Are morphological criteria sufficient for the identification of circulating tumor cells in renal cancer?

Amin El-Heliebi1, Thomas Kroneis1, Evelyn Zöhrer1, Johannes Haybaeck2, Katja Fischereder3, Karin Kampel-Kettner3, Richard Zigeuner3, Hannelore Pock4, Regina Riedl5, Rudolf Stauber4, Jochen Bernd Geigl6, Berthold Huppertz1, Peter Sedlmayr1† and Carolin Lackner2*

Abstract

**Background:** Single circulating tumor cells (CTCs) or circulating tumor microemboli (CTMs) are potential biomarkers of renal cell cancer (RCC), however studies of CTCs/CTMs in RCC are limited. In this pilot study we aimed to evaluate a novel blood filtration technique suited for cytomorphological classification, immunocytochemical and molecular characterization of filtered, so called circulating non-hematologic cells (CNHCs) - putative CTCs/CTMs - in patients with RCC.

**Methods:** Blood of 40 patients with renal tumors was subjected to ScreenCell® filtration. CNHCs were classified according to cytomorphological criteria. Immunocytochemical analysis was performed with antibodies against CD45, CD31 and carbonic anhydrase IX (CAIX, a RCC marker). DNA of selected CNHCs and respective primary tumors was analysed by array-CGH.

**Results:** CNHC-clusters with malignant or uncertain malignant cytomorphological features - putative CTMs - were negative for CD45, positive for CD31, while only 6% were CAIX positive. Array-CGH revealed that 83% of malignant and uncertain malignant cells did represent with a balanced genome whereas 17% presented genomic DNA imbalances which did not match the aberrations of the primary tumors. Putative single CTCs were negative for CD45, 33% were positive for CD31 and 56% were positive for CAIX.

**Conclusions:** The majority of CNHC-clusters, putative CTMs, retrieved by ScreenCell® filtration may be of endothelial origin. Morphological criteria seem to be insufficient to distinguish malignant from non-malignant cells in renal cancer.

**Keywords:** Circulating tumor cells, Circulating tumor microemboli, Renal cancer, ScreenCell®, Array comparative genomic hybridization (array-CGH), Circulating endothelial cells

**Background**

Hematogenous dissemination of single tumor cells (circulating tumor cells, CTCs) or tumor microemboli (circulating tumor microemboli, CTM) is an important mechanism involved in the formation of metastases, the major cause of cancer related death [1]. Results of numerous studies indicate the prognostic value of CTC detection and enumeration in many human cancers. This has been confirmed in recent meta analyses for breast [2] and colorectal cancer [3]. Besides enumeration, results of the molecular analyses of CTCs may be used to predict treatment response [4]. Investigation of CTCs may hold great promise to inform individualized treatment strategies as well as to increase the knowledge about the metastatic process in itself. Renal cell cancer (RCC) is the most frequent solid lesion of the kidney and accounts for 3% of all cancer cases worldwide. Approximately 20% of primary localized RCC will develop metastatic disease.
negatively impacting on patient survival [5]. Therapeutic options are limited by resistance of RCC to chemotherapy and radiation, but have recently been improved by the advent of targeted therapies [6, 7]. As in other malignancies, CTCs may also be valuable prognostic and predictive biomarkers of renal cancers, however studies of CTCs in RCC are limited (reviewed in [8]).

Several techniques for the detection and enumeration of CTCs have been developed during the last years and the list is continuously growing. Many methods use epithelial antigenic properties of cancer cells to detect and isolate them from blood by immunomagnetic or microfluidic based enrichment methods (reviewed in [9]). However, many of these detection systems are not commercially available and/or economically accessible. Another obstacle is the epithelial antigen-based detection of CTCs. Epithelial to mesenchymal transition (EMT) is believed to represent an integral component of the metastatic process in which cancer cells down regulate the expression of epithelial markers in favour of mesenchymal markers, a process linked to the stemness of cancer cells and increased chemoresistance [10-13]. Hence such CTCs may therefore escape detection. Recently filtration based size exclusion technologies have been developed [14, 15] which allow for antigen-independent isolation of CTCs from blood based on their larger size and cytomorphological features in comparison to hematological cells [16]. Some of these methods like the ScreenCell® filtration system are commercially available. CTCs can be isolated from diluted blood in a single step using a translucent polycarbonate membrane. Following filtration they can be further analysed by light microscopy and immunocytochemistry [16-21] but molecular data on putative CTCs/CTMs are limited [21-23].

Using the ScreenCell® filtration system and array-CGH technology we aimed to evaluate if morphological criteria [17] are sufficient to identify CTCs/CTMs in blood of renal cancer patients. Here we report for the first time chromosomal analysis of circulating non-hematological cell clusters cytomorphologically resembling CTMs by array-CGH.

**Methods**

**Ethical statement**

The study was approved by the ethics committee of the Medical University of Graz (reference EK: 19–239 ex 07/08). Written informed consent was obtained from all patients. The human iliac arterial endothelial primary cells (HIAEC) (generously provided by Dr. I. Lang-Olip, Medical University of Graz, Austria) derived from an organ donor. Ethical approval was granted by the ethics committee of the Medical University of Graz (reference EK: 19–293 ex 07/08). The ethics committee waived the need for written informed consent as the donor fulfilled presumed-consent according to Austrian Hospitals Act.

**Patients and tissues**

Thirty consecutive patients diagnosed of renal cell carcinomas (25 clear cell and 5 papillary carcinomas) and for control purposes 10 patients with benign renal tumors (1 renal cyst, 2 angiomyolipoma, 6 oncocytoma and 1 cystic nephroma) diagnosed between 2010 and 2011 were included in the study. All patients underwent surgical resection of the tumor. Clinical and histopathological parameters of the study cohort are compiled in Table 1. Blood samples from 20 healthy volunteers were used as age and sex matched negative controls. Formaldehyde (4% m/V buffered solution)-fixed and paraffin-embedded (FFPE) tumor specimens were retrieved from the BioBank of the Medical University of Graz, Austria. In 40% of the cases tumor tissue snap frozen in liquid nitrogen was available. All cancer patients were classified according to the TNM Classification of Malignant Tumors of the International Union

**Table 1 Clinical and histopathological characteristics of the study cohort**

| Parameter | Patients, n; (%) |
|-----------|-----------------|
| Age, median; (range) | 68; (30–83) |
| Men | 28; (70) |
| Women | 12; (30) |
| Benign tumors | 10; (25) |
| Renal cysts | 1; (2.5) |
| Angiomyolipoma | 2; (5) |
| Oncocytoma | 6; (15) |
| Cystic Nephroma | 1; (2.5) |
| Tumor size (cm), median; (range) | 2.5; (1.0-6.0) |
| Malignant tumors | 30; (75) |
| Clear cell RCC | 25; (62.5) |
| Papillary RCC | 5; (12.5) |
| Tumor size (cm), median; (range) | 4; (1.3-12.0) |
| Tumor differentiation | |
| G1 | 6; (20) |
| G2 | 16; (53.3) |
| G3 | 8; (26.7) |
| Venous invasion (in cases of potentially invasive tumors) | 6; (16) |
| Distant metastasis (in cases of potentially invasive tumors) | 1; (3) |
| Type of surgical intervention | |
| Open partial nephrectomy | 24; (60) |
| Open nephrectomy | 6; (15) |
| Transperitoneal nephrectomy | 4; (10) |
| Laparoscopic nephrectomy | 3; (7.5) |
| Laparoscopic partial nephrectomy | 2; (5) |
| CT targeted biopsy | 1; (2.5) |
Against Cancer (UICC) [24]. Seventeen (57%) RCC tumors were staged T1a, five (17%) were classified as T1b, four (13%) as T2a and four (13%) as T3a, respectively. Six (20%) of RCC tumors were graded G1, 16 (53%) G2 and 8 (27%) G3, respectively. In the tumors of six patients (16%) venous invasion was found.

Blood sample collection
From each patient a total of 4 blood samples were collected in 2 x 6 ml EDTA tubes (Greiner Bio-One, Kremsmünster, Austria) from a peripheral vein. The first sample was drawn one day prior to surgery (time point A), the next time points of collection were once, during surgery shortly after removal of the tumor (time point B), one day after surgery (time point C) and eight days after surgery (time point D).

DNA extraction of primary tumor tissue
Genomic DNA was extracted from snap frozen tumor tissue or if not available, from FFPE tumor tissue using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) as described by the manufacturer. DNA quantity and quality was assessed by Nanodrop measurement (Thermo Fisher Scientific, Waltham, USA) and DNA fragmentation was analysed by agarose gel electrophoresis [25].

Cell lines and evaluation of the sensitivity of the ScreenCell® filtration device
The female renal cell adenocarcinoma cell line 769-P was purchased from ATCC (Manassas, USA) and cultivated in RPMI supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, 100U/ml penicillin and 100 μg/ml streptomycin (all cell culture supplies from Gibco, Invitrogen, Carlsbad, USA). The human iliac arterial endothelial primary cells (HIAEC) (generously provided by Dr. J. Lang-Olip, Medical University of Graz, Austria) were grown in endothelial growth medium-2 (EGM-2) (Lonza, Walkersville, USA).

The sensitivity of the ScreenCell® filtration method was evaluated in nine independent experiments by spiking cultured renal cell cancer cells (769-P cell line) into blood samples from a healthy volunteer. Confluent 769-P cells were harvested by trypsinization using 0.05% Trypsin-EDTA buffer (PAA, Pasching, Austria). Then defined numbers (three batches of 2, 10 and 50 individual cells) were picked under an inverted microscope (Zeiss Axiovert M 200, Munich, Germany) using a micromanipulator (MMJ, Zeiss; CellTram vario, Eppendorf, Hamburg, Germany) equipped with microcapillaries (inner diameter of 20 μm) (TransferTip, Eppendorf). Picked cells were directly transferred into the blood sample which was then processed by filtration and hematoxylin staining. Cells were enumerated and recovery rates were calculated.

ScreenCell® filtration
All blood samples were processed within 4 hours after collection of blood as recommended by the manufacturer (ScreenCell®, Paris, France). Filtration was carried out as previously described [15] with minor modifications. Briefly, blood was diluted 8 fold with red blood cell lysis buffer (ScreenCell®, Paris, France) and incubated for 10 min at RT, with gentle agitation after 3 and 6 min. Per patient and time point 4 filtrations, each corresponding to the processing of 2 ml of whole blood were performed using vacuum tubes as described by the manufacturer. Thereafter the filters were rinsed with 2 ml of sterile phosphate-buffered saline (PBS pH 7.4) and collected from the device. The filters were counterstained with hematoxylin (Merck, Darmstadt, Germany) for 5 min and blued for a few seconds with NH3-H2O (0.06% m/V), washed in distilled water, air dried and mounted on a glass slide for evaluation by light microscopy. Of 14 selected cases (11 patients with RCC, 1 with oncocytoma, and 2 cases with benign tumors) three of the four filters remained unstained and were stored at -20°C until further immunocytochemical analysis.

Cytomorphological analyses of filtered cells
Stained filters were analysed by light microscopy by a board certified cytopathologist (CL) using the cytomorphological criteria recently issued by a panel of ten expert cytopathologists for the classification of circulating non-hematological cells (CNHC) filtered from blood using the method “isolation by size of epithelial tumor cells” (ISET) as published by Hofman et al. [17]. This filtration technology is based on comparable principles for isolation of CTCs/CTMs from blood, i.e. size exclusion by filtration through a translucent polycarbonate membrane filter with 8 μm pore size using vacuum suction, as the ScreenCell® system. Therefore cytomorphological criteria established with ISET filtration may also be used to evaluate filtered cells on ScreenCell® filters. The proposed criteria by Hofman have also been applied to renal cell carcinoma in one study [17]. According to these criteria filtered cells >8 μm are classified as CNHC with malignant (CNHC-MF), uncertain malignant (CNHC-UMF) and benign features (CNHC-BF) (Figure 1A-F).

CNHC occurring as cellular aggregates (clusters) were classified as CNHC-MF (Figure 1A) if at least four of the following criteria were present:

- Anisonucleosis (ratio >0.5)
- Nuclei larger than 3 times the calibrated 8 μm pore size of the membrane
- Irregular nuclear outline
- Presence of tridimensional cellular sheets
- High nuclear/cytoplasmic ratio
CNHC clusters were defined as CNHC-UMF when less than 4 of these criteria were seen (Figure 1B), whereas CNHC-BF did not show any of these characteristics (Figure 1C).

Single CNHC (Figure 1D-F) were classified as CNHC-MF (Figure 1D) if all 3 of the following cytological criteria were present:

- Nuclei larger than 3 times the calibrated 8 μm pore size
- Irregular nuclear outline
- High nuclear/cytoplasmic ratio

If less than 3 of these criteria were present, the single cell was CNHC-UMF (Figure 1E). CNHC-BF cells showed none of the cellular features described above (Figure 1F). The number of cells was assessed by counting nuclei of single CNHCs and within CNHC clusters. The total numbers of CNHCs are listed in Table 2.

**Immunocytochemical characterization of filtered cells**

After filtration the filters were dissembled from the device, air-dried over night at room temperature (RT) and then fixed in 4% m/V formaldehyde (Labonord, Templemars, France) for 10 min. After rehydration in Tris-buffered saline (TBS) for 10 min, the filters were incubated in TBS-Triton (TBS containing 0.2% Triton X-100) (Merck, Darmstadt, Germany) for 5 min. Following a washing step with TBS, hydrogen peroxidase blocking solution (Dako, Glostrup, Denmark) was applied for 10 min followed by incubation with Ultra V Block (Dako, Glostrup, Denmark) at RT for 5 min. To circumvent the effect of a possible EMT-associated low expression of epithelial antigens like cytokeratins in CTCs/CTMs and because renal cell cancers only weakly express epithelial cell adhesion molecule (EpCAM) [26], carbonic anhydrase IX (CAIX) was used as a marker. This protein is overexpressed in 100% of clear cell renal cell carcinomas and 57% of papillary renal cell carcinomas [27] but is not expressed in normal renal tissue [28].

---

**Figure 1** Immunocytochemical analysis of CNHCs with antibodies against the hematological marker CD45. Cellular aggregates (clusters) of non-hematological cells (CNHC) isolated from blood of patients with renal tumors by ScreenCell® filtration (A-C) with cytomorphological features of malignant (CNHC-MF) (A), uncertain malignant (CNHC-UMF) (B), and benign cells (CNHC-BF) (C) as previously defined [17]. None of the CNHC are detected by CD45 antibodies, whereas single lymphocytes are CD45-positive (indicated by arrows in A and C). Examples of filter pores (8 μm in diameter) are marked by arrow heads (B). Single CNHC isolated by ScreenCell® filtration (D-F) with cytomorphological features of malignant (−MF) (D), uncertain malignant (−UMF) (E), and benign cells (−BF) (F) are CD45-negative. However, leucocytes are stained with CD45 antibodies (arrows in D and F).
Table 2 Summary of the results of cytomorphological classification of CNHCs detected by ScreenCell® filtration in 8 ml of venous blood of patients with benign (n = 10), malignant (n = 30) renal tumors and healthy controls (n = 20)

| Patient number | Histological diagnosis | Clinical data | Time point A* | Time point B** | Time point C*** | Time point D**** |
|----------------|------------------------|---------------|---------------|----------------|-----------------|-----------------|
|                |                        | TNM Grade     | MF UMF BF     | MF UMF BF     | MF UMF BF       | MF UMF BF       |
| 26             | angiomyolipoma         | - - no        | 0 2 0 0       | 0 0 0 0       | 0 0 0 0         | 0 0 0 0         |
| 33             | angiomyolipoma         | - - no        | 0 0 0 0       | 0 0 0 0       | 0 0 0 0         | 0 0 0 0         |
| 29             | benign cyst            | - - -         | 0 0 0 0       | 10 0 0 0      | 0 0 30          | 2 0 0 0         |
| 18             | cyst. nephroma         | - - -         | 51 4 0 2      | 0 0 127 30    | 30 380 0        |                 |
| 3              | oncocytozyma           | - - no        | 0 560 2       | 0 0 0 0       | 0 0 0 0         | 0 251 195       |
| 4              | oncocytozyma           | - - no        | 0 0 0 2       | 0 0 40 8      | 0 2 3           |                 |
| 6              | oncocytozyma           | - - no        | 0 3 0 0       | 0 0 2 35      | 0 19 11         |                 |
| 14             | oncocytozyma           | - - no        | 0 0 0 0       | 39 0 37 8     | 0 21 5 0        |                 |
| 17             | oncocytozyma           | - - no        | 2 136 0       | 0 0 1 0       | 2 0 35          |                 |
| 38             | oncocytozyma           | - - no        | 0 0 4 0       | 0 0 0 0       | 0 0 0 0         |                 |
| 1              | ccRCC T2aNOM0          | 2 yes         | 0 0 0 0       | ND ND ND      | ND ND ND ND     |                 |
| 2              | ccRCC T3aNOM0          | 2 yes         | 0 0 0 0       | 0 3 10 0      | 0 0 0 0         |                 |
| 7              | ccRCC T2aNOM0          | 2 no          | 0 0 0 0       | 0 0 0 6       | 0 ND ND ND      |                 |
| 9              | ccRCC T1bNOM0          | 2 no          | 3 19 5 0      | 0 0 0 ND      | ND ND ND ND     |                 |
| 10             | ccRCC T1NOM0           | 2 no          | 16 0 10 0     | 0 0 0 0       | 3 0 22 16       |                 |
| 11             | ccRCC T1NOM0           | 2 no          | 0 0 0 2       | 0 0 ND ND     | ND ND ND 0      |                 |
| 13             | ccRCC T3aNOM0          | 3 yes         | 0 19 0 0      | 0 22 158 30   | ND ND ND ND     |                 |
| 15             | ccRCC T1aNOM0          | 2 no          | 0 0 0 0       | 0 0 0 0       | 0 0 0 0         |                 |
| 16             | ccRCC T1aNOM0          | 2 no          | 62 56 0       | 0 0 0 0       | 0 0 0 0         |                 |
| 19             | ccRCC T1aNOM0          | 1 no          | 1 0 0 0       | 0 0 0 0       | 0 0 3 5         |                 |
| 20             | ccRCC T3aNOM0          | 3 yes         | 13 5 0 0      | 0 0 20 319 58 | ND ND ND ND     |                 |
| 22             | ccRCC T1aNOM0          | 1 no          | 0 32 0 1      | 0 0 11 0 0    | ND ND ND ND     |                 |
| 23             | ccRCC T1aNOM0          | 1 no          | 1 0 0 0       | 1 0 2 10      | 5 12 15         |                 |
| 24             | ccRCC T1NOM0           | 1 no          | ND ND ND      | 0 0 5 0       | 1 0 0 0         |                 |
| 25             | ccRCC T1aNOM0          | 2 no          | 0 1 0 0       | 0 0 1 20      | ND ND ND ND     |                 |
| 28             | ccRCC T1bNOM0          | 1 no          | 0 0 0 0       | 645 0 5 5     | 25 0 36 12      |                 |
| 30             | ccRCC T1NOM0           | 3 no          | 0 0 0 31 227 4 | 0 0 0 0      | 0 67 64         |                 |
| 32             | ccRCC T1aNOM0          | 2 no          | 1 0 0 1       | 0 0 3 26 15   | ND ND ND ND     |                 |
| 34             | ccRCC T1aNOM0          | 2 no          | 0 0 0 0       | 26 51 0 18    | 0 ND ND ND      |                 |
| 35             | ccRCC T1aNOM0          | 2 no          | 0 0 0 0       | 0 ND ND ND    | 0 20 0          |                 |
| 36             | ccRCC T1aNOM0          | 3 no          | 2 125 3 0     | 0 0 2 1 7     | 0 0 0 0         |                 |
| 40             | ccRCC T1aNOM0          | 3 no          | 0 23 0 0      | 0 0 0 0       | 0 15 0 0        |                 |
| 41             | ccRCC T1aNOM0          | 3 no          | 0 0 0 0       | 0 0 1 0       | 3 74 4          |                 |
| 42             | ccRCC T1NOM0           | 1 no          | 0 0 0 0       | 0 0 22 44     | 58 124 98       |                 |
| 27             | pap.RCC T1bNOM0        | 3 no          | 0 0 0 0       | 0 0 0 0       | 0 1 6 0         |                 |
| 8              | pap.RCC T1NOM0         | 2 no          | ND ND ND      | ND ND ND      | 0 0 0 0         |                 |
| 12             | pap.RCC T3aNOM0        | 3 yes         | 0 0 5 0       | 0 0 11 0 0    | ND ND ND ND     |                 |
| 31             | pap.RCC T1aNOM0        | 2 no          | 0 0 0 0       | 0 0 ND ND     | ND ND ND ND     |                 |
| 37             | pap.RCC T2aNOM0        | 2 no          | 0 0 100 19 79 | 18 1 0 4      | ND ND ND ND     |                 |
| 39             | reg RCC T2aNOM0        | 2 yes         | 0 0 1 1 0     | 0 1 0 0       | 1 0 0 0         |                 |
| C1              | Control                | - - -         | 0 0 1 -       | - - - -       | - - - -         |                 |
The filters were incubated with primary antibodies directed against CAIX (rabbit IgG, NB100-417, Novus Biologicals, Littleton, USA; 3.5 μg/ml), CD31 (mouse IgG1, M0823, Dako, Glostrup, Denmark; 1 μg/ml) or CD45 (mouse IgG1, M0855, Dako, Glostrup, Denmark; 2.4 μg/ml) diluted in Antibody Diluent (Dako, Glostrup, Denmark) for 30 min at RT followed by application of primary antibody enhancer (Dako, Glostrup, Denmark) for 15 min. Thereafter the filters were incubated with labelled polymer-HRP Anti-Mouse + Anti-Rabbit (Dako, Glostrup, Denmark) for 10 min. AEC substrate chromogen (Dako, Glostrup, Denmark) was applied for 10 min and rinsed off with distilled water prior to counterstaining with hematoxylin (Merck, Darmstadt, Germany) as described above. Respective non-immune rabbit IgG and isotype-matched monoclonal antibodies were used as controls. Cultured 769-P and HIAEC cells were spiked in blood, filtered and stained like patient samples and served as positive or negative control for staining, enumeration and array-CGH experiments.

**Laser capture microdissection and whole genome amplification of selected filtered cells**

Isolation of selected CNHCs was carried out by laser capture microdissection (P.A.L.M., Zeiss, Munich, Germany). Before laser capture microdissection, all non-target cells and cellular debris were removed by laser ablation. The polycarbonate filter was then cut with a pulsed laser beam and the membrane and target cells were catapulted into the lid of a 200 μl PCR tube, which contained 10 μl of WGA-4 fragmentation and lysis buffer (#WGA4, Sigma-Aldrich, St. Louis, USA) (Figure 2). An exemplary laser capturing is shown in Additional file 1: Movie 1. For control purposes neutrophilic granulocytes, which were subjected to the same filtration and staining procedures like patient samples, were as well isolated by laser capture microdissection and forwarded to whole genome amplification (WGA). WGA was performed as previously described [29] with minor modifications using the GenomePlex Single Cell Whole Genome Amplification Kit (Sigma-Aldrich, St. Louis, USA). Briefly, the captured cells were digested in 10 μl fragmentation and lysis buffer, followed by GenomePlex library preparation. Amplification was performed by adding 7.5 μl of 10× amplification master mix, 48.5 μl nuclease free water and 5 μl WGA DNA polymerase. After whole genome amplification, DNA was purified using the GenElute PCR Clean-Up Kit (Sigma-Aldrich, St. Louis, USA). DNA concentration and purity

---

Table 2 Summary of the results of cytomorphological classification of CNHCs detected by ScreenCell® filtration in 8 ml of venous blood of patients with benign (n = 10), malignant (n = 30) renal tumors and healthy controls (n = 20)

|   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| C2 | Control | - | - | - | 0 | 0 | 0 | - | - | - | - | - | - |
| C3 | Control | - | - | - | 0 | 2 | 2 | - | - | - | - | - | - |
| C4 | Control | - | - | - | 83 | 100 | - | - | - | - | - | - | - |
| C5 | Control | - | - | - | 0 | 0 | 0 | - | - | - | - | - | - |
| C6 | Control | - | - | - | 0 | 0 | 0 | - | - | - | - | - | - |
| C7 | Control | - | - | - | 0 | 0 | 12 | - | - | - | - | - | - |
| C8 | Control | - | - | - | 0 | 0 | 0 | - | - | - | - | - | - |
| C9 | Control | - | - | - | 0 | 1 | 0 | - | - | - | - | - | - |
| C10 | Control | - | - | - | 0 | 0 | 0 | - | - | - | - | - | - |
| C11 | Control | - | - | - | 0 | 1 | 1 | - | - | - | - | - | - |
| C12 | Control | - | - | - | 0 | 0 | 0 | - | - | - | - | - | - |
| C13 | Control | - | - | - | 0 | 1 | 2 | - | - | - | - | - | - |
| C14 | Control | - | - | - | 0 | 0 | 0 | - | - | - | - | - | - |
| C15 | Control | - | - | - | 0 | 0 | 0 | - | - | - | - | - | - |
| C16 | Control | - | - | - | 0 | 27 | 5 | - | - | - | - | - | - |
| C17 | Control | - | - | - | 0 | 1 | 7 | - | - | - | - | - | - |
| C18 | Control | - | - | - | 0 | 0 | 0 | - | - | - | - | - | - |
| C19 | Control | - | - | - | 0 | 0 | 0 | - | - | - | - | - | - |
| C20 | Control | - | - | - | 0 | 1 | 0 | - | - | - | - | - | - |

* One day before surgery.
** During surgery, after the removal of the tumor.
*** One day after surgery.
**** Eight days after surgery.
ND Not done (sample not available).
was determined by Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, USA). DNA quality was assessed by multiplex PCR as previously described [25]. Samples showing three to four DNA bands between 100 and 400 bp were regarded as of high DNA quality.

Array-CGH
Array-CGH was performed using SurePrint G3 Human CGH Microarrays 8x60K (Agilent Technologies, Santa Clara, USA). Prior to labelling, genomic DNA derived from snap frozen and FFPE tissues as well as the corresponding reference DNA were digested by Alu I and Rsa I (Promega, Fitchburg, USA) according to the supplier’s instructions. Amplified DNA of CNHCs and the corresponding amplified reference DNA (Promega, Fitchburg, USA) showed mean DNA size distributions of 400 - 600 bp and therefore provided suitable templates for array-CGH without any digestion step. The purified samples were labelled with the Bioprime Array CGH Genomic Labeling System (Life Technologies, Carlsbad, USA). Briefly, 250 ng of sample DNA and 250 ng of female or male reference DNA (Promega, Fitchburg, USA) were labelled with dCTP-Cy5 and dCTP-Cy3 (GE Healthcare, Little Chalfont, UK), respectively. Subsequently, DNA was purified with Amicon Ultracel-30 filters (Millipore, Billerica, USA) and mixed in equal amounts. The mixture was hybridized to a 60 K array ON using the Oligo aCGH/ChIP-on-chip Hybridization kit (Agilent Technologies, Santa Clara, USA). Following hybridization, the array was washed and scanned (Agilent Technologies, Santa Clara, USA) as recommended by the manufacturer. Data analysis was performed with Agilent Genomic Workbench Lite Edition 6.5.0.18. (Agilent Technologies, Santa Clara, USA). The following settings were used for tumor tissues: ADM-2, threshold 8.0, with at least 10 consecutive oligos with an absolute log ratio of 0.22. For amplified DNA the algorithm ADM-2, threshold 9.3 was used with at least 100 consecutive oligos with an absolute log ratio of 0.30.

**Definition of thresholds and controls for array-CGH**
As it was our purpose to characterize CNHC clusters, pools of leucocytes and 769-P cells were used to test the detection limits of genetic aberrations of the array-CGH method. 769-P cells and leucocytes were subjected to ScreenCell® filtration, microdissected from the filters, the DNA was subjected to WGA and further analysed by array-CGH. There was concordance of gains and losses of chromosomal DNA detected in amplified pools of ten 769-P cells and non-amplified DNA from cultured 769-P cells [30] (Figure 3). From this comparison we estimated that deletions of approximately ~6.8 Mb can be detected in pools of ten 769-P cells (chromosome 9, genomic position 16.2-23.0 Mb) which is sufficient to indicate larger scale aberrations in RCC [31]. The DNA of isolated pools of 10 leucocytes from blood of a healthy individual, representing a balanced genome, was used to set the thresholds for the detection limits of gains and losses by array-CGH in our study. In contrast to cell cultured cells, the array-CGH profiles of amplified DNA of CNHCs demonstrated slightly noisier ratio profiles, as we expected if going from an artificial cell culture system to clinical samples. By applying the above mentioned threshold settings, gains and losses could be reliably detected (Figure 3).

**Statistical analysis**
We investigated if the presence or absence of CNHC types (categorized as binary variables) differed between time points A–D, using Chi-square tests. Furthermore, median, minimum and maximum were used to describe the number of CNHCs of each type and for every time
point. The associations between numbers of CNHCs of each type with tumor size, venous invasion and differentiation grade were explored using nonparametric methods. A p-value of <0.05 was considered to indicate statistical significance. All p-values were regarded in an explorative sense. The statistical calculations were performed using the SPSS software package, version 20.0 (IBM, Armonk, USA).

Results

Spiking experiments

The average number of recovered 769-P cells for 50, 10 and 2 spiked cells were 45.3 (SD 2.1), 8.7 (SD 1.5), and 1.7 (SD 0.6), respectively. The average recovery rates of 769-P were 91%, 87% and 83% for 50, 10 and 2 spiked 769-P cells, respectively which compares to sensitivity rates published by Desitter et al. for the ScreenCell® filtration device [15].

Cytomorphological analysis of CNHC types in patients with renal tumors and healthy controls

Overall CNHCs of the MF-type were detected more frequently in renal cancer patients (29%) as compared to healthy controls (0%) (p = 0.014). CNHC-MF were also found in 20% of cases with benign renal tumors which was not significantly different from the frequency found in healthy controls (p = 0.103). However in healthy controls CNHC-MF were not found. CNHC-UMF and –BF types were identified in the blood of 29% and 21% of the renal cancer patients, in 50% and 20% of benign renal tumor patients as well as in 40% and 40% of healthy individuals (Figure 4).

A summary of the results of the cytomorphological analyses and the number of cells of all patients and time points is provided in Table 2.

Overall, one day before surgery (time point A) 26%, 34%, and 21% of the blood samples were positive for CNHC-MF, -UMF and –BF, respectively. However, during surgery, after removal of the renal tumor (time point B) CNHC-MF, -UMF and -BF were found in only 18%, 20% and 13% of samples, respectively. One day after surgery (time point C) there was a significant increase in the number of blood samples positive for CNHC-MF (40%, p = 0.040), -UMF (43%, p = 0.045) and BF (43%, 0.004). Eight days after surgery (time point D) 48% of the samples were positive for CNHC-UMF and 38% were positive for CNHC-BF, whereas in only 31% of samples CNHC-MF were found (Figure 5).
The cytomorphological CNHC types, CNHC-MF, -UMF, -BF either as single cells or clusters were found at every time point in patients regardless of the histological tumor diagnosis (Table 2).

The median number of CNHC-MF per 8 ml of blood was 3 cells (range: 1–62) before surgery (time point A), 2 cells (range: 1–31) during the surgery (time point B), 3 cells (range: 1–37) one day after surgery (time point C), and 3 cells (range: 1–38) eight days after surgery (time point D) (Figure 6A). The median number of CNHC-UMF per 8 ml of blood was 19 cells (range: 1–560) at time point A, 33 cells/8 ml (range: 1–645) at time point B, 18 cells/8 ml (range: 1–319) at time point C and 21 cells/8 ml (range 2–380) at time point D (Figure 6B). Cells with benign features represented with a median number of 4 cells/8 ml (range: 1–100) at time point A, 5 cells/8 ml (range: 3–51) at time point B, 20 cells/8 ml (range: 3–58) at time point C and 15 cells/8 ml (range: 3–195) at time point D (Figure 6C). In renal cancer patients there was no correlation between histopathological tumor parameters (tumor size, grade of differentiation) and detection of CNHC regardless of type or time point examined (Additional file 2: Table S1 and Additional file 3: Table S2). However, one day after surgery (time point C) higher numbers of CNHC-MF were detected in patients with RCC with venous invasion as compared to those cases without venous invasion (p = 0.013). This was not found at any of the other time points investigated (Additional file 4: Table S3).

In the blood of healthy controls no CNHC-MF were detected. In three out of 20 cases (15%) we found CNHC clusters, primarily of the BF- (14 clusters) and UMF-types (6 clusters). In 7 out of 20 cases (35%) we found single CNHCs, mostly of the BF- (13 cells) and less frequently the UMF-types (5 cells). Overall, 0%, 40%, and 40% of the blood samples from healthy controls were positive for CNHC-MF, -UMF and -BF, respectively (Table 2). All detected CNHCs were negative for CAIX or CD45. However, 4 CNHC clusters of the –BF type and 2 of the –UMF type were positive for CD31.

Immunocytochemical analysis of CNHC in patients with renal tumors

Results of the immunocytochemical analyses are compiled in Table 3.

On the 53 filters stained with antibodies against CD45 (Figure 1A-F), hematological cells including lymphocytes and polymorphic nuclear granulocytes showed a positive staining, whereas the CNHC-MF, -UMF and -BF, either present as cell clusters (Figure 1A-C) or as single cells (Figure 1D-F) were without exception negative.

On the 46 filters stained with antibodies against the RCC marker CAIX (Figure 7), 28 CNHC clusters were
detected. None exhibited cytological features of malignancy, 16 were classified as CNHC-UMF and 12 were CNHC-BF. Of the 16 CNHC-UMF clusters, one (6%) was decorated by CAIX antibodies (Figure 7A), 15 (94%) were negative (Figure 7B), as was the case with the 12 CNHC-BF (Figure 7C). Nine single CNHC-MF were detected, 5 (56%) of which exhibited positive cytoplasmic reactivity (Figure 7D), whereas 4 (44%) were negative (Figure 7E). Only 4 single CNHC-UMF and one CNHC-BF were found and these cells were also CAIX-negative (Figure 7F and 7G, respectively).

Immunocytochemical analysis of 14 filters with antibodies against the endothelial cell marker CD31 (Figure 8) yielded 3 CNHC-MF- and 17 CNHC-UMF cell clusters, all of which were CD31 positive (Figure 8A and B, respectively), as were 8 (62%) of the 13 CNHC-BF clusters (Figure 8C). Of single cells, only one cell with UMF was CD31 positive.

**Array-CGH**

After WGA, 55 out of 115 (48%) stained microdissected cells yielded DNA products. According to the multiplex PCR, the DNA quality of 49% (27 of 55) of these cells was suitable for array-CGH. Six of CNHC-MF- and 6 of CNHC-UMF-types were selected for array-CGH analysis. All passed multiplex quality control and yielded informative array-CGH profiles. The DNA of the CNHC clusters and the respective renal tumor tissues, one case of papillary RCC (Figure 9), two cases of clear cell RCC (Figure 10 and Additional file 5: Figure S1), one oncocytoma (Figure 10) as well as one cystic nephroma (Additional file 5: Figure S1) were subjected to array-CGH analysis. The results are summarized in Table 4.

The chromosomal aberrations found in the DNA of the tumor tissues are in keeping with the genetic alterations reported for ccRCC, papRCC, and oncocytoma in the literature [31-33] and copy number aberration database.

| Single CNHCs positive with CD45/CAIX/CD31 antibodies | CNHC clusters positive with CD45/CAIX/CD31 antibodies |
|-----------------------------------------------------|-------------------------------------------------------|
|           | CD45  | CAIX | CD31    | CD45  | CAIX | CD31    |
| CNHC-MF   | 0/3   | 5/9   | 0/1     | 0/5   | None detected | 3/3   |
| CNHC-UMF  | 0/4   | 0/4   | 1/3     | 0/12  | 1/16 | 17/17   |
| CNHC-BF   | 0/2   | 0/1   | 0/6     | 0/8   | 0/12 | 8/13    |

**Table 3 Summary of the immunocytochemical analysis of CNHCs with antibodies against CD45, CD31, and CAIX**

[Figure 7](#) Immunocytochemical analysis of CNHCs with antibodies against the RCC marker CAIX. Clusters of CNHCs cytomorphologically classified as uncertain malignant (−UMF) with cytoplasmic positive staining with antibodies against the RCC marker CAIX (A). Clusters of CNHC-UMF and -BF without reactivity for CAIX antibodies (B and C, respectively). A single CNHC-MF with positive cytoplasmic (D) and without staining for CAIX (E). Single CAIX-negative CNHC-UMF and -BF (F and G, respectively).
DNA generated from a benign renal cyst of one patient (patient # 18) was of insufficient quality for analysis by array-CGH. Irrespective of the cytomorphological type, most of the CNHC clusters showed a balanced genome. In only two of the 12 (17%) CNHC clusters, one of which was a MF- and the other one was a UMF- cytomorphological type chromosomal aberrations could be identified which did not match the patterns of chromosomal changes found in the respective renal tumors (Table 4).

**Discussion**

We evaluated the feasibility and utility of the ScreenCell® filtration system for the detection of CTCs and CTMs in the blood of patients with RCC. We investigated if morphological features can be used to discriminate between malignant and non-malignant cells by applying array-CGH.

We found the ScreenCell® filtration an easy to perform procedure which allowed for the detection of large cells (i.e. diameter of > 8 μm) after filtration of 8 ml of diluted blood in 60% of patients with benign and in 53% with malignant renal tumors one day before surgical intervention. According to their cytomorphological features, these cells either occurring as single cells or cellular clusters consisting of at least 3 cells were classified as CNHC-MF, -UMF or -BF using diagnostic criteria recently published by a panel of expert cytopathologists [17]. Surprisingly, each type of CNHCs was found in blood of patients with benign renal tumors including renal cysts, cystic nephroma, angiomyolipomas, oncocytomas as well as malignant papillary or clear cell RCCs. The presence of CNHCs did not correlate with histopathological features of the respective tumors including tumor size, histological diagnosis and grade of tumor differentiation. However, in patients with renal cancer a correlation between the CNHC-MF numbers and histological venous invasion was found at time point C (one day after surgery). At time point A (one day before surgery) CNHC-MF were also more frequently detected in renal cancer patients as compared to healthy controls.

Immunocytochemical analysis revealed that single cells or cellular clusters found on the filters could be regarded as CNHCs because they were invariably negative for CD45, an established marker for hematological cells [35]. Indeed, the results of the CD31 immunocytochemical staining and genetic analysis seem to indicate that most of the cellular CNHC clusters identified in our study may represent aggregations of circulating endothelial cells (CECs) rather than CTMs. More than half (62%) of the clusters of CNHCs-BF-type and all of the CNHC of the -MF and -UMF types found on the filters analysed with antibodies against CD31 were positive, whereas only 6% of the CNHC-UMF- and none of the CNHC-BF clusters were positive on the filters stained for the RCC marker CAIX [36,37]. Although with lower staining intensity as compared to endothelial cells, neutrophilic granulocytes, some lymphocytes, monocytes and platelets can also be stained by the CD31 antibodies [38]. Therefore we cannot exclude that the CNHC clusters may contain some of these cell types as well. However, this is not supported by their cytomorphological features and negative staining results with CD45-antibodies.

In contrast to what was observed for the CNHC clusters, 56% of the single CNHC-MF on the filters analysed with antibodies against the renal cancer marker CAIX [36,37] were positive and might thus represent “true” CTCs. Only 1 out of 10 single CNHC (10%) was positive for CD31. Unfortunately, only DNA of insufficient quality could be generated from single CNHCs of any type precluding array-CGH characterisation of these cells.

Analysis of limited amounts of DNA, as in the analysis of CTCs is faced with several technical problems. Procedures of fixation and staining can decrease DNA quality and interfere with the whole genome amplification (WGA).
Several WGA methods are available to generate sufficient quantities of DNA for array-CGH, all of which are prone to amplification bias [40-42]. We therefore used the GenomePlex library technology which has been shown to exhibit no nucleotide related amplification bias [43,44]. In addition, the reference DNA was also amplified using GenomePlex library technology to further minimize the amplification bias [45]. Array-CGH profiles from amplified DNA of few cells tend to be “noisier” than compared to non-amplified DNA [39,43]. Although higher resolutions are reported with dense array platforms [43], copy number variations as small as 6.8 Mb could be identified in our study which seems appropriate to detect large scale aberrations described in RCC [31].

CTMs have been described in several CTC isolation procedures [19,46,47], in particular with techniques relying on size filtration [11,17,20]. Based on their cytological features some were designated “atypical”, “uncertain malignant” or “morphologically doubtful” [17,48,49]. However, endothelial cells, megakaryocytes as well as large monocytes may be difficult to distinguish from “true” CTC or CTM [17]. The reason why circulating benign...
cells display cytological atypia is not known. Reactive changes might be introduced by shear forces in circulation and/or during filtration.

In some studies the ScreenCell® filtration technique was applied to isolate CTC/CTM from the blood of patients with adrenocortical, prostate, colon and breast cancer or malignant melanoma [15,50,51]. These cells have been characterized by immunocytochemistry or telomeric analysis. However, array-CGH data or results from immunocytochemical testing with CD31 antibodies have not been reported. Here we describe for the first time array-CGH analysis of CNHC clusters cytomorphologically.

**Figure 10** Array-CGH profiles of the DNA of an oncocytoma or clear cell RCC and the respective CNHCs. The DNA of the oncocytoma represents with typical losses at chromosomes 1, 14, 17, 22 and Y (red profile). Array-CGH profile of the DNA of a CNHC-MF cluster (photograph A) reveals a gain of 1p and loss of chromosome 9 (profile A), whereas the profiles of another CNHC-MF cluster (photograph B) and a CNHC-UMF cluster (photograph C) indicated no detectable copy number variations (profiles B and C, respectively). The DNA of the clear cell RCC of patient #16 reveals gains of 1p, 2p, 5q, 17 and a loss of 3p (green profile) commonly observed in clear cell RCC. The array-CGH profile of the DNA of a CNHC-MF (photograph D) of patient #16 is balanced (profile D). All clusters were hematoxylin stained. As a control, the DNA of an isolated pool of 10 leucocytes from blood of a healthy individual showed a balanced genome (leucocyte control). Gains and losses of the X- and Y-chromosomes (green profile, profile A and D) do not reflect true copy number variations. They result from differences between the sex of the reference and the samples DNA (i.e. male patient DNA was hybridized against a female reference DNA thereby resulting in a loss of X and gain of Y chromosome). Bars above the x-axis are considered to be gains, below the x-axis losses of DNA.
Table 4 Summary of the results of array-CGH profiling of the DNA of primary tumors and respective CNHCs

| Patient number | Items analysed by array-CGH | Chromosomal aberrations identified by array-CGH |
|----------------|----------------------------|-----------------------------------------------|
|                |                            | Gains | Losses |
| 14             | Oncocytoma                 | None detected | 1, 14, 17, 22, Y |
|                | CNHC-MF (A)                | 1p     | 9     |
|                | CNHC-MF (B)                | Balanced | Balanced |
|                | CNHC-UMF (C)               | Balanced | Balanced |
| 16             | Clear cell RCC            | 1p, 2p, 5q, 17 | 3p     |
|                | CNHC-MF (D)                | Balanced | Balanced |
| 18             | Cystic nephroma           | Not analysed* | Not analysed* |
|                | CNHC-UMF (C)**             | Balanced | Balanced |
|                | CNHC-UMF (D)**             | Balanced | Balanced |
| 30             | Clear cell RCC**          | 5q, 7  | 3p     |
|                | CNHC-UMF (A)**             | Balanced | Balanced |
|                | CNHC-UMF (B)**             | Balanced | Balanced |
| 37             | Papillary RCC             | 6p, 7, 16, 17q, 20 | Y     |
|                | CNHC-MF (A)                | Balanced | Balanced |
|                | CNHC-MF (B)                | Balanced | Balanced |
|                | CNHC-MF (C)                | Balanced | Balanced |
|                | CNHC-UMF (D)               | None detected | 3p, 4q |

* Unfortunately the quality of the DNA generated from the tumor tissue was not suitable for array-CGH due to insufficient quality.

** Array-CGH profiles are available as additional information (Additional file 5: Figure S1).

resembling CTMs. Array-CGH analysis revealed that the majority of the clusters of the CNHC-MF as well as the CNHC-UMF types did not show chromosomal aberrations and had a balanced genome. The DNA of one CNHC-UMF cluster isolated from a patient with pap. RCC and one CNHC-MF cluster from a patient with oncocytoma showed distinct chromosomal abnormalities on array-CGH which, however, did not match the aberrations found in the respective primary tumors (Figures 9 and 10, respectively) nor in a copy number aberration database (www.progenetix.net) [34]. It might be speculated that these CNHC clusters represent true CTMs. The reason for the differences of the chromosomal aberrations in the CNHCs and the respective tumors is not known, however, tumor heterogeneity [52] might be one of the factors responsible.

Data from animal models and patients with non-small-cell lung cancer indicate that cellular aggregates in blood may consist of CTCs associated with non-neoplastic cells like stromal cells which could enhance CTC survival and the metastatic process [23,53]. It has been estimated that array-CGH can detect gains and losses in mixed populations of tumor and non-neoplastic cells, if more than 20-25% of the population consist of tumor cells [54,55]. Therefore we cannot exclude that the CNHC of -MF or -UMF types without chromosomal aberrations described in our study also contain low numbers of CTCs that were not detectable by array-CGH.

Our results indicate that CNHC can be isolated from blood of patients with renal tumors using the ScreenCell® system. The majority of the isolated clusters may be of endothelial origin as indicated by positive staining with CD31 antibodies. These putative endothelial cell aggregates have hitherto not been reported in patients with renal tumors. It might be speculated that they mirror active angiogenesis in the tumors or during wound healing after surgery [56]. Increased numbers of CECs, probably shed from activated or damaged tumor vessel walls [57,58] and circulating endothelial progenitor cells derived from the bone marrow have been described in the blood of cancer patients and may importantly contribute to cancer growth and metastasis (reviewed in [58-60]). CECs are also found in several other clinical syndromes with vascular injury as well as in response to chemotherapy and anti-angiogenic treatment [61,62]. Therefore CECs and/or endothelial progenitor cells are considered biomarkers of vascular damage. Recently published data suggest CECs as prognostic [63] as well as predictive markers for response to anti-angiogenic therapy in prostate [64] and metastatic renal cell cancer [65-67]. However, their application as biomarkers in clinical practice has been limited by the difficulty to reliably detect them by flow cytometry [68]. Although limited by the relatively small number of cases analysed, the results of our study might indicate that detection of CECs may be facilitated by filtration based and immunocytochemical augmented methods. In this respect it is interesting that we detected a significant increase of the percentage of blood samples positive for all types of CNHCs one day after surgery as compared during surgery. This was also found for CNHC-UMF and –BF-positive samples, but not CNHC-MF-containing samples eight days after surgery.

Conclusions

For patients with renal tumors cytomorphological classification alone seems not to be sufficient to allow for reliable distinction of epithelial CNHCs - the putative CTCs or CTMs - from endothelial CNHCs. As also suggested by others, reliable detection of CTCs or CTMs should thus be confirmed by immunocytochemical and/or molecular biological methods [17,69].

Additional files

Additional file 1: Movie 1. An exemplary laser capture microdissection.
Additional file 2: Table S1. Correlation of tumor size and number of CNHCs.
Additional file 3: Table S2. Relationship of tumor grade and number of CNHCs.

Additional file 4: Table S3. Relationship of venous invasion and number of CNHCs.

Additional file 5: Figure S1. Array-CGH profiles of the DNA of a clear cell RCC and the respective CNHCs or CNHCs derived from a patient with a cystic nephroma. The DNA of the clear cell RCC of patient #30 reveals gains of Sq, 7 and losses of 3p, changes commonly observed in clear cell RCC (red profile). The array-CGH profile of the DNA of two CNHC-UMF clusters (photograph A and B) of patient #30 represent with balanced genomes (profile A and B). The array-CGH profile of the two CNHC-UMF clusters (photograph C and D) revealed balanced genomes (profile C and D). All clusters were hematoxylin stained. The DNA of the respective renal tissue was not suited for array-CGH analysis due to insufficient quality. Gains and losses of the X- and Y-chromosomes do not reflect true copy number variations. They result from differences between the sex of the reference and the samples DNA (i.e. male patient DNA was hybridized against a female reference DNA thereby resulting in a loss of X and gain of Y chromosome and vice versa). Bars above the x-axis are considered to be gains, below the x-axis losses of DNA.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
CL, PS, RZ, and BH designed the study. Data was collected and assembled by AE, TK, EZ, JH and CL. The interpretation and analysis was performed by AE, TK, CL, PS, BH, JBG, RR, JH. The samples and patient data were reviewed by KS, KKK, RZ, HP and RS. The manuscript was drafted by AE, CL, PS. All authors read and approved the final manuscript.

Acknowledgements
The authors wish to thank Ralph Koenig for his help with the graphical design of the figures, Martina Auer, Eva Maria Hoffman and Daniel Kummer for their technical advice. The tissue samples used in this project have been provided by Biobank Graz.

Author details
1Institute of Cell Biology, Histology & Embryology, Medical University of Graz, Harrachgasse 21/VI, 8010 Graz, Austria. Institute of Pathology, Medical University of Graz, Auenbruggerplatz 25, 8036 Graz, Austria. 2Department of Urology, Medical University of Graz, Auenbruggerplatz 5/6, 8036 Graz, Austria. 3Department of Internal Medicine, Division of Gastroenterology and Hepatology, Medical University of Graz, Auenbruggerplatz 15, 8036 Graz, Austria. 4Institute for Medical Informatics, Statistics and Documentation, Medical University of Graz, Auenbruggerplatz 2, 8036 Graz, Austria. 5Institute of Human Genetics, Medical University of Graz, Harrachgasse 21/VI, 8010 Graz, Austria.

Received: 24 May 2013 Accepted: 13 September 2013

References
1. Paterlini-Brechot P, Benali NL: Circulating tumor cells (CTC) detection: clinical impact and future directions. Cancer Lett 2007, 253:180–204.
2. Zhao S, Liu Y, Zhang Q, Li H, Zhang M, Ma W, Zhao W, Wang J, Yang M: The prognostic role of circulating tumor cells (CTCs) detected by RT-PCR in breast cancer: a meta-analysis of published literature. Breast Cancer Res Treat 2011, 130:809–816.
3. Rahbari NN, Aigner M, Thorlund K, Mollberg N, Motschall E, Jensen K, Dierer MK, Buchler MW, Koch M, Weitz J: Meta-analysis shows that detection of circulating tumor cells indicates poor prognosis in patients with colorectal cancer. Gastronetrology 2010, 138:1714–1725.
4. De Albuquerque A, Kubis A, Istoel B, Ernst D, Boese-Landgraf J, Breier G, Stamminger G, Ferres N, Kaul S. prognostic and predictive value of circulating tumor cell analysis in colorectal cancer patients. J Transl Med 2012, 10:222-5876-10-222.
5. Pichler M, Hutterer GC, Chronikey TF, Jesche J, Kampil-Keftner K, Rehak P, Pummer K, Ziegler R: External validation of the Leibovich prognostic score for nonmetastatic clear cell renal cell carcinoma at a single European center applying routine pathology. J Urol 2011, 186:1773–1777.
6. Escudier B, Eisen T, Stadler WM, Szczylik C, Oudard S, Staeler H, Negrier S, Chevreau C, Desai AA, Rolland F, Demirkov T, Hutson TE, Gore M, Anderson S, Hoffena G, Shank M, Penna C, Latthia C, Bukowski RM: Sorafenib for treatment of renal cell carcinoma: final efficacy and safety results of the phase III treatment approaches in renal cancer global evaluation trial. J Clin Oncol 2009, 27:3312–3318.
7. Motzer RJ, Hutson TE, Tomczak P, Michaelson MD, Bukowski RM, Oudard S, Negrier S, Szczylik C, Pili R, Bjarnason GA, Garcia-del-Muro X, Soranjan A, Soltilka E, Wilding G, Thompson JA, Kim SH, Chen X, Figlin RA: Overall survival and updated results for sunitinib compared with interferon alfa in patients with metastatic renal cell carcinoma. J Clin Oncol 2009, 27:3584–3590.
8. Small AC, Gong Y, Oh WK, Hall SJ, van Rijn CJ, Galsky MD: The emerging role of circulating tumor cell detection in genitourinary cancer. J Urol 2012, 188:21-26.
9. Parkinson DR, Dracopoli N, Petyt BG, Compton C, Cristofanilli M, Deisseroth A, Hayes DF, Kapke G, Kumar P, Lee JS, Liu MC, McCormack R, Mikulsik S, Nagahara L, Pantel K, Pearson-White S, Pommnuo EA, Rodmpton LT, Schade AE, Scher HI, Sigman CC, Kelkoff GJ: Considerations in the development of circulating tumor cell technology for clinical use. J Transl Med 2012, 10:138-5876-10-138.
10. Christiansen JJ, Rajasekaran AR: Reassessing epithelial to mesenchymal transition as a prerequisite for carcinoma invasion and metastasis. Cancer Res 2006, 66:8319–8326.
11. Krebs MG, Hou JM, Sloane R, Lancashire L, Nonaka D, Ward TH, Backen A, Clark G, Hughes A, Ranson M, Blackhall FH, Dive C: Analysis of circulating tumor cells in patients with non-small cell lung cancer using epithelial marker-dependent and -independent approaches. J Thorac Oncol 2012, 7:306–315.
12. Raimondi C, Gradilone A, Naso G, Vincenzi B, Petracca A, Nicolazzo C, Palazzo A, Saltarelli R, Spremberg F, Cortesi E, Gazzaniga P: Epithelial-mesenchymal transition and stemness features in circulating tumor cells from breast cancer patients. Breast Cancer Res Treat 2013, 130:449–455.
13. Joosse SA, Pantel K: Biologic challenges in the detection of circulating tumor cells. Cancer Res 2013, 73:8–11.
14. Zheng S, Lin HK, Lu B, Williams A, Qater R, Cote RJ, Tai YC: 3D microfilter device for viable circulating tumor cell (CTC) enrichment from blood. Biomiedc Icmunner 2011, 13:203–213.
15. Desitter I, Guerrouahen BS, Benali-Furet N, Wechsler J, Janne PA, Kuang Y, Yangita M, Wang L, Berkowitz JA, Distel RJ, Cayre YE: A new device for rapid isolation by size and color recognition of rare circulating tumor cells. Anticancer Res 2011, 31:427–441.
16. Vona G, Sable A, Loucha M, Strik V, Romana S, Schutze K, Capron F, Franco D, Pazzagi M, Vekemans M, Mach P, Brechot C, Paterlini-Brechot P: Isolation by size of epithelial tumor cells: a new method for the immunomorphological and molecular characterization of circulating tumor cells. Am J Pathol 2000, 156:673–683.
17. Hofman VJ, Ilie M, Bonnetouta C, Selva E, Long E, Molina T, Vignaud JM, Flejou JF, Lantejoul S, Patton E, Butori C, Mouaroud M, Poulidoux N, Bahadoran P, Siboni S, Genuara N, Santini J, Venissac N, Moreau J, Vieu P, Hofman PA: Cytopathologic detection of circulating tumor cells using the isolation by size of epithelial tumor cell method: promises and pitfalls. Am J Clin Pathol 2011, 135:146–156.
18. Hofman V, Bonnetouta C, Ilie M, Vieu P, Vignaud JM, Flejou JF, Lantejoul S, Patton E, Mouaroud M, Butori C, Selva E, Poulidoux N, Siboni S, Kelhaf S, Venissac N, Jais JP, Moreau J, Molina TJ, Hofman PA: Preoperative circulating tumor cell detection using the isolation by size of epithelial tumor cell method for patients with lung cancer is a new prognostic biomarker. Clin Cancer Res 2011, 17:827–835.
19. Hou JM, Krebs MG, Lancashire L, Sloane R, Backen A, Swain RK, Priest LJ, Greystoke A, Zhou C, Morris K, Ward T, Blackhall FH, Dive C: Clinical significance and molecular characteristics of circulating tumor cells and circulating tumor microemboli in patients with small-cell lung cancer. J Clin Oncol 2012, 30:525–532.
20. Khosla L, Backen A, Sloane R, Menaese L, Ryder D, Krebs M, Board R, Clark G, Hughes A, Blackhall F, Vallee JW, Dive C: A pilot study to explore circulating tumour cells in pancreatic cancer as a novel biomarker. Br J Cancer 2012, 106:508–516.
21. Pinzani P, Salvadori B, Simi L, Bianchi S, Distante V, Cataliotti L, Pazzagi M, Orlando C: Isolation by size of epithelial tumor cells in peripheral blood.
of patients with breast cancer: correlation with real-time reverse transcriptase-polymerase chain reaction results and feasibility of molecular analysis by laser microdissection. Hum Pathol 2006, 37:711–718.

22. Vona G, Estea L, Beroud C, Damotte D, Capron F, Nalpary B, Mineur A, Franco O, Lacour B, Poil S, Brechet C, Paterlini-Brechot P: Impact of cytometricological detection of circulating tumor cells in patients with liver cancer. Hepatology 2004, 39:703–707.

23. Pailler E, Adam J, Barthelemy A, Oulhenn M, Auger N, Valient A, Borget J, Planchar D, Taylor M, Andre F, Soria JC, Veilh P, Besse B, Farace F: Detection of circulating tumor cells harboring a unique ALK rearrangement in ALK-positive Non-small-cell lung cancer. J Clin Oncol 2013, 31:2273–2281.

24. Sobin LH, Gospodarowicz MK, Wittekind C: TNM Classification of Malignant Tumours (UICC International Union Against Cancer). 7th edition. Hoboken, NJ, USA: John Wiley & Sons; 2009.

25. van Beers EH, Joosse SA, Ligtenberg MJ, Fles R, Hogervorst FB, Verhoef S, Nederlof PM: Rearrangement in ALK-positive Non-small-cell lung cancer. Br J Cancer 2012, 107:18392–18397.

26. Liu L, Qian J, Singh H, Meiers I, Zhou X, Bostwick DG: Identification of small gains and losses in single cells after whole genome amplification on tiling oligo arrays. Nucleic Acids Res 2008, 36:e89.

27. Gupta R, Balzer B, Picken M, Osunkoya AO, Shet T, Alsabeh R, Luthringer D, Franço D, Lacour B, Pol S, Brechot C, Paterlini-Brechot P: The leukocyte common antigen family. Cytogenet Genome Res 2012, 131:1229–1239.

28. Mathiesen RR, Fjelldal R, Liestol K, Due EU, Geigl JB, Riethdorf S, Borgen E, Strict regulation of CAIX(G250/MN) by HIF-1α in clear cell renal cell carcinoma. Int J Cancer 2001, 94:1071–1077.

29. Frangioni JV, Hsu PM, Huang Y: Circulating tumor cells of patients with breast cancer. J Natl Cancer Inst 2002, 94:1057–1065.

30. Gruber K, Winkel R, Blaszczyk A, De Giorgi V, Panelos J, Paglierani M: Comparative genomics of renal oncocytomas. Am J Med Genet A 2013, 161A:405–409.

31. El-Heliebi S, Zhang S, Martignoni G, Cheng L, Presti JC Jr, Moch H, Reuter VE, Huynh D, Waldman FM: Diagnostic implications of transcription factor Pax 2 protein and transmembrane enzyme complex carbonic anhydrase IX immunopositivity in adult renal epithelial neoplasms. Am J Surg Pathol 2009, 33:241–247.

32. Halfhill RE, De Weijert MC, Verhaegh GW, Schalken JA, Oosterwijk E: Comparative genomics for genetic analysis of renal oncocytomas. Genes Chromosomes Cancer 2003, 36:361–374.

33. Patard JJ, Moch H, Reuter VE, Pisansky TM, Testa JR, Su BL, Fanburg-Smith JC, Renshaw AA, Eble JN: Cytogenetic profile predicts prognosis of patients with clear renal cell carcinoma. J Clin Oncol 2009, 27:746–753.

34. Binzinger M, Elbe JH, Zhang S, Martignoni G, Cheng L: Gains of chromosomes 7, 17, 12, 16, and 20 and loss of Y occur early in the evolution of papillary renal cell neoplasia: a fluorescent in situ hybridization study. Mod Pathol 2003, 16:1053–1059.

35. Sambucini G, Larghi A, Sezer O, De Groen PC, Shaffer LG, Bejjani BA: Three-dimensional telemanic analysis of isolated circulating tumor cells (CTCs) defines CTC subpopulations. Transl Oncol 2013, 6:61–65.

36. Gerlinger M, Rowan AJ, Horswell S, Larkin J, Endesfelder D, Gronroos E, Fuhrmann C, Schmidt-Kittler O, Stoecklein NH, Petat-Dutter K, Vay C, Bockler K, Reinhardt R, Raag T, Klein CA: High-resolution array comparative genetic hybridization of single micrometastatic tumor cells. Nucleic Acids Res 2008, 36:e39.

37. Geigl JB, Obenauf AC, Waldispuehl-Geigl J, Hoffmann EM, Auer M, Hormann M, Fischer M, Trajanoski Z, Schenk MA, Baumbusch LO, Speicher MR: Identification of small gains and losses in single cells after whole genome amplification on tiling oligo arrays. Nucleic Acids Res 2009, 37:e105.

38. Puth T, Delaney AD, Famoud A, Filotte B, Griffith M, Li H, Qian J, Farace F: A direct comparison of Cell Search and ISET for circulating tumour-cell detection in patients with metastatic carcinoma. Br J Cancer 2011, 105:847–853.

39. De Giorgi V, Pinzani P, Salvianti F, Panelos J, Paglierani M: Comparative genomic aberration data. Bioinformatics 2001, 17:1229–1229.

40. Mathiesen RR, Fjelldal R, Liestol K, Due EU, Geigl JB, Riethdorf S, Borgen E: Comparative plasticity of CAIX(G250/MN) by HIF-1α in clear cell renal cell carcinoma. Int J Cancer 2001, 94:1057–1077.

41. Cytogenetic profile predicts metastatic carcinoma. Br J Cancer 2012, 107:18392–18397.

42. Fuhrmann C, Schmidt-Kittler O, Stoecklein NH, Petat-Dutter K, Vay C, Bockler K, Reinhardt R, Raag T, Klein CA: High-resolution array comparative genetic hybridization of single micrometastatic tumor cells. Nucleic Acids Res 2008, 36:e39.

43. Geigl JB, Obenauf AC, Waldispuehl-Geigl J, Hoffmann EM, Auer M, Hormann M, Fischer M, Trajanoski Z, Schenk MA, Baumbusch LO, Speicher MR: Identification of small gains and losses in single cells after whole genome amplification on tiling oligo arrays. Nucleic Acids Res 2009, 37:e105.

44. Fiegler H, Geigl JB, Langer S, Rigler D, Porter K, Unger K, Carter NP, Speicher MR: High-resolution array CGH analysis of single cells. Nucleic Acids Res 2007, 35:e15.

45. Doran T, Sawczuk IS, Postorek J, Wiernik PH, Dutcher JP: Detection of circulating tumor cells in cutaneous melanoma. J Invest Dermatol 2010, 130:2440–2447.

46. Pinzani P, Scatena C, Salvianti F, Corsini E, Canu L, Poli G, Paglierani M, Piccin V, Pazzaglia M, Nesi G, Mannelli M, Luconi M: Detection of circulating tumor cells in patients with adrenocortical carcinoma: a monocentric preliminary study. J Clin Endocrinol Metab 2013, 98:E371–E378.

47. Adebayo Awe J, Xu MC, Wechsler J, Benali-Fontey N, Carey YE, Saranchuk J, Drenghoven DJ, Ma S, Three-dimensional telemanic analysis of isolated circulating tumor cells (CTCs) defines CTC subpopulations. Transl Oncol 2013, 6:61–65.

48. Gerlinger M, Rowan AJ, Horswell S, Larkin J, Endesfelder D, Gronroos E, Martinez P, Matthews N, Stewart A, Tarpey P, Varela I, Phillimore B, Begum S, McDonald NQ, Butler A, Jones D, Raine K, Latimer C, Santos CR, Nohadani M, Blund AC, Spencer-Dene B, Clark G, Picketing L, Stamp G, Gore M, Szallasi Z, Downward J, Futreal PA, Swanton C: Intronarthritis and heterogeneous branch evolution revealed by multiregion sequencing. N Engl J Med 2012, 366:883–892.

49. Duda DG, Duyverman AM, Kohono M, Snuderl M, Steller EJ, Fukumura D, Jain RK: Malignant cells facilitate lung metastasis by bringing their own soil. Proc Natl Acad Sci U S A 2010, 107:21677–21682.

50. Ballif BC, Lorenz EA, Sundin K, Linicic M, Gaskin S, Coppinger J, Kathoky CD, Shaffer LG, Beijani BA: Detection of low-level mosaicism by array CGH in routine diagnostic specimens. Am J Med Genet A 2006, 140:2757–2767.

51. Patel A, Kang SH, Lennon PA, Li YF, Rao PN, Abuzzahab L, Shaw C, Chinchalkar AC, Cheung SW: Validation of a targeted microarray microarray for the clinical evaluation of recurrent abnormalities in chronic lymphocytic leukemia. Am J Hematol 2008, 83:540–546.

52. Park Y, Kim Hana T, Takagi R, Kato R: Does surgery for breast cancer induce angiogenesis and thus promote metastasis? Oncology 2011, 81:199–205.

53. Chang YS, di Tomaso E, McDonald DM, Jones R, Jain RK, Munn LL: Mosaic blood vessels in tumors: frequency of cancer cells in contact with flowing blood. Proc Natl Acad Sci U S A 2000, 97:14608–14613.

54. Mancuso P, Calleri A, Bertolini F: Circulating endothelial cells and circulating endothelial progenitors. Recent Results Cancer Res 2012, 195:163–170.
59. Ribatti D, Ranieri G, Basile A, Azzariti A, Paradiso A, Vacca A: Tumor endothelial markers as a target in cancer. Expert Opin Ther Targets 2012, 16:1215–1225.

60. Strijbos MH: Circulating tumour cells and circulating endothelial cells as biomarkers in oncology. Acta Clin Belg 2011, 66:332–336.

61. Shaked Y, Ciarrocchi A, Franco M, Lee CR, Man S, Cheung AM, Hicklin DJ, Chaplin D, Foster FS, Benezra R, Kerbel RS: Therapy-induced acute recruitment of circulating endothelial progenitor cells to tumors. Science 2006, 313:1785–1787.

62. Shaked Y, Henke E, Roodhart JM, Mancuso P, Langenberg MH, Colleoni M, Daenen LG, Man S, Xu P, Ermnenegger U, Tang T, Zhu Z, Witte L, Strieter RM, Bertolini F, Voest EE, Benezra R, Kerbel RS: Rapid chemotherapy-induced acute endothelial progenitor cell mobilization: implications for antiangiogenic drugs as chemosensitizing agents. Cancer Cell 2008, 14:263–273.

63. Strijbos MH, Gratama JW, Schmitz PI, Rao C, Onstenk W, Doyle GV, Miller MC, de Wit R, Tenstappen LW, Steigel S: Circulating endothelial cells, circulating tumour cells, tissue factor, endothelin-1 and overall survival in prostate cancer patients treated with docetaxel. Eur J Cancer 2010, 46:2027–2035.

64. Matsusaka S, Suenaga M, Mishima Y, Takagi K, Terui Y, Mizunuma N, Hatake K: Circulating endothelial cells predict for response to bevacizumab-based chemotherapy in metastatic colorectal cancer. Cancer Chemother Pharmacol 2011, 68:763–768.

65. Gruenwald V, Beutel G, Schuch-Jantsch S, Reuter C, Karyl P, Ganser A, Haubritz M: Circulating endothelial cells are an early predictor in renal cell carcinoma for tumor response to sunitinib. BMC Cancer 2010, 10:695.

66. Mancuso A, Di Paola ED, Leone A, Catalano A, Calabro F, Cerbone L, Zivi A, Messina C, Alonso S, Vigna L, Caristo R, Sternberg CN: Phase II escalation study of sorafenib in patients with metastatic renal cell carcinoma who have been previously treated with anti-angiogenic treatment. BJU Int 2012, 109:200–206.

67. Vrolijk L, van der Veldt AA, de Haas RR, Haenen JR, Schuurhuis GJ, Kuk DJ, van Cuijisen H, Verheul HM, van den Eertwegh AJ, Hoekman K, Boven E, van Hinsbergh VW, Broxterman HJ: Increased numbers of small circulating endothelial cells in renal cell cancer patients treated with sunitinib. Angiogenesis 2009, 12:69–79.

68. Hernandez-Yanez M, Heymach J, Zurita AI: Circulating biomarkers in advanced renal cell carcinoma: clinical applications. Curr Oncol Rep 2012, 14:221–229.

69. Pantel K, Denoeve E, Nocca D, Coffy A, Vendrell JP, Maudelonde T, Riethdorf S, Alix-Panabieres C: Circulating epithelial cells in patients with benign colon diseases. Clin Chim Acta 2012, 58:536–540.

doi:10.1186/1479-5876-11-214

Cite this article as: El-Heliebi et al: Are morphological criteria sufficient for the identification of circulating tumor cells in renal cancer? Journal of Translational Medicine 2013 11:214.