Lung Cancer 134 (2019) 147–150

Contents lists available at ScienceDirect

Lung Cancer

journal homepage: www.elsevier.com/locate/lungcan

Circulating tumor cell clusters are a potential biomarker for detection of non-small cell lung cancer

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ARTICLE INFO

Keywords:
Circulating tumor cells
Liquid biomarkers
Non-small cell lung cancer
Lung cancer screening

ABSTRACT

Objectives: Circulating tumor cell (CTC) clusters (≥2 CTCs in aggregate) detected in the peripheral blood have predictive value in solid cancers, including non-small cell lung cancer (NSCLC). The goal of the study was to investigate the presence of CTC clusters in NSCLC patients and in high-risk screening subjects having no or benign nodules in a screening low-dose CT (LDCT).

Materials and methods: In a prospective pilot trial, 7.5 ml peripheral blood was collected from treatment-naïve NSCLC patients, LDCT screening subjects (55–80 years, ≥30 pack-year smoking history) with no (Lung-RADS 1) or benign lung nodules (Lung-RADS 2), and healthy never-smoking controls. CTCs were enriched by size, also allowing CTC cluster isolation. For CTC identification and enumeration, immunofluorescence staining was performed for cytokeratins (CK) 8/18 and/or 19, EpCAM, CD45, and nuclei were stained with DAPI. Clinicopathological data were collected, and LDCT interpreted by the American College of Radiology Lung-RADS criteria.

Results: CTC clusters were detected in 12/29 (41.4%) of all NSCLC patients, but not found in 31 high-risk screening subjects with Lung-RADS 1 or Lung-RADS 2 ($P < 0.05$). Since non-clustered, single CTCs were detectable in both groups of NSCLC patients (100%) and in 18/31 (58.1%) of high-risk screening subjects. No CTCs were detected in 20 healthy control subjects.

Conclusion: This pilot study suggests that CTC clusters are a useful and specific liquid biomarker to further explore for screening by LDCT and risk stratification of NSCLC patients. Future prospective studies with higher subject numbers will need to be performed.

1. Introduction

Non-small cell lung cancer (NSCLC) accounts for ~85% of lung malignancies, which are the leading cause of cancer-related deaths worldwide. In 2011, the National Lung Cancer Screening Trial (NLST) demonstrated in subjects at high-risk that mortality can be reduced 20% by screening with low-dose CT (LDCT) [1]. The U.S. Preventive Services Task Force recommends LDCT screening in individuals 55–80 years of age with ≥30 pack-year smoking history, current smokers or who have quit within the last 15 years (grade B evidence). However, LDCT screening has the limitation of a very high rate of false-positive lung nodule findings [1]. Many lung nodules are further evaluated with radiographic imaging or invasive interventions, leading to unnecessary radiation exposures, morbidities and costs. A clinically applicable biomarker reducing the high false-positive rate in LDCT imaging will fill an unmet need in lung cancer screening.

Circulating tumor cells (CTCs) are considered to have detached from a primary tumor and are consistently found in the blood of cancer patients. CTCs are defined as cancer-associated cells in the blood that express cytokeratins (CKs) 8/18 and/or 19 and EpCAM (epithelial cell markers) and that do not express CD45 (a leukocyte marker), with a well-defined nucleus identifiable with DAPI. Using appropriate detection technologies such as size-based isolation, CTCs are frequently observed in aggregates, and these CTC clusters (≥2 CTCs; also described as tumor microemboli) have shown to harbor a unique tumor biology and microenvironment with significantly enhanced metastatic potential

Abbreviations: CTC, circulating tumor cell; NSCLC, non-small cell lung cancer; LDCT, low-dose computed tomography; Lung-RADS, lung imaging reporting and data system

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https://doi.org/10.1016/j.lungcan.2019.06.016
Received 3 May 2019; Received in revised form 10 June 2019; Accepted 15 June 2019

Table 1
Subjects' characteristics and analyses for CTC clusters and non-clustered CTCs.

	N	CTCs present (*N*)	CTC mean (± SEM); median (range)	CTC clusters present (*N*)	CTC clusters mean (± SEM); median (range)
Total # of subjects:	**80**				
NSCLC	**29**	**29 (100%)**	**23.62 (± 1.65); 23 (11-40)**	**12 (41.4%)**	**0.69 (± 0.19); 0 (0-4)**
Age (median/range)	71 (48-91)				
Gender	13 (44.8%)				
• Females	16 (55.2%)				
• Males					
AJCC stages (8th ed.)	18 (37.9%)	18 (100%)	18.22 (± 1.35); 18 (11-33)	6/18 (33.3%)	0.56 (± 0.22)
• *I/II*	11 (62.1%)	11 (100%)	32.45 (± 1.56)	6/11 (54.5%)	0 (0-3)
• *III/IV*			33 (23-40)		0.91 (± 0.37)
					1 (0-4)
Histology	15 (51.7%)	15 (100%)	25.67 (± 2.62)	6 (40%)	0.60 (± 0.27)
• *AC*	11 (37.9%)	11 (100%)	28 (11-40)	5 (45.5%)	0 (0-4)
• *SCC*	3 (10.4%)	3 (100%)	22.55 (± 2.27)	1 (33.3%)	0.73 (± 0.27)
• *LCNEC*			23 (13-34)		0 (0-2)
			17.33 (± 0.67)		1.00 (± 1.00)
			18 (16-18)		0 (0-3)
LDCT screening subjects	**31**	**18 (58.1%)**	**2.45 (± 0.49); 2 (0-8)**	**0**	**0**
Age (median/range)	63 (55-74)				
Gender	18 (58.1%)				
• *Females*	13 (41.9%)				
• *Males*					
• *No nodule*	7 (22.6%)				
• *(Lung-RADS 1)*	24 (77.4%)				
• *Benign nodule*					
• *(Lung-RADS 2)*					
Healthy never-smokers	**20**	**0**	**0**	**0**	**0**
Age (median/range)	44 (28-69)				
Gender	10 (50%)				
• *Females*	10 (50%)				
• *Males*					

Abbreviations: LDCT: low-dose computed tomography; Lung-RADS: lung imaging reporting and data system; NSCLC: non-small cell lung cancer; CTCs: circulating tumor cells; SEM: standard error of the mean; AC: adenocarcinoma; SCC: squamous cell carcinoma; LCNEC: large-cell neuroendocrine carcinoma.

in comparison to non-clustered, single CTCs [2]. Consistent with findings in other cancers, presence of CTC clusters in the blood has been reported to be associated with poor prognosis of NSCLC patients [3].

Based on our finding that non-clustered CTCs are sometimes detectable in high-risk lung cancer screening subjects with no nodule (Lung-RADS 1) or with benign lung nodule (Lung-RADS 2), we hypothesized that CTC clusters might be specific for patients with NSCLC. This would make CTC clusters a potential and by itself unique biomarker for NSCLC detection by LDCT in high-risk screening subjects.

2. Materials and methods

2.1. Subjects' selection criteria

The study was conducted in accordance with the Declaration of Helsinki, with approval by the Institutional Review Board (IRB) of the University of Missouri and Truman VA Hospital (IRB2010166, IRB2004401-VA). All subjects gave informed consent before participation. Recruitment timeframe was from July 2017 to August 2018. All NSCLC patients were treatment-naïve. Eligibility for lung cancer screening with LDCT was defined as the U.S. Preventive Services Task Force recommends (grade B evidence): 55–80 years, ≥30 pack-year smoking history, current smokers or quit within last 15 years. Screening subjects with no or benign lung nodules (Lung-RADS 1 and 2) were included. Subjects with concurrent/past history of cancer were excluded. Healthy controls were never-smoking volunteers.

2.2. Clinicopathological data

Data were prospectively collected. The staging manual of the American Joint Committee on Cancer (AJCC), 8th Edition, was used. Screening LDCTs were performed without intravenous contrast on ≥64-row multidetector CT scanners (Somatom; Siemens AG, Germany). LDCTs were categorized by Lung-RADS™ (Version 1.0) criteria imaging and reporting data system developed by the American College of Radiology.

2.3. CTC detection and identification

Blood draws were done before biopsies were performed to avoid cancer cell detachment by tissue manipulation. The first 6 ml of blood were discarded to avoid epithelial contamination from skin puncture. Then, 7.5 ml of blood were collected in CellSave® tubes (Menarini-Silicon Biosystems, Huntingdon Valley, PA). Within 1 h, CTCs were enriched with an established microfilter platform that has been successfully used for CTC cluster detection [4–6]. Blood was passed through filters with a syringe pump (KD Scientific Legato 110 CMT, Analytical West Instruments, CA). Enriched cells were characterized by immunofluorescence staining on the filter with an antibody cocktail (CK8/18/19-FITC, EpCAM-PE, CD45-Cy5), and anti-fade mounting medium with DAPI (Cell Signaling Technology, Danvers, MA) was added for nuclear staining. In alignment with the FDA-approved definition, a CTC was defined as $CK^{pos}/EpCAM^{pos}/CD45^{neg}$ with a $DAPI^{pos}$ nucleus [7]. Clusters were defined as ≥2 CTCs in aggregate [2].

2.4. Statistical analysis

Specimens and clinical data were collected prospectively, with the analytic personnel blinded to the outcome. Tests performed were non-parametric Kruskal-Wallis test and Fisher's exact test. Significance statements refer to a P value of < 0.05. Analyses were performed using Prism version 5.00 (GraphPad, La Jolla, CA).

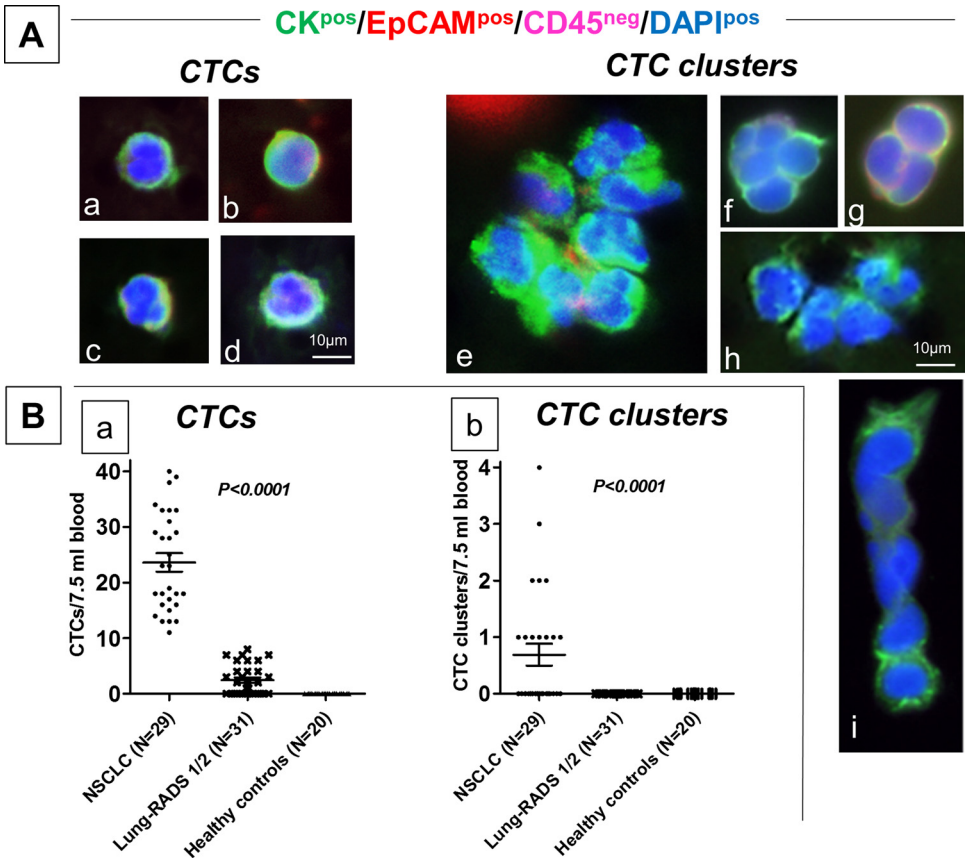

Fig. 1. (A) CTCs and CTC clusters found in NSCLC patients. CTCs were detected by drawing 7.5 ml of peripheral blood. CTCs were enriched by microfilter isolation, and immunofluorescence staining performed for cytokeratins (CK) 8/18 and/or 19, EpCAM, CD45, and the nucleus identified with DAPI (a–d). CTC clusters were defined as an aggregated group of ≥2 CTCs. CTC clusters were observed in different shapes: spherical (e,f), triangular (g,h), or linear (i). (B) CTC clusters are exclusively detected in NSCLC patients. CTCs analysis was performed in NSCLC patients, high-risk screening subjects undergoing LDCT with no (Lung-RADS 1)/benign lung nodules (Lung-RADS 2), and healthy controls (total $N = 80$). Mean counts (bars ± standard error of the mean) of (a) non-clustered CK^{pos}/$EpCAM^{pos}$/$CD45^{neg}$CTCs, and (b) CTC clusters in the different groups are presented. P values was calculated by non-parametric Kruskal-Wallis test.

3. Results

3.1. Study groups and detection of CTCs and CTC clusters

In a prospective trial, 7.5 ml of blood was analyzed from a total of 80 subjects: 29 treatment-naïve NSCLC patients, 31 high-risk lung cancer screening subjects with no lung nodules (Lung-RADS 1; 7 (22.6%)) or benign nodules (Lung-RADS 2; 24 (77.4%)) on LDCT, and 20 healthy never-smoking controls (all clinicopathological data provided in Table 1).

CTCs were enriched with a validated microfilter method that preserves CTC clusters. CTCs were identified by immunostaining as CK^{pos}/$EpCAM^{pos}$/$CD45^{neg}$ cells with a $DAPI^{pos}$ nucleus (Fig. 1A: a–d). CTC clusters were observed in various morphologic configurations: sphere-like (Fig. 1A: e,f), triangular geometries (Fig. 1A: g,h), or linear alignments of CTCs (Fig. 1A: i).

3.2. CTC clusters are detected in NSCLC, but not in screening subjects with benign findings on LDCT

All NSCLC patients had non-clustered CTCs detectable (mean count 23.62 (SEM ± 1.65); median 23 range (11–40)) (Fig. 1B: a). CTC clusters were found in 12/29 (41.4%) NSCLC patients (0.69 (± 0.19); 0 (0–4)) (Fig. 1B: b). Importantly, none of the 31 high-risk screening subjects was found to have CTC clusters.

However, 18/31 (58.1%) of screening subjects had non-clustered, single CTCs detectable (2.45 (± 0.49); 2 (0–8)). No CTCs were identified in the 20 healthy never-smoker control subjects. These data suggest that CTC clusters are exclusive to a subset of NSCLC patients, and are not found in high-risk screening subjects without or with a benign lung nodule on LDCT (Lung-RADS 1 and 2). Yet, these high-risk subjects without a cancer diagnosis still carried non-clustered, single CTCs, although at a significantly lower level than NSCLC patients (Fig. 1B: a; $P < 0.0001$; non-parametric Kruskal-Wallis test).

As expected, CTCs were identified at increasing levels from AJCC stages I/II ($N = 18$) to III/IV ($N = 11$) ($P < 0.0001$; unpaired t test). A tendency towards a higher CTC cluster count was noted from stage I/II (0.56 (± 0.22), 0 (0–3)) to III/IV (0.91 (± 0.37) 1 (0–4)), however this trend did not reach level of significance ($P = 0.2956$). In addition, no significant correlations between CTC cluster results and other clinical parameters (tumor sizes (pT), lymph node status (pN), NSCLC histologic subtypes, or presence of lymphovascular invasion were observed.

4. Discussion

Despite recent implementation of novel targeted agents, five-year survival of lung cancer remains at a low rate of 19%. Screening of high-risk subjects with LDCT can reduce lung cancer-related mortality by 20%, but the high false-positive rate of lung nodules leads to morbidities from interventions, unnecessary imaging, and costs [1]. Liquid biomarkers, including CTCs, have been suggested to improve accuracy of LDCT lung cancer screening, which would ultimately reduce morbidities and costs. In a prospective study on high-risk screening subjects (aged 55–80 and smoking history ≥30 pack-years) undergoing LDCT and patients diagnosed with NSCLC, we demonstrate that presence of CTC clusters (≥2 CTCs aggregate) is limited to NSCLC patients only. However, non-clustered CTCs can be detected in high-risk screening subjects with no (Lung-RADS 1) or benign nodules (Lung-RADS 2) on LDCT. Therefore, CTC clusters have a potential role as liquid biomarkers in LDCT screening due to their specificity to NSCLC.

In our cohort of lung cancer screening subjects with no or benign nodules, single CTCs were identified in some individuals at low levels. Similarly, non-clustered CTCs were also described in women with benign conditions undergoing breast cancer screening [6]. The reason for these false-positive rates are unclear, but could be due to another undiagnosed cancer (such as colorectal or prostate) since screening

populations are obviously at risk for other malignancies. In particular the high-risk LDCT subjects with CTCs will need to be followed longitudinally. Although we did not observe CTC clusters in all NSCLC patients (41.4%), their exclusiveness to NSCLC and occurrence in early stages makes CTC clusters attractive for detection of at least a subset of NSCLCs. In the present pilot study, we intended to compare presence of CTC clusters between patients with NSCLC and a group with similar risk factors (defined by age and smoking history) that has no cancer (Lung-RADS 1 and 2). Yet, a CTC cluster analysis on screening subjects with probably benign (Lung-RADS 3) and suspicious lung nodules (Lung-RADS 4A/B/X) will need to be performed, as this will further clarify the specificity of CTC clusters for NSCLC diagnosis.

In the present study a microfilter for size-based cell isolation was applied for CTC detection [4–6]. In contrast to other technologies, such as flow cytometry, size-based isolation reliably detects CTC clusters [5]. CTC clusters, sometimes referred to as tumor microemboli, have been described to be predictive of cancer outcome in patients with NSCLC, small cell lung (SCLC), breast, colorectal, gastric, pancreatic, and liver cancer [2,3]. The tumor biology of a CTC cluster is thought to be significantly different from a single CTC in the blood stream. Molecular studies showed that within a cluster, CTCs interact amongst themselves and other cells (immune cells, fibroblasts, platelets), enhancing the potential for survival [8]. CTC clusters undergo epithelial-mesenchymal transition (EMT), a critical process for metastasis [9]. In the present study, we observed CTC clusters in various appearances (spherical, triangular, and linear). These different morphologic configurations can favorably influence their complex, pro-metastatic microenvironment, and therefore affect cluster survival and development to a solid metastasis [10]. Further molecular studies will need to shed light on the role of CTC clusters in cancer metastasis biology.

In summary, our pilot study suggests higher specificity of CTC clusters than non-clustered CTCs for detection of NSCLC in lung cancer screening. Future large-scale longitudinal studies will need to reveal the clinical applicability of CTC clusters in LDCT screening and personalized NSCLC therapy management.

5. Conclusions

Integration of clinically applicable liquid biomarkers in NSCLC screening and personalized care would add significant value and potentially improve outcome. In contrast to non-clustered CTCs, we found CTC clusters exclusively in a subset of NSCLC patients, but not in high-risk screening subjects with no or benign nodules on LDCT. This pilot study suggests that CTC clusters in the peripheral blood are a useful biomarker to explore for screening and further risk stratification of NSCLC patients. Future confirmative studies on the role of CTC clusters in NSCLC screening with higher patient numbers will need to be performed.

Funding

This study is supported by a Mizzou Advantage Interdisciplinary Research Grant (MAIRG) (J.T.K. and G.L.).

Acknowledgements

We are very grateful to all subjects and their families for their voluntary participation, and to Blanche L. Lasta, RN, and Ken Baker, RN, for assistance in trial conductance.

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