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Sensitive and Direct Detection of Circulating Tumor Cells by Multimarker μ-Nuclear Magnetic Resonance[1,2]

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Abstract

Identifying circulating tumor cells (CTCs) with greater sensitivity could facilitate early detection of cancer and rapid assessment of treatment response. Most current technologies use EpCAM expression as a CTC identifier. However, given that a significant fraction of cancer patients have low or even absent EpCAM levels, there is a need for better detection methods. Here, we hypothesize that a multimarker strategy combined with direct sensing of CTC in whole blood would increase the detection of CTC in patients. Accordingly, molecular profiling of biopsies from a patient cohort revealed a four-marker set (EpCAM, HER-2, EGFR, and MUC-1) capable of effectively differentiating cancer cells from normal host cells. Using a point-of-care micro–nuclear magnetic resonance (μNMR) system, we consequently show that this multimarker combination readily detects individual CTC directly in whole blood without the need for primary purification. We also confirm these results in a comparative trial of patients with ovarian cancer. This platform could potentially benefit a broad range of applications in clinical oncology.

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Introduction

Tumor biopsies are routinely performed for diagnosis, prognosis, treatment selection, or, increasingly, for the assessment of treatment response. However, the cost, morbidity, and measurement intervals associated with biopsies are often limiting; thus, less invasive alternatives are being examined. For the last few years, circulating tumor cells (CTCs) have received significant attention as surrogate markers [1–3]. Indeed, emerging evidence suggests that both the absolute number of CTCs and the changes in their number can serve as prognostic or predictive markers [4–9]. As a result, more than 300 clinical trials are currently investigating CTCs in an attempt to correlate their presence and concentration with treatment outcome (www.clinicaltrials.gov). While the introduction of commercial kits has facilitated CTC assessment, their heavy reliance on EpCAM as a single marker has been limiting. It is generally accepted that EpCAM-based detection has low sensitivity in EpCAM-negative cancers, which may explain why up to 70% of patients with metastatic epithelial malignancies fail to exhibit detectable CTCs using such methods. This is especially the case for aggressive tumor cells, which often downregulate EpCAM during epithelial-mesenchymal transition [4]. Such findings underscore the importance of identifying alternative CTC metrics.

To date, a variety of experimental CTC detection methods have been described using different biological markers. However, because most of these methods rely on immunocytochemical or polymerase chain reaction–based analyses, they often require primary purification and subsequent skilled processing and interpretation [3]. There is thus an urgent need to translate cancer-related discoveries into practical clinical and research tools for cancer investigation and treatment. To achieve this, low-cost strategies that yield robust, quantitative, and fully objective data within a point-of-care setting are required [10]. Given that some trials are starting to stratify and tailor patient therapy based

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on CTC changes (clinicaltrials.gov ID: NCT00382018), this is becoming increasingly important.

Here, our aim was to address three central questions in CTC detection: 1) Can detection be performed directly in whole blood so as to allow rapid point-of-care use? 2) Can current EpCAM-based detection paradigms be improved by using a combination of molecular markers? And 3) how much more sensitive would such a method be when applied to clinical samples? With these questions in mind, we hypothesized that direct detection of CTCs in whole blood (i.e., eliminating the need for primary CTC isolation, during which cells are often lost) using a multimarker combination would not only serve to increase detection sensitivity but also would allow detection within a point-of-care setting.

We investigated this hypothesis in the present study by making use of a highly sensitive micro–nuclear magnetic resonance (μNMR) technology that is able to perform cellular profiling directly in whole blood without the need for cell isolation. Detection was achieved through the *combined* and *simultaneous* sensing of four cancer markers: EpCAM, HER-2, EGFR, and MUC-1. This new approach, coined "quad-μNMR," is fast and can be effectively used in a point-of-care setting. It also obviates the need for skilled cytology analysis and data interpretation, a step that is often limiting in busy clinical and laboratory environments. Using human blood spiked with cancer cell lines differentially expressing EpCAM, we show considerably higher CTC detection rates with quad-μNMR than with other currently used techniques. These findings were later corroborated in a comparative clinical study of advanced-stage ovarian cancer. The assay's superior performance is particularly evident from its ability to detect EpCAM-negative ($EpCAM^{neg}$) cells. The described technology is now poised to enhance CTC assessment in both preclinical and clinical settings.

Materials and Methods

Cell Culture and Sample Preparation

Tumor cell lines were cultured in flasks according to manufacturer's recommendations and supplemented with 10% fetal bovine serum (FBS) before subsequent harvesting using trypsin. To determine cell numbers, a 10-μl aliquot of cells was placed on a hemocytometer plate, and counts were performed using a conventional inverted microscope. Cells for each experiment were counted in triplicate, and average values were used as the final spiked value. For experiments using whole blood, a known number of tumor cells were spiked into 7 ml of whole blood. For sensitivity experiments, each tube was spiked with 200, 100, 50, and 25 SKBR3 or SKOV3 tumor cells. Whole blood was obtained from healthy volunteers and placed in tubes containing ethylenediaminetetraacetic acid (Becton Dickinson, Franklin Lakes, NJ) or into CellSave preservative tubes (Veridex LLC, Raritan, NJ). CTC experiments using CellSearch to detect spiked cancer cells were conducted at an independent, outside laboratory blinded to the μNMR results.

Preparation of TCO-Modified Antibodies

Monoclonal antibodies against EpCAM, MUC-1, HER-2, and EGFR were conjugated with (*E*)-cyclooct-4-enyl 2,5-dioxopyrrolidin-1-yl carbonate (TCO-NHS), as previously reported (Table W1) [11]. Briefly, the reaction was carried out with 0.5 mg of antibody in the presence of 1000 equivalents of TCO-NHS in phosphate-buffered saline (PBS) and 10% dimethylformamide for 3 hours at room temperature. Unreacted TCO-NHS was then washed using 2 ml of Zeba desalting columns (Thermo Fisher, Rockford, IL), and antibody concentrations were determined by absorbance measurement.

Preparation of Tetrazine-Modified Nanoparticles

Cross-linked iron oxide (CLIO) nanoparticles were prepared, and tetrazine (Tz)-CLIO conjugation was performed (as previously reported [11]) in PBS containing 5% dimethylformamide for 3 hours at room temperature. Excess Tz-NHS was removed by gel filtration using Sephadex G-50 (GE Healthcare, Pittsburgh, PA).

Sample Processing and Labeling with Tz-Modified Nanoparticles

Each whole blood sample (7 ml) was lysed, and the cell pellet was resuspended in buffer (100 μl of PBS/1% FBS). TCO-modified monoclonal antibodies (10 μg/ml) were added, and the pellet was incubated at room temperature for 20 minutes. Samples were then washed twice, and antibody binding was revealed using magnetic nanoparticles (100 nM Tz-CLIO) for 10 minutes at room temperature. Excess Tz-CLIO was removed by washing (centrifugation) twice in 1 × PBS containing 1% FBS, before being resuspended in 20 μl of PBS for μNMR measurements. The overall labeling and incubation procedure took approximately 30 minutes.

Clinical Subjects

μNMR expression profiles from the biopsies of 58 patients with confirmed epithelial malignancies were obtained as part of an institutional review board–approved study. These patients had been referred for clinical biopsy of an intra-abdominal lesion after an abnormal computed tomography or ultrasound, at the Massachusetts General Hospital Abdominal Imaging and Intervention suites. Also included were six patients with benign diagnosis, as determined by repeat core biopsies, imaging [12], and clinical information, as previously described [13]. The median age of the malignant cohort was 65 years (range, 25-91 years). The cancer subtypes consisted of breast (n = 4), gastrointestinal (n = 17), genitourinary (n = 4), gynecologic (n = 7), pancreatic (n = 10), lung (n = 9), and poorly differentiated adenocarcinoma (n = 7).

Collection of peripheral blood for comparative quad-μNMR and CellSearch CTC detection was performed using a convenience sample of 15 patients with ovarian cancer, who were receiving care at the Gillette Center for Gynecologic Oncology, Massachusetts General Hospital. The study was approved by the Dana Farber/Partners Cancer Care Institutional Review Board, and informed consent was obtained from all participants. Two clinicians (C.M.C. and R.W.), blinded to the μNMR and CellSearch results, reviewed each subject's documented clinical, imaging, and pathology data. Peripheral blood samples were also obtained from five healthy volunteers.

Design of Experiments

For the present study, our experiments were primarily focused on 1) optimizing the detection conditions, 2) comparing single and multimarker detection strategies, and 3) comparing the detection sensitivity of μNMR to the standard clinical assay, CellSearch.

1) Detection was optimized by examining a variety of different sample processing and cell fixation procedures. Three aliquots of SKBR3 cells (14,000, 7000, and 1400 cells) were each spiked into 7 ml of whole blood in triplicate. HER-2 antibody (10 μg/ml)

labeling was then performed for each cell concentration experiment under the following conditions: a) whole blood treated with a red blood cell (RBC) lysis procedure (Qiagen, Valencia, CA), b) whole blood treated with a Ficoll density gradient (Miltenyi Biotec, Auburn, CA) to separate mononucleated cells, and c) whole blood with no additional processing. The effect of cell fixation was examined by targeting the HER-2 biomarker in SKBR3 (as described above); this was done by fixing cells in whole blood either immediately before or after spiking varying concentrations of SKBR3 cells (140/ml, 1100/ml, and 6300/ml) into 7 ml of whole blood.

2) To compare single *versus* multimarker detection, five 7-ml aliquots of whole blood were collected from one single healthy individual for each of 12 experiments. Each blood tube was spiked with equal and known numbers of cancer cells. The samples were then processed and distributed into two tubes, one labeled "test" (containing antibody) and the other labeled control (no antibody). To each tube designated as "test," a single aliquot of antibody against EpCAM, MUC-1, HER-2, or EGFR was added (10 μg/ml), with the exception of the tube receiving the quad marker (this tube received antibodies for all four markers). All samples were then processed for nanoparticle labeling (100 nM) and analyzed by a single operator, according to sample preparation and sample analysis protocols. For each biomarker, the "μNMR Value" was calculated as the signal obtained from the "test" sample divided by the signal obtained from the corresponding "control" sample. "μNMR Value" ratios for both single markers and for the quad marker were obtained for 12 different cell lines (Figure 2).

3) Detection sensitivity was examined by titration, using varying numbers of SKBR3 or SKOV3 cells spiked into a whole blood sample obtained from a healthy donor. The performance of μNMR was then compared to the standard clinical assay, CellSearch. The experiment was carried out using a split-sample procedure where identical samples were processed separately with either μNMR or CellSearch. The enumeration and identification of tumor cells identified by CellSearch were performed independently at Brigham and Woman's Hospital (Boston, MA). In the quad-μNMR experiment, cell recovery was calculated based on the number of nanoparticles detected on the tumor cell surface. In separate validation experiments, bulk cell loss was also assessed by flow cytometry; specifically, we counted the tumor cells and then used their size and fluorescent tags to gate and separate the cells from leukocytes (data not shown).

μNMR Measurements

Measurements were conducted using a portable μNMR system recently developed for point-of-care operations [13,14]. The polarizing magnetic field strength was 0.5 T. The transverse relaxation times were measured in 1- to 2-μl sample volumes. Carr-Purcell-Meiboom-Gill pulse sequences with the following parameters were used: echo time, 3 milliseconds; repetition time, 4 seconds; number of 180° pulses per scan, 900; number of scans, 7. All measurements were performed in triplicate, and data are displayed as mean ± standard error of mean.

Data Analysis and Statistics

T_2 values were obtained in triplicate using the μNMR system. R_2 values were calculated as the inverse of the mean T_2 values. The effect of media in the μNMR readout was accounted for by subtracting the R_2 value of the media (1 × PBS/1% FBS) from either the values obtained from the test (i.e., samples receiving antibody and CLIO for labeling) or from the control (i.e., samples receiving only CLIO for background signal determination). The μNMR readout was subsequently calculated by dividing $\Delta R_2^{\text{sample}}$ by $\Delta R_2^{\text{control}}$ to account for any nonspecific binding of CLIO to cells. The μNMR value served as an expression level of the biomarkers.

Receiver operating characteristic (ROC) analysis was performed for individual markers and for marker combinations. An Az (area under the ROC curve) of 0.5 was used to indicate no differences between two groups, whereas an Az of 1.0 was used to indicate a perfect separation between the groups. The optimal cutoff value for identifying malignant status was then defined as the point on the ROC curve with the minimum distance between the 0% false-negative and the 100% true-positive.

A paired Student's *t* test was used to evaluate the statistical significance between the percent cell recovery obtained by μNMR and that obtained by the CellSearch system. A 2 × 2 contingency analysis, using the Fisher exact test, was used to evaluate μNMR for its ability to detect CTCs in whole blood samples from both patients and healthy controls.

Results

Defining the Detection Signature for Quad-μNMR

The heterogeneous nature of biomarker expression levels is a well-known phenomenon in cancer, and this served as the rationale for considering multiple, rather than single, markers for CTC detection. To begin, we initially obtained samples from a cohort of patients (n = 58) who had undergone fine needle aspiration of their cancers. These samples were then profiled for a number of markers including MUC-1, EGFR, B7-H3, HER-2, Ki-67, EpCAM, Vimentin, CK18, and p53. Statistical analysis (Spearman correlation and ROC curve) indicated that diagnostic information (malignant *vs* benign) could be achieved by using four key markers: EpCAM, MUC-1, HER-2, and EGFR (Figure 1). Although analysis of each protein individually revealed that a notable fraction of the samples were negative for single proteins (EpCAM, 34.4%; HER-2, 32.7%; MUC-1, 32.7%; and EGFR, 31.0%; Figure 1*A*), combined marker analysis was able to correctly identify 99.2% of samples as malignant.

With this information, we developed a μNMR strategy for directly sensing CTCs in whole blood. This was achieved by first incubating blood samples with a cocktail of four *trans*-cyclooctene (TCO)–labeled antibodies against each protein target, washing the cells, and then identifying antibody-positive cells using Tz-decorated magnetic nanoparticles. This "cocktail" method of targeting cancer cells not only increases the chances of detecting single marker-negative patients but also results in much higher magnetic nanoparticle binding per cell; this leads to increased μNMR signals and better discrimination from background (Figure 1*B*). The method also eliminates the need for CTC capture or isolation; instead, CTCs are measured directly from a pellet of host cells. The nanoparticles also serve as an amplification strategy, influencing the relaxation times of billions of surrounding water molecules; these changes can be subsequently measured by μNMR.

Optimization of μNMR for CTC Detection

Our initial aim was to optimize sample preparation to achieve direct detection of cells in whole blood, while maximizing the μNMR signal. We thus empirically discovered that cell detection in whole

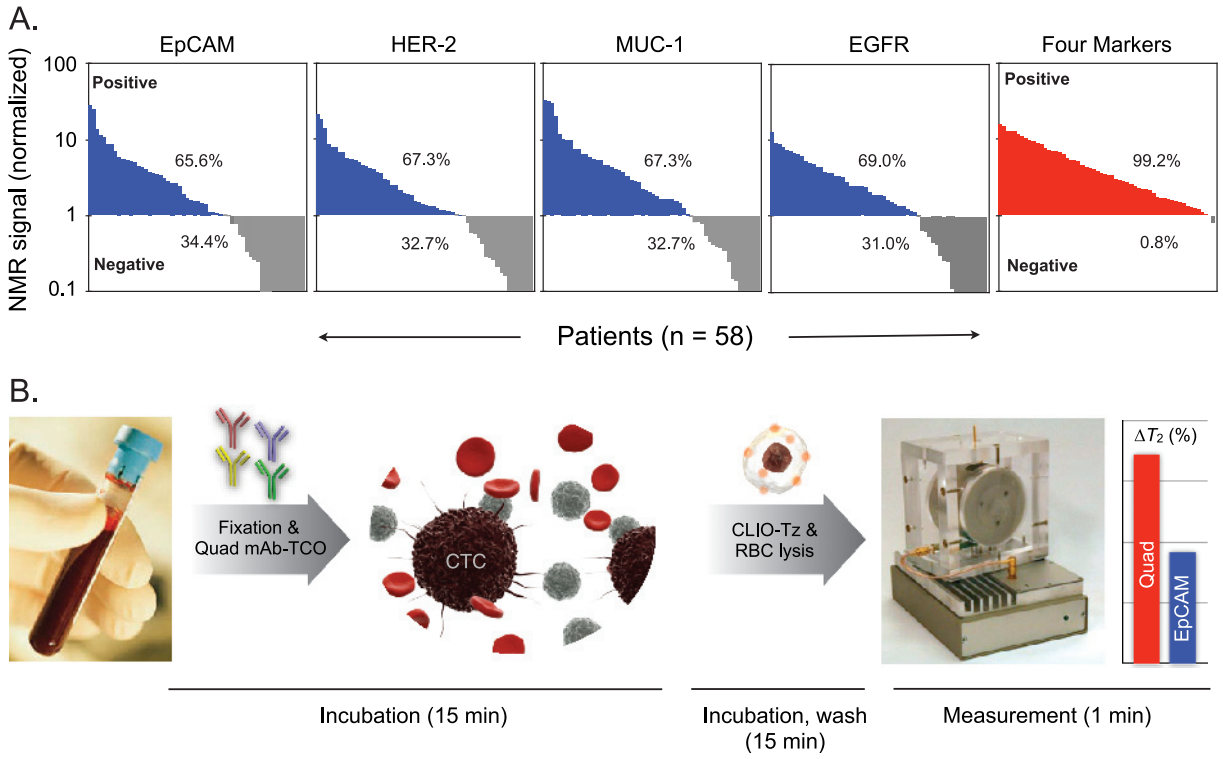

Figure 1. Clinical rationale and quad-μNMR schematic. (A) Biomarker expression, as assessed by μNMR, showed that a significant percentage of cancer patients ($n = 58$) were negative for EpCAM (34.4%), HER-2 (32.7%), MUC-1 (32.7%), or EGFR (31.0%). Combining these markers, however, enabled identification of nearly all cancer patients (99.2%). (B) Schematic of the quad-μNMR system. TCO-labeled antibodies are added to whole blood. RBC are then lysed and the cells spun down before reaction with Tz-containing magnetic nanoparticles. The process of labeling antibodies and targeting nanoparticles requires less than 30 minutes. Biomarker measurements are then taken using the μNMR device (shown).

blood was best after RBC lysis (Figure W1) and that fixation before antibody labeling resulted in more reproducible measurements of CTCs (Figure W2). Next, to compare single-marker *versus* multi-marker detection, whole blood samples from a healthy individual were spiked with 12 different types of epithelial cancer cells representing a spectrum of EpCAM expression levels (Figure 2). Before µNMR measurements, each sample was incubated either with a single antibody-TCO or with a cocktail containing all four antibody TCOs (quad marker). From this comparison, we found that the µNMR signal was highest for the quad marker compared to each single biomarker (Figure 2), even in the EpCAM-overexpressing group (e.g., SKBR3; Figure 2*A*). This increase in µNMR values is likely the result of more efficient nanoparticle binding to cells. Differences in µNMR signals were particularly pronounced in cell lines lacking (or expressing low levels of) EpCAM (e.g., MDA 436, HS578T, BT 549, and PACA-2; Figure 2, *B* and *C*); these cells showed much higher values for quad-µNMR than for single EpCAM. This also held true for cell lines with medium EpCAM expression, which likewise showed higher µNMR values for quad-µNMR than for single EpCAM. Such a finding has important implications for the detection of CTCs in cancers with mesenchymal transition or in $EpCAM^{neg}$ cancers.

Detection Sensitivity in Whole Blood

We next performed a series of blinded experiments to determine the detection sensitivity of CTC in whole blood. Blood samples (7 ml; similar to clinical CTC protocols) were spiked with known amounts of cancer cells to achieve a concentration spectrum ranging from ~30 CTC/ml to 0 CTC/ml. Triplicate samples were then simultaneously processed for µNMR and CellSearch measurements. The latter measurements were performed independently, in a blinded fashion, in a clinical service laboratory at an affiliated institution. The detection rates of quad-µNMR in 7-ml blood samples for different CTC concentrations were as follows: 86/200 spiked cells, 41/100 spiked cells, 18/50 spiked cells, and 8/25 spiked cells (Figure 3*A*). Overall, the percentage of tumor cells (number) detected after sample processing ranged from 43% at the higher CTC concentration (~30 cells/ml) to 32% at the lower CTC concentration (~3 cells/ml) with an average rate of 38% across all concentrations. Figure 3*B* plots the detection rates against theoretical values. Using quad-µNMR, we were able to reliably detect ~3 individual CTCs per sample (in sample volumes ranging from 1 to 10 ml of blood). Furthermore, for all concentrations assessed, quad-µNMR demonstrated far more sensitive detection of CTCs than single EpCAM-µNMR (Figure 3*B*).

We next compared direct µNMR sensing to the clinically approved CellSearch system. The µNMR assay showed considerably higher detection sensitivities at all CTC concentrations and for all cell lines tested. Using CellSearch, the detection rates were, on average, 8.5/200 spiked cells, 8.5/100 spiked cells, 2.5/50 spiked cells, and 3.5/25 spiked cells. The percentage of tumor cells recovered thus ranged from 14% at the 200-cell spike (~30 cells/ml) to 4.3% at the 25-cell spike (~3 cells/ml; Figure 4), and the average recovery rate for CellSearch was 9.1% across all concentrations. These results demonstrate that the quad-µNMR detection platform outperforms CellSearch for all cell concentrations tested ($P < .05$; Figure 4*B*).

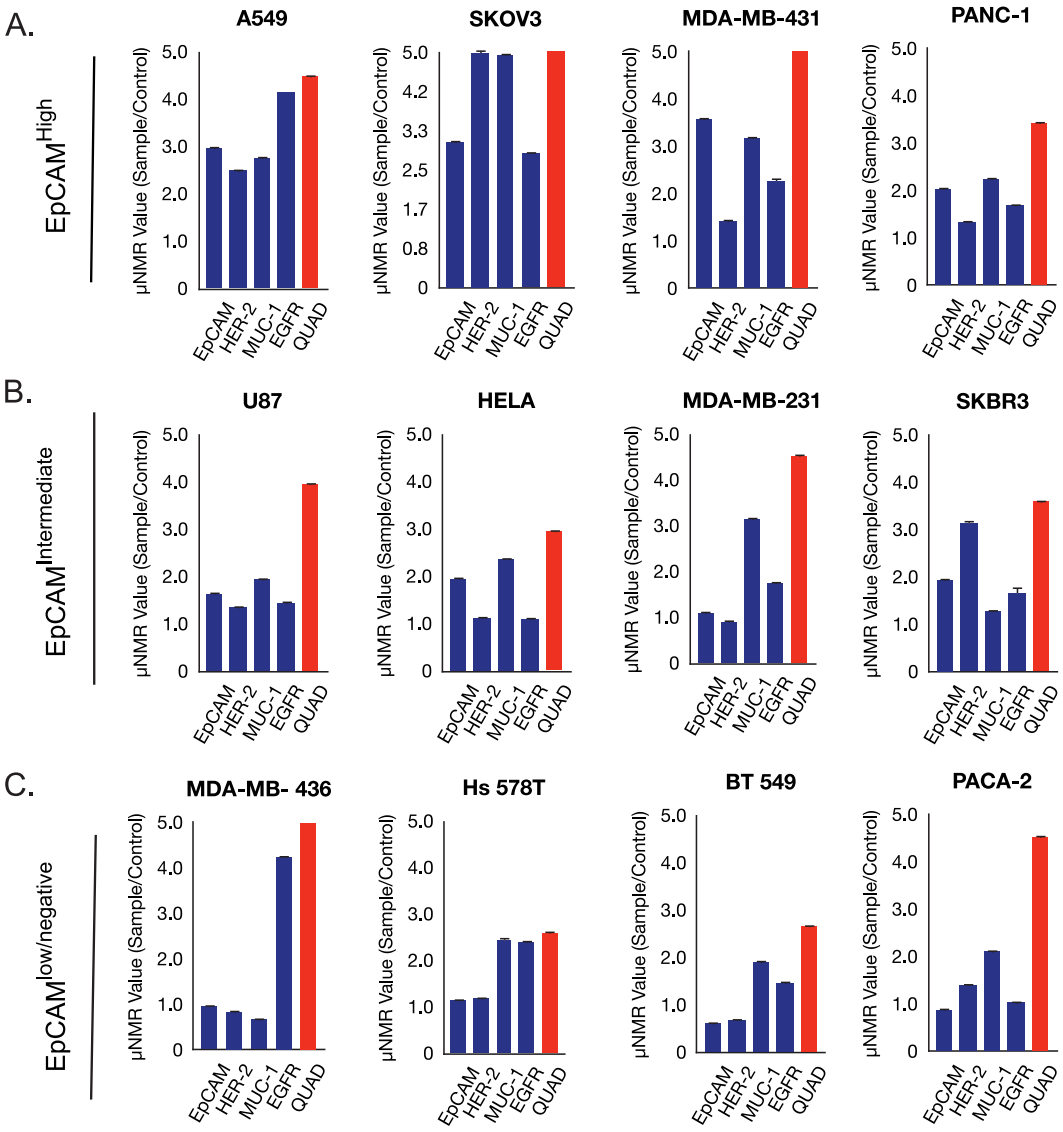

Figure 2. Comparison of single and quad marker detection using μNMR. Quad-μNMR CTC targeting (red bars) in whole blood showed higher NMR signals than single-marker CTC targeting (blue bars). For comparative purposes, the μNMR values are displayed as relative ratios. All measurements were obtained in triplicate (note narrow error bars). In all cell lines assessed, quad-μNMR consistently outperformed all single-μNMR detections. Most notably, quad-μNMR even outperformed EpCAM-μNMR in high- (A), intermediate- (B), and low/negative- (C) EpCAM-expressing cell lines.

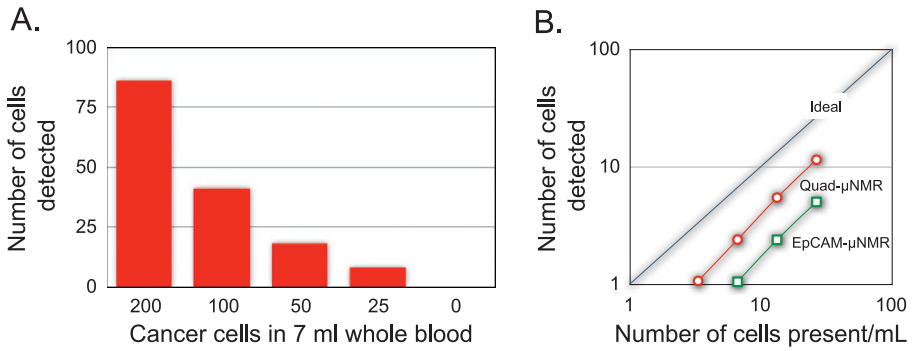

Figure 3. Detection sensitivity of quad-μNMR in whole blood compared with the clinical standard. Varying numbers of cancer cells (i.e., 200, 100, 50, and 25 cells) were spiked separately into 7-ml samples of blood from a healthy donor. Quad-μNMR measurements are shown as mean values. Quad-μNMR was able to detect 86/200 spiked cells, 41/100 spiked cells, 18/50 spiked cells, and 8/25 spiked cells. All μNMR measurements were done in triplicate for both test and control samples. (A) Quad-μNMR outperformed single marker EpCAM-μNMR for all cell concentrations assessed. (B) Observed values are plotted against theoretical values (gray line).

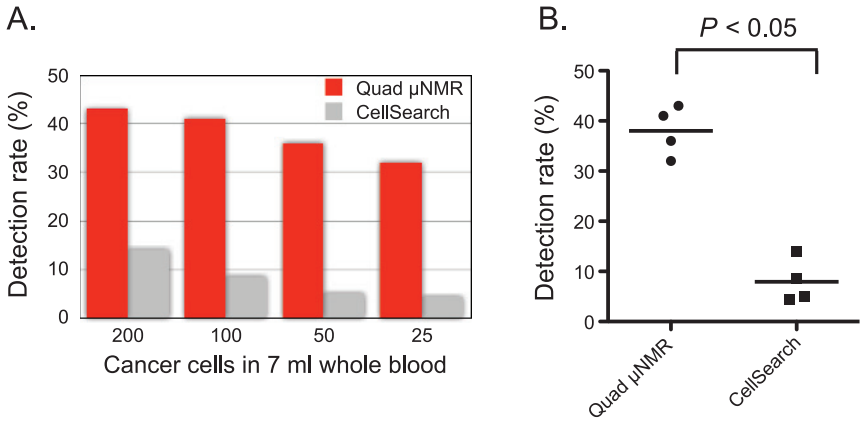

Figure 4. Comparison of quad-μNMR to the clinical standard. (A) Using quad-μNMR detection, the average recovery rate was 38% across the various cell concentrations assessed (i.e., 200, 100, 50 and 25 spiked cells). Identical experiments performed using the CellSearch detection system showed an average recovery rate of just 9.1% across all concentrations, ranging from 14% at the 200-cell spike (~30 cells/ml) to 4.3% at the 25-cell spike (~3 cells/ml, i.e., below the five-cell detection threshold recommended by CellSearch). (B) Compared to the clinically used CellSearch system, quad-μNMR showed higher CTC detection sensitivity ($P < .05$) for all cell concentrations assessed.

Comparative Clinical Study

To determine the clinical utility of the quad-μNMR method, we conducted a feasibility study in patients with advanced-stage ovarian cancer (Table 1). We reasoned that given their widespread systemic disease, these patients were all likely to have CTCs. As a negative control, we also enrolled 10 normal subjects with no evidence of cancer. Peripheral blood samples from these subjects were processed for both quad-μNMR and CellSearch. Figure 5*A* compares the quad-μNMR signals from cancer patients with those of normal controls. Of 15 patients, 13 (87%) showed measurable CTCs by quad-μNMR (Figure 5, *A* and *B*). Average CTC counts (determined with either quad-μNMR or CellSearch) were higher in more advanced cases (stage IV, platinum-resistant, and progressive disease) as well as in patients not undergoing active therapy for various reasons (e.g., hospice bound, critical illness). Quad-μNMR was even positive in patients with nonserous (and presumably less EpCAM-dependent) histologies (counts, 13-75). Overall, quad-μNMR was found to be capable of detecting significantly larger numbers of CTC compared to CellSearch. Indeed, using the standard CellSearch cutoff of five CTCs or more per 7-ml blood, only one patient (7%) was identified as CTC positive (Figure 5*B*); at a cutoff of two CTCs per 7-ml blood, only three patients (20%) were positive, whereas 87% of patients were positive by quad-μNMR.

Discussion

Accurate detection and profiling of CTC in blood has been touted as the "holy grail" in cancer diagnosis. Unfortunately, single marker (EpCAM)–based detection technologies are not ideal [15]. The US Food and Drug Administration–approved CellSearch, for instance, relies on signal derived from EpCAM and reportedly misses $EpCAM^{neg}$ cells [16–18]. Moreover, in the absence of cancer-specific markers, a systematic analysis of miss rates across large cohorts of patients has been difficult to obtain. We thus hypothesized that a multimarker detection paradigm would likely increase cancer cell detection sensitivity. Our results show that quad marker detection of cancer cells in blood is more sensitive than conventional EpCAM-based detection, both for μNMR and for clinical systems (CellSearch). In addition, analysis of cancer cell lines with variably expressed EpCAM (low, intermediate or high) served as a better approximate to the clinical reality evident from reported literature [13,19]. For the cell lines tested, and in cells where EpCAM is present, we show a 400% increase in detection sensitivity of μNMR over the clinical method. In low-EpCAM-expressing cell lines, μNMR was even more sensitive. For example, μNMR was found to be 645% more sensitive in MDA-MB-436, a BRCA1 mutant breast adenocarcinoma cell line associated with epithelial-mesenchymal

Table 1. Summary of Patient Data.

Characteristic	Number	Percentage	CTC by Cell Search, Mean (Range)	CTC by μNMR, Mean (Range)
No. patients	15		2 (0-18)	40 (0-170)
Age (years)				
Median	62			
Range	36-92			
Ovarian histology				
Serous	10	66	3 (0-18)	48 (0-170)
Endometrioid	1	6.8	0	0
Transitional	1	6.8	0	16
Carcinosarcoma	1	6.8	0	75
Mucinous	1	6.8	0	15
Poorly differentiated	1	6.8	0	13
Stage				
IIIC	5	34	1 (0-3)	33 (0-76)
IV	10	66	2 (0-18)	44 (0-170)
Surgical debulking				
Optimal	11	73.2	2 (0-18)	41 (0-170)
Suboptimal	3	20	1 (0-4)	45 (18-97)
None	1	6.8	0	13
Median time from diagnosis (months)	28			
Therapy				
Active	11	73.2	0.4 (0-3)	27 (0-76)
None	4	26.8	6 (0-18)	76 (19-170)
Platinum				
Sensitive	9	60	2 (0-18)	36 (0-170)
Resistant	6	40	1 (0-4)	46 (15-97)
Refractory	0	0	0	0
Disease course				
Response	4	26.8	0	8 (0-18)
Stable	3	20	1.3 (0-3)	47 (15-76)
Progression	8	53.2	3 (0-18)	54 (15-170)

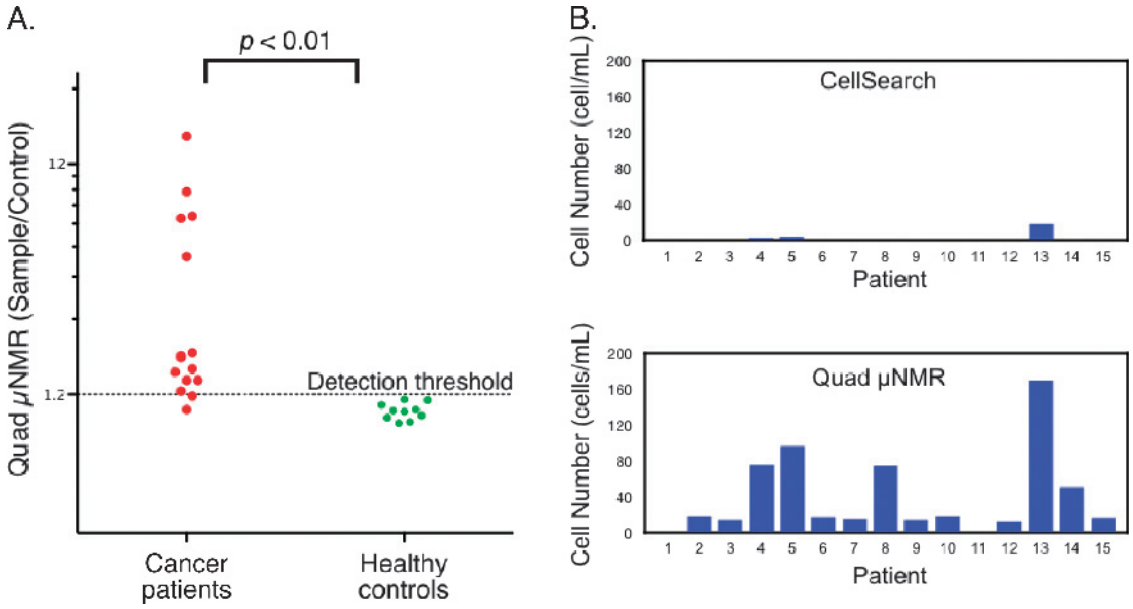

Figure 5. Detection of CTC levels in patient samples using quad-μNMR. (A) CTC levels in the peripheral blood of 15 advanced-stage (IIIC or IV) ovarian cancer patients compared with those of 10 healthy individuals, as assessed by quad-μNMR. (B) A comparison of CTC levels in samples determined by either quad-μNMR or CellSearch. A positive result was obtained for 13 of 15 cancer patients using quad-NMR, whereas only 1 of 15 patients (patient 13) was clearly positive with CellSearch. The dynamic range of CTC levels obtained using quad-μNMR was 0 to 170.

transition and aggressive clinical behavior, which is sensitive to PARP inhibition [20]. The accurate and timely monitoring of therapy (e.g., PARP inhibition)-induced changes in low-EpCAM-expressing CTC is one specific scenario in which the μNMR approach, if validated in human trials, could outperform existing CTC detection strategies. This could lead to improvements in pharmacodynamic assessment for both cancer drug discovery and clinical research.

There are two novel aspects to the work presented here: a) the use of μNMR technology for direct measurements of rare cells in whole blood and b) the preclinical validation of a quadruple CTC marker signature with promising clinical utility. Prior μNMR iterations using cancer tissue involved aliquoting parent samples into vials for sequential analyses of each marker [13]. Here, we were able to optimize the assay for whole blood analysis by performing RBC lysis and by using a cocktail of TCO-modified monoclonal antibodies [11]. By subsequently adding Tz-modified magnetic nanoparticles, which concentrate and cluster on the cell surface (with additive effects resulting in higher signals), malignant cells could be readily distinguished from host cells using magnetic resonance. The μNMR technology could then measure the presence of these particles in blood with high sensitivity.

In the present study, we tested quad-μNMR in advanced-stage ovarian cancer patients. Cancer antigen-125 (CA-125) is a protein that can be elevated in ovarian cancer cells and as such has been used, although not approved, as a diagnostic cancer marker. The problem with CA-125, however, is that it is not a reliable indicator of the disease and thus is not widely recommended for routine testing of ovarian cancer. As an alternative, CTCs have been identified as novel and promising biomarkers to improve ovarian cancer diagnosis and monitoring of treatment response and recurrence. CTCs have been recently measured in ovarian cancer using the CellSearch method, and an arbitrary cutoff threshold of two CTCs per 7.5-ml blood [21,22]. Whereas such studies have underscored the feasibility of CTC detection and demonstrated a correlation between CTC number and progression-free survival [22], they have also raised questions regarding CTC detection sensitivity. For example, in one recent study of 216 patients with ovarian cancer, randomized into two chemotherapy arms, only 14% met the criteria for CTC positivity (more than two CTCs per 7.5 ml using CellSearch) [22]. To compare the detection sensitivity of CellSearch with quad-μNMR in the present study, we focused our experiments on advanced-stage patients who were more likely to have detectable levels of CTCs. Our results showed that using the quad-μNMR system, 87% (13/15) of patients had measurable CTCs, compared to only 7% with the CellSearch system (using the recommended five-CTCs cutoff; at a two-CTCs cutoff, detection increased to 20%). The only patient positive for CTC using traditional CellSearch criteria (18 CTCs per sample) was also found to have the highest CTC levels in the cohort, as assessed using quad-μNMR (170 CTC/sample). All patients with at least one CTC detectable by CellSearch (27%) also had detectable CTCs on quad-μNMR. Based on the preclinical validation presented here, the higher CTC values obtained by the quad-μNMR technology likely reflect the system's improved recovery of CTCs together with its higher detection sensitivity. Table W2 provides a summary of pertinent clinical information and patient CTC levels. Although a larger trial is now required to gain further insight into the clinical potential of the new CTC detection technology, it is already clear that a strong correlation exists between CTC (as detected by quad-μNMR) and the clinical scenario, including CA-125 levels. Quad-μNMR's superior CTC detection over and above an EpCAM-based approach may improve clinicians' ability to monitor their patients for tumor response or recurrence.

There are additional advantages to using the μNMR technology for CTC detection. First, it requires relatively small blood samples for detection of scant cells. Our experimental data show that we can reliably and repeatedly detect concentrations of three individual CTCs per sample. Because these measurements are performed in cell pellets and in 50- to 100-μl volumes, similar measurements could be

performed in both larger blood volumes (to maximize the chance of detecting rare cells) as well as in blood samples as small as 100 µl. Using µNMR, we show an average CTC detection rate of 38%; this percentage is approximately four to six times higher than that obtained using the CellSearch system. It is likely that this detection sensitivity could be yet further increased by using nanoparticles with higher magnetic moments and by continuing to optimize the device. Second, the portability of the assay is truly point-of-care and readily adaptable to the clinical scenario. Its modular use (TCO-labeled antibodies) could also be easily modified to include other cancer marker subsets. The use of this strategy for some targets may be obvious (TTF-1 for lung cancer, PSMA for prostate cancer populations), but perhaps the biggest improvement in detection sensitivity will come from the detection of other emerging targets such as underglycosylated and immaturely glycoslyated cancer cells [23], circulating DNA [24], and/or of unique endothelial cell populations (e.g., breast cancer patients often have distinct CD34/VEGFR1 populations in their peripheral circulation). Finally, µNMR represents a platform technology that could be multiplexed onto chips for the detection of other diagnostic targets even beyond that of protein markers, for example, circulating DNA or exosomes. Although these possibilities were not addressed in the present study, this is an area of ongoing investigation within our laboratory.

Advances in CTC detection and profiling will undoubtedly contribute to our understanding of human cancer biology. The µNMR assay in the present study will likely benefit such investigations. A key remaining question is whether CTC levels and characteristics will be useful for clinical decision making. The implications of such decisions are not trivial and demand robust and accurate profiling; this is simply not possible using a single universal marker.

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Table W1. List of Antibodies and Their Associated Characteristics Relevant to the Study.

Marker	Clone	Species	Isotype	MW	MW (TCO)	TCO Valency	Company
EpCAM	158206	Mouse	IgG2b	150,600 ± 300	152,100 ± 200	9.7	R&D Systems (Minneapolis, MN)
HER2	Trastuzumab	Human	IgG1	148,700 ± 300	152,300 ± 400	23.2	Genentech (San Francisco, CA)
EGFR	Cetuximab	Hu/Ms	NA	152,400 ± 300	153,800 ± 200	9.2	Imclone Systems (New York, NY)
MUC-1	M01102909	Mouse	IgG1	151,900 ± 200	152,600 ± 200	4.8	Fitzgerald Ind. (Acton, MA)

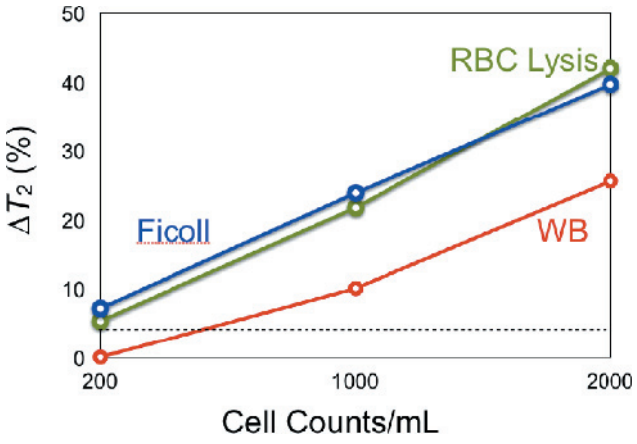

Figure W1. Effect of media on μNMR detection. Three aliquots of SKBR3 cells (200/ml, 1000/ml, and 2000/ml) were each spiked into 7-ml samples of whole blood. Cancer cells were then targeted using the HER-2 antibody in untreated whole blood (red line), whole blood treated with Ficoll (blue line), and whole blood treated with a RBC lysis procedure (green line). Compared to the other methods, RBC cell lysis treatment resulted in higher detectable μNMR signals and was thus adopted for subsequent comparisons.

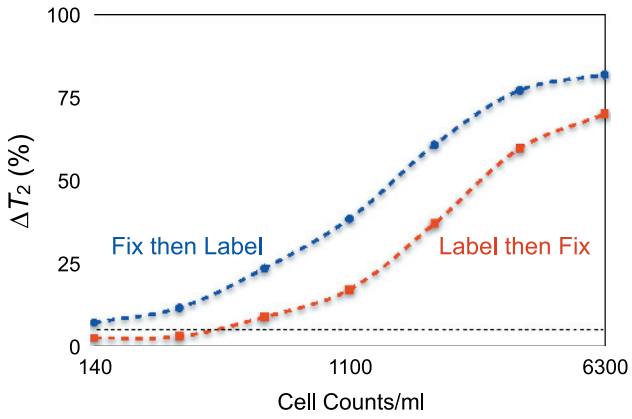

Figure W2. Effect of fixation on μNMR detection. Three aliquots of SKBR3 cells (140/ml, 1100/ml, and 6300/ml) were spiked into 7-ml samples of whole blood for duplicate experiments. In these experiments, cell fixation was done either before (blue line) or after (red line) labeling with the HER-2 antibody. μNMR data show that cell fixation before labeling produces better results.

Table W2. Selected Clinical Scenarios Illustrating the Potential Utility of Quad-μNMR.

Clinical Scenario	Subject No.	CA-125	Cell Search CTC	Quad-NMR CTC	Quad Value
Stage IV	8	11	0	15	1.38
	11	17	0	13	1.24
	6	57	0	16	1.5
	7	99	0	75	6.96
	2	139	0	19	1.82
	1	145	0	0	1.03
	5	145	0	18	1.73
	15	307	0	15	1.38
	12	1123	18	170	15.9
	4	1142	4	97	9.09
Progressive disease	14	48	0	17	1.55
	6	57	0	16	1.5
	7	99	0	75	6.96
	2	139	0	19	1.82
	15	307	0	15	1.38
	12	1123	18	170	15.9
	4	1142	4	97	9.09
	9	1707	0	19	1.77
Any CellSearch positive	13	136	1	51	4.75
	3	895	3	76	7.11
	12	1123	18	170	15.9
	4	1142	4	97	9.09