Somatic mutations of the EGFR, KRAS and BRAF genes: homogeneity in single cells from cell lines and heterogeneity in circulating epithalial tumor cells (CETCs) as determined using the cobas® z 480 analyzer

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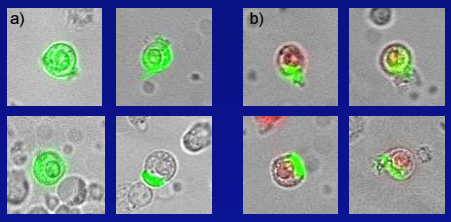
Abstract

Background: Targeted therapies directed specifically against somatic mutations in genes involved in signalling pathways have been shown to improve outcome compared with cytotoxic chemotherapies in patients with advanced tumors carrying the respective mutations.
Purpose/Objectives: Identification of such mutations is performed in formalin fixed material from the primary tumor. However, such material is not always available and, even more importantly, cells with metastatic potential must be released and travel via the blood during the course of disease to reach their distant loci. Using maintrac®, a non-dissipative approach avoiding enrichment steps, CETCs can be detected and individually isolated in almost all patients with lung, colon cancer and melanoma and therefore can provide a liquid biopsy to monitor the course of disease. We, here, report on the successful analysis of such isolated cells for gene mutations in tumor driver genes EGFR, KRAS and BRAF.
Materials and Methods: Blood from patients with non small cell lung cancer, colon cancer and malignant melanoma was analyzed for cells positive for epithelial antigen (EpCAM) using the maintrac® approach, which avoids cell selection, and an image anlysis system or laser scanning cytometry for detection. Between 8-20 EpCAM positive cells from each patient were isolated individually using a semiautomated capillary approach and deposited one by one into micro cups. The DNA was subsequently amplified by whole genome amplification and assayed using either the cobas® EGFR mutation test, the cobas® KRAS mutation test or the cobas® BRAF V600 mutation test.
Results: DNA could be amplified from all individually isolated cells. An EGFR mutation was detected in 12% of isolated tumor cells from a patient with non-small cell lung cancer, the KRAS mutation was detectable in 28% of cells from a patient with colon cancer and the BRAF mutation in 100% of cells from a BRAF mutated cell line and in 50% of evaluable cells from a patient with melanoma.
Conclusions: Individually isolating epithelial tumor cells from the peripheral blood from patients with non-small cell lung cancer, colon cancer and melanoma allows not only detection of driver mutations in circulating tumor cells but also to determine the frequency of mutated cells. The results were confirmed by single cell analysis of a BRAF mutated cell line. This proves that at least part of the CETCs derived from the tumor. They can, in the future, be used as markers of response to the action of drugs and contribute insight into how resistance may be acquired.

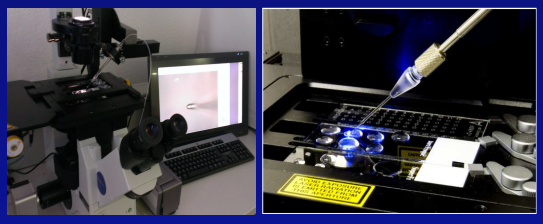
Methods

Blood samples (2-7 ml) from patients with NSCLC, metastatic colon cancer and malignant melanoma were drawn into normal blood count tubes with EDTA as an anticoagulant. For determining the number of circulating epithelial tumor cells (CETCs), tumor cells were stained with a FITC-conjugated epithelial cell adhesion molecule antibody (EpCAM) and enumerated using image analysis in a Scan-R fluorescence microscope. Vital CETCs were defined as EpCAM-positive cells, lacking in nuclear propidium iodide (PI) staining and with intact morphology.

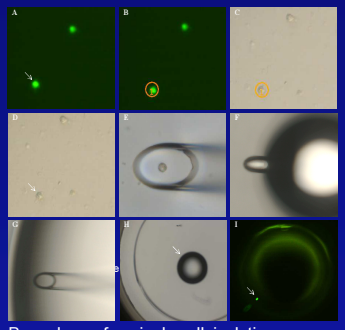
In a further step 8-20 vital cells stained with membrane EpCAM and excluding PI as an avitality marker among the white blood cells were selected individually using a semiautomated capillary approach (CellEctor Plus, MMI, Zurich, Switzerland) and deposited one by one into micro cups. For mutation analysis the DNA was subsequently amplified using *Ampli1*™ (silicon biosystems) or REPLI-g (Qiagen) Whole Genome Amplification for Single Cell Kit and assayed using the cobas® EGFR, KRAS and BRAF V600 Mutation Test Kits (Roche) and cobas z480 (Roche).



Images of a) vital (EpCAM green-positive and propidium iodide red-negative); b) dead (EpCAM green-positive and propidium iodide red-positive) circulating epithelial tumor cells.



Semiautomated capillary device for aspiration of individual cells from cell suspensions (slides in the front) and deposition in individual cups (slides in the background)



Procedure of a single cell isolation

Results

Spiking experiment for determining the sensitivity of our approach

Percentage of spiked cells (SK-Mel-28)	B-RAF mutation analysis
100 %	Mutation detected
75 %	Mutation detected
50 %	Mutation detected
25 %	Mutation detected
12,5 %	Mutation detected
6,25 %	Mutation detected
3,125 %	Mutation detected
1,56 %	Mutation detected
0,78 %	Mutation not detected

Negative and positive controls for determination of the specificity.

Probes	WGA	B-RAF mutation analysis
2 x leukocytes	silicon biosystems	Mutation not detected
2 x leukocytes	Qiagen	Mutation not detected
2 x Sk-Mel-28 cells	Qiagen	Mutation detected

Individually isolated single CETCs analyzed for their heterogeneity in the oncogenes BRAF (melanoma), KRAS (colorectal cancer) and EGFR (NSCLC).

	Number of isolated CETCs with wild type (%)	Number of isolated CETCs with detected mutation (%)	Invalid samples (%)
Patient 1: Colorectal Cancer (KRAS)	5/7 (71.4)	2/7 (28.6) (Codon 61)	--
Patient 2: Colorectal Cancer (KRAS)	--	2/4 (50)	2/4 (50)
Patient 1: Malignant melanoma (BRAF)	3/8 (37.5)	3/8 (37.5) (V600)	2/8 (25)
Patient 2: Malignant melanoma (BRAF) under treatment with Vemurafenib	4/4	--	--
Non-small cell lung cancer (EGFR)	5/8 (62.5)	1/8 (12.5) (Exon 20)	2/8 (25)

We were capable of detecting the fraction of cells among the tumor cells carrying the respective mutation. One patient with initial diagnosis of BRAF V600 mutation in primary tumor had no mutated CETCs under targeted treatment. This will allow in the future to monitor changes of these properties during targeted therapy and thus to determine the effect of this therapeutic approach directly in the relevant part of the tumor.

Conclusion

Individually isolating epithelial tumor cells from the peripheral blood from patients with non-small cell lung cancer, colon cancer and melanoma allows not only detection of driver mutations in circulating tumor cells but also to determine the frequency of mutated cells. The results were confirmed by single cell analysis of a BRAF mutated cell line. This proves that at least part of the CETCs are from the tumor. They can, in the future, be used as markers of response to the action of drugs and contribute insight into how resistance may be acquired.