Abschlussbericht

Thema des Forschungsprojektes

Detection and characterization of circulating tumor cells from patients with advanced breast, urothelial and renal cancer during the course of immune checkpoint therapy

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Datum und Unterschrift der Projektleiterinnen

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Zusammenfassung des Projektvorhabens

Die Einführung von Immuncheckpoint-Inhibitoren (ICI) in der Therapie des Urothelkarzinoms der Harnblase (mUCB) hat im Vergleich zur Chemotherapie zu verlängerten Ansprechraten geführt. Auch im metastasierten triple-negativen Mammakarzinom (mTNBC) konnte durch Kombination von ICI-Therapie mit Standardchemotherapie ein verlängertes Progressions-freien Überleben beobachtet werden. Verschiedene ICI-enthaltende Kombinationstherapien werden darüber hinaus auch beim metastasierten klarzelligen Nierenzellkarzinom (mccRCC) eingesetzt. Leider profitiert ein Teil der Patienten nicht von diesen Therapien. Deshalb werden Biomarker, die die Vorhersage für ein Therapieansprechen unterstützen, dringend benötigt. Sowohl die Expression von Immuncheckpoint-Proteinen, wie PD-L1 und PD-1 in Tumor- und Immunzellen, als auch der Immunstatus der Patienten oder die Mutationslast der Tumoren sind wichtige, aber nicht immer ausreichende Parameter, die für die Stratifizierung der Patienten herangezogen werden können. Problematisch ist weiterhin die Tatsache, dass sich die Expression dieser Marker, die bisher vorwiegend in Primärtumoren bestimmt werden, im Verlauf der Tumorprogression verändern kann, Metastasenbiopsien jedoch nicht in ausreichendem Maße zur Verfügung stehen.

In den letzten Jahren ist für eine Vielzahl von Tumorentitäten gezeigt worden, dass der Nachweis von zirkulierenden Tumorzellen (circulating tumor cells – CTCs) besonders im metastatischen Stadium der Erkrankung prognostische Bedeutung besitzt. Deshalb wird weltweit intensiv daran gearbeitet, den Wert von CTCs und deren C®harakterisierung als prädiktiven Marker zur Stratifizierung der Patienten u.a. für ICI-Therapien aufzudecken.

Im vorliegenden Antrag soll deshalb getestet werden, ob die Detektion von zirkulierenden Tumorzellen und deren Charakterisierung hinsichtlich wichtiger Immuncheckpoint-Regulatoren, wie z.B. PD-L1, für ein Echtzeit-Monitoring des Therapieansprechens bei Patienten mit mUCB, mccRCC und mTNBC) herangezogen werden können. Als Goldstandard für die Bestimmung und Messung der PD-L1-Expression der CTCs soll in der vorliegenden Studie das auf der Expression von epithelialen Proteinen beruhende CellSearch-Gerät zum Einsatz gelangen. Um aber auch CTCs zu detektieren, die aufgrund der fehlenden Expression epithelialer Proteine, z.B. durch epitheliale-mesenchymale Transition mit dem CellSearch-Gerät nicht detektiert werden können, soll das auf Zellgröße und Plastizität basierende Parsortix-System vergleichend eingesetzt werden.

Results

Detection of CTCs by CellSearch™ and Parsortix, micromanipulation and collection of single CTCs for single cell transcriptomic and genomic characterization

Breast cancer patients

Twenty two blood samples from 10 patients with metastatic triple negative breast breast cancer (mTNBC) treated at UKE with the combination of the azetolizumab [immune checkpoint inhibitor (ICI) anti PD-L1 (programmed death-ligand 1) antibody] and nab-paclitaxel (chemotherapy) were analyzed for CTCs by CellSearch® (Menarini Silicon Biosystems, Bologna, Italy). Among them, 8 samples from 5 patients were analyzed with the Parsortix® device. Only 4/10 (40%) patients had CTCs with two of them ≥5 CTCs/7.5 ml. From these 4 patients, 2 or more samples were analyzed. For patient 1 (Figure 1), CTC results at 3 different time points are shown and CTC numbers measured with both devices raised strongly. CTC clusters (Figure 2) suggested to be associated with a more aggressive behavior of the tumors, were predominantly found by using the Parsortix® approach, although the total number of CTCs was higher using the CellSearch® system. This patient died 9 weeks after the start of atezolizumab/nab paclitaxel treatment. Figure 1 also indicates how many CTCs and leukocytes could be collected by single cell micromanipulation for following down-stream analyses. After processing the blood samples by Parsortix® without a fixation step, CTCs were send to our collaboration partner for RNA sequencing. In contrast, CTCs isolated after CellSearch® processing including a fixation and permeabilization steps are no longer suited for RNA analyses, but were collected for genomic analysis [copy number alterations (CNA) or next generation sequencing (NGS)] after whole genome analysis (WGA).
In order to circumvent this limitation of the Cellsearch system, first experiments were started to compare the commonly used CellSave tubes (Menarini) with newly developed RNA rescue tubes (Menarini). CTCs isolated from 2 blood samples processed in parallel with one or the other tube are now being analyzed for RNA integrity.
From a second patient, 2 blood samples were analyzed within two weeks by CellSearch® and the number of CTCs increased from 0 to 2/7.5 ml. From 2 other mBC patients, repeated blood samples stayed CTC-negative for 2 or 3 months, respectively.

From all blood samples, plasma was collected for subsequent analyses of cell-free analytes such as cell-free and exosomal nucleic acids.
Moreover, PBMCs (peripheral blood mononuclear cells) were enriched by Ficoll density gradient centrifugation from all blood samples for further characterization of blood cells such as immune cells, circulating endothelial cells and fibroblasts.

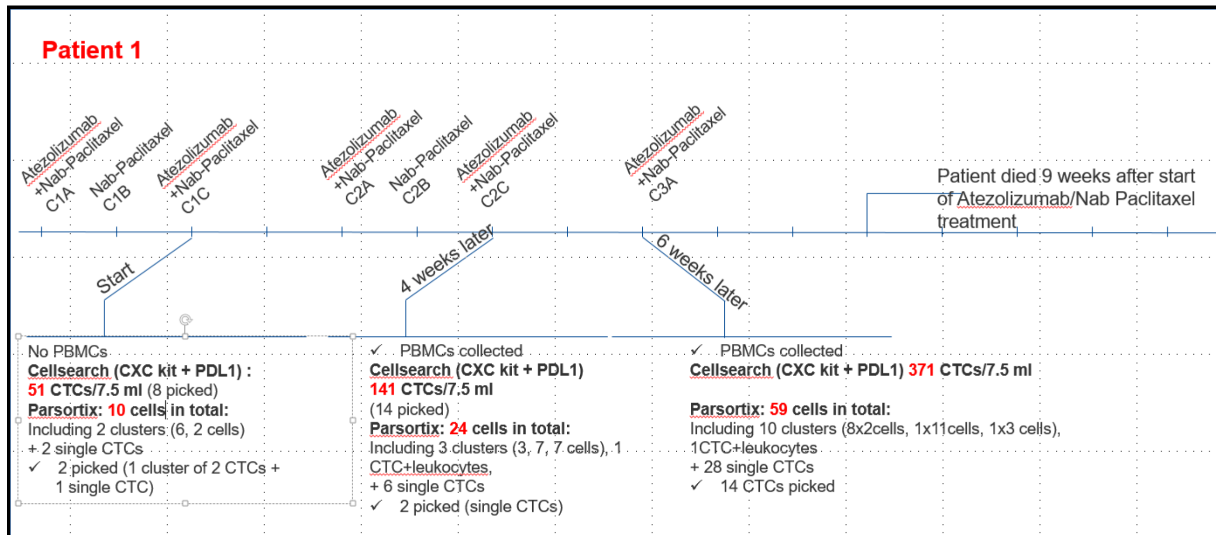

Figure 1: Analyses of CTCs from a patient with metastatic breast cancer receiving azetoliziumab/nab paclitaxel treatment. Number of detected CTCs in red.

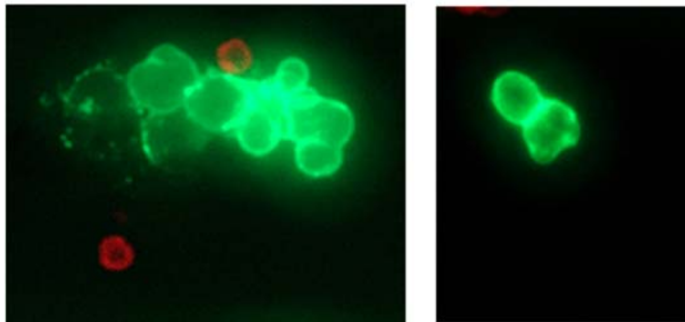

Figure 2: Clusters of at least 6 (left) and 2 CTCs (right) from patient 1. Immunofluorescent staining was performed by an antibody cocktail (Alexa 488-conjugated antibodies against EpCAM, EGFR and HER2, green), CD45 immunostaining in red.

In parallel, in collaboration with the University of Heidelberg/DKFZ Heidelberg, we followed 2 mTNBC patients treated by different therapies including ICI therapy for 16 or 24 months with 7 and 24 blood analyses, respectively, by CellSearch®. All but one of these samples revealed to be CTC-positive (patient A: at maximum 480 and patient B 494 CTCs/7.5 ml). Whether decreases or increases of CTC numbers at different time points and CTC PD-L1 expression are associated with the particular therapies and whether different CTC populations will be removed or survive will be investigated by evaluation of the clinical data and genomic analyses of CTCs after WGA and CNA analysis/NGS or by mass spectrometry.

Furthermore we determined PD-L1 expression of these CTCs. Most CTCs were PD-L1 negative or weakly positive.

Testing of a new anti-PD-L1antibody

Previously, we established an immunofluorescence assay to detect PD-L1 expression of CTCs (Bergmann S et al. Oncoimmunology 2020). Unfortunately, the phycoerythrin (PE)-conjugated antibody (E1L3N®, CellSignalling) recognizing the intracellular domain of PD-L1 and used in the fourth channel of the CellSearch® system is no longer available. Therefore we compared the results of this antibody with those obtained with the antibody D8T4X (extracellular domain-specific) rabbit mAb PE-conjugate (CellSignalling) for the urinary bladder carcinoma cell lines RT4 (negative control) and 647V (positive control). As expected, all cells of the cell line 647V presented with a strong (3+) intensity of immunofluorescence while RT4 cells were PD-L1 negative (Figure 3 left, below). As known from Western blot analyses and immunofluorescent stainings (data not shown here), PD-L1 staining of triple negative breast cancer cells MDA-MB-231 was weaker and heterogeneous compared to 647V cells (Figure 3 left, above). Counting each 500 cells, the distribution of different intensities of immunofluorescence in CellSearch® is shown in Figure 3 (right) for MDA-MB-231 and 647V cells. Thus, we decided to use the D8T4X antibody for further analyses of PD-L1 expression of CTCs in this study.

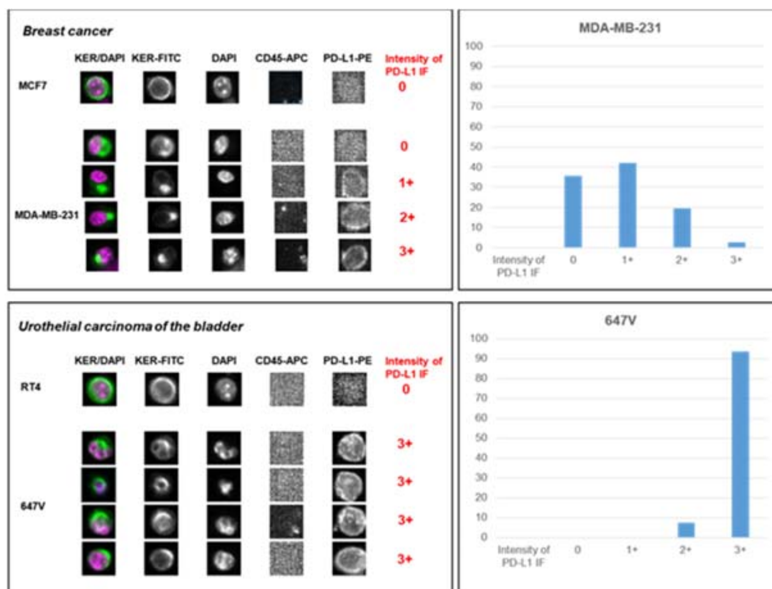

Figure 3: Enrichment of cell line cells from breast cancer and urothelial carcinoma of the bladder by using the CellSearch® system. Left: CellSearch® images for breast cancer and urinary bladder cell line cells with different intensities of PD-L1 immunostainings, KER: keratin, DAPI: 4',6-Diamidino-2-phenylindol; FITC: fluorescein; APC: allophycocyanine; PE: phycoerythrin), IF: immuno-fluorescence

Urinary bladder cancer (UCB)

During the project time we only received blood samples from 3 patients who presented with progressive UCB disease under ICI therapy. Blood samples from 2 of these patients were CTC negative and one patient had 1 CTC/7.5 ml blood by CellSearch® analysis. Therefore down-stream analysis of CTCs from these patients was not possible. Nevertheless, we collected plasma and PBMCs for further analyses of cell-free liquid biopsy components and immunophenotyping of PBMCs.

Renal cell cancer (RCC)

We did not receive blood samples from RCC patients progressive under ICI.

Conclusions and outlook

In this project we were able to analyze blood samples from metastatic breast and UCB patients for circulating tumor cells and CTC PD-L1 expression. We could collect CTCs from 3 patients received at different time points during treatment for subsequent transcriptomic and/or genomic analyses. CTC counts and PD-L1 expression of CTCs can now be correlated with the clinical data of the patients and with therapy responses. Explorative down-stream analyses of collected CTCs will help identifying those cells that are escaping from therapy and evaluating their genomic characteristics. Plasma and PBMC analyses will provide us with additional important results in terms of therapy response.