III and IV NSCLC. The authors found no correlation between tumour stage and the number of CTCs detected (27). Our detection rates, in comparison to other studies, support the idea that the release of CTCs is not related to tumour stage. A study by Wendel *et al.* (2012) found no significant differences in the median number of CTCs in patients with different stages of NSCLC detected by using a fluid phase biopsy approach (28). However, there is contradictory evidence since Krebs *et al.* (2011) found that the number of CTCs correlated with NSCLC stage. Patients at a higher NSCLC stage, had a greater number of CTCs. CTCs were detected using a semi-automated, epithelial cell adhesion molecule-based immunomagnetic technique (5). The conflicting evidence may be due to the different methods of CTC detection and that CTCs detected by different methods may have different biological characteristics.

In our study, patients negative for CTCs had a higher mortality rate during the study period, with 40% (N=2) in patients negative or CTCs *vs.* 22.2% (N=4) % in patients positive for CTCs (Table III). Additionally, in patients who died the median survival was shorter in those who were positive for CTCs. These results differ from other studies where patients who tested positive for CTCs had a worse prognosis. Krebs *et al.* (2011) found that prognosis was significantly worse in patients with stage III and IV NSCLC who had more circulating tumour cells (5).

These differences in survival may reflect the way that CTCs are analyzed. ScreenCell detects CTCs based on size exclusion, not allowing larger epithelial cells to pass through the micropores of the filtration device. On the other hand, Krebs *et al.* (2011) used an antigen-based technique. It may be that although ScreenCell is detecting CTCs it may not detecting clinically relevant CTCs that have the biological properties to influence the survival of NSCLC by establishing metastatic disease. Additionally there may be differences in the characteristics of CTCs in early-stage lung cancer and more advanced NSCLC (5). An alternative hypothesis that could explain the lower mortality rate would be that CTC detection in early-stage NSCLC could prime the immune system to respond more effectively against CTCs.

Our results indicate that detection of CTCs using ScreenCell is not necessarily prognostic and the detection of CTCs should not preclude the patient from undergoing surgical resection. The limitations of our study is the small cohort size, as well as analysing retrospective data. Additionally, our analysis was performed based on whether the patient had CTCs or not rather than looking at the effect of the total number of CTCs on prognosis.

In conclusion, our results do not support the idea that detection of CTCs using ScreenCell equates to a worse prognosis in comparison to patients who tested positive for CTCs using ScreenCell. Detection of CTCs by ScreenCell should not prevent patients from undergoing surgery with curative intent. More research is needed into this field to investigate the application of ScreenCell in other forms of cancers and in particularly to check whether in more advanced cancers this technology does show a correlation between CTC detection and poor prognosis. Additionally, it would be useful to look in the future at the different biological characteristics of CTCs detected by different methods.

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