

Multivalent Binding and Biomimetic Cell Rolling Improves the Sensitivity and Specificity of Circulating Tumor Cell Capture



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Abstract

Purpose: We aimed to examine the effects of multivalent binding and biomimetic cell rolling on the sensitivity and specificity of circulating tumor cell (CTC) capture. We also investigated the clinical significance of CTCs and their kinetic profiles in patients with cancer undergoing radiotherapy treatment.

Experimental Design: Patients with histologically confirmed primary carcinoma undergoing radiotherapy, with or without chemotherapy, were eligible for enrollment. Peripheral blood was collected prospectively at up to five time points, including before radiotherapy, at the first week, mid-point and final week of treatment, as well as 4 to 12 weeks after completion of radiotherapy. CTC capture was accomplished using a nanotechnology-based assay (CarioCyte) functionalized with aEpCAM, aHER-2, and aEGFR.

Results: CarioCyte was able to detect CTCs in all 24 cancer patients enrolled. Multivalent binding via poly(amidoamine)

dendrimers further improved capture sensitivity. We also showed that cell rolling effect can improve CTC capture specificity (% of captured cells that are CK⁺/CD45⁻/DAPI⁺) up to 38%. Among the 18 patients with sequential CTC measurements, the median CTC decreased from 113 CTCs/mL before radiotherapy to 32 CTCs/mL at completion of radiotherapy ($P = 0.001$). CTCs declined throughout radiotherapy in patients with complete clinical and/or radiographic response, in contrast with an elevation in CTCs at mid or post-radiotherapy in the two patients with known pathologic residual disease.

Conclusions: Our study demonstrated that multivalent binding and cell rolling can improve the sensitivity and specificity of CTC capture compared with multivalent binding alone, allowing reliable monitoring of CTC changes during and after treatment. *Clin Cancer Res*; 1–9. ©2018 AACR.

Introduction

Circulating tumor cells (CTCs) are an important biomarker in cancer management (1, 2), as CTCs have provided prognostic information in patients with breast (3–5), prostate (6–8), colorectal (9–11), and lung cancers (4, 12). Despite the initial enthu-

siasm, however, clinical applications of CTCs remain limited. This is in part due to the relatively low sensitivity and specificity of existing CTC capture assays. CTCs are extremely rare, consisting of as few as one in a billion cells in the blood (1–4). To capture, enrich, and then correctly identify the small number of tumor cells in blood is a difficult process. Existing CTC technologies rely on capturing cells with antibodies against cancer-specific epithelial markers such as epithelial cell adhesion molecule (EpCAM; refs. 2, 5–8). However, affinity-based capture methods are limited by the low binding affinity of the antibodies to cells, as well as the heterogeneity of expression of the target antigens (9–11). Because of such limitations, the CTC capture rate of existing assays is typically less than a few cells per milliliter of peripheral blood (12). Another limitation of existing technologies is their low specificity (percentage of cells captured that are tumor cells), as many leukocytes are captured due to nonspecific binding (13, 14). Such low specificity limits the use of CTCs as liquid biopsy specimens for genomic analysis and precision medicine.

We have previously shown that multivalent binding and biomimetic cell rolling can potentially improve CTC capture in preclinical studies (15, 16). In addition, our group and others have demonstrated that the use of multiple antibodies targeting tumor cells, that is, aEpCAM, aHER2, and aEGFR (their cocktail is collectively termed as ABmix in this article), can also improve CTC capture (17–19). Using these strategies, we have engineered a CTC

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Translational Relevance

Circulating tumor cell (CTC) is an important cancer biomarker, but its application has been limited by the low sensitivity and specificity of the existing assays. Our study examined the use of multivalent binding and biomimetic cell rolling to improve the sensitivity and specificity of CTC detection. Using blood samples from a diverse cohort of patients receiving cancer radiotherapy treatment, we demonstrated that multivalent binding improves CTC capture sensitivity, and cell rolling improves CTC detection specificity. Furthermore, our data suggest that CTC changes during treatment are predictive of radiotherapy response. Our findings can be applied to CTC detection that is clinically significant.

device, CapiroCyte, to improve capture of CTCs. In this study, we aimed to examine the effects of multivalent binding, cell rolling, and multiple antibodies on CTC capture using clinical samples. Another objective of this study was to investigate the changes in CTCs in patients undergoing radiotherapy. There is a general lack of studies examining CTCs in patients receiving radiation treatment (20, 21). Moreover, patients undergoing radiotherapy can provide important information on CTC kinetics during cancer treatment. Finally, we aimed to examine CapiroCyte assay in a diverse (diagnosis) cohort of patients to provide preliminary data on the generalizability of this technique in multiple cancers.

Materials and Methods

Study design

Patients were enrolled on prospective, Institutional Review Board (IRB)-approved study protocols at University of Illinois at Chicago (UIC) (UIC #2013-1033) and the University of North Carolina (UNC)/Lineberger Comprehensive Cancer Center (LCCC1408, NCT02449837). Written informed consents were obtained from all of the patients. The study was conducted according to the Declaration of Helsinki. To be eligible for inclusion, patients had to have a histologically confirmed rectal, cervical, prostate, or head and neck primary carcinoma. Oncologic treatment with radiotherapy per standard of care in the Department of Radiation Oncology at UNC, with or without chemotherapy, was also required. All enrolled individuals provided written, informed consent. Control blood samples from age-matched healthy donors were obtained from Interstate Blood Bank (Memphis, TN), and samples from younger healthy donors were obtained under a separate IRB-approved protocol (UIC #2012-0139).

Patient care was provided, and blood was collected at UNC in Chapel Hill (UNC), and CTC analysis was performed at UIC. Demographic, clinical and treatment data were collected, including patient age, gender, ethnicity, histological subtype, smoking status, concurrent chemotherapy, radiotherapy received, radiotherapy response, disease status at follow-up, and survival (Supplementary Table S1). Posttreatment determination of response to radiotherapy was performed by pathologic assessment when available, and/or radiologic evaluation using Response Evaluation Criteria in Solid Tumors (RECIST). All care providers at UNC were blinded to CTC quantitative analyses, while laboratory

personnel at UIC were blinded to patient characteristics and treatment response. Demographic data for healthy control groups, such as donor age, gender, and ethnicity, were also collected as listed in Supplementary Table S2.

Blood collection and processing

Pretreatment, baseline blood specimens (pre-radiotherapy) for CTC analysis were collected within 1 week before starting radiotherapy, typically on the day of CT simulation for radiotherapy planning or on the day of pretreatment patient set-up. During radiotherapy, specimens were collected at up to three time points, including during the first week of radiotherapy (1W-RT), mid-way through radiotherapy (Mid-RT), and during the last week of radiotherapy (End-RT). Posttreatment (Post-RT) blood specimens were drawn 4 to 12 weeks after the completion of radiotherapy during follow-up clinic appointments.

Approximately 12 mL of whole peripheral blood, drawn from either healthy donors or patients with cancer, was collected into heparin-treated BD Vacutainer tubes to prevent coagulation, except for the first patient enrolled, whose baseline specimen was collected into EDTA-treated BD Vacutainer tubes. Blood specimens were kept at ambient temperature, and shipped from UNC to UIC via overnight express to analyze the specimens within 24 hours after blood collection. Mononuclear cells, including CTCs in buffy coat were separated from whole blood using Ficoll-Paque Plus (Stemcell Technologies Inc., Vancouver, Canada) as described previously (17). After washing the buffy coat twice with 2% FBS-containing PBS, the recovered cells were suspended in 0.2 mL of the complete DMEM media and used for subsequent experiments.

CapiroCyte surface functionalization by immobilization of capture agents

CTCs were measured from the clinical blood samples using CapiroCyte (Fig. 1). This novel CTC detection platform integrates multiple cancer-specific antibodies immobilized on the surfaces functionalized with dendrimers. Under static conditions without flow, the device is indicated as CapiroCyte-S. This assay was compared to CapiroCyte-D, which integrated E-selectin on the surface to induce cell rolling and thus capture CTCs under dynamic flow conditions. Generation 7 (G7) poly(amidoamine) (PAMAM) dendrimers, and all other chemicals, unless noted otherwise, were obtained from Sigma-Aldrich.

Figure 1 depicts the process for CapiroCyte fabrication for CTC capture. The dendrimers, E-selectin, and antibody mixtures (ABmix) were fabricated using surface chemistries as previously described (15, 22) on epoxy-functionalized glass surfaces (Super-Epoxy2, TeleChemo International Inc.). Briefly, surface functionalization was performed using 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide/N-hydroxysulfosuccinimide; EDC/NHS)-based amine-coupling chemistry (15) to sequentially immobilize heterobifunctional polyethylene glycol (PEG, COOH-PEG-NH₂), G7, partially carboxylated PAMAM dendrimers, and antibody mixtures (ABmix) of aEpCAM/TROP1 and aHER-2/TROP1 (R&D Systems), and aEGFR/N-20 (Santa Cruz Biotechnology) for generation of the CapiroCyte-S assay. Human recombinant E-selectin molecules (R&D Systems) were additionally immobilized through covalent bonding to generate the CapiroCyte-D assay. The surfaces were characterized using X-ray photoelectron spectroscopy and fluorescence microscopy to confirm the successful surface functionalization, as reported previously (15–17). The

functionalized surfaces were kept at 4°C, and the experiments using the surfaces were performed within 1 week after the surface preparation.

CTC capture assay

Buffy coat from each blood specimen was divided into two equal parts, one of which was mixed with DMEM media for capture of CTCs under static conditions without flow (CarioCyte-S) and the other directly used for CTC detection under flow (CarioCyte-D). For CarioCyte-D, flow chamber experiments were performed as previously reported (16). Suspension of the isolated buffy coat was injected into the in-house (UIC mechanical shop) flow chamber, which was composed of two connected channels [60 mm (*L*) × 10 mm (*W*) × 0.125 mm (*D*) for each channel; Fig. 1G]. The CarioCyte-D capture of the cells was continuously observed under flow at 25 μ L/min, and then washed with DMEM for 20 minutes and PBS for 15 minutes at 90 μ L/min.

To identify CTCs among the surface-captured cells, a series of immunostaining assays were performed. After fixation with 4% paraformaldehyde for 15 minutes, all captured cells were treated with 0.2 w/v% triton X-100 (penetrating buffer) for 10 minutes to enhance the antibody penetration. To prevent nonspecific binding, whole slides were treated with 2 w/v% BSA solution for 30 minutes before immunostaining. The cells were then sequentially stained with the following antibodies: (i) rabbit antibody against human pan-cytokeratin (CK; 1:50, abcam 9377), (ii) AlexaFluor 594-conjugated secondary antibody against anti-CK (1:100, Invitrogen), (iii) rabbit antibody against human CD45 (1:500, BD Biosciences 555480), and (iv) AlexaFluor 488-conjugated secondary antibody against anti-CD45 (1:100, Invitrogen). The 4',6-diamidino-2-phenylindole (DAPI)-included mounting media (VectaShield Laboratories, Inc.) was also used to stain the nuclei of mononuclear cells and prevent photo-bleaching during analysis.

Standard immunostaining against CK (epithelial marker), CD45 (leukocyte marker), and DAPI (nuclei marker) was performed to identify CK⁺/CD45⁻/DAPI⁺ CTCs among captured cells on the surface (23). The entire capture surface with immuno-

stained cells were scanned using a Zeiss 701 confocal laser scanning microscope equipped with a motorized stage (CLSM, Carl Zeiss, Germany). The 405-nm line of a 30-mW diode UV laser, 488-nm line of a 25-mW multi-line Ar laser, and 561-nm line of a 20-mW solid-state diode laser were used for the excitation of DAPI, AlexaFluor 488, and AlexaFluor 568, respectively. Images were captured using a 20 × /0.8 Plan-Apochromat objective. The scanning speed (at 1 fps) and image quality were constantly controlled with built-in ZEN 2010 software (Zeiss). The number of CK⁺/CD45⁻/DAPI⁺ CTCs on each of the capture surfaces was counted using an integral particle analyzer of ImageJ (NIH). Cells were counted only when they met the criteria set at threshold of 125, pixel² size of >50, and circularity of 0.75–1.00. The capture specificity was calculated by the ratios of the CK⁺/CD45⁻/DAPI⁺ CTC counts per total DAPI⁺ cells, including leukocytes and CTCs.

Statistical analysis

Statistical analyses were performed using SPSS version 21.0 for Windows (IBM Corp.). The difference in absolute CTC numbers between Pre-RT and End-RT for all patients was compared using the paired Wilcoxon signed-rank test. The Mann-Whitney test was used to compare Pre-RT CTC count between different patient tumor and nodal subgroups. The Friedman test was used to determine the statistical difference of absolute CTC levels obtained by the three different CTC capture platforms (PEG-aEpCAM, PEG-ABmix, and G7-ABmix) evaluated. Results were considered statistically significant if the *P* value is <0.05 (two-tailed).

Results

Patient characteristics

A total of 24 patients with cancer with pathologically confirmed rectal (*n*=1), cervical (*n*=1), prostate (*n*=1), larynx (*n*=2), oral cavity (*n*=2), paranasal sinus (*n*=3), or oropharynx (*n*=14) carcinomas were enrolled from June 2014 to December 2014. Patient demographic and clinical information is summarized in Supplementary Table S1. The majority of patients had locally advanced lymph node-positive disease (71%), but only one of 24

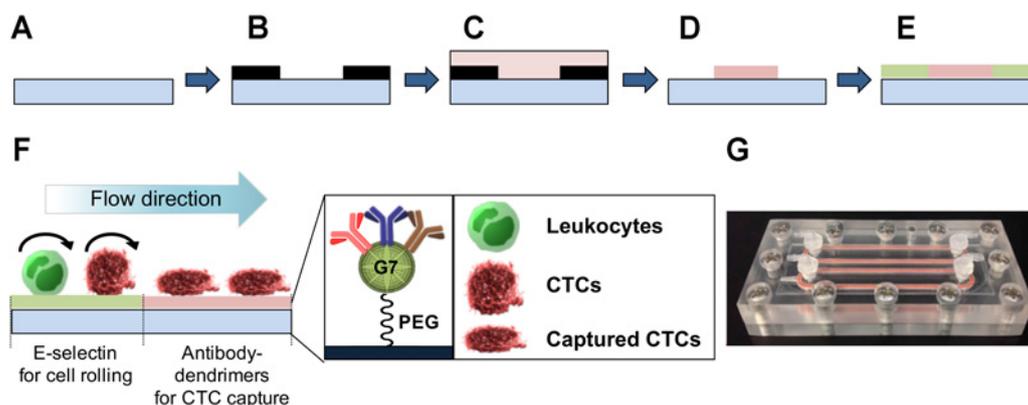


Figure 1.

Schematic diagram of CarioCyte fabrication and its capture mechanism. **A** and **B**, The surface of an epoxy-functionalized glass slide was defined with a patterned polydimethylsiloxane (PDMS) gasket. **C**, The CarioCyte-S surface was then functionalized by a sequential immobilization of PEG, G7 PAMAM dendrimers, and antibody mixtures using the EDC/NHS chemistry. **D** and **E**, For CarioCyte-D, the gasket was removed (**D**), followed by backfilling with E-selectin **E**. **F**, After flowing cells including leukocytes and CTCs were recruited onto the surface by E-selectin-mediated cell rolling, CTCs were isolated from the rolling cells using antibody-dendrimer-coated surface. **G**, The two-channel fluidic system fabricated in house was used for this clinical pilot study.

patients (4%), who had a diagnosis of prostate cancer, had metastatic cancer (M1). Nearly all patients received chemotherapy, most commonly with a concurrent regimen (79%), and two of 24 patients (8%) with nonmetastatic disease received induction chemotherapy before the start of definitive radiotherapy.

At least one on-treatment specimen was collected for subsequent CTC analysis after baseline measurement for a total of 22 of 24 enrolled patients (92%). A final blood draw before completion of radiotherapy was collected from 19 patients (79%). Sixteen patients (67%) had blood specimens collected 4 to 12 weeks after radiotherapy. The median follow-up time since the date of study enrollment before the start of radiotherapy was 10.3 months (range, 4 to 17 months).

CTC capture in patients with cancer undergoing radiotherapy

Blood specimens from 10 healthy participants (21–33 years) without a cancer history were used to establish the detection thresholds (Fig. 2A) for CTC capture. In the control population, the number of cells falsely detected as CTCs using CapiroCyte-S and CapiroCyte-D were 9.7 ± 0.9 and 2.4 ± 1.5 cells per mL, respectively, which is significantly lower than the counts we measured from patients' blood samples. Subsequent analysis using CapiroCyte-D of an age-matched, healthy donor population (>50 years) resulted in a broader range of epithelial cells per mL, consistent with previously published work by Renier and colleagues (24). However, the difference between the median numbers of the captured epithelial cells from two healthy donor groups (2 vs. 1 per mL for <35 years and >50 years, respectively) was negligible (Supplementary Fig. S2).

CapiroCyte was able to detect CTCs from all 24 patients with cancer (100%) from blood samples obtained before the initiation of radiotherapy. With the CapiroCyte-S assay, CTC counts ranged from 4 to 1,134 CTCs per mL of whole blood, with a mean and median of 230 ± 62 (SE, standard error of the mean) and 90 CTCs per mL of whole blood, respectively (Fig. 2B). With the addition of E-selectin to the surface (the CapiroCyte-D assay), the mean and median CTC counts were 200 ± 47 and 88 CTCs per mL, respectively, with the counts ranging from 19 to 849 CTCs per mL of whole blood (Fig. 2C). The potential variability of the CapiroCyte-D assay was tested using duplicated blood draw and healthy donors' blood samples. Supple-

mentary Fig. S1 shows that the CapiroCyte-D assay exhibits a high level of reproducibility in detecting clinical CTCs (correlation between duplicated samples of $R^2 = 0.9745$, $P < 0.0001$). Of note, the first patient enrolled was excluded from analysis with CapiroCyte-D because the blood sample was treated with EDTA, which interferes with accurate E-selectin-integrated CTC enumeration as cell rolling does not occur in Ca^{++} and Mg^{++} -deficient conditions (17).

Multivalent binding and multi-antibody approach improve CTC capture sensitivity

To examine the effects of multivalent binding and multi-antibody capture on CTC capture, the CapiroCyte was compared using three different CTC capture surfaces: (i) PEG-aEpCAM; (ii) PEG-ABmix; and (iii) G7-ABmix conjugates (Fig. 3A). A pairwise comparison of each the capture surfaces was performed to determine the contribution of each to the overall enhancement of CTC capture sensitivity. Compared with PEG-aEpCAM (the orange dotted line), the addition of other antibodies (ABmix that includes aEGFR and aHER-2), although modest, enhanced CTC capture by 22.3%. An additional enhancement of CTC capture was obtained when multivalent binding through G7 PAMAM dendrimers (by ~ 1.8 -fold), which was further improved when the dendrimers and ABmix used together (G7-ABmix) by 2.1-fold (Fig. 3B).

Biomimetic cell rolling improves CTC capture specificity

As seen in Fig. 3C, the CTC counts obtained using CapiroCyte-D (with cell rolling) were similar to those of CapiroCyte-S (without cell rolling; $R^2 = 0.9681$) with the same capture surface, indicating that the introduction of cell rolling does not significantly affect CTC detection sensitivity. However, the use of cell rolling notably enhanced the capture specificity and reduced nonspecific capture of leukocytes (shown as green- and blue-stained cells; Fig. 3D). The specificity of CapiroCyte-D (0.66–37.54%) was improved by 6.9-fold on average and up to 170-fold, compared to that of CapiroCyte-S (0.05%–6.78%). The fluorescence images after immunostaining of CTCs (shown as purple-stained cells) clearly show the difference between the absence of E-selectin in the CapiroCyte-S platform (Fig. 3E) and the presence of E-selectin in the CapiroCyte-D platform (Fig. 3F).

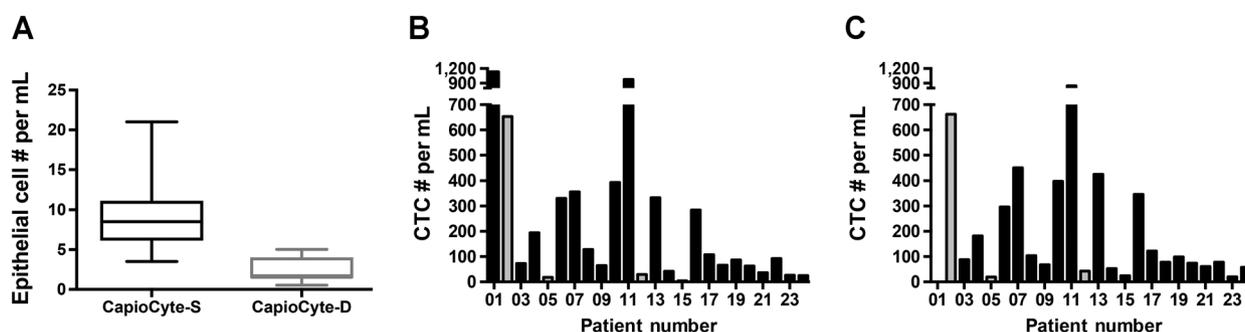


Figure 2.

CTC capture in patients with cancer undergoing radiotherapy. **A**, Epithelial cell counts obtained using blood samples from healthy donors. On the basis of these counts falsely counted as CTCs, the thresholds of CTC counts using CapiroCyte-S and CapiroCyte-D were set at 9.7 ± 0.9 and 2.4 ± 1.5 cells per mL (mean \pm SE), respectively. **B**, Significant CTC counts per mL blood from all patients ($N = 24$) obtained using CapiroCyte-S. The CTC counts from patients with non-head and neck cancer are shown in gray (patient #02: colorectal, patient #05: cervical, and patient #12: prostate) bars. **C**, Significant CTC counts per mL blood from patients ($N = 23$) obtained using CapiroCyte-D. Note that the CTC count for the first patient was not included as the blood sample was treated with EDTA, instead of heparin, destabilizing the rolling response of the cells.

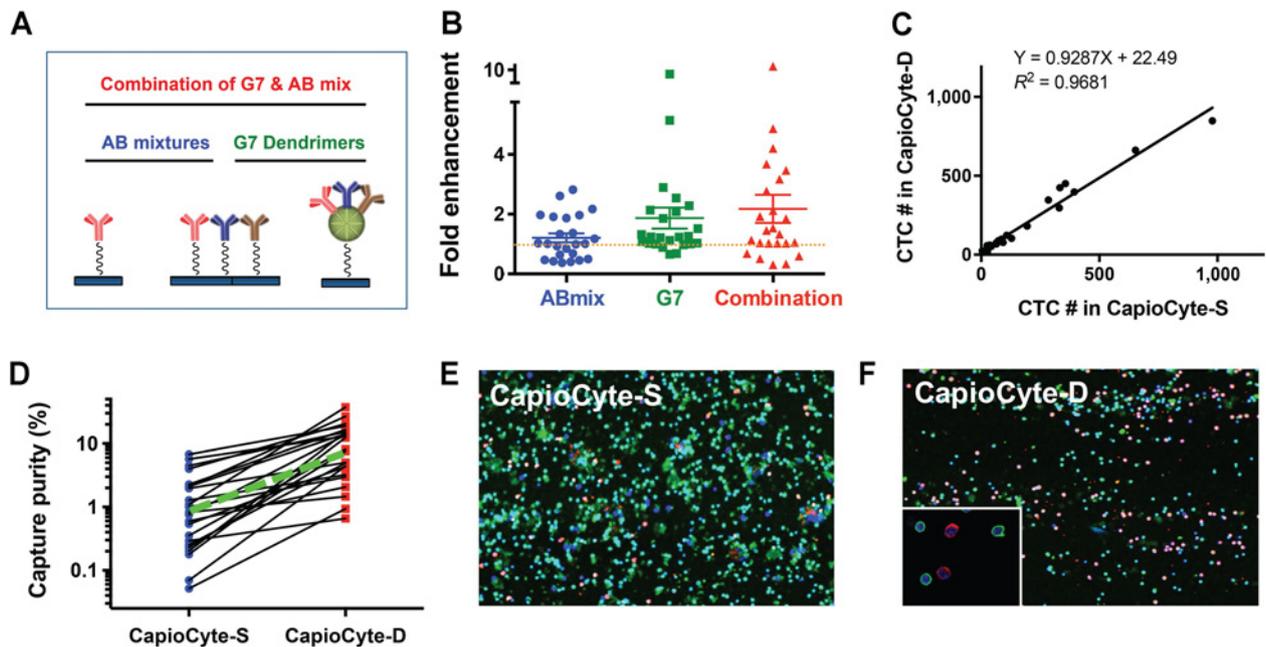


Figure 3.

Enhanced CTC capture sensitivity and specificity via multivalent binding and biomimetic cell rolling using CapioCyte-D. **A–B**, Enhanced CTC capture sensitivity via multivalent binding and multi-antibody approach. **A**, Schematic representation of the effect using antibody mixture (blue), G7 dendrimers (green), and combination of the two (red). **B**, Fold enhancements of antibody mixture, G7 dendrimers, and combination of the two, relative to the CTC counts captured on the control surface coated with aEpCAM only (orange dotted line). **C–F**, Enhanced CTC capture specificity via E-selectin-mediated cell rolling to CapioCyte-D, with addition of E-selectin-mediated cell rolling to CapioCyte-S. **C**, Comparison of the CTC counts measured using CapioCyte-D and CapioCyte-S. **D**, Significantly enhanced CTC capture purities (%) among all captured cells using CapioCyte-D (0.66%–37.54%), compared with those using CapioCyte-S (0.05%–6.78%). This result indicates that the capture specificity of CapioCyte-D was dramatically enhanced via E-selectin-mediated cell rolling. The green line indicates the median values of the data for CapioCyte-S and CapioCyte-D. **E, F**, Representative fluorescence images for CTC count analysis. CTCs (shown in purple) were captured with significantly less leukocytes (green) using CapioCyte-D, compared with CapioCyte-S. An image ($\times 63$ magnification) of the captured CTCs on CapioCyte-S at $\times 63$ magnification is inserted on the representative image of CapioCyte-S at $\times 20$ magnification. All red average lines indicate the mean \pm standard error (SE).

CapioCyte-D using multivalent binding detected the highest number of CTCs at Pre-RT and at End-RT from the clinical blood samples (Table 1). At Pre-RT, there was a significantly higher number of CTC captured with the G7-ABmix surface (199.6 on average of the entire cohorts of the patients), compared with the PEG-aEpCAM (166.1, $P = 0.017$) and the PEG-ABmix (145.0, $P < 0.001$) surface platforms, respectively. This difference in CTC counts between different surface preparations persisted when CTC levels were measured at End-RT [G7-ABmix (47.7 on average) versus PEG-aEpCAM (33.0), $P = 0.002$; G7-ABmix versus PEG-ABmix (36.1), $P < 0.001$]. A comparison of absolute CTC numbers obtained by all three methods demonstrated significantly higher CTC capture for CapioCyte-D (199.6 for Pre-RT, 47.7 for End-RT, $P < 0.001$).

This result supports the conclusion that the effects of multivalent binding on CTC capture sensitivity remains with the use of biomimetic cell rolling.

CTC count is correlated with radiotherapy treatment response

All 23 patients (100%) examined with CapioCyte-D had detectable CTCs in their blood at the Pre-RT, at an average of 200 ± 47 CTCs per mL, significantly higher than 2.0 ± 1.9 CTCs counted per mL of blood found in the samples from three healthy donors ($P < 0.0001$). The concern of false-positive reading was mitigated considering that the highest CTC count from healthy donors were lower than the lowest count from patients. At baseline before the start of radiotherapy, a higher CTC count was not associated with a higher tumor burden as

Table 1. Enhanced CTC capture using G7 dendrimers with cancer-specific antibody mixtures

	Pre-RT CTCs per mL whole blood (N = 23)			End-RT CTCs per mL whole blood (N = 18)		
	PEG-aEpCAM	PEG-ABmix	G7-ABmix ^a	PEG-aEpCAM	PEG-ABmix	G7-ABmix ^a
Mean	166.1	145.0	199.6	33.0	36.1	47.7
Median	42.0	48.0	88	16.0	21.0	31.8
Range	8.5–813.5	16.0–510.5	18.5–848.5	2.0–201.0	0.5–130.5	2.0–149.5
SD	234.7	36.4	46.6	45.1	32.2	38.5
SEM	48.9	24.5–250.8	59.3–321.3	10.6	7.6	9.1
IQR	19.8–208.5	145.0	199.6	12.1–40.8	13.5–53.8	20.1–67.1

Abbreviations: ABmix, antibody mixtures of aEpCAM, aHER-2, aEGFR; aEpCAM, anti-epithelial cellular adhesion molecule; G7, dendrimer generation 7; IQR, interquartile range; PEG, polyethylene glycol; RT, radiotherapy.

^aSignificantly higher absolute CTC count ($P < 0.001$) when compared with other two-capture surface methods, per Friedman statistical testing.

Table 2. Patient characteristics, sequential CTC analysis, and response to radiotherapy (N = 15)

Histology	AJCC stage (TNM classification)	CTCs per mL (whole blood)			CTC change	RT response	CTCs per mL		Clinical status
		Pre-RT	RT ^a	Post-RT (<3 mos)			Post-RT (>6 mos)		
Rectal SCC	IIA (T3N0M0)	662	70	6	Reduction	CR	50	LRR	
Oropharynx SCC	IVA (T2N2bM0)	182	274	76	Elevation	PR/pRD	6	NED	
Oropharynx SCC	IVA (T3N2bM0)	297	197	45	Reduction	CR	—	NED	
Oropharynx SCC	IVA (T2N2aM0)	450	75	40	Reduction	CR	—	NED	
Oropharynx SCC	IVA (T2N2bM0)	104	86	39	Reduction	CR	—	NED	
Oropharynx SCC	IVA (T4aN2cM0)	849	33	51	Elevation	PR/pRD	—	LRR/DM	
Larynx SCC	III (T3N1M0)	424	106	23	Reduction	PR	—	LRR	
Oropharynx SCC	IVB (T1N3M0)	346	35	35	Reduction	CR	—	NED	
Paranasal SNUC	III (T3N0M0)	122	59	54	Reduction	CR	—	NED	
Oropharynx SCC	III (T1N1M0)	78	35	38	Reduction	CR	2	NED	
Oropharynx SCC	IVA (T2N2bM0)	74	35	25	Reduction	CR	—	NED	
Paranasal SNUC	IVA (T4N0M0)	61	29	8	Reduction	CR	—	NED	
Larynx SCC	IVA (T3N2bM0)	78	69	6	Reduction	CR	—	NED	
Oropharynx SCC	IVA (T3N2bM0)	20	49	2	Reduction	CR	11	DM	
Oropharynx SCC	IVA (T2N2bM0)	58	51	3	Reduction	CR	—	NED	

Abbreviations: AJCC, American Joint Committee on Cancer; CR, complete response; DM, distant metastasis; LRR, locoregional recurrence; mos, months; NED, no evidence of disease; PR, partial response; pRD, pathologic residual disease; RT, radiotherapy; SCC, squamous cell carcinoma; SNUC, sinonasal undifferentiated carcinoma; TNM, tumor, lymph node, metastasis.

^aMean of up to three independent CTC measurements during radiotherapy.

significantly lower if the CTC-negative patients' results were included, given the detection rates were only 5% to 26%.

CTC count is correlated with clinical outcomes

Four patients had serial blood specimens drawn for CTC enumeration after completion of radiotherapy in the post-treatment follow-up period (Post-RT). When evaluating the clinical outcomes of these patients, there was further evidence suggesting CTC changes were associated long-term clinical outcomes (Table 2). The patient with anal SCC (Fig. 4D) had a nadir CTC count of 6 CTCs/mL 1 month after completing definitive chemoradiation, substantially decreased from baseline Pre-RT CTC count of 662 CTCs/mL (Fig. 4C) and had a complete clinical response on rectal exam at 1 month and complete radiographic response on PET/CT scan at 2 months posttreatment (Fig. 4E). However, at 6-month follow-up, CTC count rose to 50 CTCs/mL (Fig. 4C). Clinical exam detected a recurrent nodular tumor in the rectum, and PET/CT scan confirmed increased FDG-uptake associated with rectal wall thickening (Fig. 4F). The patient subsequently underwent salvage surgical resection of his recurrent cancer.

CTC changes in the follow-up period were also associated with the development of distant disease or durable complete response to radiation (Table 2). For example, a patient with oropharynx SCC had no evidence of recurrent disease locoregionally during follow-up exam and imaging, but subsequently developed biopsy-proven lung metastases in the setting of a rising CTC level, from a nadir of 2 to 11 CTCs/mL at 1 month and 7 months post-RT, respectively. Also, a patient with 1-month post-RT count of 32 CTCs/mL demonstrated a persistent decline in CTC levels during follow-up that was associated with a long-term durable response to radiotherapy. In the setting of no clinical or radiographic evidence of recurrence, the CTC count decreased to 4 then 2 CTCs/mL at 6 months and 1-year post-RT, respectively. Interestingly, the oropharynx SCC patient with persistent nodal disease who had a decline but required salvage neck dissection for persistent nodal disease at radiotherapy had a drop to 6 CTCs/mL after salvage neck dissection, with no evidence of radiographic or clinical recurrent disease during follow-ups.

Discussion

Detection of CTCs has been technically challenging, primarily due to both the rarity of the cells and the difficulty in capturing these cells using a single antibody. We have previously shown that antibodies immobilized on PAMAM dendrimer surfaces capture cells with high sensitivity and specificity through multivalent binding effects (15, 17, 18). Compared with linear polymer controls, dendrimers have a higher density of reactive sites available for antibody conjugation and a flexible, three-dimensional structure that permits multiple points of contact between the capture surfaces and CTCs at the nanoscale (15, 29–31). When one of the dendrimer-bound antibodies binds a cell, it causes a conformation change of the dendrimer and enables the other antibodies on the same dendrimer to engage the same cell, leading to localized multivalent binding. We showed that such multivalent binding can increase binding affinity up to 1×10^6 -fold (15). In this study, we have validated our hypothesis that multivalent binding can improve CTC capture sensitivity using clinical samples (Fig. 3A and B). We showed that CapioCyte with antibody-dendrimer (multivalent binding) surface captured significantly more CTCs than the same system with antibody-PEG surfaces only.

There have also been strong preclinical data suggesting that the use of a combination of antibodies, rather than just anti-EpCAM, can also improve CTC capture (9, 17, 19). In this study, we compared a mixture of antibodies against EpCAM, EGFR, and HER-2, to EpCAM-only capture. EGFR and HER-2 are overexpressed by various cancer cells and correlates with cancer progression (32, 33). Because of potential nonspecific binding of hematological cells, other mesenchymal markers, such as CD44 and N-cadherin, known to be overexpressed by post-EMT CTCs and/or circulating cancer stem cells were not considered in this study (9). We showed that CapioCyte containing a mixture of antibodies captured more CTCs than CapioCyte containing just aEpCAM, confirming the higher sensitivity of the multi-antibody for approach (Fig. 3A and B).

Our group has also shown that the specificity of CTC capture can be improved with the cell rolling effect (16, 17). With cell rolling, the flow velocity of CTCs as well as leukocytes will be

reduced, which can improve target-specific binding and reduce nonspecific binding. Indeed, we showed that CapiroCyte-D, which induces cell rolling using E-selectin, captured CTCs with higher specificity than CapiroCyte-S, which does not induce cell rolling. Our finding validates our preclinical data and our hypothesis (Fig. 3C–F). Overall, our data showed that the use of multivalent binding, antibody mixture, and cell rolling improves the sensitivity and specificity of CTC capture using CapiroCyte. This study also demonstrated that CapiroCyte containing all three components is a highly sensitive CTC capture assay.

Another key objective of our study was to examine the changes in CTC in patients undergoing radiotherapy. Radiotherapy is an important treatment for cancer, as more than 50% of all cancer patients are estimated to receive radiotherapy during their disease course (34, 35). CTCs, as a biomarker, has the potential to enable response-based adaptive radiotherapy as well as assist in treatment decisions. To our knowledge, we are the first to demonstrate CTC capture in 100% of the patients who are receiving radiotherapy (Fig. 4A and B). In previous studies using CellSearch for CTC capture, the presence and the number of CTCs have been shown to be associated with higher disease stage and poorer prognosis (20, 36, 37). Interestingly, there was no association between pre-RT CTC levels and tumor or nodal stage in our study. A possible explanation for this discrepancy is the enhanced detection sensitivity and specificity of CapiroCyte-D compared with CellSearch (Fig. 4B), which may not have been able to capture CTCs in patients with more favorable disease courses. The CTC counts using CapiroCyte-D were significantly higher than those of CellSearch described in the literature, particularly from patients with HNSCC cancer (Fig. 4B; refs. 25–28). A recent study evaluating CellSearch in HNSCC was only able to detect CTCs in 1 of 3 patients and had a low capture sensitivity (5 CTCs per 7.5 mL; ref. 26), compared with CapiroCyte-D (mean 200 CTCs per 1 mL). Because the CellSearch system employs only a single antibody against EpCAM bound to rigid beads, we attribute higher sensitivity of CapiroCyte-D to biomimetic rolling on E-selectin, the mixture of three different antibodies against epithelial surface markers, and the nanostructured surface that mediates the multivalent binding.

Although the absolute number of CTCs did not appear to correlate with disease status in our study, the comparison of an individual's CTC count before and after the initiation of radiotherapy suggests that CTC monitoring may be able to identify patients who are responding to radiotherapy during the course of treatment. In the majority of patients, CTC levels significantly decreased over the course of radiotherapy and were correlated with patients' clinical response ($P = 0.001$, Fig. 4A). However, the CTC elevations during and after radiotherapy in the two patients with oropharynx cancers were associated with an incomplete response to treatment. Both of these patients had pathologically proven residual carcinoma detected on surgical excision of radiographically concerning residual lymphadenopathy after definitive radiotherapy. Furthermore, posttreatment CTC changes seemed to correlate with long-term patient outcomes after radiotherapy, as rising CTC levels were associated with local recurrence or distant metastasis, whereas persistently declining CTC levels were correlated with persistent, durable disease control. Although some published reports suggest an increase in CTC numbers during the earliest stages of

radiotherapy (38), our results are consistent with a number of studies reporting correlation of CTC numbers with treatment outcome (7, 20). Thus, a simple liquid biopsy using CapiroCyte-D, may allow for earlier and more specific prediction of therapeutic efficacy in patients with cancer undergoing radiotherapy, as well as early detection of recurrent disease in the posttreatment setting, especially in those patients with oropharynx primary malignancies.

In summary, we have demonstrated that the use of cell rolling and multivalent binding with multiple antibodies improves the CapiroCyte assay's specificity and sensitivity, respectively. We have also shown that CTC changes during radiotherapy treatment are correlated with patients' clinical response, suggesting that CTCs may be an important and predictive biomarker for radiotherapy treatment. Our findings are being further validated in a larger cohort of patients.

Disclosure of Potential Conflicts of Interest

M.J. Poellmann is an employee of Capiro Biosciences, Inc. T. Zhang reports receiving speakers bureau honoraria from Exelixis, is a consultant/advisory board member for AstraZeneca, Bayer, Exelixis, Genentech, Janssen, and Sanofi, and reports receiving commercial research grants from Acerta Pharma, Janssen, Merrimack, Novartis, OmniSeq, Pfizer, PGDx, and StemCentRx. A.Z. Wang is an employee of and has ownership interests (including patents) at, and reports receiving commercial research grants from Capiro Biosciences. S. Hong is an employee of and has ownership interests (including patents) at, and reports receiving commercial research grants from Capiro Biosciences. No potential conflicts of interest were disclosed by the other authors.

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