Molecular characterization of circulating tumor cells enriched by a microfluidic platform in patients with non-small cell lung cancer

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Background

Lung cancer is the leading cause of cancer related death worldwide. Despite of recent advances in the treatment of non-small cell lung cancer (NSCLC), the most common histological type, the prognosis of the patients remains poor, with a 5-year survival rate of 63% in patients with localized disease, and just 7% when the disease has already spread.
Circulating tumor cells (CTCs) are widely accepted to be precursors of distant metastasis, and may early on indicate progression of the disease and treatment failure. In the present study, we asked whether in addition to well-established gene markers for epithelial CTCs (EpCAM, CK19), further markers characteristic for ciliated lung cells, for epithelial-to-mesenchymal transition (EMT) and for cancer stem cells (CSC), could indicate the presence of CTCs and may have prognostic relevance in this type of cancer.

Methods

Peripheral blood was taken after written informed consent at primary diagnosis or at start of treatment from patients with confirmed NSCLC. Control blood samples came from healthy donors without a history of cancer. The study was approved by the Ethic Committee of the Medical University of Vienna (EK366/2003 and EK2266/2018).

Each 15ml blood was drawn in Vacuette EDTA tubes (Greiner Bio-One) and processed on the same day using microfluidic Parsortix™ cassettes with acritical step size of 6.5µm (Angle plc, UK). Spike-in controls were performed by adding each 100 fluorescently labeled PC-9 and NCI-H1975 NSCLC cells to healthy donor blood. The enriched cells were partly lysed and partly transferred on poly-lysine coated glass slides.

CTCs on glass slides were identified after immunofluorescent staining of EpCAM, and CK4, 5, 6, 8, 10, 13, and 18. Counterstaining of co-purified leukocytes was performed using anti-CD45. The stained cells were analyzed with the CellCelector microscope (Automated Lab Solutions, Jena, Germany).

The total RNA was extracted from the lysed cells (RNeasy Micro Kit, Qiagen) and converted into cDNA (SuperScript VILO, Invitrogen). After a specific pre-amplification (TaqMan PreAmp Master Mix, Life Technologies) of all 14 target genes (Table 1), qPCR was performed in duplicates (ViiA7 Real-Time PCR System, Life Technologies). To evaluate the prognostic significance of each marker, the "optimal" cut point for the Ct-value was determined using the function "surv_cutpoint" from the R-package Survminer (version 0.4.2) providing a value of a cut point that corresponds to the most significant relation with overall survival[1].

Epithelial	EMT	CSC	Lung	other
EPCAM	FAM83A	NANOG	BPIFA1	UCHL1
CK19	PTHLH	PROM1		GRP
	ERBB 3	MET		TERT
	TWIST1			CDH5

Table1: 14 gene markers for qPCR

Results

Patients and blood samples

A total of 118 patients were included in the study, with a mean age of 66.4 years and balanced representation of the sexes. Median follow-up time was 7 months (range 0-15 months). The blood samples were taken at primary diagnosis in 67 (56.8%) cases, and in 39 (33.1%) at progression of the disease. In 12 (10.2%) cases, the time of sampling was not documented.

Recovery of spiked PC-9 and NCI-H1975 cells

After adding each 100 labeled tumor cells to a donor blood sample, on average 67 PC-9 cells and 83 NCI-H1975 cells were captured in the microfluidic cassette (Figure 1A).

Qith qPCR, no gene expression specific for CDH5, PTHLH, FAM83A, and CK19 was observed in the un-spiked samples (n=2); in contrast, the presence of spiked tumor cells was indicated by a positive qPCR amplification signal of these markers. BPIFA1 and GRP were neither detected in the donor blood nor in the spiked sample. All other gene markers were detected both in the un-spiked as well as in the spiked blood sample, albeit at different gene expression levels (Figure 1B).

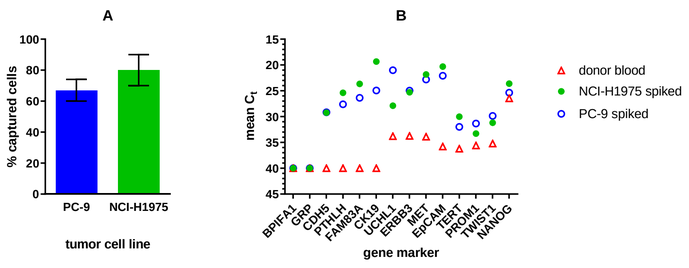

Figure 1: (A) Mean rate of PC-9 and H1975 NSCLC tumor cells captured in the Parsortix™ microfluidic separation cassette with a critical step size of 6.5µm.
(B) Mean Ct-values of the selected gene markers in Parsortix™ enriched donor blood samples with and without PC-9 and NCI-H1975 tumor cells. A Ct-value of 40 indicates a negative qPCR result.

Gene expression levels in NSCLC patients and healthy donors

The transcript levels of the selected markers were evaluated in the enriched blood samples from further 30 healthy donors and from 118 NSCLC patients, confirming the absence of FAM83A, GRP and BPIFA1 transcripts in the healthy blood samples. After applying a diagnostic threshold by adding 2*SD to the mean Ct of "false-positive" healthy control samples for the other markers, EpCAM was the most frequently detected transcript in NSCLC, with 53.7% positive samples at primary diagnosis and 25.6% at recurrence (Figure 2)

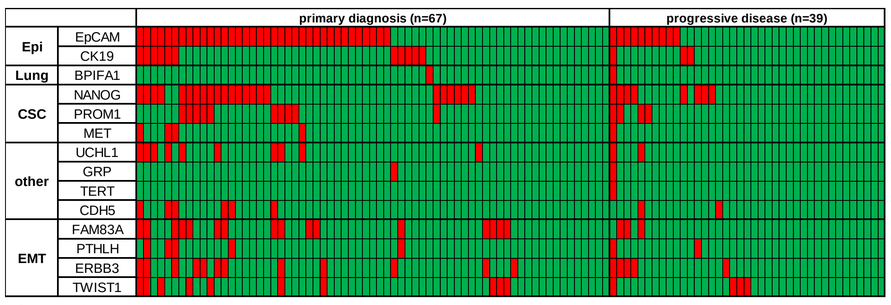

Figure 2: Heat map for 14 transcripts in NSLC blood samples. Red squares indicate gene expression beyond the diagnostic threshold level.

Prognostic significance of NANOG+ and PROM+CTCs

Gene expression levels of EpCAM, CK19, NANOG, PROM1, MET, and PTHLH beyond the optimal prognostic cut point was associated with worse OS (Figure 3).

However, the multivariate Cox regression analysis revealed that only the presence of NANOG (HR 2.97, 95%CI 1.25-7.05, p=0.014) and PROM1 (HR 3.01, 95%CI 1.21-7.50, p=0.018) transcripts beyond the prognostic threshold value were independently related with OS.

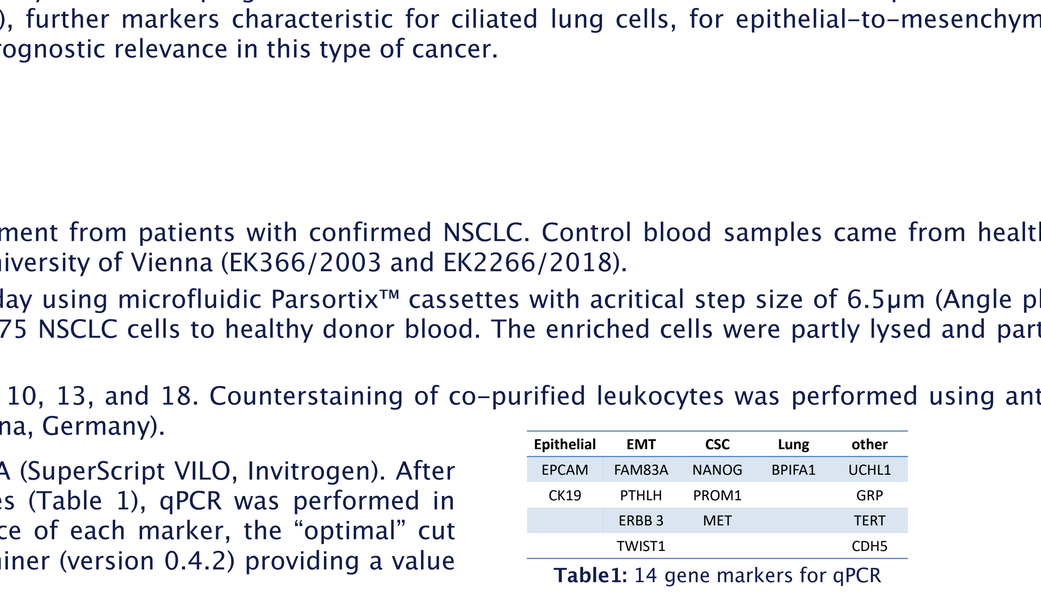

Figure 3: Overall survival plots of patients with univariate significant gene expression levels stratified by the prognostic threshold. P-values correspond to log-rank tests.

NANOG gene expression may probably be more prognostic than CTC counts

Immunofluorescent staining was performed in 9 cases. In 2/9 cases no CTCs were found; the median CTC number in the 7 CTC+ cases was 18 (range 10-155) per 7.5ml blood. The agreement between epithelial protein expression detected by IF and by qPCR was substantial (chi-square 89%, Cohens κ=0.61). However, a high CTC count was not necessarily associated with short OS: all patients with extremely short survival were rather NANOG-positive by qPCR than had epithelial CTCs (Figure 4).

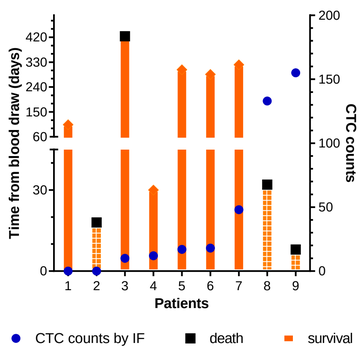

Figure 4: Swimming pool plot of 9 patients with epithelial CTCs detected by IF staining and presence or absence of NANOG gene expression in the same sample.
Head-arrow indicates that the patient was still living at study completion, and a black square that the patient has died. Blue circles indicate the absolute CTCs numbers by IF. The length of the bars indicate the overall survival after the blood draw, with checkered bars for patients with NANOG gene expression levels beyond the threshold value.

Conclusions

The present study further underlines the relevance of the molecular characterization of CTCs by qPCR. Our multi-marker analysis highlighted the prognostic value of CSC- and EMT-related transcripts, which remained significantly related to worse prognosis, independent from the presence of EpCAM transcripts

References

1. Kassambara A, Kosinski M, Przemyslaw B, Scheipl F. 2020 survminer: Drawing Survival Curves using "ggplot2.". <https://cran.r-project.org/package=survminer>.