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Frequency and clinical impact of preoperative circulating tumor cells in resectable non-metastatic lung adenocarcinomas

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A R T I C L E I N F O

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A B S T R A C T

Objectives: Despite successful surgery, 30–50% of patients with resectable non-small cell lung cancer develop tumor recurrence within 5 years of surgery.
Materials and methods: In this prospective study, we performed CTC enumerations in 40 patients with non-metastatic lung adenocarcinoma (NMLA) using a size-based microfilter. Additionally, cfDNA isolated from plasma was analyzed in 35 out of 40 patients.
Results: CTCs were identified in 15 out of 40 patients (37.5%) with a range of 1–44 cells, whereas mutated cfDNA was only detected in 3 out of 35 patients (8.6%). Disease-free survival (DFS) was significantly associated with CTC positivity (log-rank $p = 0.025$), grading (log-rank $p = 0.019$), tumor stage (log-rank $p = 0.025$) and lymph node status (log-rank $p = 0.029$). Multivariate analysis, including tumor stage and grading, showed that CTC positivity ($p = 0.006$), grading (0.039) and tumor stage ($p = 0.022$) were independently associated with DFS.
Conclusion: Our study found that microfilter-based CTC enumeration in NMLA patients is an independent predictor of worse DFS. The used NGS-based cfDNA characterization had limited sensitivity to be clinically informative in our study cohort. CTC assessment before surgery can thus identify NMLA patients at high risk of disease recurrence.

1. Introduction

Despite successful surgical treatment, 30–50% of patients with resectable non-small cell lung cancer (NSCLC) develop tumor recurrence within 5 years of surgery [1]. Biomarkers that can identify high-risk patients who may benefit from further systemic therapies and disease monitoring are therefore urgently needed. Liquid biopsy assays, such as circulating tumor cells (CTCs) enumeration or circulating free DNA (cfDNA) characterization may help identify biomarkers for risk stratification of disease recurrence in lung cancer patients.

Tumor cells that have left the primary tumor or metastases can be found in the circulation of patients and are called CTCs. They can provide unique biological and clinical information [2]. The prognostic value of CTCs has already been shown in several studies [3–6] and is of particular interest in those tumors where access to the primary tumor tissue is limited, as in lung cancer [7]. The CellSearch system (Veridex LLC, Rarity, NJ, USA) has been approved by the United States Food and Drug Administration (FDA) for some types of cancer [8–10], but not for lung cancer, and has revealed a rather moderate sensitivity in lung cancer compared to other enrichment methods [11–13]. While CellSearch isolates and detects CTCs based on expression of epithelial cell adhesion molecules (EpCAM), it may not capture subpopulations of CTCs with low or absent EpCAM expression. Importantly, EpCAM down-regulation has been shown to frequently occur during disease

Abbreviations: cfDNA, circulating free DNA; CK, pan-cytokeratin; CT, competed tomography; CTC, circulating tumor cells; DAPI, 4 6-diamidino-2-phenylindole; DFS, disease-free survival; EDTA, ethylenediaminetetraacetic acid; EGFR, epidermal growth factor receptor; EpCAM, epithelial cell adhesion molecule; FFPE, formalin-fixed paraffin-embedded; H & E, hematoxylin-eosin; HR, hazard ratio; KRAS, kirsten rat sarcoma viral oncogene homolog; LVI, lympho-vascular invasion; NA, not available; NACT, neoadjuvant chemotherapy; NGS, next generation sequencing; NMLA, non-metastatic lung adenocarcinoma; NSCLC, non-small cell lung cancer; OS, overall survival

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progression and metastasis in epithelial cancers [14]. Size-based enrichment techniques like the one used in the current study can overcome this limitation. Our platform is based on a filtration approach, which isolates CTCs larger than 8 μm [15,16]. As a result, our microfilter has the capacity to enrich heterogeneous CTC subpopulations independent of epithelial marker expression. In fact, other size-based enrichment techniques, such as the ISET (RareCells, Paris, France) platform, have shown an increased sensitivity in advanced stage NSCLC patients compared to the CellSearch system [12].

Most of the CTC studies so far have been performed in advanced-stage cancer patients. While some studies in early-stage breast cancer have already provided important insights into the potential clinical utility of CTCs as an early prognostic marker, studies addressing the prognostic role of CTCs in early-stage NSCLC are still limited [17–21]. One reason for the limited number of studies in early stage disease is that isolation of CTCs is more challenging due to their rarity.

The objectives of this study were a) primarily evaluate the presence of size-based captured CTCs and their clinical impact in non-metastatic lung adenocarcinoma (NMLA) patients prior to surgery, and b) secondarily, assess clinical relevance of next generation sequencing (NGS) based cfDNA from plasma in these patients.

2. Material and methods

2.1. Study design

We performed a prospective study of NMLA patients who were planned for curative pulmonary resection between 2015 and 2016 at the Division of Thoracic and Hyperbaric Surgery at the Medical University of Graz. In all patients, histological diagnosis was established preoperatively either by CT-guided fine needle puncture/or biopsy in case of peripheral tumors or by bronchoscopic biopsy in case of centrally located tumors with bronchial infiltration. Blood samples were collected before surgery and consisted of one CellSave tube (Veridex, LLC) for CTC analysis and one vacutainer containing ethylenediaminetetraacetic acid (EDTA) for plasma preparation. Blood for plasma collection was processed within 2 h and that in CellSave tubes was processed within 24 h. The study complied with the Declaration of Helsinki and the protocol was approved by the local Institutional Review Board and all patients signed informed consents (25-003ex12/13).

2.2. CTC collection and analysis

Seven and a half ml volume of blood was drawn in CellSave tubes and processed on the CTC device as previously described, following technical details and validation of the size-based microfilter assay [15,16]. Briefly, blood was diluted 1:1 with phosphate-buffered saline and fixed with a final concentration of 1% formalin (Sigma-Aldrich, Vienna, Austria) for 10 min. After the mild fixation, blood was processed through the microfilter at a constant flow rate of 75 ml/hour using a motorized syringe pump. Following filtration, CTC identification and enumeration were carried out by double immunofluorescence staining including a pan-cytokeratin (CK) antibody and CD45 antibody. Filters containing CTCs were placed onto a glass microscope slide and treated with blocking buffer consisting of 5% normal goat serum (Life Technologies, Vienna, Austria) and 3% Triton X-100 (Sigma Aldrich) at room temperature for 30 min. Next, samples were incubated with primary antibodies, mouse anti-CD45 (Ready-to-use; DAKO, Glostrup, Denmark) and polyclonal rabbit anti-cytokeratin (1:300; DAKO) at room temperature for 1 h. Samples were then incubated with secondary antibodies; goat anti-mouse Alexa 594 for CD45 and goat anti-rabbit Alexa 488 for CK (both at 1:100 dilution, Life Technologies Inc.) at room temperature for 1 h. Finally, samples were counterstained with 4, 6-diamidino-2-phenylindole (DAPI, Invitrogen) and mounted on coverslips with ProLong Gold Antifade mounting media (Life Technologies). The entire area of the microfilter was viewed under a confocal laser-scanning microscope (Zeiss, Oberkochen, Germany), and CTCs were identified as nucleated, CK+/CD45- cells. Results of CTC enumeration were expressed as the number of CTCs per 7.5 ml of blood. In this study, a sample was considered to be positive if even a single CTC was identified.

2.3. cfDNA isolation and next generation sequencing

Whole blood (9 ml) was collected in EDTA vacutainer tubes (BD Biosciences, Heidelberg, Germany) and 0.225 ml of a 10% neutral buffered solution containing formaldehyde (4% weight per volume, Sigma-Aldrich, Vienna, Austria) was added immediately after blood draw. Plasma was isolated from blood samples by centrifugation at 200*g* for 10 min with brake and acceleration powers set to zero. The supernatant was then removed and centrifuged at 1600*g* for an additional 10 min. Plasma was then stored in 2 ml tubes at −70 °C. cfDNA was isolated from 1 ml of plasma using the QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

2.4. DNA extraction from archival primary tumors

Formalin-fixed paraffin-embedded (FFPE) tumor tissue blocks were retrieved from the Biobank of the Medical University of Graz. One hematoxylin-eosin (HE) stained and 6 unstained 5 μm thick sections were cut and tumor tissue was marked on the H & E slide by a board-certified pathologist (LB). Tumor tissue was scratched off the unstained slides and DNA was extracted using the Maxwell FFPE Tissue LEV DNA Purification Kit (Promega GmbH, Mannheim, Germany) according to the manufacturer's protocol.

2.5. Ion torrent targeted next generation sequencing (NGS)

DNA was quantified using Picogreen fluorescence and the Qubit instrument (Thermo Fisher Scientific, Waltham, MA, USA). Up to 30 ng of the isolated cfDNA or 10 ng DNA isolated from FFPE tissue were then used to prepare NGS libraries with the Ion Ampliseq Colon and Lung Research Panel v2 primer pool (available from www.ampliseq.com) and the Ion Ampliseq Library kit (#4480442, Thermo Fisher Scientific). NGS libraries were quantified and sequenced on the Ion Proton benchtop sequencer to a yield of more than 1 million reads per sample, resulting in an average depth of ∼8.000 reads per amplicon. Sequencing data was mapped to hg19 using Torrent Mapper and variant calling was performed using the Torrent Variant caller version 5.2 (https://github.com/iontorrent/TS). Validation of the assay confirmed reliable detection of known variants down to 1% allelic frequency.

2.6. Statistical analysis

Statistical analyses were performed using the IBM SPSS 23 software for Windows (IBM Corp, New York, USA). Continuous variables were reported as medians (25th-75th percentile), whereas categorical variables were summarized as absolute frequencies and percentages. Associations between two categorical variables were analyzed with the chi-square test (expected cell counts > = 5) or Fisher's exact test (expected cell counts < 5). Means of continuous variables were compared with Wilcoxon's rank-sum test or Kruskal-Wallis test. The median follow-up time was estimated with the reverse Kaplan-Meier method [22]. Overall survival (OS) was calculated from the date of surgery to the date of patients' death from any cause. Disease-free survival (DFS) was assessed as the time from surgery to the time of recurrence (distant or local metastasis or death from any cause). In case the corresponding event was not observed, the censoring date was the last day of follow-up. Survival rates were calculated using the Kaplan-Meier method, and the differences were analyzed using the log-rank test. Multivariate

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