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Multiplex Gene Expression Profiling of In Vivo Isolated Circulating Tumor Cells in High-Risk Prostate Cancer Patients

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BACKGROUND: Molecular characterization of circulating tumor cells (CTCs) is important for selecting patients for targeted treatments. We present, for the first time, results on gene expression profiling of CTCs isolated in vivo from high-risk prostate cancer (PCa) patients compared with CTC detected by 3 protein-based assays—CellSearch®, PSA-EPISPOT, and immunofluorescence of CellCollector® in vivo-captured CTCs—using the same blood draw.

METHODS: EpCAM-positive CTCs were isolated in vivo using the CellCollector from 108 high-risk PCa patients and 36 healthy volunteers. For 27 patients, samples were available before and after treatment. We developed highly sensitive multiplex RT-qPCR assays for 14 genes (*KRT19*, *EpCAM*, *CDH1*, *HMBS*, *PSCA*, *ALDH1A1*, *PROM1*, *HPRT1*, *TWIST1*, *VIM*, *CDH2*, *B2M*, *PLS3*, and *PSA*), including epithelial markers, stem cell markers, and epithelial-to-mesenchymal-transition (EMT) markers.

RESULTS: We observed high heterogeneity in gene expression in the captured CTCs for each patient. At least 1 marker was detected in 74 of 105 patients (70.5%), 2 markers in 45 of 105 (40.9%), and 3 markers in 16 of 105 (15.2%). Epithelial markers were detected in 31 of 105 (29.5%) patients, EMT markers in 46 of 105 (43.8%), and stem cell markers in 15 of 105 (14.3%) patients. EMT-marker positivity was very low before therapy (2 of 27, 7.4%), but it increased after therapy (17 of 27, 63.0%), whereas epithelial markers tended to decrease after therapy (2 of 27, 7.4%) compared with before therapy (13 of 27, 48.1%). At least 2 markers were expressed in 40.9% of patients, whereas the positivity was 19.6% for CellSearch, 38.1% for EPISPOT, and 43.8% for CellCollector-based IF-staining.

CONCLUSIONS: The combination of in vivo CTC isolation with downstream RNA analysis is highly promising as a high-throughput, specific, and ultrasensitive approach for multiplex liquid biopsy-based molecular diagnostics.

Prostate cancer (PCa)[6] is the second most lethal cancer among men; however, despite the widespread use of prostate-specific antigen (PSA) screening, 15%–30% of newly diagnosed PCas are classified as high-risk tumors *(1)*. Local therapy is increasingly performed in high-risk patients and 10-year cancer-specific survival rates range between 88% and 97%. However, >50% of the surgically treated high-risk patients will experience a biochemical disease recurrence *(2)*, and 11% will develop metastatic disease within 5 years *(3)*.

Very recently, the US Food and Drug Administration (FDA) cleared 2 novel Conformité Européene in vitro diagnostic (CE-IVD) assays for the early detection of PCa based on classic peripheral tumor markers such as Prostate Health Index (PHI, Beckman Coulter) and prostate cancer antigen 3 (PCA3) *(4–6)*. Another liquid biopsy test, the ExiDx Prostate (IntelliScore), was developed to help rule out high-grade PCa before an initial prostate biopsy *(7)*.

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[6] Nonstandard abbreviations: PCa, prostate cancer; CTC, circulating tumor cells; BCR, biochemical disease recurrence; gDNA, Genomic DNA; DAPI, 4′,6-diamidino-2-phenylindole; EMT, epithelial-to-mesenchymal transition; LOD, limit of detection; IF, immunofluorescence.

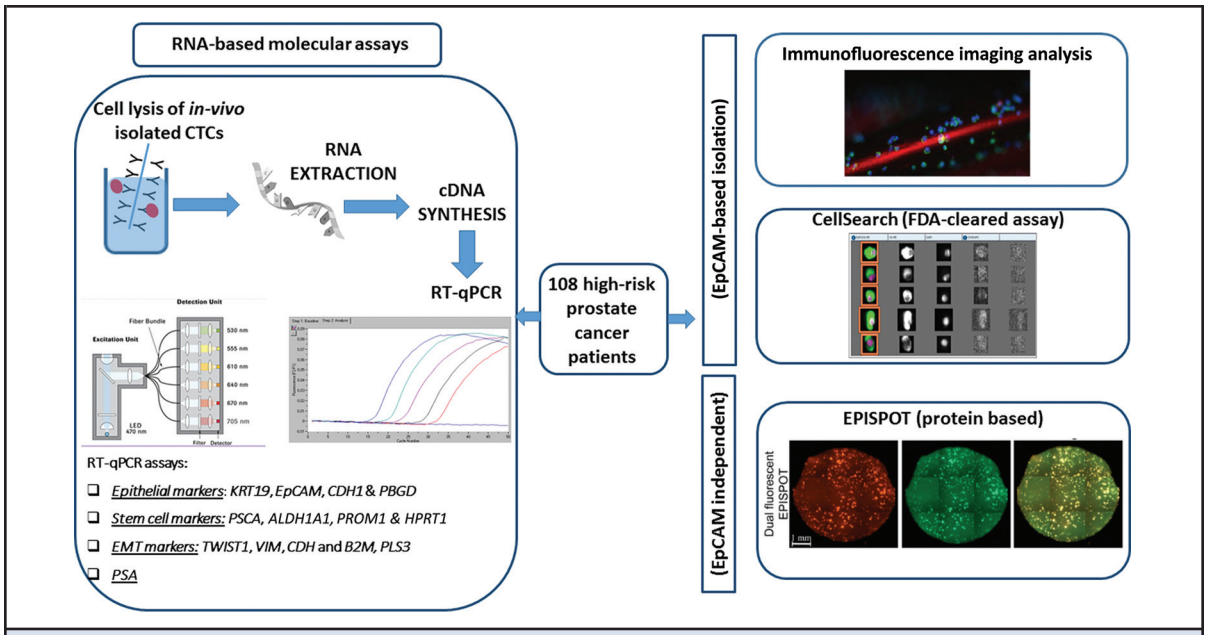

Fig. 1. Schematic workflow of the present study.

Liquid biopsy based on the analysis of circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), circulating miRNAs, and exosomes provides a source of valuable biomarkers using noninvasive blood tests. The presence of CTCs in peripheral blood has been linked to worse prognosis and early relapse in numerous clinical studies *(8)*. Imaging systems at the protein level or molecular assays are used for CTC detection and molecular characterization *(9, 10)*.

In vivo isolation of CTCs is now feasible through a novel technology—by inserting a nanodetector (GILUPI CellCollector) into the patient's arm vein via a standard 20-gauge needle *(11–14)*. Circulating EpCAM-positive tumor cells bind in vivo to anti-EpCAM antibodies that are covalently attached to the CellCollector, while the functionalized surface prevents the nonspecific binding of other blood constituents. This approach overcomes the restrictions of a limited blood sample because, during its 30-min in vivo application, the CellCollector comes into contact with a higher quantity of blood than that found in blood samples using in vitro methods.

We present here, for the first time, multiplex gene expression profiling of in vivo isolated CTCs in high-risk PCa patients, which was compared to CTC detection by 3 protein-based assays—CellSearch, the current FDA-cleared "gold standard" *(15)*; PSA-EPISPOT, which detects viable CTCs *(16)*; and immunofluorescence (IF) staining of in vivo-captured CTCs by use of the CellCollector *(11–14)*.

Materials and Methods

PATIENTS

High-risk PCa patients (PSA ≥20 ng/mL and/or biopsy Gleason score ≥8 and/or clinical tumor stage ≥2c) without evidence of overt metastases were screened for CTCs before and after therapy. In total, 73 patients were screened for CTCs 1 day before surgery or radiotherapy and 3–5 months after surgery (in the case of radiotherapy, the samples were taken 3–5 months after the end of radiation). Blood samples were taken for CellSearch and EPISPOT analysis and in parallel the CellCollector was applied for 30 min in the vein of each patient. A group of 36 healthy blood donors was used as noncancerous control; in 18 of the healthy donors, the CellCollector was applied in the vein, exactly as in PCa patients (in vivo isolation), and in 18 cases, the CellCollector was added in tubes containing peripheral blood from healthy donors (HD) (in vitro isolation). The study was carried out in accordance with the World Medical Association Declaration of Helsinki. All patients gave written informed consent for participation in this study and the publication of results. An outline of the whole study is shown in Fig. 1.

MOLECULAR ANALYSIS OF IN VIVO ISOLATED CTCs

In vivo isolation of CTC, RNA isolation, and cDNA synthesis. For in vivo isolation of CTCs, the CellCollector (GILUPI, Germany) was inserted through a conventional cannula (32 mm) and incubated for 30 min in the pa-

tient's vein. Afterward, the antibody-coated surface area of the CellCollector was washed twice with PBS (10 mmol/L $Na_2HPO4 \cdot 2H_2O$, 1.8 mmol/L KH_2PO4, 137 mmol/L NaCl, 2.7 mmol/L KCl), cut with a sterile scalpel, transferred into a 2-mL tube, stored in 1 mL Trizol LS (Ambion, Life Technologies), and transported to the University of Athens laboratory from different participants' sites at room temperature. Isolation of total RNA from Trizol was performed according to the manufacturer's instructions. The high-capacity RNA to cDNA kit (Applied Biosystems) was used for reverse transcription in a 20 μL of total volume reaction. A negative control was included in each experiment to ensure that there was no contamination by genomic DNA (gDNA). All cDNA samples were kept at −20 °C until further molecular analysis.

Multiplex RT-qPCR gene expression analysis of in vivo isolated CTCs. We first developed and analytically validated 3 multiplex RT-qPCR assays for the quantitative determination of (*a*) *Epithelial markers*: *CK-19 (KRT19)*, *EpCAM*, *E-cadherin (CDH1)*, and *HMBS* (reference gene); (*b*) *Stem cell markers: PSCA*, *ALDH1A1*, *CD133 (PROM1)*, and *HPRT1* (reference gene); and (*c*) *EMT markers: TWIST1*, *Vimentin (VIM)*, *N-cadherin* (*CDH2)*, and *B2M* (reference gene). All multiplex RT-qPCR reactions were performed in the LightCycler 2.0 (IVD Instrument, Roche). We further developed and validated 2 singleplex RT-qPCR assays for (*i*) *PSA* and (*ii*) *Plastin-3* (*PLS3*). Primers and dual hybridization probes were designed de novo in silico. Each probe set included a 3′-fluorescein (F) donor probe and a 5′-LC acceptor probe. A color compensation test was performed by using pure dye spectra so that spectral overlap between dyes was corrected *(17)*. A positive control (cDNA from SK-BR-3, PC-3, and VCaP cell line) and a negative control (PCR-grade H_2O) were included in all runs. RT-qPCR was performed for 45 cycles.

Immunofluorescence staining of in vivo isolated CTCs. CTCs in the CellCollector were stained for pan-keratins *(8, 18, 19)*, PSA, and CD45, as recently described *(14)*.

CellSearch analysis. For CellSearch, 7.5 mL of venous blood was collected into CellSave tubes and processed using the CellSearch Circulating Tumor Cell Kit (Menarini Diagnostics) according to the manufacturer's instructions *(14)*.

PSA-EPISPOT assay. First, RosettSep™ (StemCell Technologies) reagent was used to isolate CTCs from 13–15 mL of EDTA blood, and the PSA-EPISPOT assay was performed as previously described *(14, 16)*.

Statistical analysis. Agreement between the developed RT-qPCR assays and the other methods (CellSearch, PSA-EPISPOT, and IF-staining) was assessed using the McNemar test and the Cohen κ coefficient, κ (SPSS Statistics, version 23.0). $P < 0.05$ was considered statistically significant.

Results

MOLECULAR ANALYSIS OF IN VIVO ISOLATED CTCs

Development and analytical validation of multiplex RT-qPCR gene panels. We first developed 3 novel multiplex RT-qPCR assays after careful selection of the target genes. Before applying these assays in clinical samples, we performed extensive optimization experiments and validated their analytical performance as described below in detail. In total, 105 samples were positive for all reference genes tested and were eligible for analysis.

Multiplex epithelial markers assay. A novel multiplex RT-qPCR assay was developed for *KRT19*, *EpCAM*, *CDH1*, and *HMBS* gene transcripts. First, we tested the analytical specificity of all oligonucleotides when only 1 individual gene target was present as a template. Each primer and dual hybridization probe pair amplified specifically only the corresponding target amplicon (Fig. 2A). The analytical sensitivity was evaluated by estimating the LOD using quantification calibrators containing a known number of copies/μL prepared as previously described *(18)*. For each gene target, a calibration curve was generated using serial dilutions of external standards in triplicate for each concentration, ranging from 10^5 to 10 copies/μL. The LOD for the epithelial markers assay was 10 copies/μL (Fig. 2B).

The diagnostic specificity of the developed assay was evaluated by analyzing peripheral blood samples from 36 healthy individuals. The assay was highly specific (Fig. 3A), as only 1 of 36 (2.8%) samples was positive for *KRT19* and 1 of 36 (2.8%) samples for *EpCAM*. *CDH1* transcripts were not detected in any sample (see Fig. 1A in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol64/issue2), whereas *HMBS* transcripts were detected in all samples because of the presence of contaminating leukocytes as expected.

Multiplex stem cell markers assay. A novel multiplex RT-qPCR assay was developed for *PSCA*, *ALDH1*, *PROM1*, and *HPRT1* gene transcripts. We assessed analytical specificity as mentioned above and we did not observe any nonspecific interactions between the 16 oligonucleotides used (4 for each gene target) (Fig. 2A). The analytical sensitivity of the assay was evaluated as above. The LOD was 10 copies/μL for all markers except *HPRT1*, which was 100 copies/μL (Fig. 2B).

The assay was highly specific, as only 2 of 36 (5.6%) HD samples were positive for *ALDH1* (Fig. 3A; see Fig. 1B in the online Data Supplement), whereas *PSCA* and *PROM1* transcripts were not detected in any sample.

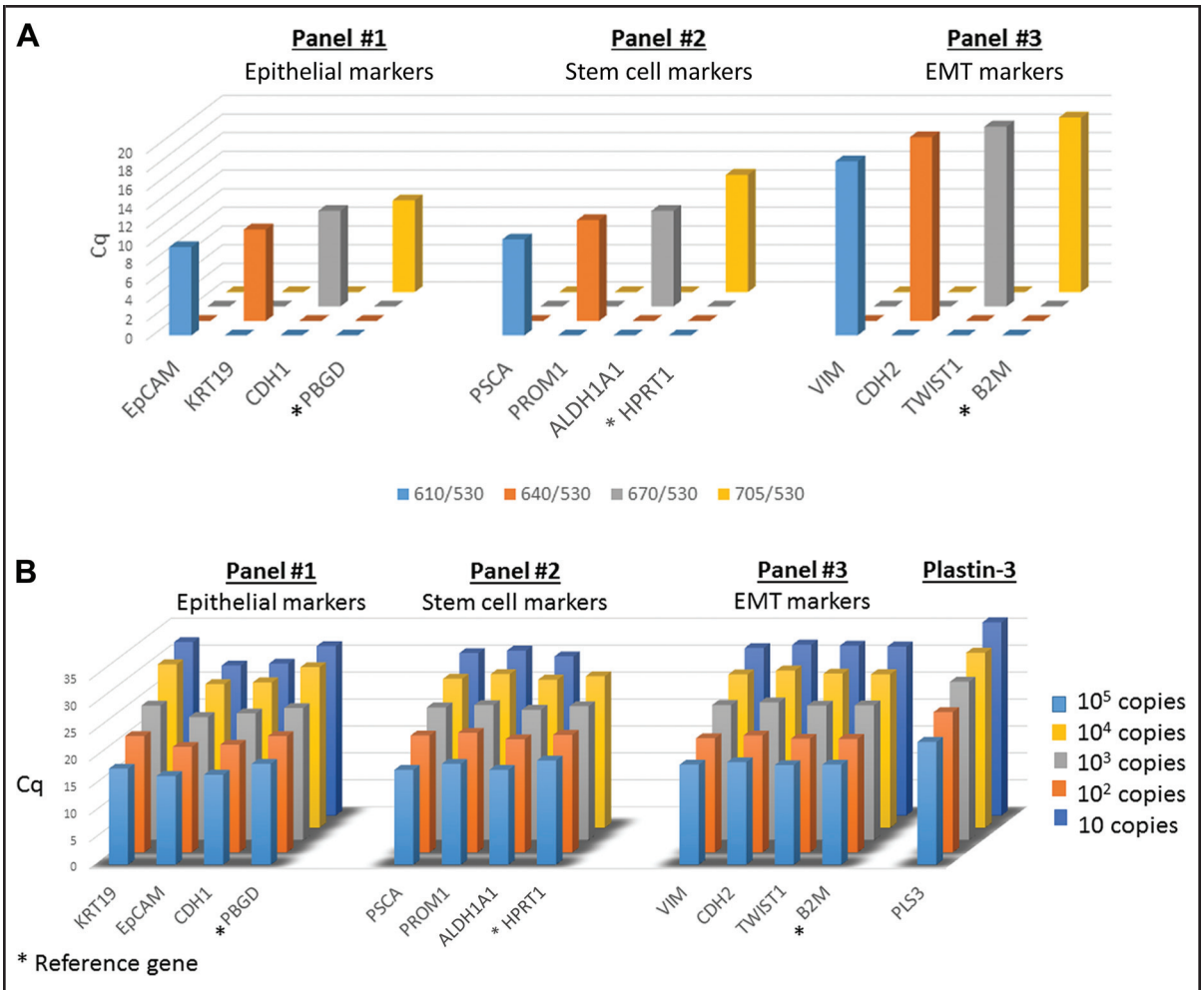

Fig. 2. Analytical specificity and sensitivity of the developed RT-qPCR assays.

(2A) The analytical specificity for each multiplex RT-qPCR assay was checked in synthetic samples, using all oligonucleotides when only one individual gene target was present as a template. (2B) The analytical sensitivity for each multiplex RT-qPCR assay was checked by estimating the LOD, using quantification calibrators containing a known number of copies/μL. For each gene target, a calibration curve was generated using serial dilutions of external standards in triplicate for each concentration, ranging from 10^5 copies/μL to 10 copies/μL. All experiments were run in triplicate.

HPRT transcripts were detected in all samples due to the presence of contaminating leukocytes as expected.

Multiplex EMT markers assay. A multiplex RT-qPCR assay was developed for *TWIST1*, *VIM*, *CDH2*, and *B2M* gene transcripts. We assessed analytical specificity and sensitivity as mentioned above. We did not observe any nonspecific interactions (Fig. 2A), and the LOD for the EMT markers was 10 copies/μL (Fig. 2B). The diagnostic specificity was evaluated as above. *CDH2* and *VIM* were expressed in both cancer cells and PBMCs; the expression of these genes was normalized with respect to *B2M* expression (reference gene). ΔCq values were calculated by using Cq values for each gene (*CDH2* and *VIM*) and the corresponding *B2M* for each sample. Relative quantification was based on the ΔΔCq method *(19)*. We defined a sample as *CDH2*- or *VIM*-positive based on the fold change of *CDH2* or *VIM* expression in comparison with the corresponding values obtained for the control group. The cutoff value was estimated according to the expression of *CDH2* or *VIM* in the in vivo isolated samples of 18 healthy individuals that were analyzed in exactly the same way as the patient's peripheral blood samples (see Fig. 1C in the online Data Supplement).

Single-plex assays: plastin-3 and PSA. Single RT-qPCR was performed for *PLS3* and *PSA*. Primers and TaqMan

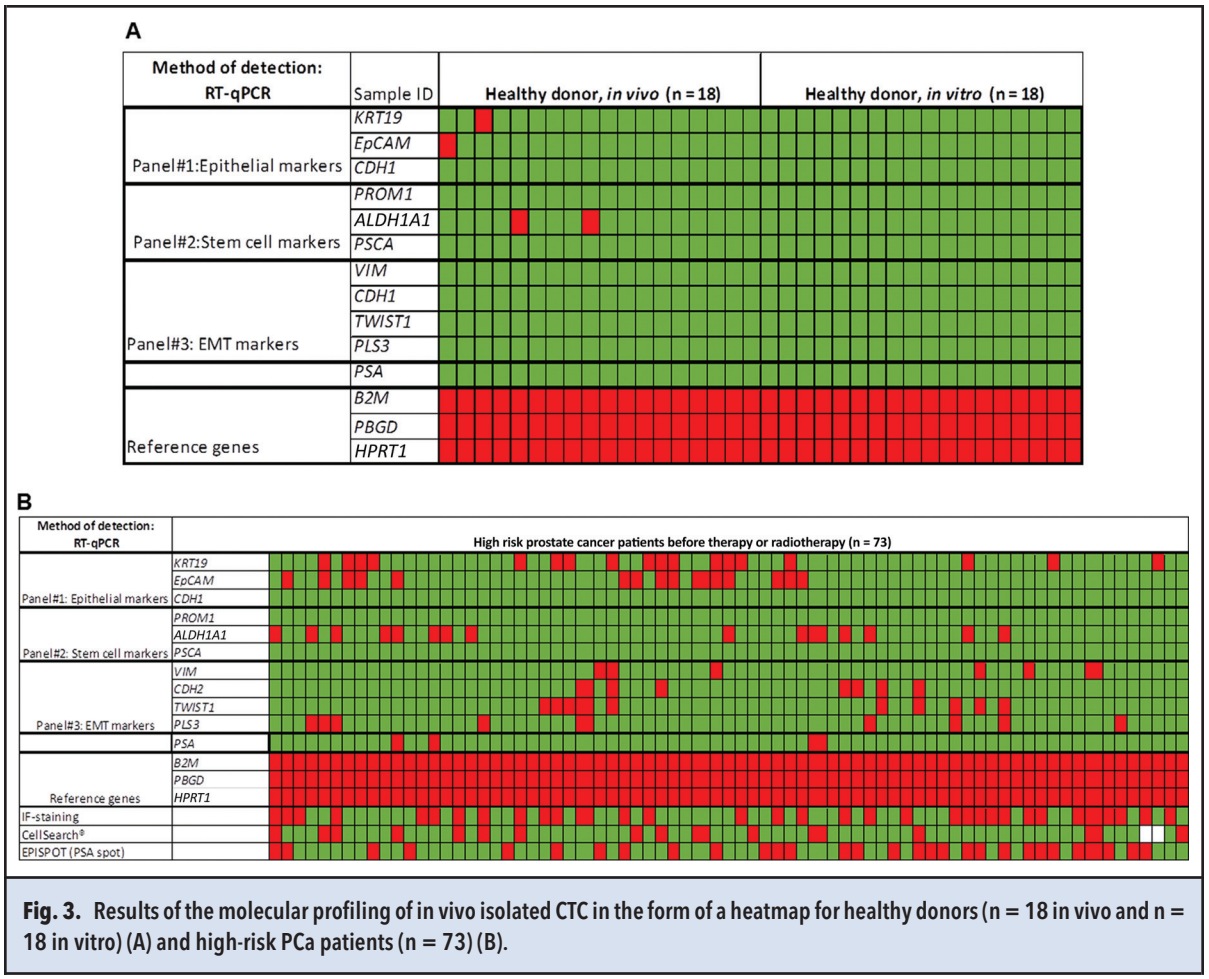

Fig. 3. Results of the molecular profiling of in vivo isolated CTC in the form of a heatmap for healthy donors (n = 18 in vivo and n = 18 in vitro) (A) and high-risk PCa patients (n = 73) (B).

probe were de novo in silico designed for *PLS3*. To evaluate the sensitivity of the *PLS3* assay, serial dilutions of a *PLS3* PCR product of known concentrations ranging from 10 to 10^5 copies/μL were used and calibration curves were prepared. The LOD for *PLS3* was 10 copies/μL (Fig. 2B). *PSA* transcripts were quantified according to a previously reported assay *(20)* (see Fig. 1D in the online Data Supplement).

GENE EXPRESSION OF IN VIVO ISOLATED CTCs

The expression of at least 1 marker was detected in the vast majority of patients but not in healthy individuals (Table 1 and Fig. 3). More specifically, 74 of 105 patients (70.5%) were positive for at least 1 marker, whereas at least 2 markers were positive in 45 of 105 patients (40.9%), and 3 markers in 16 of 105 patients (15.2%). In in vivo isolated $EpCAM^+$ CTC samples, 31 of 105 (29.5%) were positive for at least 1 epithelial marker, 46 of 105 (43.8%) were positive for at least 1 EMT marker, and 15 of 105 (14.3%) were positive for at least 1 stem cell marker. *VIM* was positive in 25 of 105 patients (23.8%), whereas all samples were negative for *CDH1*, *CDH2*, and *PSCA* expression. We observed a high heterogeneity in gene expression in the captured CTCs for each individual patient. None of the patients was positive for all genes tested, and, in many patients, only one of these genes was expressed (Fig. 3B). *PLS3* transcripts were detected in 10 of 105 patient samples (9.5%), whereas *PSA* transcripts were detected in only 6 of 105 patient samples (5.7%).

MOLECULAR CHARACTERIZATION OF IN VIVO ISOLATED $EpCAM^+$ CTCs BEFORE AND AFTER THERAPY IN HIGH-RISK PROSTATE CANCER PATIENTS

For 27 patients, in vivo isolated samples kept in Trizol were available both before surgery or radiotherapy and after treatment (the samples were taken 3–5 months after the end of radiation). We evaluated gene expression in this group to detect changes occurring in gene expression during therapy. It is important to mention that we detected almost the same percentage of positive events in the 2 groups (before and after therapy) (Fig. 4).

Table 1. Multiplex gene expression profiling of in vivo isolated EpCAM$^+$ CTCs in high-risk prostate cancer patients and healthy donors by the developed RT-qPCR assays.

RT-qPCR Gene target	PCa patients (n = 105) Positive (%)	Healthy donors, in vivo (n = 18) Positive (%)	Healthy donors, in vitro (n = 18) Positive (%)
KRT19	23 (21.9%)	1 (5.5%)	0 (0%)
CDH1	0 (0%)	0 (0%)	0 (0%)
EPCAM	16 (15.2%)	1 (5.5%)	0 (0%)
PROM1	0 (0%)	0 (0%)	0 (0%)
ALDH1A1	15 (14.2%)	2 (11%)	0 (0%)
PSCA	0 (0%)	0 (0%)	0 (0%)
VIM[a]	25 (23.8%)	0 (0%)	0 (0%)
TWIST1	22 (20.9%)	0 (0%)	0 (0%)
CDH2[a]	21 (20%)	0 (0%)	0 (0%)
PLS3	10 (9.5%)	0 (0%)	0 (0%)
PSA	6 (5.7%)	0 (0%)	0 (0%)
At least 1 gene	74/105 (70.5%)	4 (22.2%)	0 (0%)
At least 2 genes	43/105 (40.9%)	0 (0%)	0 (0%)
At least 3 genes	16/105 (15.2%)	0 (0%)	0 (0%)

[a] Overexpression.

Interestingly, although the expression of EMT markers was very low before therapy, 2 of 27 (7.4%), this expression tended to increase after therapy 17 of 27 (63.0%) (McNemar test $P = 0.004$, $\kappa = -0.149$). On the contrary, the expression of epithelial markers tended to decrease after therapy 2 of 27(7.4%) compared to the expression before therapy 13 of 27(48.1%) (McNemar test $P = 0.007$, $\kappa = -0.147$). Unexpectedly, stem cell markers (especially *ALDH1*) were expressed only in the group of patients before surgery or radiotherapy 9 of 27 (33.3%) (Fig. 4).

MOLECULAR ANALYSIS OF IN VIVO ISOLATED CTCs IN COMPARISON WITH 3 DIFFERENT ISOLATION AND PROTEIN-BASED DETECTION SYSTEMS

CTC enrichment and enumeration from blood samples were additionally performed by 3 different isolation and protein-based detection systems: (*a*) CellSearch, (*b*) PSA-EPISPOT, and (*c*) direct IF-staining of CTCs captured in vivo on the CellCollector. Our findings at the gene expression level for in vivo isolated CTCs were compared with these 3 different assays. In all cases, CTCs were isolated from peripheral blood from the same blood draw.

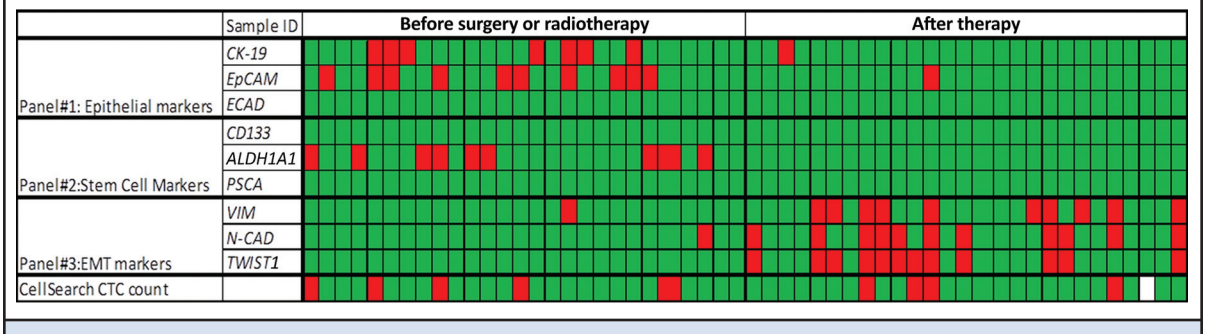

Fig. 4. Molecular profiling of in vivo isolated CTC of PCa patients before surgery and after treatment (n = 27).

Table 2. Comparison between RT-qPCR assays, IF-staining, CellSearch system and PSA-EPISPOT analysis for the detection of CTCs in PCa.

		IF-staining			CellSearch® analysis			PSA-EPISPOT analysis		
RT-qPCR		–	+	Total	–	+	Total	–	+	Total
At least 1 gene positive	–	17	14	31	27	3	30	17	14	31
	+	42	32	74	55	17	92	48	26	74
Total		59	46	105	82	20	102	65	40	105
Concordance		**49/105 (46.7%) $P < 0.001$**			**44/102 (43.1%) $P < 0.001$**			**43/105 (40.9%) $P < 0.001$**		
At least 2 genes positive	–	28	31	59	50	9	59	37	25	62
	+	31	15	46	32	11	43	28	15	43
Total		59	46	105	82	20	102	65	40	105
Concordance		43/105 (40.9%) $P = 0.795$			**61/102 (59.8%) $P < 0.001$**			52/105 (49.5%) $P = 0.784$		
KRT19	–	42	40	82	64	16	80	50	32	82
	+	17	6	23	18	4	22	15	8	23
Total		59	46	105	82	20	102	65	40	105
Concordance		**48/105 (45.7%) $P = 0.003$**			68/102 (66.6%) $P = 0.864$			**58/105 (55.2%) $P = 0.019$**		
Epithelial markers	–	39	35	74	60	12	72	45	29	74
	+	20	11	31	22	8	30	20	11	31
Total		59	46	105	82	20	102	65	40	105
		50/105 (47.6%) $P = 0.058$			68/102 (66.6%) $P = 0.121$			56/105 (53.3%) $P = 0.253$		
Stem cell markers	–	52	38	90	71	16	87	54	36	90
	+	7	8	15	11	4	15	11	4	15
Total		59	46	105	82	20	102	63	40	105
		60/105 (57.2%) $P < 0.001$			75/102 (73.5%) $P = 0.442$			**58/105 (55.2%) $P < 0.001$**		
EMT markers	–	34	25	59	48	9	57	35	24	59
	+	25	21	46	34	11	45	30	16	46
Total		59	46	105	82	20	102	65	40	105
		55/105 (52.4%) $P = 0.445$			**59/102 (57.8%) $P = 0.012$**			51/105 (48.6%) $P = 0.885$		

Bold values indicate $p < 0.05$.

Comparison results are summarized in Table 2. Comparison studies with the CellSearch, PSA-EPISPOT, and IF-staining on the CellCollector demonstrated a higher sensitivity for the CellCollector/RT-qPCR assays. When 1 gene-transcript of the RT-qPCR assays was positive, only 44 of 102 samples (43.1%) were in agreement with CellSearch, 43 of 105 samples (40.9%) were in agreement with the PSA-EPISPOT, and 49 of 105 samples (46.7%) were in agreement with IF. When 2 gene transcripts of the RT-qPCR assays were positive, 61 of 102 samples (59.8%) were in agreement with CellSearch, 52 of 105 (49.5%) were in agreement with the PSA-EPISPOT, and 43 of 105 (40.9%) were in agreement with IF.

However, all these methods were using different isolation systems and different markers for CTCs. The best agreement was observed only in the case where similar markers were used; when we compared the results using the CellSearch (based on $EpCAM^+$ and keratin expression of CTCs) with the molecular assay for *KRT19*

(EpCAM$^+$ isolated in vivo samples) an agreement of 68 of 102 (66.6%) (64 both negative and 4 both positive) was observed (Table 2). When we included *EpCAM* expression in this comparison, 8 samples were positive exclusively for *EpCAM* by RT-qPCR but negative for *KRT19*. Four of these were found positive in the CellSearch and 4 negative, and so the agreement when using both these epithelial markers (*EpCAM* and *KRT19*) was 68 of 102 (66.6%), 60 negative and 8 positive, for both assays. As expected, the sensitivity was increased when both epithelial markers (*KRT19* and *EpCAM*) were used (30 of 102, 29.4%) as compared to *KRT19* alone (22 of 102, 21.6%).

Combining the results of all molecular assays, we found that at least 2 markers were expressed in 46 of 105 patients (43.80%), whereas the positivity for CellSearch was 20 of 102 (19.6%), for EPISPOT 40 of 105 (38.1%), and for CellCollector-based IF-staining 46/105 (43.8%).

Discussion

Molecular analysis of CTCs holds great promise to unravel the biology of cancer cell dissemination and identify gene targets and signaling pathways relevant to therapeutic interventions. Understanding the tumor heterogeneity of CTCs by multimarker profiling allows for a better understanding of the mechanisms involved in cancer progression with potential implications for improving treatment strategies *(18, 21, 22)*. Molecular characterization of CTCs has been explored so far at the gene expression *(18, 22–24)*, DNA methylation *(25, 26)*, and DNA mutation levels *(27–32)*. Large-scale translational trials will hopefully drive CTC analysis toward more widespread clinical use *(33)*.

In vivo isolation of CTC technology was first used in patients with metastatic PCa, in which tumor-associated transcripts of *EGFR* and *PSMA* were detected in 42.8% and 14.3%, respectively, of the analyzed samples *(12)*. Using dPCR, mutations in the *KRAS* and *EGFR* genes relevant for treatment decisions could be detected in in vivo-captured CTCs in lung cancer *(13)*. Recently, our group has shown that in nonmetastatic PCa patients, CTC detection can be significantly improved when combining the CellSearch, IF analysis of in vivo-isolated CTCs using the CellCollector and the PSA-EPISPOT assay *(14)*.

Molecular assays for studying gene expression on CTCs take full advantage of the extreme sensitivity and specificity of RT-qPCR. Multiplexing requires the presentation of evidence demonstrating that accurate quantification of multiple targets in a single tube is not compromised. This concern is of particular importance when targets of appreciably lower abundance are coamplified with highly abundant targets *(17)*. The use of reliable multimarker RT-PCR assays can increase the sensitivity and specificity of CTC detection and give the ability to save precious samples and reduce the cost and time of analysis *(17, 24, 34, 35)*. Our group has developed assays for simultaneous detection of the expression of various genes in CTCs based on RT-qPCR *(17)* and liquid bead array *(23, 36)*.

In metastatic prostate cancer, CTC enumeration using the CellSearch system has been cleared by the FDA *(15)*, and CTC counts were found to be superior to PSA concentration measurement in predicting overall survival *(37)*. In contrast, the clinical relevance of CTCs in nonmetastatic PCa is unclear.

In this study, for the first time, we combined in vivo CTC isolation with downstream molecular RNA analysis. This combination is less prone to preanalytical errors that commonly result from the instability of CTCs in peripheral blood during transportation of samples to central laboratories for downstream CTC analysis, because the captured CTCs are immediately lysed in Trizol, where the nucleic acids are stable. We developed and validated 3 novel multiplex RT-qPCR assays for detecting gene expression of epithelial, stem cell and EMT markers, and PSA in tumor cells captured in vivo by the CellCollector in peripheral blood of nonmetastatic high-risk PCa patients. We further compared our findings by analyzing the peripheral blood of the same patients and the same blood draw with CellSearch, EPISPOT, and IF-staining. Our findings revealed an unexpectedly high incidence of CTCs by molecular assays. On the contrary, published reports using the CellSearch system showed positivity rates of 5%–27% *(38)*.

When we compared gene expression in paired samples before and after surgery or radiotherapy, CTC rates detected by the molecular assays did not display a statistically significant difference after surgery (74.1% vs 66.6%). However, it is important to note that EMT markers were detected in only 7% of patient samples before therapy but in 63.0% of samples after therapy, a finding indicating the survival of EMT cells during therapy. Stem cell markers were found to be expressed only in the group of patients before surgery or radiotherapy, whereas epithelial markers were detected in a small percentage of patient samples (7.4%) after therapy. This indicates that after the removal of the primary tumor, fewer epithelial EpCAM$^+$ CTCs would circulate in the blood stream but there are many cancer cells that circulate with a different phenotype. Because EpCAM-based enrichment alone cannot detect all CTC subpopulations, the detection of multiple genes by molecular assays is quite important.

Our experiments are focused solely on gene expression at the mRNA level, which can be very important in cancer as this is clearly verified by the success of mRNA-based approaches for breast cancer, such as Oncotype Dx

(based on RT-qPCR), Mammaprint assay (mRNA level, FDA-cleared assay), PAM50, and many others.

However, regulation of protein expression is complex and is not controlled only by mRNA expression; miRNAs, lnRNAs, as well as epigenetic regulation by DNA methylation in the promoter region of tumor suppressor genes, can affect protein expression. Detection of multiple protein markers on CTCs is not feasible because the number of antibodies that can be used in parallel in IF experiments is limited. A mass spectrometry-based proteomics approach is still not sensitive enough to be applied to CTCs; however, very recently, a microfluidic western blot for an 8-plex protein panel for individual CTCs derived from estrogen receptor-positive (ER+) breast cancer patients has been described *(39)*.

Each CTC assay consists of an enrichment and a detection step. In the present study, for enrichment, we used an in vivo device that captures EpCAM-positive cells in the blood during a 30-min exposure, as described in our previous reports *(13, 14)*. The tumor nature of the captured EpCAM+ cells has been demonstrated by genomic analyses *(13)*. For CTC detection, we used RT-qPCR for tumor-associated transcripts, which is one of the key technologies used in CTC research *(8)*, and this approach has been validated in our previous CTC publications *(18, 22)*. The specificity of the present transcripts for the detection of tumor cells in blood has been demonstrated in our study by careful analyses of blood from healthy controls. To the best of our knowledge, the combination of the in vivo capture device with multiplex RT-qPCR for CTC detection is novel, and we have followed the internationally accepted procedure of testing new CTC devices.

Our findings suggest that in addition to CTCs expressing epithelial antigens, a subgroup of CTCs have an EMT phenotype that could preexist but remained undetected when using detection assays based exclusively on epithelial markers. We now know that CTCs are highly heterogeneous; thus, it is expected that different subgroups of CTCs would be detected by different methodologies. Our comparison studies of RT-qPCR with the CellSearch, EPISPOT, and IF-staining have shown poor agreement. EMT markers were the most frequently expressed, and this could explain the low concordance with CellSearch, which is fully based on epithelial markers. It will be interesting to elucidate in future studies whether these EMT-transitioned CTCs are clinically important or whether they are they apoptotic cells. In a very recent study, it was shown that cell-surface vimentin (CSV)-guided CTC enumeration may hold prognostic value and should be further validated as a possible measurement of prostate cancer progression toward the deadly, androgen-independent form *(40)*. Our findings suggest that detection systems based on only epithelial-cell surface markers, such as EpCAM and cytoskeletal proteins, such as CKs *(40)*, are not the ideal for evaluating and characterizing heterogeneous CTCs with an EMT phenotype. The gene expression assays described here also could be used in combination with the use of additional antibodies such as CSV for CTC isolation *(40)*.

In conclusion, the combination of in vivo isolation of CTC with downstream highly specific and sensitive RT-qPCR assays is minimally invasive, capable of high throughput, and suitable for the development of molecular diagnostic applications. The relevance of the molecular analyses described in this study with respect to the clinical outcome needs to be validated.

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