

# In Situ Detection and Quantification of AR-V7, AR-FL, PSA, and *KRAS* Point Mutations in Circulating Tumor Cells

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**BACKGROUND:** Liquid biopsies can be used in castration-resistant prostate cancer (CRPC) to detect androgen receptor splice variant 7 (AR-V7), a splicing product of the androgen receptor. Patients with AR-V7-positive circulating tumor cells (CTCs) have greater benefit of taxane chemotherapy compared with novel hormonal therapies, indicating a treatment-selection biomarker. Likewise, in those with pancreatic cancer (PaCa), *KRAS* mutations act as prognostic biomarkers. Thus, there is an urgent need for technology investigating the expression and mutation status of CTCs. Here, we report an approach that adds AR-V7 or *KRAS* status to CTC enumeration, compatible with multiple CTC-isolation platforms.

**METHODS:** We studied 3 independent CTC-isolation devices (CellCollector, Parsortix, CellSearch) for the evaluation of AR-V7 or *KRAS* status of CTCs with in situ padlock probe technology. Padlock probes allow highly specific detection and visualization of transcripts on a cellular level. We applied padlock probes for detecting AR-V7, androgen receptor full length (AR-FL), and prostate-specific antigen (PSA) in CRPC and *KRAS* wild-type (wt) and mutant (mut) transcripts in PaCa in CTCs from 46 patients.

**RESULTS:** In situ analysis showed that 71% (22 of 31) of CRPC patients had detectable AR-V7 expression ranging from low to high expression [1–76 rolling circle products (RCPs)/CTC]. In PaCa patients, 40% (6 of 15) had *KRAS* mut expressing CTCs with 1 to 8 RCPs/CTC. In

situ padlock probe analysis revealed CTCs with no detectable cytokeratin expression but positivity for AR-V7 or *KRAS* mut transcripts.

**CONCLUSIONS:** Padlock probe technology enables quantification of AR-V7, AR-FL, PSA, and *KRAS* mut/wt transcripts in CTCs. The technology is easily applicable in routine laboratories and compatible with multiple CTC-isolation devices.

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Liquid biopsies gained enormous momentum in recent years. Minimally invasive blood tests that detect circulating tumor cells (CTCs)<sup>11</sup> and cell-free circulating tumor DNA are highly beneficial compared with invasive tissue biopsies (1), but they face challenges in their usability to guide treatment selection. Although several techniques have been developed to allow characterization of CTCs and circulating tumor DNA (2–6), they have not been put into widespread clinical use. Recently, however, promising studies have been published, investigating the role of androgen receptor splice variant 7 (AR-V7) in prostate cancer (7, 8). The authors showed that patients with AR-V7-positive CTCs had greater benefit of taxane-based therapy compared with enzalutamide or abiraterone therapy (8–10). This indicates that the assessment of CTC-based AR-V7 status may serve as a treatment-selection biomarker in metastatic castration-resistant prostate cancer (CRPC) patients. However,

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<sup>11</sup> Nonstandard abbreviations: CTC, circulating tumor cell; AR-V7, androgen receptor splice variant 7; CRPC, castration-resistant prostate cancer; PaCa, pancreatic cancer; mut, mutant; wt, wild type; AR-FL, androgen receptor full length; PSA, prostate-specific antigen; RCP, rolling circle products; NHT, novel hormonal therapy.

published assays require sophisticated technologies for CTC isolation, which are available only at specialized centers (8), or are based on pooled (CTC) lysates that do not yield any quantitative information on the number of CTCs in circulation or the fraction of cells expressing clinically relevant AR-V7 transcripts (7, 10).

In parallel to splice variants, determining *KRAS*<sup>12</sup> point mutations in CTCs from pancreatic cancer (PaCa) patients could be used to improve diagnostics and stratification. Mutational activation of *KRAS* occurs in approximately 95% of pancreatic ductal adenocarcinoma cases (11) and is associated with worse prognosis (12), making it a potential biomarker. PaCa has a high mortality rate because of late diagnosis, often at advanced stages with metastatic disease (13, 14). Owing to the difficult access of the primary tumor with its high desmoplastic reaction in its microenvironment and false-negative biopsy results, CTC characterization could represent an important additional tool in diagnosis and treatment monitoring as a tumor-specific marker, as well as a prognostic marker suitable for stratification of these patients.

Thus, there is an urgent need for reliable and easily accessible technologies to investigate CTCs and their expression profile or mutational status. Here, we aimed at developing (a) an assay that adds clinically relevant molecular information of CTCs such as AR-V7 or *KRAS* status and (b) is compatible with multiple CTC isolation platforms. Our assay is based on padlock probe technology (15). Padlock probes consist of oligonucleotides that specifically bind to their target sequence (e.g., AR-V7, *KRAS*) and form a DNA circle that is amplified by rolling circle amplification (16). These amplified products are subsequently targeted by fluorescent probes, resulting in bright quantifiable signals. Padlock probes are highly specific, allowing differentiation between sequences differing in only a single base [e.g., splice variants or mutant (mut) vs wild type (wt)] (15). Here, we report an mRNA-based in situ padlock probe approach that allows visualization and quantification of prostate-specific transcripts (AR-V7), androgen receptor full length (AR-FL), prostate-specific antigen (PSA), and *KRAS* point mutations directly within single CTCs. Enrichment of CTCs was performed with the EpCAM-dependent in vivo CellCollector device, the CellSearch system, and the EpCAM-independent microfluidic Parsortix device.

## Materials and Methods

### CELL LINES

The following prostate cancer cell lines were used: VCaP (kindly provided by Martina Auer, Medical University

Graz, Austria), LNCaP (kindly provided by Martine Mazel, Montpellier, France), PC-3, and 22RV1 (ATCC). The CAPAN-1 and BxPC-3 [European Collection of Authenticated Cell Cultures (UK)] cell lines were used for PaCa experiments. Cells were cultured in cell culture flasks as recommended by the distributor. Cell lines received from other laboratories (VCaP and LNCaP) were verified by short tandem repeat analysis using the PowerPlex 16 System Kit (Promega) and were tested free of *Mycoplasma* (Biotool).

### SEEDING OF CELL LINES

Cultured cells were seeded on slides as previously described (17). Seeding of cells onto CellCollectors (Gilupi) is described in the Materials and Methods section of the Data Supplement that accompanies the online version of this article at <http://www.clinchem.org/content/vol64/issue3>.

### SPIKING OF CELL LINES INTO HEALTHY DONOR BLOOD

For spiking experiments, cell line cells were counted in a Neubauer chamber and spiked into blood. If defined cell counts were needed, cells were spread to a petri dish filled with medium, manually counted, picked under a light microscope, and spiked into blood.

### PATIENT SAMPLES AND ETHICS

The study enrolled patients from 4 centers. Patients with histologically confirmed CRPC were enrolled at the Division of Clinical Oncology, Medical University Graz (Austria), University Medical Center Hamburg-Eppendorf (Germany), and the Erasmus MC Cancer Institute (the Netherlands). Histologically confirmed PaCa patients were treated at Karolinska Institutet Hospital (Sweden). Patient characteristics are summarized in Tables 1 and 2 of the online Data Supplement. Healthy donor and patient blood samples were obtained in accordance with the World Medical Association Declaration of Helsinki, and all patients gave written informed consent. Each center received approval of the study from their ethical committees (Medical University Graz EK28-177-ex-15/16; Karolinska Institutet Hospital Dnr2013/1783-31/3; University Medical Center Hamburg PV3779; Erasmus MC MEC-2016-464). For samples from the Erasmus MC Cancer Institute, written informed consent was obtained for only the blood samples and their analysis and explicitly excluded any detailed clinical data.

### IN VIVO CTC ISOLATION: CELLCOLLECTOR

CellCollectors were applied in 13 patients with CRPC and in 15 patients with PaCa as previously described (18). In short, an intravenous catheter (20 gauge, BD Venflon) was placed into the patients' cubital vein, and the CellCollector was carefully inserted until the wire was extended 2 cm into the vein. After 30 min, the Cell-

<sup>12</sup> Human Genes: *KRAS*, *KRAS* proto-oncogene, GTPase; *ACTB*, actin beta.

Collector was removed, washed 3 times in 1×PBS, fixed in 100% acetone for 10 min, air-dried for 5 min, and stored at −20 °C until further usage.

#### **CTC ISOLATION: PARSORTIX**

Thirty-eight blood samples were drawn from 17 CRPC patients into standard 7.5-mL EDTA vacutainers or CellSave® (Menarini-Silicon Biosystems) preservation tubes. Each patient, therefore, provided a matched sample of EDTA and CellSave blood for further analysis. Two patients donated an additional set of blood samples on a second visit. CTCs were enriched from EDTA blood using the size-dependent, label-free Parsortix system (ANGLE plc) as described previously (19, 20). A separation pressure of 99 mbar was chosen to process the blood samples. After cell harvest into a cytospin funnel on a glass slide, samples were spun down (190g, 5 min) via cytocentrifuge, dried overnight at room temperature, and stored at −80 °C. More detailed information on the general separation mechanism of the Parsortix is found in the Materials and Methods section of the online Data Supplement.

#### **CTC ANALYSIS USING THE CELLSEARCH SYSTEM**

The patient cohort of the University Medical Center Hamburg-Eppendorf (Germany) was analyzed with Parsortix and CellSearch in parallel. Patient blood samples were collected into CellSave preservation tubes and processed with the CellSearch system using the CellSearch® CXC Kit (21). This specific kit uses anti-CK-fluorescein, diaminopyrolylindole 4,6-diamino, 2-pyrolylindole, and anti-CD45-APC to identify CTCs and leukocytes. All analyses were performed by trained CellSearch analysts using the CellTracks Analyzer II. The AR-FL status was assessed in the fourth fluorescence channel of the CellSearch with a phycoerythrin-labeled Celltracks anti-androgen receptor antibody (Janssen Diagnostics). To test whether CellSearch-processed samples can be analyzed by in situ analysis, 1 patient sample from the Erasmus MC Cancer Institute was processed with the CellSearch system and forwarded for in situ analysis. The blood was collected in an EDTA tube and processed within 24 h on the CellSearch system. After enrichment of the CTCs with the CellSearch CTC Profile Kit (Menarini Silicon Biosystems), the enriched cell fraction was cytocentrifuged on slides (first, 300g, 4 min; after removal of the remaining fluid, 1000g, 1 min) using 4-mL CytoSystem funnels (Hettich Laboratory technology) supported by a magnetic device. The sample was then dried and fixed with 3.7% formaldehyde (Sigma) for 15 min. After fixation, the slides were dried again and run through an ethanol series. Afterward, slides were stored at −20 °C before being forwarded for in situ analysis.

#### **IN SITU REACTIONS**

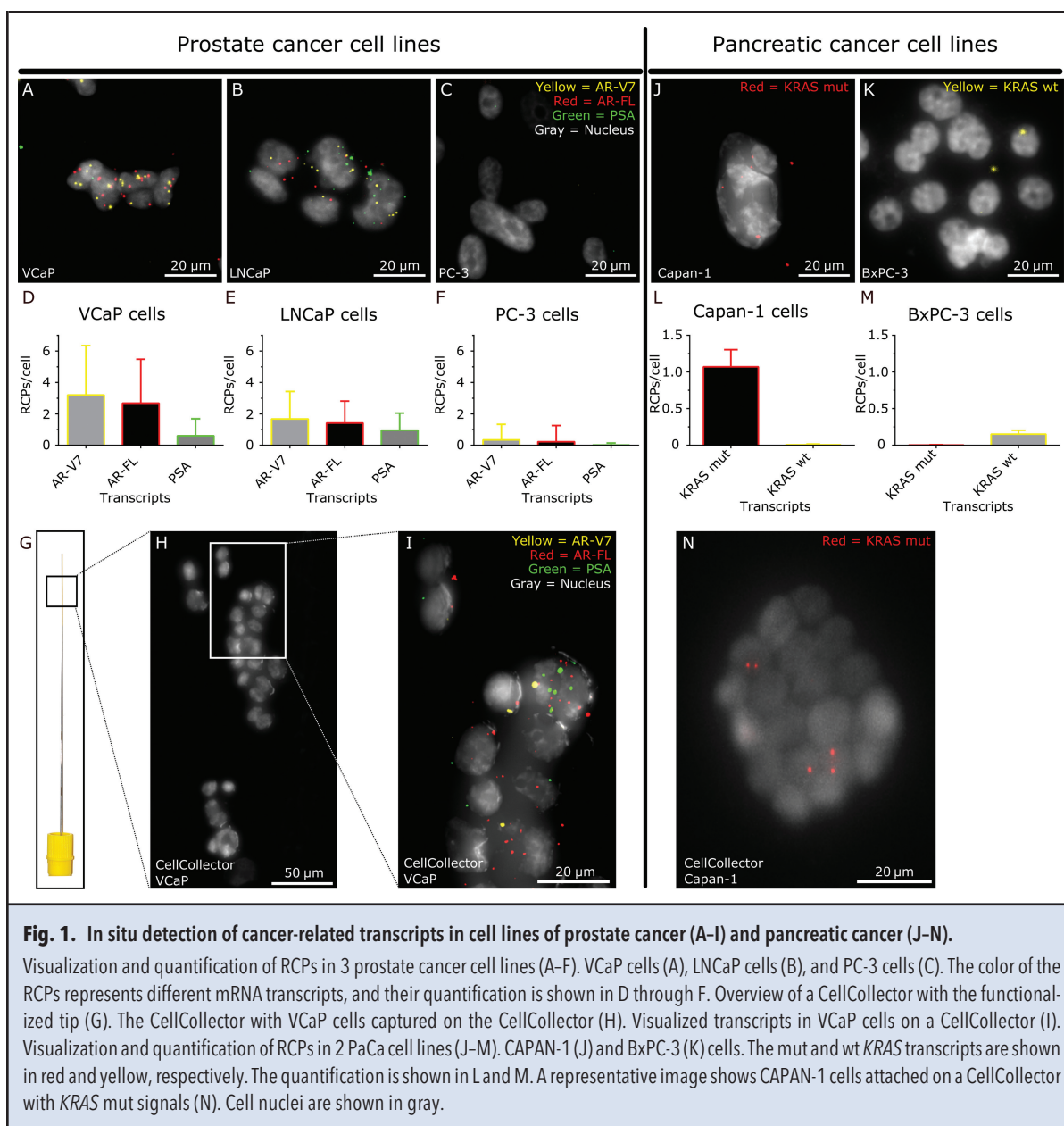
Oligonucleotides for prostate cancer were designed according to published guidelines by Weibrecht et al. using CLC Main Workbench software (CLC Bio Workbench version 7.6, Qiagen) (17). Sequences with the GenBank accession number NM\_000044.3 (AR-FL), FJ235916.1 (AR-V7), and NM\_001030047 (PSA) were retrieved from the National Center for Biotechnology Information. For AR-FL and PSA, single padlock probes were used. As expression levels of AR-V7 are generally low (1.2 and 0.4 copies/cell in VCaP and LNCaP, respectively) (22), we amplified the signals by an array of AR-V7 padlocks. Details for the padlock probes can be found in the Materials and Methods section of the online Data Supplement. Oligonucleotides for PaCa were designed as previously described for *KRAS* codon 12 (23). A mixture of padlock probes targeting the housekeeping gene *ACTB*, *KRAS* codon 12 wt, and mut G12S, G12R, G12C, G12D, G12V, and G12A were combined with immunofluorescence staining of cytokeratin and CD45.

For cytokeratin immunostaining, we combined antibodies directed against a broad panel of cytokeratins, namely, the C-11 (Exbio), AE1/AE3 (Thermo Fisher Scientific), and DC-10 (Exbio) antibodies, which detect multiple keratins. The C-11 clone reacts with cytokeratins 4, 5, 6, 8, 10, 13, and 18; the AE1/AE3 clone recognizes cytokeratins 1, 2, 3, 4, 5, 6, 7, 8, 10, 14, 15, 16, and 19; and the DC-10 clone reacts with cytokeratin 18. A detailed protocol for immunostaining can be found in the Materials and Methods section of the online Data Supplement. All oligonucleotides are summarized in Table 3 of the online Data Supplement.

In situ reactions were performed as described previously (24) with slight modifications, including the doubling of all enzyme concentrations for in situ reactions of patient material, as blood cells show an inhibitory effect on PCR reactions (25). The in situ reactions for the CellCollectors were performed in glass pipettes as described in the Materials and Methods section of the online Data Supplement.

For imaging of a CellCollector, the wire was placed and fixed onto a metal frame object slide with an imaging window (Carl Zeiss) using tape or fixed onto a wire holder (Gilupi). Then, wires were scanned across the whole length of the functional part. To image the whole surface of the CellCollector, it was rotated in 90° angles until fully processed. To verify rolling circle products (RCPs) to be specific, each signal was checked in all other fluorescent channels. Signals present in all channels were considered unspecific (17) (see Fig. 1 in the online Data Supplement).

Quantification of RCPs was performed with CellProfiler software (version 2.1.1) (26). We modified the previously published quantification pipeline (27). A detailed description can be found in the Materials and Methods section of the online Data Supplement.



## Results

### AR-V7, AR-FL, AND PSA CAN BE VISUALIZED AND QUANTIFIED IN PROSTATE CANCER CELL LINES

Three prostate cancer cell lines were selected for validation of specificity and sensitivity of padlock probes. AR-V7, AR-FL, and PSA were detected in the “positive” cell lines VCaP and LNCaP and were not expressed in the “negative” PC-3 cell line cells (Fig. 1). The terms positive and negative refer to previously published data as detectable and not detectable mRNA, respectively (22). For VCaP cells, the average numbers of RCPs/

cell for AR-V7, AR-FL, and PSA were 3.2 (range, 0–20), 2.7 (range, 0–19), and 0.6 (range, 0–7), respectively. The corresponding numbers of RCPs for the LNCaP cells were 1.7 RCPs/cell (range, 0–17) for AR-V7, 1.4 (range, 0–6) for AR-FL, and 1.0 (range, 0–7) for PSA. PC-3 cells yielded an average RCP/cell of 0.3 (range, 0–9) for AR-V7 and 0.2 (range, 0–11) for AR-FL, but negligible expression of PSA with 0.0 (range, 0–2) RCPs/cell. In total, 252 VCaP, 108 LNCaP, and 186 PC-3 cells were evaluated and RCPs quantified by CellProfiler software. The in situ results reflect previously published data (22). VCaP cells

seeded onto the CellCollector showed results comparable with cells on a slide (Fig. 1).

### IN SITU AR-V7 DETECTION CAN BE COMBINED WITH IMMUNOSTAINING FOR PANCK AND CD45 OF PARSORTIX-PROCESSED PROSTATE CANCER CELL LINE CELLS

To evaluate whether our novel approach can also be applied to samples obtained from other CTC isolation platforms, spiked prostate cancer cell line cells were enriched from whole blood by the EpCAM-independent Parsortix system (20). 22VRv1 and PC-3 cells were chosen for these experiments, having high and low AR-V7 expression, respectively. After enrichment, cells were processed via in situ padlock probe technology and immunofluorescent staining of panCK and CD45. This combination of mRNA and protein analysis allows for effective discrimination between CTCs and background blood cells (see Fig. 2 in the online Data Supplement). Accordingly, both cell lines showed expression of panCK and no expression for CD45. 22VRv1 cells also exhibited clear AR-V7 signals, whereas PC-3 cells were AR-V7-negative. Based on these data, we concluded in situ padlock probes can be successfully combined with immunostaining after enrichment with the Parsortix system.

### KRAS WT AND MUT TRANSCRIPTS CAN BE VISUALIZED AND QUANTIFIED IN PACA CELL LINES

To validate specificity and sensitivity of padlock probes and antibodies, experiments were performed using the 2 pancreatic cell lines CAPAN-1 [EpCAM(+), panCK(+), CD45(-), and *KRAS* G12V homozygous mutation] and BxPC3 (*KRAS* wt) (28, 29). As expected, CAPAN-1 displayed homozygous expression of *KRAS* mut (1.1 RCP/cell), as well as positive protein staining for panCK and negative staining for CD45. BxPC3 cells expressed *KRAS* wt (0.2 RCP/cell) and had a low false-positive rate for the mutation (0.004 RCP/cell) (Fig. 1).

### AR-V7, AR-FL, AND PSA CAN BE DETECTED IN CTC ISOLATED BY CELLCOLLECTOR

We assessed the CTC status of 13 patients with CRPC by applying in situ padlock probe technology on cells attached to the CellCollector. Multiplex in situ analysis for expression of AR-V7, AR-FL, and PSA was directly performed on CTCs attached to the surface of the CellCollector. An overview of the results is provided in Table 4 of the online Data Supplement. In all, 62% (8 of 13) of the patients were CTC-positive (range, 1–15 CTCs/patient). In situ analysis revealed that 54% (7 of 13) of patients had CTCs with AR-V7 mRNA expression (range, 1–10 RCPs/CTC) (Fig. 2). AR-FL-positive CTCs were detectable in 39% (5 of 13) of patients (expression range, 1–9 RCPs/CTC), and PSA-positive CTCs were found in 23% (3 of 13) of patients (expres-

sion range, 1–2 RCPs/cell) (Fig. 2). Representative CTCs on the CellCollector are shown in Fig. 2.

### DIFFERENT SUBTYPES OF CTC CAN BE IDENTIFIED BY IN SITU AR-V7 DETECTION AND PANCK IMMUNOSTAINING OF CTC ISOLATED BY PARSORTIX

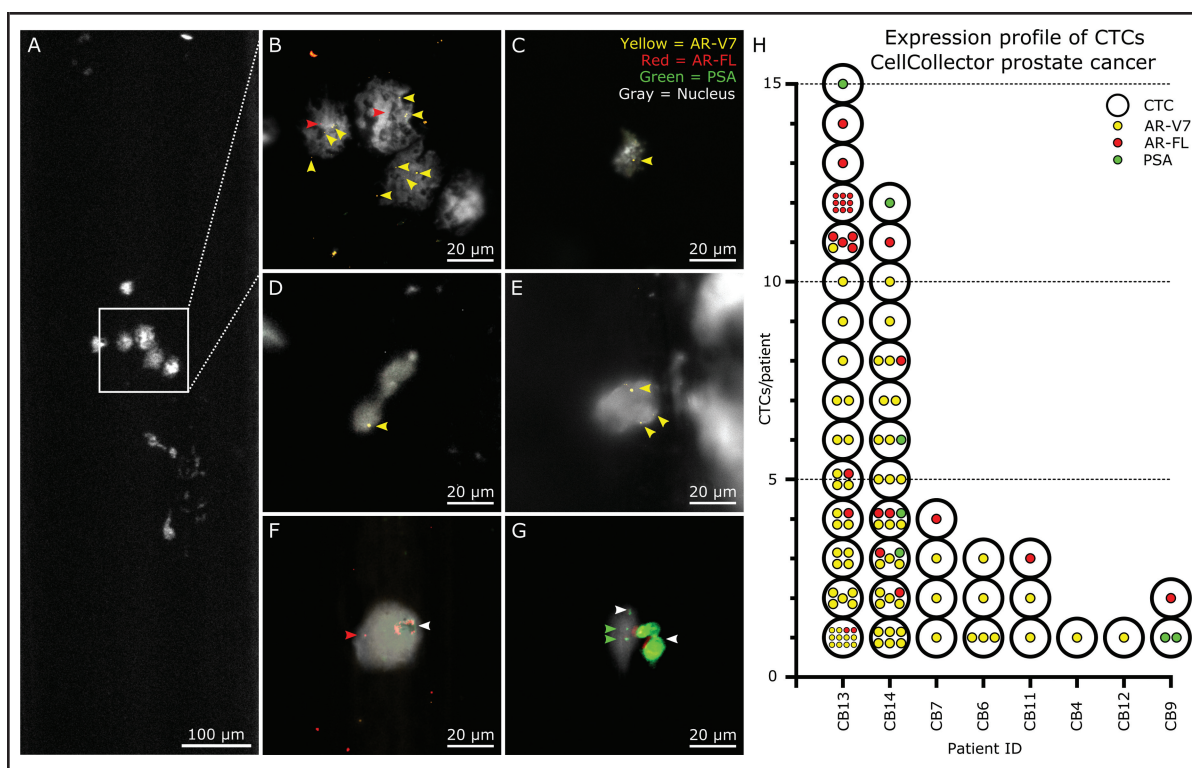
We evaluated the CTC status of 17 patients (19 samples) with CRPC using the Parsortix system to isolate CTCs. CTCs were evaluated by applying in situ padlock probe technology for AR-V7 in combination with panCK and CD45 immunostaining. In all, 89% of the samples (17 of 19) were positive for CTCs (range, 1–158 CTCs/7.5 mL of blood). In situ analysis revealed that 79% of samples (15 of 19) also contained CTCs with AR-V7 mRNA expression (range, 1–30 RCPs/CTC) (Fig. 3). We observed 3 types of CTCs by combining in situ AR-V7 detection with panCK immunostaining: (a) panCK(+)/AR-V7(-), (b) panCK(-)/AR-V7(+), and (c) panCK(+)/AR-V7(+). An overview of the distribution of the different CTC subtypes is shown in Fig. 3. Images of representative CTCs are shown in Fig. 4. In all, 84% of the samples (16 of 19) were positive for panCK(+)/AR-V7(-) CTCs, 68% of samples (13 of 19) were positive for panCK(+)/AR-V7(+) CTCs, and 32% of samples (6 of 19) were positive for panCK(-)/AR-V7(+) CTCs. In rare cases, single cells were detected showing staining for both, panCK and CD45, similar to the results of others (30). These cells were not counted as CTCs. An overview of the results is provided in Table 4 of the online Data Supplement.

### AR-V7 CAN BE DETECTED IN CTC ISOLATED BY CELLSEARCH

One CRPC sample was processed with the CellSearch System and analyzed by in situ padlock probe technology for AR-V7 expression. In total, 557 AR-V7(+) CTCs/7.5 mL of blood were detected, with AR-V7 expression ranging from 1 to 76 RCPs/CTC (mean, 10 RCPs/CTC) (Fig. 5).

### KRAS WT AND MUT TRANSCRIPTS CAN BE DETECTED IN CTC ISOLATED BY CELLCOLLECTOR

The CTC status of 15 patients with PaCa was characterized by in situ padlock probe technology combined with immunostaining for CK18/panCK and CD45, directly on patient cells attached to the CellCollector. Representative CTCs on the CellCollector are shown in Fig. 6. In all, 47% of the patients (7 of 15) were CTC-positive (range, 1–3 CTCs/patient). The expression of *KRAS* and panCK/CK18 showed inpatient and interpatient heterogeneity (Fig. 6). The *KRAS* mut expression ranged from 1 to 8 RCPs/CTC, and *KRAS* wt ranged from 1 to 2 RCPs/CTC. The overall rate of *KRAS* mutated CTCs was 75% (9 of 12). In all, 50% (6 of 12) of CTCs detected were also positive for cytokeratin protein, demonstrating that in situ padlock probes can be successfully



**Fig. 2. Prostate cancer CTCs captured by the CellCollector.** Visualized transcripts are marked by arrowheads (AR-V7, yellow; AR-FL, red; and PSA, green). Overview image of CTCs captured on the CellCollector (A). CTCs with AR-V7 and AR-FL expression (B). CTCs with AR-V7 expression (C-E). A CTC-expressing AR-FL (F) and a CTC with PSA expression (G). Diagram showing the number of CTCs and their respective expression profile of each CTC-positive patient (H). The expression level of each CTC (black circle) is represented as a colored spot corresponding to a single RCP (AR-V7, yellow; AR-FL, red; and PSA, green). Cell nuclei are shown in gray. Unspecific staining patterns are marked by white arrowheads.

combined with immunostaining using the CellCollector. CD45 immunostaining showed only weak staining of mononuclear cells attached to the wire with stronger staining of a certain lymphocyte subpopulation. The *KRAS* mutation status of the corresponding primary tumor DNA in 5 of the CTC-positive patients was further tested with a diagnostic PCR analysis. A similar mutation pattern could be observed (see Fig. 3 of the online Data Supplement). An overview of the results is provided in Table 5 of the online Data Supplement.

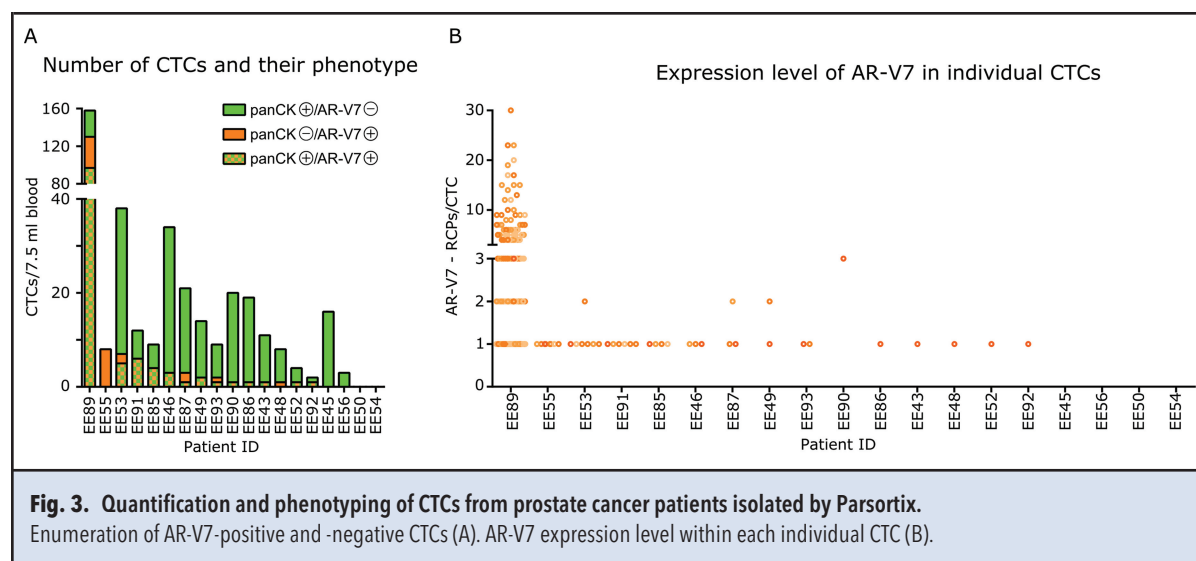
#### CHARACTERISTICS OF THE PATIENT COHORTS

As this study aimed to develop a CTC-based assay (e.g., AR-V7 detection), patients with advanced disease were enrolled to obtain a high number of CTCs. Basic clinical data were obtained from the patients with additional follow-up data from a subset of patients. A detailed overview of both patient cohorts for each individual patient can be found in Tables 1 and 6 of the online Data Supplement. An overview of PSA response is shown in a waterfall plot (see Fig. 4 of the online Data Supplement).

#### Discussion

We evaluated the feasibility and utility of in situ padlock probe technology for the analysis of clinically relevant splice variants and point mutations in CTCs isolated by 3 independent enrichment technologies. Our data show that CTCs from prostate cancer patients can be analyzed for AR-V7, AR-FL, and PSA expression and that CTCs from PaCa patients can be analyzed for *KRAS* point mutations. We further demonstrate the feasibility of combining immunostaining (panCK and CD45) with in situ padlock probe technology. Our approach allows for quantification of CTC and simultaneous interrogation of clinically relevant markers.

Our padlock approach for AR-V7 mRNA detection shows robust signals in isolated CTCs. A strength of the method is the possibility to easily quantify expression levels by simply counting the fluorescent signals. Another advantage is the option to investigate nucleic acids without lysis of CTCs and, therefore, allowing the quantification of CTCs, which serves as a prognostic marker. In



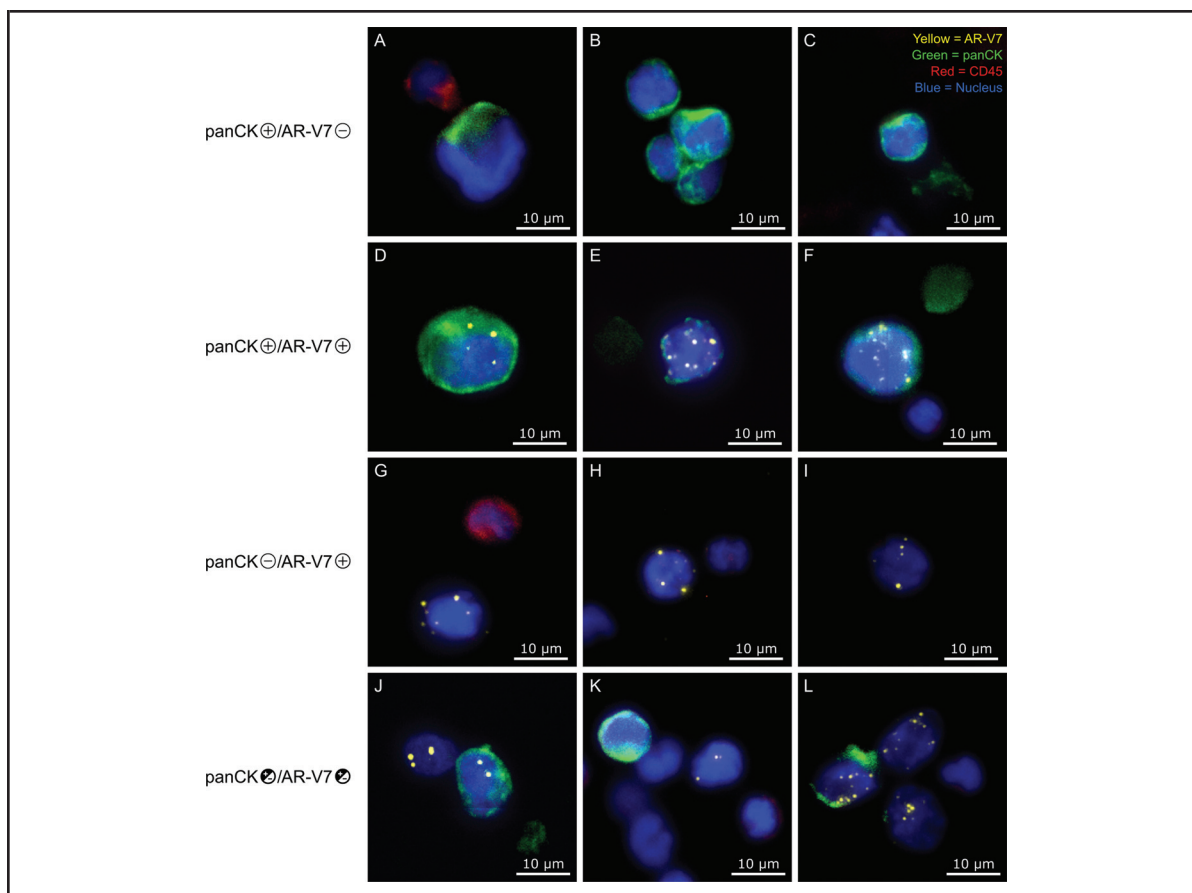
**Fig. 3. Quantification and phenotyping of CTCs from prostate cancer patients isolated by Parsortix.** Enumeration of AR-V7-positive and -negative CTCs (A). AR-V7 expression level within each individual CTC (B).

addition, heterogeneity among CTCs can be easily visualized. Although the AR-V7 mRNA expression is shown to correlate with worse clinical outcomes in recent literature, we cannot simply conclude that every mRNA transcript will be translated to protein (31). Indeed, Scher et al. showed that resistance toward novel hormonal therapy (NHT) depends on the nuclear-specific location of AR-V7 protein rather than on cytoplasmic localization (32). To investigate the location of AR protein in CTCs, we analyzed a subset of CellSearch-processed samples. The CellSearch samples were immunostained for AR in addition to panCK and CD45 (see Table 4 in the online Data Supplement) as recently published (33). The used AR antibody targets the N-terminal part of the protein, thereby also detecting AR-V7 but without the possibility to discriminate between the full length and splice variant. Interestingly, the patient with one of the highest AR-V7(+) CTC counts showed localization of AR protein in the nucleus of CTCs analyzed by CellSearch (see Table 4 in the online Data Supplement). This indicates that high expression of AR-V7 may correlate with active AR-V7 protein in the nucleus. A recent publication showed that another splice variant may also play an important role in the NHT resistance mechanism. Kohli et al. stated that initial annotation of the splice variant AR-V9 needs to be reevaluated, as they revealed by short- and long-read sequencing that the sequences of the 3'-UTR of AR-V7 and AR-V9 overlap (34). This implies that our AR-V7 assay may partially also detect AR-V9. Nevertheless, the authors also found that AR-V9-expressing cells had similar therapeutic resistance toward the NHT drug enzalutamide as the AR-V7-expressing cells (34). The patients we enrolled in our study were selected for establishing the in situ assay, rather than an evaluation of patient outcome. To do so,

our patients were selected for advanced stage, rather than a clearly defined timepoint or treatment regimen. For example, most of the recruited CRPC patients already acquired resistance toward enzalutamide and abiraterone. Our rationale was to increase the probability of AR-V7 detection among the patients, as AR-V7 positivity increases in later lines of therapy to approximately 20% of CRPC patients (8). Our assay for the quantification of AR-V7 expression may become an important tool to stratify patients into AR-V7 “high” or “low” expressors or to supply a cutoff value for potential resistance to NHT. However, our assay needs validation in a prospective clinical study.

Moreover, we successfully characterized the mutation status of *KRAS* in CTCs of PaCa patients. The number of CTCs detected ranged from 1 to 3 CTCs per patient. In this study, all pancreatic tumors were surgically resectable with relatively little tumor burden, so the number of CTCs was expected to be low. For those with advanced disease, for whom we would expect a higher frequency of CTCs, a low CTC count might not be sufficient for monitoring progression; however, it could still be useful for diagnostics.

In our hands, the CellCollector has proven to be a usable in vivo CTC enrichment device. The application into a cubital vein for 30 min is somewhat more laborious than simple blood drawing, but the wire can be easily processed and stored for at least 1 month at  $-20^{\circ}\text{C}$  without any noticeable degradation of in situ signals. Furthermore, for performing in situ reactions and follow-up screening of the CellCollector, only a routine molecular biology lab, equipped with a fluorescent microscope, is needed. Processing and screening time of the CellCollector is short and generates results within 48 h. Recent data show that the CellCollector can detect

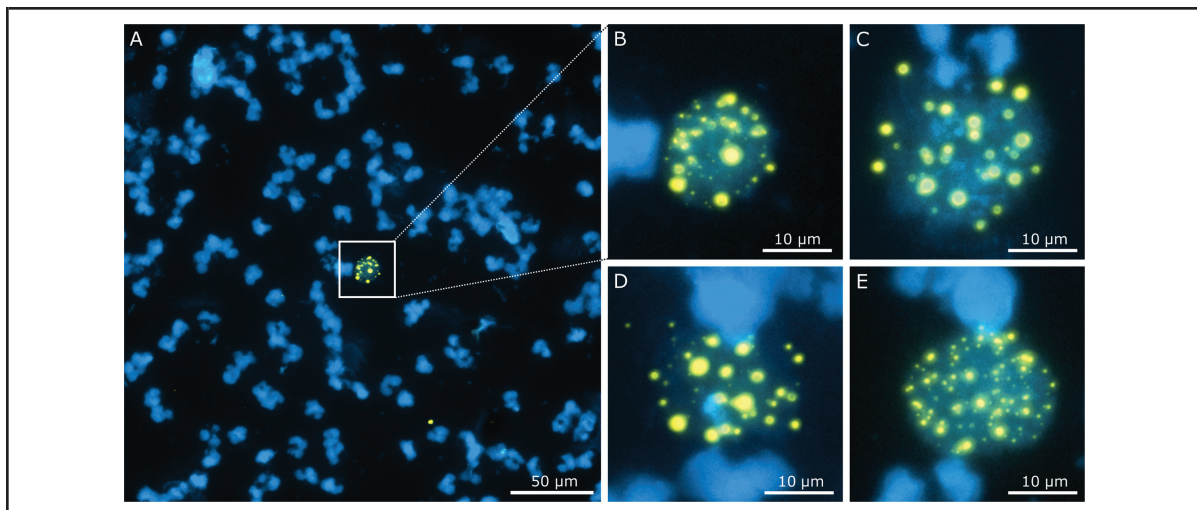


**Fig. 4. In situ detection of AR-V7 in CTCs from prostate cancer patients isolated by Parsortix.**

CTCs and a CTC cluster with positive immunostaining for panCK but lack of AR-V7 expression (A-C). panCK(+) and AR-V7(+) CTCs with varying degree of AR-V7 expression (4–20 AR-V7 RCP signals) (D–F). panCK(–) and AR-V7(+) CTCs (G–I). Mixed populations of CTCs and clusters, with and without panCK and AR-V7 expression (J–L). AR-V7 (yellow), panCK (green), leukocyte common antigen (CD45, red), and nucleus (blue).

higher rates of patients positive for CTCs as compared with other technologies (18). The CTCs are firmly attached to the CellCollector and will not be lost during the in situ process. Indeed, the cells cannot be released from the wire without their lysis. For more elaborate single-cell molecular characterization (e.g., next-generation sequencing), a novel CellCollector was developed, namely, the Catch & Release CellCollector (Gilupi). The Catch & Release CellCollector allows EpCAM-based capturing of cells and their release by a releasing buffer for subsequent single-cell analysis, but it is not yet cleared for use in patients (35). As the CellCollector is based on anti-EpCAM antibodies for CTC isolation, it may miss several other CTC subpopulations with downregulated epithelial markers (36). To evade limitation by EpCAM, we combined the in situ padlock approach with the EpCAM-independent Parsortix platform (20), demonstrating that the in situ padlock approach is generally applicable to different isolation platforms. By combining

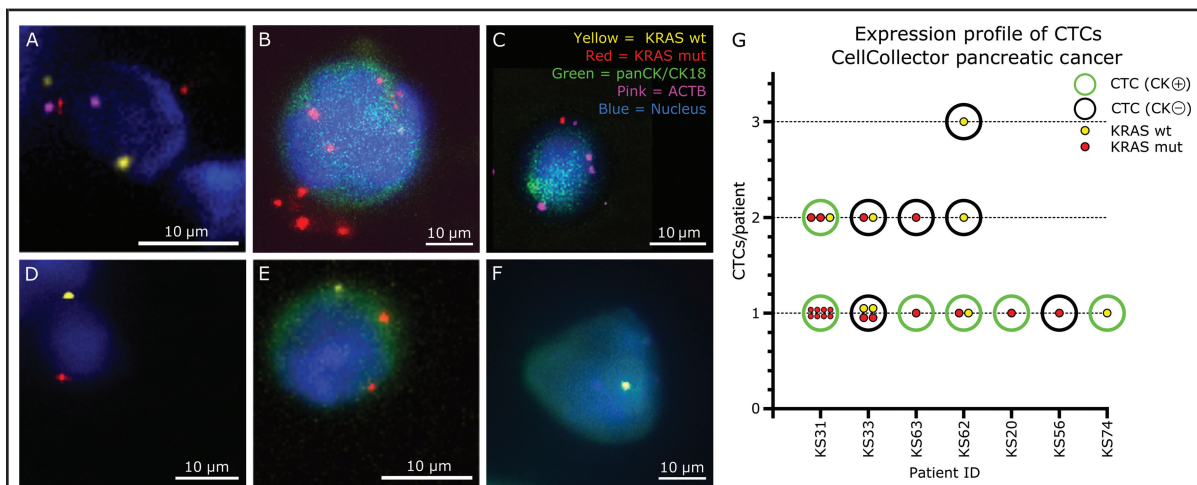
the in situ AR-V7 detection with the Parsortix system, we observed heterogeneous cytokeratin expression of CTCs. Heterogeneity among CTCs is well described, including a wide range of differentiation states from epithelial to mesenchymal types (37). With this approach, in situ padlock probes allowed the detection of several CTCs that had no sign of cytokeratin expression, suggesting cells in an active state of epithelial to mesenchymal transition. These CTCs actively expressed AR-V7 or *KRAS* transcripts and would have been “missed” by EpCAM-dependent assays. It might be hypothesized that these CTCs are of high clinical relevance. We observed 6 CRPC patients with panCK(–)/AR-V7(+) CTCs. Five of these 6 patients also showed panCK(+)/AR-V7(+) CTCs (see Table 4 in the online Data Supplement), making it difficult to distinguish which of the AR-V7-positive populations is “most relevant” for clinical resistance toward enzalutamide and abiraterone. Nevertheless, this reflects the selection of resistant clones toward enzalut-



**Fig. 5.** In situ detection of AR-V7 of CTCs isolated by CellSearch from 1 prostate cancer patient. Overview of cells retrieved from a CellSearch cartridge and cytocentrifuged on a slide (A). Representative images of AR-V7(+) CTCs (B-E).

amide and abiraterone treatment (38). In situ padlock probes can thereby add an extra layer of information to CTCs, including diagnostic data and proof of CTC origin. Indeed, the traditional definition of CTCs (positive for EpCAM/panCK and negative for CD45) becomes extended by molecular characterization. As we have shown with our combined in situ AR-V7 and panCK/CD45 immunostaining approach, CTCs can be clearly

identified and grouped into different subtypes. The sole immunostaining profile is insufficient to stratify CTCs into different subtypes. Moreover, recent studies identified cells in circulation with common epithelial/CTC-like features and balanced copy number profiles (39). Others have shown that CK+/CD45- cells can be found in healthy volunteer samples. The clinical relevance of these cells is currently unknown (40). This un-



**Fig. 6.** In situ detection of mRNA transcripts combined with immunofluorescence in CTCs of pancreatic cancer patients captured by the CellCollector.

Representative images of CTCs from PaCa patients (A-F). Visualized transcripts are marked according to color, yellow signals = *KRAS* wt, red signals = *KRAS* mutant (mt), and pink signals = *ACTB* control. The nucleus is displayed in blue, and the green staining corresponds to CK18 or panCK immunostaining. The top- and bottom-left CTCs were both CK18-negative. Diagram showing the number of CTCs and their respective *KRAS* expression profile of each CTC-positive patient (F). Yellow dots = *KRAS* wt signals and red dots = *KRAS* mut signals.

derlines the importance of molecular characterization in liquid biopsies beyond the traditional EpCAM/panCK immunostaining. Deeper molecular insight into different CTC populations may lead to a better understanding of how metastatic disease and therapy resistance form and which cells contribute to their formation.

As a proof of principle, we also applied our in situ AR-V7 approach to CellSearch-enriched CTCs from 1 patient. Although just a single case, we clearly identified CTCs with high AR-V7 expression, suggesting that the in situ padlock probe approach is applicable to multiple independent CTC enrichment technologies.

In conclusion, we have shown detection and quantification of clinically relevant molecular markers in single CTCs using our in situ approach. We thereby could add additional information beyond simple CTC enumeration, which likely will improve the predictive and prognostic value of CTC analysis in the clinic.

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