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IASLC 2015
Denver, 6th-9th September

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An effective *in vivo* Liquid Biopsy tool for high-yield isolation of circulating tumor cells

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Abstract **Background**: Analysis of tumor biopsy material only represents accessible tumor and can define the state of cancer only at the time of diagnosis. This approach neglects tumoral heterogeneity changes occurring during disease progression. However, during systemic therapies tumors undergo molecular changes and usually develop resistance mechanisms. Reevaluation of tumors after therapy, at disease progression and before new treatment initiation would be informative for the selection of appropriate next steps. However, re-biopsies are not often feasible and can cause morbidity. Liquid biopsy, i.e. isolating and analyzing circulating tumor cells (CTCs), can be an additional source of diagnosis, prognosis, evaluation of treatment efficacy, and molecular tumor evolution and metastatic sites. Commonly, CTCs are isolated from small blood volumes (5-10 ml) in *in vitro* approaches. This approach has limited sampling volume particularly in detecting low frequency CTCs. To overcome this limitation, the GILUPI CellCollector®, an intravascular in-dwelling device, screens a large volume (>1 liter) of blood for CTCs directly in the vein of the cancer patient. The device has specific monoclonal antibodies attached to pull down epithelial derived CTCs. Here, we demonstrate the application of the GILUPI CellCollector® in the assessment of CTCs in non-small cell lung cancer (NSCLC) patients at different tumor stages.

Patients and Methods: In this study a total of 59 non-small cell lung cancer (NSCLC) patients, stage IA to IIIB, were applied for CTC isolation before (n=59) and one week after surgery (n=25). Also 7 non-cancer patients were included in the study. CTC validation and enumeration was conducted by immunofluorescence (IF) microscopy. Following this, for (n=24) cancer patients isolated CTCs were analyzed for mutations in the KRAS gene commonly found in NSCLC using the PointMan DNA mutation enrichment assay. Primary tumor tissue was analyzed for the same mutations to investigate concordance. For (n=21) CTC samples a direct comparison with CELLSEARCH® was performed. **Results**: In this study a significantly higher isolation efficacy compared to the FDA-cleared CELLSEARCH® System could be shown. No CTCs could be found in non-cancer patients. The overall sensitivity of the CellCollector DC01 is 89%. Comparing the pre-surgical isolation rate of 84% with the post-operative rate of 62.5% show an expected result as the number of CTCs only slowly decreases after operation. **Conclusions**: The GILUPI CellCollector® overcomes blood volume limitations of other CTC extraction approaches and thereby increases the diagnostic sensitivity of CTC isolation. It allows CTC enumeration, molecular characterization, and biomarker expression analysis, which could help guide treatment strategies and monitoring therapy efficacy.

GILUPI CellCollector® - an *in vivo* CTC isolation technology

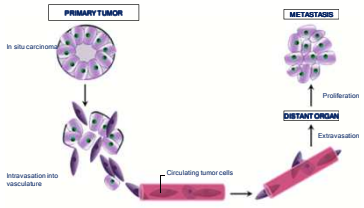

Figure 1: CTCs and cancer progression, Schematic model of metastatic model. Modified from Paris *et al*., 2013.

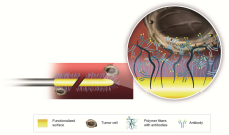

Figure 2: The device. The functionalized surface of the stainless steel wire consists of a gold layer and a hydrogel which bears covalently bound antibody against epithelial cell surface marker EpCAM.

Insertion into peripheral arm vein of a patient

Figure 3: The device application. Insertion of the GILUPI CellCollector® through an indwelling cannula into a peripheral arm vein for 30 min. During the application the 20 mm long functionalized tip comes into direct contact with the blood circulation and captures CTCs via EpCAM binding.

Results

Patient recruitment

1st Cohort
- 34 NSCLC patients (stage IA - IIIB) and 7 non-cancer subjects
- 21 of them compared with CELLSEARCH®

2nd Cohort
- 25 patients enrolled
  - 25 NSCLC patients stage IA to IIIB, were applied for CTC isolation two-times before (n=50) and one week after surgery (n=25)

CTC enumeration

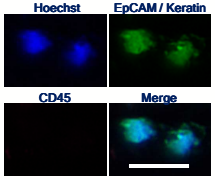

Figure 5: Immunofluorescence analysis. CTCs were identified via positive EpCAM/Keratin, Hoechst and negative CD45 staining as well as morphological features. Scale bar: 50µm.

Clinical correlation

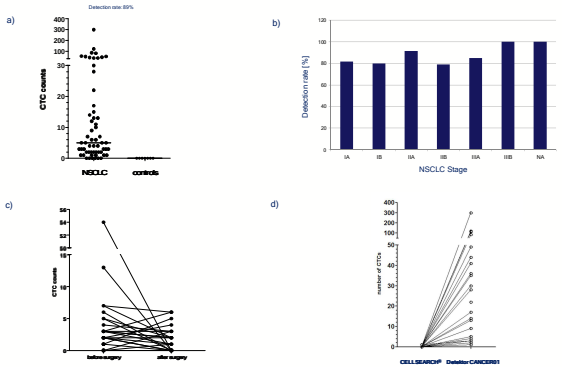

Figure 6: a) Results of *in vivo* CTC isolation rate with the GILUPI CellCollector® DC01 from 59 NSCLC patients and 7 control subjects (non-cancer patients). b) CTC isolation rate in different NSCLC stages of 59 patients. c) CTC isolation rate before and after surgery (n=25). d) Comparison of the *in vivo* (GILUPI CellCollector®) and *in vitro* (CELLSEARCH®) CTC isolation methods. (n=21). In figure a) median is shown.

Molecular Analysis

Figure 7: The PointMan DNA enrichment technology:

WT: Enriching primers (grey) containing blocking moiety complementary to the wild type sequence bind with high avidity to sample DNA and extend. Amplifying primers (orange) prime at sites flanking the sequence of interest but are blocked from extension by the high avidity of the extended enriching primers.

Variant: Enriching primers do not efficiently anneal and extend due to mismatch at the *3'* end and stringency of PCR conditions. Amplifying primers therefore extend right through the region of interest, without hindrance.

WT: In the subsequent cycle, only products from the enriching primers are available for further replication. This replication is terminated by the blocking moieties and the product does not contain any further priming sites.

Variant: Products of amplifying primers are available for priming and extension.

WT: Lack of priming sites for either set of primers ensures only linear replication of starting template.

Variant: Exponential amplification of PCR products is possible.

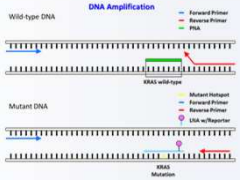

Figure 8: Schematic of PNA/LNA clamp directed PCR developed by the JWCC. Top, PNA/wt DNA complex, with no amplification. Bottom, amplification of DNA template containing KRAS mt using the dual-labeled LNA probe that recognizes and hybridizes to KRAS mt codon.

PointMan DNA enrichment technology:
- KRAS mutations can be detected in CTC samples and the corresponding primary tumor tissue
- Certain samples show WT in the primary tumor, but KRAS mutations in the CTC sample
- Some samples show inconclusive results
- Further investigations are in progress to develop a robust method for mutational analysis of CTCs

PNA/LNA clamp directed PCR:
- In progress

Summary

- **Detection rate of 89%** for *in vivo* captured CTCs with the GILUPI CellCollector®
- Detection of CTCs could be shown at all **occurred tumor stages**
- The implementation of the GILUPI CellCollector® into clinical practice may improve early detection, prognosis and therapy monitoring of lung cancer patients.
- Besides enumeration, the method allows the molecular analysis of CTCs, enabling personalized treatment management