Direct comparison of size-dependent versus EpCAM-dependent CTC enrichment at the gene expression and DNA methylation level in head and neck squamous cell carcinoma

Martha Zavridou1, Sophia Mastoraki1, Areti Strati1, George Koutsodontis2, Apostolos Klinakis3, Amanda Psyrrī2 & Evi Lianidou1*

We directly compared two different approaches used for Circulating Tumor Cell (CTC) isolation, a size-dependent microfluidic system versus an EpCAM-dependent positive selection for downstream molecular characterization of CTC both at the gene expression and DNA methylation level in Head and Neck Squamous Cell Carcinoma (HNSCC). A size-dependent microfluidic device (Parsortix, ANGLE) and an EpCAM-dependent positive immune-magnetic isolation procedure were applied in parallel, using 10 mL PB from 50 HNSCC patients and 18 healthy donors. Total RNA was isolated from enriched CTCs and RT-qPCR was used to study the expression levels of CK-19, PD-L1, EGFR, TWIST1, CDH2 and B2M (reference gene). Real time methylation specific PCR (MSP) was used to study the methylation status of RASSF1A and MLL3 genes. In identical blood draws, the label-free size-dependent CTC-isolation system was superior in terms of sensitivity when compared to the EpCAM-dependent CTC enrichment, since a significantly higher percentage of identical PB samples was found positive at the gene expression and DNA methylation level, while the specificity was not affected. Our results indicate that future studies focused on the evaluation of clinical utility of CTC molecular characterization in HNSCC should be based on size-dependent enrichment approaches.

Liquid biopsy provides a valuable source of biomarkers on prognosis and response to treatment of cancer patients4 and has recently shown a significant potential even for early cancer diagnosis and screening5. Isolation of circulating tumor cells (CTCs) from peripheral blood (PB) and their further downstream molecular characterization at the DNA, RNA and protein level is very important for reliable liquid biopsy analysis3. However, the identification and molecular characterization of CTCs is very challenging since these cells are extremely rare, and the amount of available sample for analysis in most cases is very limited1,6.

A variety of molecular assays have been developed for CTCs detection and molecular characterization. Molecular assays are based on total RNA isolation from CTCs and subsequent mRNA quantification of specific genes, and gDNA isolation for mutation analysis and DNA methylation studies5. CTC molecular characterization at the gene expression level has the potential to elucidate the critical signaling pathways involved in metastasis biology and even improve patient management. We have shown many years ago that the detection of CK-19 expression in CTCs has prognostic significance in both early and metastatic breast cancer5-7. Beyond gene expression, DNA methylation analysis in CTCs has a high potential to provide novel epigenetic biomarkers for diagnosis, prognosis, risk assessment, and disease monitoring in many types of cancer8. Based on this, we selected

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1Analysis of Circulating Tumor Cells Lab, Department of Chemistry, University of Athens, Athens, 15771, Greece.  
2Oncology Unit, 2nd Department of Internal Medicine - Propaedeutic, Attikon University Hospital, National and Kapodistrian University of Athens, Athens, Greece.  
3Biomedical Research Foundation Academy of Athens, Athens, 11527, Greece. *email: lianidou@chem.uoa.gr
two tumor-suppressor genes namely, KMT2C/MLL3 and RASSF1A and evaluated their methylation status in CTCs of HNSCC patients. Recently, it was shown that down-regulation of lysine-specific methyltransferase 2 C (KMT2C/MLL3), a putative tumor suppressor leads to epigenetic and expression changes of DNA repair genes. We also selected RASSF1A, since it has been shown that a high frequency of RASSF1A methylation is associated with more aggressive tumour phenotype among different cancer types, while a recent meta-analysis suggested that there was a significant association between aberrant RASSF1A methylation and Head and Neck Squamous Cell carcinoma (HNSCC).

CTC-enrichment procedures are necessary prior to their molecular characterization, mainly for the reduction of background due to the presence of peripheral blood mononuclear cells (PBMCs). However, CTCs are highly heterogeneous and can have a different profile in different types of cancer. CTC heterogeneity and their phenotypic variation complicate their enrichment and subsequent phenotypic and molecular characterization. Moreover, the effect of pre-analytical conditions and the establishment of quality control procedures in each analytical step is very important and very critical for CTC molecular characterization both at the gene expression and DNA methylation level.

Over the past few years, a plethora of CTC isolation technologies based on their different biological and physical characteristics have been developed, including immune-magnetic, microfluidic, size dependent and function-based methods. In the CellSearch™ system (Menarini Silicon Biosystems), the only Food and Drug Administration (FDA) approved approach for CTC detection and enumeration so far, CTCs enrichment is based on a positive selection targeting EpCAM-positive cells. However, it is obvious that in all EpCAM-based CTC-capture systems, EpCAM-negative CTC subpopulations are non-detectable. This can have serious implications in cases where CTCs are characterized by a phenotypic plasticity that mainly reflects an epithelial to mesenchymal transition state (EMT). EMT results in down-regulation of epithelial markers like EpCAM and simultaneous up-regulation of mesenchymal markers. The lack of expression of epithelial markers on CTCs due to EMT has resulted in the development of novel procedures for CTCs isolation that are based on label-free microfluidic devices, size-based filtration, or combination of microchips and positive selection through specific antibodies. Antibody combinations on immunomagnetic beads when compared to anti-EpCAM antibodies enable capturing of a larger number of CTCs.

Head and Neck Squamous Cell carcinoma (HNSCC) is a devastating disease and novel treatments are urgently needed. Molecular characterization of CTCs can be a powerful prognostic tool while serial assessments of CTCs at different time points during treatment may guide treatment decisions. EMT is a common phenomenon in HNSCC progression, thus EpCAM-based approaches can be suboptimal for CTC isolation. We have recently shown that in HNSCC the detection of CTCs overexpressing PD-L1 provides important prognostic information and could be used for the selection of personalized medicine and treatment monitoring.

The aim of the present study was to select the optimal enrichment system for CTC downstream molecular characterization in HNSCC. For this reason we directly compared the performance of a label-independent size-based microfluidic device versus an EpCAM-based CTC enrichment system using identical blood draws, and downstream molecular characterization both at the gene expression and DNA methylation level.

Results

RNA-based analysis. Comparison between size-dependent and EpCAM-dependent CTC-enrichment at the gene expression level. We first evaluated in a quantitative way the performance of these two different CTC enrichment approaches by downstream RNA-based CTC analysis run in parallel, using exactly the same procedure and the same RT-qPCR assays in material extracted from 50 HNSCC patients and 18 HD. Our results clearly reveal a significant difference in the expression levels of B2M (reference gene) in CTCs isolated through Parsortix versus EpCAM-dependent CTC enrichment. In all molecular assays, reference genes are selected on the basis that they are expressed at the same level both in the CTCs and in the PBMC fraction, as we have previously described.

More specifically, as shown in Fig. 1A, Cq values for B2M were significantly lower in the EpCAM-based CTC-enrichment, indicating a higher number of B2M transcripts that definitely are due to a higher number of non-specific contaminant cells which are in their absolute majority PBMCs co-isolated with CTCs. B2M expression did not differ between HD and HNSCC within each CTC enrichment system as expected, since this reference gene is expressed at similar levels both in CTCs and PBMC, and the number of CTCs is very low (Fig. 1A).

Size-dependent CTC-enrichment. All samples isolated with the size-dependent microfluidic device (Parsortix) were of excellent RNA quality as this was verified by our RT-qPCR assay for B2M. Relative fold change values (2^{-ΔΔCt}) for PD-L1 normalized according to B2M for individual samples are shown in Fig. 1B. Relative PD-L1 expression in the Parsortix was 0.48 (range: 0–2.39) in HD, 0.71 (range: 0–2.48) in PD-L1 negative HNSCC patients and 5.94 (range: 3.07–11.63) in PD-L1 positive HNSCC patients. Relative fold change values (2^{-ΔΔCt}) for TWIST1, and CDH2 normalized according to B2M for individual samples are shown in Fig. 1C,D. Mean fold change of TWIST1 expression in the Parsortix was 0.75 (range: 0–5.35) in HD, 0.10 (range: 0–2.04) in TWIST1 negative HNSCC patients and 22.79 (range: 18.90–982.29) in TWIST1 positive HNSCC patients. Mean fold change of CDH2 expression in the Parsortix was 0.45 (range: 0–1.95) in HD, 0.13 (range: 0–1.35) in CDH2 negative HNSCC patients and 22.26 (range: 4.08–58.49) in CDH2 positive HNSCC patients. Our results for all genes tested are also shown in a heatmap (Fig. 2); PD-L1 was overexpressed in 9/50 (18.0%) and CK-19 in 11/50 (22.0%) cases.
In 4/11 (36.4%) of CK-19 positive samples PD-L1 was also overexpressed. EGFR expression was not detected in any sample 0/50 (0.0%). Concerning EMT markers, TWIST1 was overexpressed in 5/50 (10%), and CDH2 in 3/50 (6%). None of 18 samples of the control group (HD) was found positive for mRNA expression for all genes tested.

**EpCAM-dependent CTC enrichment.** Using EpCAM-dependent CTC enrichment, all samples were also of excellent RNA quality as this was certified by RT-qPCR for \(\beta_{2}M\). Relative fold change values \((2^{-\Delta\Delta Cq})\) for TWIST1 normalized according to \(\beta_{2}M\) are shown in Fig. 1C. Mean fold change of TWIST1 expression in the EpCAM-based enrichment was 0.92 (range: 0–3.16) in HD, 0.12 (range: 0–1.48) in TWIST1 negative HNSCC patients and 10.63 (range: 3.2–31.12) in TWIST1 positive HNSCC patients. Relative fold change values \((2^{-\Delta\Delta Cq})\)

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**Table 1.** Direct comparison of gene expression in HNSCC and HD in CTC enriched using size-dependent enrichment (Parsortix) vs EpCAM-dependent CTC enrichment system using identical blood draws. (Red: gene expression, green: no expression).

| CTC enrichment | Gene Target | Healthy individuals (n=18) | HNSCC patients (n=50) |
|----------------|-------------|----------------------------|-----------------------|
| Parsortix (10mL PB) | B2M | | |
| | CK19 | | |
| | PD-L1 | | |
| | EGFR | | |
| | TWIST1 | | |
| | CDH2 | | |
| EpCAM-based (10mL) | B2M | | |
| | CK19 | | |
| | PD-L1 | | |
| | EGFR | | |
| | TWIST1 | | |
| | CDH2 | | |

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**Figure 1.** RT-qPCR results using size-dependent enrichment (Parsortix) and EpCAM-dependent CTC enrichment for: (A) \(\beta_{2}M\) (reference gene); (B) CD-45 (specific leukocyte marker) (C) Relative fold change values \((2^{-\Delta\Delta Cq})\) for reference gene (\(\beta_{2}M\)) and target gene (PD-L1) for healthy individuals and HNSCC patients’ CTC samples; (D) Relative fold change values \((2^{-\Delta\Delta Cq})\) for reference gene (\(\beta_{2}M\)) and target gene (TWIST1); (E) Relative fold change values \((2^{-\Delta\Delta Cq})\) for reference gene (\(\beta_{2}M\)) and target gene (CDH2) Red horizontal line: cutoff value. Different colors represent individual patients.

**Figure 2.** Direct comparison of gene expression in HNSCC and HD in CTC enriched using size-dependent enrichment (Parsortix) vs EpCAM-dependent CTC enrichment system using identical blood draws. (Red: gene expression, green: no expression).

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In 4/11 (36.4%) of CK-19 positive samples PD-L1 was also overexpressed. EGFR expression was not detected in any sample 0/50 (0.0%). Concerning EMT markers, TWIST1 was overexpressed in 5/50 (10%), and CDH2 in 3/50 (6%). None of 18 samples of the control group (HD) was found positive for mRNA expression for all genes tested.

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**EpCAM-dependent CTC enrichment.** Using EpCAM-dependent CTC enrichment, all samples were also of excellent RNA quality as this was certified by RT-qPCR for \(B2M\). Relative fold change values \((2^{-\Delta\Delta Cq})\) for TWIST1 normalized according to \(B2M\) are shown in Fig. 1C. Mean fold change of TWIST1 expression in the EpCAM-based enrichment was 0.92 (range: 0–3.16) in HD, 0.12 (range: 0–1.48) in TWIST1 negative HNSCC patients and 10.63 (range: 3.2–31.12) in TWIST1 positive HNSCC patients. Relative fold change values \((2^{-\Delta\Delta Cq})\)
analyses. RASSF1A magnetic enrichment; MLL3 in 0/31 (0%). DNA methylation analysis was performed in 31 samples isolated using the EpCAM-dependent immuno-magnetic enrichment system. More specifically, we studied the expression levels of EGFR, RASSF1A and MLL3 in 3/43 (7%) and MLL3 methylation in 7/43 (16.3%) samples.

A direct comparison study, using identical blood draws, between a label-independent size-based microfluidic enrichment system and an EpCAM-based CTC enrichment system. At least one gene

for CDH2 normalized according to B2M are shown in Fig. 1D. Mean fold change of CDH2 expression in the EpCAM-dependent enrichment was 0.28 (range: 0–1.42) in HD, 0.07 (range: 0–1.41) in CDH2 negative HNSCC patients and 2.29 (range: 2.25–2.33) in CDH2 positive HNSCC patients. Mean fold change of PD-L1 expression in the EpCAM-dependent enrichment was 0.84 (range: 0–1.54) in HD, 0.55 (range: 0–1.66) in PD-L1 negative HNSCC patients and 3.24 (range: 1.72–4.99) in PD-L1 positive HNSCC patients. Our results are shown for all genes tested in a heatmap (Fig. 2); CK-19 and EGFR expression were not detected in any sample tested 0/50 (0%), while TWIST1 was overexpressed in 4/50 (8%), and CDH2 in 2/50 (4%) samples. PD-L1 overexpression was detected in 4/50 (8%) cases. None of 18 samples of the control group (HD) was found positive for mRNA expression for all genes tested.

In HNSCC CK-19 positive CTCs were only detected when enriched using the label-independent Parsortix approach but not in the EpCAM-dependent immunomagnetic CTC enrichment (Fig. 2). PD-L1 overexpression was detected in 9/50 (18.0%) cases after using the Parsortix device but only in 4/50 (8.0%) cases after using EpCAM-dependent CTC-enrichment. Three (#29, #47 and #48) out of four samples found positive for PD-L1 overexpression after using EpCAM-based CTC enrichment were also positive after size-dependent enrichment. CTCs are heterogeneous, so it is possible that in these patients there were populations of PD-L1 positive CTCs that were both EpCAM+ and EpCAM−. EGFR mRNA was not detected in any sample tested, using both approaches.

DNA methylation analysis. Size-dependent CTC-enrichment. Ten peripheral blood samples from healthy donors were processed following exactly the same procedure as the HNSCC samples in order to verify the diagnostic specificity of the DNA methylation analysis assays. None of the HD samples was found positive for RASSF1A and MLL3 methylation (0/10, 0%). DNA methylation analysis was performed in 43 samples isolated using the Parsortix microfluidic device; RASSF1A methylation in 3/43 (7%) and MLL3 methylation in 7/43 (16.3%) samples.

EpCAM-dependent CTC enrichment. Ten peripheral blood samples from healthy donors were processed following exactly the same way as the HNSCC samples in order to verify the diagnostic specificity of the DNA methylation analysis assays. None of the HD samples was found to be positive for RASSF1A and MLL3 methylation (0/10, 0%). DNA methylation analysis was performed in 31 samples isolated using the EpCAM-dependent immunomagnetic enrichment; RASSF1A in 0/31 (0%) and MLL3 promoter methylation in 4/31 (12.9%).

Direct comparison between size-dependent and EpCAM-dependent CTC-enrichment for DNA methylation analyses. For 29 of these patients we had available material for a direct comparison between size-based and EpCAM-dependent CTC-enrichment at the DNA methylation level. The assessment of correlation for DNA methylation profile of each gene of interest between CTCs isolated using the label-independent versus the EpCAM-dependent CTC-enrichment method was performed using the χ² test for these 29 matched samples. We did not observe any statistically significant correlation for the above samples in none of the tumor-suppressor genes tested (Fig. 3).

Discussion

CTC enrichment technologies based on EpCAM present the risk of missing EpCAM-negative CTCs. Recent technological advances have now enabled CTC-enrichment based on different biological and physical properties of CTCs. Label-independent enrichment microfluidic devices for CTC enrichment have the potential to isolate successfully CTCs in an EMT state, since EMT leads to down-regulation of EpCAM.

In order to evaluate the most appropriate system for downstream molecular characterization at the gene expression and DNA methylation analysis level in CTCs from HNSCC patients, we performed for the first time a direct comparison study, using identical blood draws, between a label-independent size-based microfluidic device and an EpCAM-based CTC enrichment system. More specifically, we studied the expression levels of CK-19, PD-L1, EGFR, TWIST1, CDH2 and B2M (reference gene) and the methylation status of RASSF1A and MLL3 genes, that are all indicating the presence of CTCs. Our data clearly indicate that the CTCs population enriched

| CTC enrichment | Healthy individuals (n=10) | HNSCC patients (n=29) |
|----------------|---------------------------|-----------------------|
| Parsortix (10mL PB) | RASSF1A, MLL3 | RASSF1A, MLL3 |
| EpCAM-based immunomagnetic enrichment (10mL PB) | RASSF1A, MLL3 | RASSF1A, MLL3 |

| At least one gene | ACTB | CDH2 |
|-------------------|------|------|
| Healthy individuals (n=10) | 1 2 3 4 5 6 7 8 9 10 | 1 2 3 4 5 6 7 8 9 10 |
| HNSCC patients (n=29) | 1 2 3 4 5 6 7 8 9 10 | 1 2 3 4 5 6 7 8 9 10 |

Figure 3. Direct comparison of DNA methylation analysis markers in HNSCC (n = 29) and HD (n = 10) in CTC enriched using a size-dependent enrichment (Parsortix) vs EpCAM-dependent CTC enrichment system, using identical blood draws. (Red: positive for DNA promoter methylation, green: negative for DNA promoter methylation).
using the label-independent size-based microfluidic device is of higher purity than that of CTCs isolated using
EpCAM-based CTC enrichment; B2M levels revealed a much lower PBMC contamination in the size-dependent
approach compared to EpCAM-dependent CTC enrichment. Our results were in concordance with two recent
studies; Obermayer et al. reported that the Parsortix system was more appropriate to remove leukocytes and
allow for the subsequent molecular analysis in a high purity of the enriched cells. The Parsortix system was also
evaluated as having the highest recovery rate and the lowest leukocyte contamination, compared to two different
CTC isolation methods.

Our results indicate that distinct populations of CTCs are isolated when these two different enrichment
approaches are used. It is clear though that when the size dependent isolation was used a lot more positive events
for the presence of CTCs are detected, eg the same sample is characterized as CTC negative using EpCAM iso-
lation. We report a higher percentage of PD-L1 positive samples following CTC isolation with the Parsortix system
in comparison to the EpCAM-dependent approach. Three out of four samples found positive for PD-L1 over-
expression after using EpCAM-dependent CTC enrichment were also positive after using the Parsortix device. The
clinical significance of this finding will be soon evaluated in an independent study, involving a large number of
HNSCC patients under specific treatments. According to a recent meta-analysis, a high PD-L1 expression in the
tumour cells did not correlate with poor prognosis of patients suffering for oral squamous cells carcinoma. Most
studies published on PD-L1 expression are performed in the primary tumour and are based on immunohisto-
chemistry, and have shown a significant variation in results, limiting the use of PD-L1 expression by immuno-
histochemistry as a prognostic biomarker in clinical practice. Our results on PD-L1 mRNA overexpression in
CTCs after Parsortix enrichment indicate towards a clinical evaluation of this finding in a large number of clinical
samples.

EGFR mRNA was not detected in any sample tested, using both approaches. We did not observe any correla-
tion in the expression of EMT markers (TWIST1 and CDH2) between the Parsortix and EpCAM-dependent CTC
enrichment. According to our results, the number of patients positive for CDH2 expression is very low, therefore,
to draw any conclusion studying this gene is inappropriate, so we simply report our finding.

In HNSCC several preliminary studies have detected CTCs by using various enrichment-isolation methods
that yielded varying results with respect to the number of CTCs and the frequency of patients with positive
CTCs. In our recent study, when we evaluated the expression of PD-L1 in CTCs of HNSSC patients using
EpCAM isolation, we did not find any CK-19 mRNA expression in CTCs (data not shown). Similar to our results,
Bozec et al. have shown that according to the results obtained by the CellSearch system, CTCs are in a relatively
low proportion in patients with locally advanced HNSSC. Thus, HNSSC is not considered as an exclusively
EpCAM positive type of cancer.

In CTC samples isolated using the Parsortix microfluidic device a significantly higher percentage of samples
positive for MLL3 and RASSF1A promoter methylation was detected when compared to paired samples isolated
using the EpCAM-positive immunomagnetic approach. RASSF1A and MLL3 were found to be methylated in
CTCs at various percentages, confirming the presence of heterogeneity, even in CTCs isolated from the same
patient.

There is an urgent need for the evaluation of technologies investigating the expression, mutation and DNA
methylation status of CTCs and it is important to select the best system in every type of cancer. Lampignano et
al. developed a workflow using the Parsortix system for single CTC analysis, permitting for the first time assess-
ment of the heterogeneity of PIK3CA mutational status within patient-matched EpCAM-high and EpCAM-low
CTCs. Furthermore, Gorges et al. established reliable workflows in order to study multi-marker profiles of
single CTCs by qPCR approaches. These workflows were combined with Parsortix system enabling the recovery
of higher quality RNA. El-Heliebi et al. compared three different CTC isolation systems for gene expression
and DNA mutation analysis in CTCs of prostate cancer patients and showed also differences. The incorporation
of microfluidics into CTC isolation is now emerging for clinical applications. Many microfluidic technologies have
reported high sensitivity and specificity for capturing CTCs, however, the question still remains as to the supe-
riority in comparison to immunoaffinity based approaches, specifically to identify different CTC populations.

In conclusion, distinct populations of CTCs are isolated with these two different enrichment technologies. It is
clear though that when the size dependent isolation was used a lot more positive events for the presence of CTCs
are detected. The clinical significance of this finding will be soon evaluated in an independent prospective study,
involving a large number of HNSSC patients under specific treatments.

Materials and Methods
The experimental flowchart of the study is outlined in Fig. 4.

Peripheral blood samples collection. Fifty patients with squamous cell carcinoma of the oral cavity, oro-
pharynx, hypopharynx or larynx and eighteen healthy donors were included in this study. Two of these patients,
heavy smokers, were positive for HPV. In oral cavity and hypopharynx no HPV testing is indicated, while in some
hypopharynx cases HPV testing is not feasible because we have only cytology. Blood specimens were obtained
before initiation of treatment. The first 5 ml of blood were discarded to avoid skin epithelial cells contamina-
tion. Peripheral blood was then collected into two 10 ml K2EDTA tubes (BD Vacutainer, Plymouth, UK) and
mixed immediately after blood draw by inverting gently 10 times, maintained at room temperature (RT) and
processed within 3 h, to CTC isolation. A size-dependent microfluidic device (Parsortix, ANGLE plc, UK) and an
EpCAM-dependent positive immunomagnetic enrichment procedure were applied simultaneously, using 10 ml
PB in each case. All patients gave their informed consent for participating in this study. IRB approval for the
collection of samples was obtained from Attikon University Hospital, National and Kapodistrian University of
Athens, Greece, (EBΔ472/30–10–14). Attikon University Hospital approved the research and all methods were
performed in accordance with the relevant guidelines and regulations.
CTCs enrichment using a size-based microfluidic device. The Parsortix CE-marked system (ANGLE plc, UK), based on micro-fluidics was used to capture and then harvest CTCs from 10 mL whole blood collected in K$_2$EDTA tubes. Separation of blood components took place in a microscope slide sized disposable cassette, which contains a series of steps leaving a 6.5 μm measuring gap between the top cover and the final step. Following enrichment, CTCs were harvested in a total volume of 200 μL of PBS deposited into 1.5 mL Eppendorf tubes by applying a reverse flow to the cassette using a specific software protocol. Spiking experiments were performed using 100 cells spiked in 10 mL peripheral blood of a healthy donor using the SCC-47 NHSCC cell line. According to our results, b2M (reference gene) and CK-19 (epithelial marker) transcripts were quantified in these cells before and after spiking. B2M and CK-19 transcripts were detected in these spiked samples after enrichment using the Parsortix.

CTCs enrichment using EpCAM-dependent immunomagnetic approach. Magnetic beads, coated with the monoclonal antibody BerEP4 against the human epithelial antigen, EpCAM, were used for CTCs enrichment (Dynabeads® Epithelial Enrich, Life Technologies, USA) from 10 mL whole blood collected in K$_2$EDTA tubes as previously described.

RNA extraction and cDNA synthesis. Total RNA from CTCs was isolated using TRIZOL-LS (ThermoFisher Scientific, USA), followed by cDNA synthesis as previously described.

RT-qPCR. We used our previously developed and analytically validated RT-qPCR assays for PD-L1, keratin 19 (CK-19), epidermal growth factor receptor (EGFR) and beta-2-microglobulin (B2M) (used as a reference gene). A multiplex RT-qPCR was performed for the quantitative determination of EMT markers, cadherin 2 (CDH2), and TWIST family transcription factor 1 (TWIST1) as previously reported. All RT-qPCR reactions were performed in the LightCycler 2.0 (IVD instrument, Roche Diagnostics, Germany) following the MIQE guidelines. The amplification reaction mix for B2M contained 1 μL of the PCR Synthesis Buffer (5X), 1.2 μL of MgCl$_2$ (25 mM), 0.15 μL of dNTPs (10 mM), 0.3 μL BSA (10 μg/μL), 0.1 μL Hot Start DNA polymerase (HotStart, 5 U/μL, Promega, USA), 0.25 μL of forward and reverse primer (10 μM), 0.83 μL of hydrolysis probe (3 μM). 1 μL of cDNA was added in the PCR mix and dH$_2$O was added to a final volume of 9 μL. Protocol conditions: 1 cycle at 95 °C for 2 min, followed by 45 cycles of: 95 °C for 10 s, annealing at 58 °C for 20 s and extension at 72 °C for 20 s, and a final cooling cycle at 40 °C for 30 s. In each RT-qPCR run we used the same cDNA as a positive control in order to evaluate the accuracy and reproducibility of the results. For this purpose we aliquoted cDNA from MCF-7 cells and then stored these aliquots at −80°C. The expression levels of PD-L1, CDH2, and TWIST1 were normalized using the $2^{-ΔΔCt}$ approach in respect to the expression of B2M.
gDNA isolation from CTCs. gDNA was extracted from CTCs using the TRIZOL-LS reagent (ThermoFisher Scientific, USA) as previously described. Isolated gDNA was dissolved in 30μL of 8 mmol/L NaOH.

Sodium Bisulfite (SB) treatment. gDNA samples were treated with SB, to convert non-methylated cytosines to uracil, while methylated cytosines were not converted, using the EZ DNA Methylation Gold Kit (ZYMO Research, USA). SB-treated DNA was stored at –70°C until use. In each SB reaction, dH2O and 100% methylated DNA were included as negative and positive control respectively.

Real-time MSP. We used our previously designed and validated real time MSP assays for each gene of interest. All experiments for RASSF1A methylation analyses were performed in the LightCycler 2.0 (IVD instrument, Roche, Germany), whereas MLL3 methylation analysis was performed in the 96-well plates of LightCycler® 480 system (IVD, Roche Molecular Diagnostics, Switzerland) in a total volume of 10μL. We report a sample as methylation positive, when we detect an MSP amplification signal (Cq<40.00) and as methylation negative only in the complete absence of amplification signal.

Quality control. Quality control checks were performed in all steps prior to sample analysis. In each step of the analytical procedure we included appropriate positive and negative controls in order to ensure the quality and reproducibility of results as previously described. Before proceeding to the SB-treatment and real-time MSP, we assessed the gDNA integrity of all samples by amplifying the PIK3CA exon 20. Only samples that were positive for PIK3CA exon 20 amplification were further processed to SB treatment. After SB-treatment, converted DNA was also checked by a real-time PCR assay for β-actin (ACTB) and samples that were not amplified were excluded from the study. Human placental genomic DNA (gDNA; Sigma-Aldrich, USA) was used as a real-time MSP negative control after SB-treatment, while Universal Methylated Human DNA Standard (ZYMO Research, USA) was used as fully methylated (100%) positive control.

Statistical analysis. We performed statistical evaluation of data using SPSS (SPSS Statistics 25.0). We used the chi-square test of independence, and the Mann Whitney test (SPSS, version 25.0) to make comparisons between groups. A level of P<0.05 is considered statistically significant.

Conclusions

Our data indicate that molecular characterization of CTCs based on a label-free size-dependent isolation CTC system, gives superior results compared to the EpCAM-dependent approach, in HNSCC patients. The clinical significance of CTC detection using this approach remains to be elucidated in prospectively collected well-defined patient cohorts. Our results indicate that future studies focused on the evaluation of clinical utility of CTC molecular characterization in HNSCC should be based on size-dependent enrichment approaches.

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The authors declare no competing interests.

Additional information
Correspondence and requests for materials should be addressed to E.L.

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