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Treatment monitoring of patients with epithelial ovarian cancer using invasive circulating tumor cells (iCTCs)

Michael L. Pearl¹, Huan Dong^{1,2}, Shaun Tulley^{1,2}, Qiang Zhao^{1,2}, Marc Golightly³, Stanley Zucker^{4,5}, and Wen-Tien Chen^{1,2}

¹Division of Gynecologic Oncology, Stony Brook Medicine, Stony Brook NY 11794, USA

²Vitatex Inc., 25 Health Sciences Drive, Stony Brook, NY 11790, USA

³Department of Pathology, Stony Brook Medicine, Stony Brook, NY 11794, USA

⁴Department of Medicine, Stony Brook Medicine, Stony Brook, NY 11794, USA

⁵Department of Medicine and Research, Veterans Affairs Medical Center, Northport, NY 11768, USA

Abstract

Goals—Contemporary management of epithelial ovarian cancer (EOC) uses biomarkers to monitor response to therapy. This study evaluates the role of invasive circulating tumor cells (iCTCs) in monitoring EOC treatment in comparison with serum cancer antigen 125 (CA125).

Methods—Molecular and microscopic analyses were used to identify seprase and CD44 as tumor progenitor (TP) markers. The iCTC flow cytometry assay was optimized using blood donated by 64 healthy donors, 49 patients with benign abdominal diseases and 123 EOC patients. Serial changes in iCTCs and CA125 were measured in 129 blood and 169 serum samples, respectively, from 31 EOC patients to assess their concordance during therapy and their relationship with risk of progressive disease (PD).

Results—The assay had 97% specificity and 83% sensitivity for detecting iCTCs in blood of EOC patients. iCTCs were detected in each monitoring patient (31/31, 100%) and in 110 of the 129 blood samples (85.3%). The concordance between changes in iCTCs / CA125 levels and changes in the intervals associated with no evidence of disease (NED) were markedly stronger (specificity: CA125 93.8%; iCTCs 90.6%), whereas increases in iCTCs (79.5%) were more

Corresponding author: Wen-Tien Chen, Ph.D. Vitatex Inc., 25 Health Sciences Drive, Stony Brook, NY 11790, USA. Tel: 631 444-8482. wentien@vitatex.com.

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Author Contributions:

M.L.P., and W.C., had the initial idea of this project, wrote the manuscript, and conceived the project and supervised all research. H.D., Q.Z., and S.T. performed the majority of experiments, prepared most data presentations and wrote the manuscript. H.D. and M.G. designed the flow cytometry experiments. M.L.P., and S.Z., provided and analyzed clinical data.

Conflict of Interest Statement:

According to the policy, the six authors (H.D., S.T., Q.Z., M.G., S.Z., and M.L.P.) do not have any relevant financial relationship with a commercial interest.

sensitive than increases in CA125 (67.6%) to predict PD or relapse. Among the six patients who had greater than 6 measurements, iCTCs but not CA125 antedated changes in clinical status from PD to NED during and after chemotherapy, and predated relapse.

Conclusion—Serial measurements of iCTCs could predict therapeutic responsiveness in 31 EOC patients who underwent standard taxol / carboplatin therapy.

Keywords

Cell invasion; circulating cancer cells; CTC; metastasis detection; treatment monitoring; ovarian cancer

INTRODUCTION

Epithelial ovarian cancer (EOC) is the leading cause of death among gynecologic malignancies with approximately 22,280 cases and 15,500 deaths every year in the United States [1]. Responsiveness to contemporary management may be assessed with the use of serial imaging, but radiographic measurements often fail to detect changes in micrometastases. CA125, also described as MUC16, is the serum tumor marker most closely associated with EOC [2]. The clinical utility of serum CA125 levels has been well established to monitor treatment response to chemotherapy [3–6] or to surgery [7] in advanced EOC. Considering serum CA125 as a surrogate for tumor volume, the Gynecologic Cancer Intergroup (GCI) CA125 response criteria were found to be a better prognostic tool than the Response Evaluation Criteria in Solid Tumors (RECIST) on survival in EOC patients receiving second-line chemotherapy [3]. Nevertheless, for a fraction of patients with initial low levels of CA125, CA125 is not an accurate indicator of disease progression during chemotherapy, and there is currently no effective alternative to monitor response of these patients.

With almost 80% of primary EOC initially responding to platinum-based therapy, a prospective biomarker would need to be highly predictive of success or failure of current therapies and benefit of alternative treatment [8]. The detection of CTCs, using an anti-epithelial antibody-enrichment method followed by positive and negative antigen cell identification techniques, has been shown to be useful in distinguishing between metastatic cancer patients with favorable and unfavorable prognosis [9–11]. The CellSearch system (VERIDEX), which depends on antigenic enrichment and identification of CTCs, has been approved by the US Food and Drug Administration (FDA) for prognostic use in metastatic breast, prostate and colon cancer. However, the CellSearch could not effectively detect CTCs in patients with metastatic and recurrent ovarian cancer, nor was it effective in patients with early stage disease who nonetheless have a poor prognosis [12–15]. Other studies using laser scanning cytometry to screen epithelial cells (rather than epithelial cells captured by anti-EPCAM antibody-based enrichment) in a mononuclear cell population [16] detected an epithelial population of CTCs in early stage breast cancer and demonstrated that an increase of 10-fold or more at the end of therapy was a strong predictor of relapse. Although promising, CTC assays for epithelial cells have not yet demonstrated clinical utility (treatment monitoring) over standard tumor marker blood tests.

We have developed a functional cell enrichment / identification method capable of enriching viable CTCs one-million fold and have identified CTCs by their invasive phenotype in both metastatic and non-metastatic cancers of the breast, ovary and prostate [17–20]. This system employs a functional component that takes advantage of CTCs avidity to bind to a type I collagen matrix (Cell Adhesion Matrix [CAM]) and subsequently ingest labeled matrix protein (CAM+) within 24 hours of culture *ex vivo*, indicating the invasive phenotype of cells isolated called iCTCs. Captured iCTCs are further distinguished by microscopy and flow cytometry using antibodies against epithelial / tumor antigens (EPCAM, CA125, DPP4, CD44, seprase and cytokeratins) to confirm the tumor cell, and negative selection using antibodies against hematopoietic lineage markers (HL–: leukocyte common antigen CD45) to exclude hematopoietic cells [21].

In this study, we used molecular and microscopic analyses to identify antibodies against tumor progenitor (TP) markers, seprase and CD44 that recognize CTCs, followed by standardization of the CTC flow cytometry assay using CAM+ and TP+ positive CTC markers. The optimized assay was initially used to serially detect CAM±TP+NA+HL– circulating epithelial cells, called Epis, and CAM+TP+NA+HL– iCTCs in 31 patients who underwent standard taxol / carboplatin treatment and who were monitored to determine if Epis and iCTCs might have adequate specificity and sensitivity in detecting treatment responsiveness. We then assessed whether changes in iCTCs and CA125 were correlated with treatment response. Finally, we evaluated the clinical utility of iCTCs, with concomitantly-measured CA125 levels, to predict prognosis in six EOC patients who had 6 – 19 serial measurements of iCTCs and CA125.

METHODS

Identifying biomarkers for CTCs using qRT-PCR and microscopic analyses

Biomarkers specific for CTCs were identified based on a previous publication [22] and detailed methods described in Supplementary Methods.

Flow cytometric assay for measurement of iCTCs and Epis

CAM-avid cells from patient blood were identified as described [17, 20, 21] by multi-parameter flow cytometry (FACSCalibur, BD Biosciences). Specifically, the antibodies and reagents used were: phycoerythrin (PE)-conjugated anti-TP antibodies (anti-CD44 and anti-seprase, Vitatex), and allophycocyanin (APC)-conjugated anti-HL antibody (anti-CD45, BD Pharmingen). CTCs had nuclei that were stained with a nucleic acid (NA) dye, i.e., 7-aminoactinomycin D (7AAD), after cell fixation. Events were analyzed using FlowJo software (version 7.6.5 for Windows).

Patients, Blood collection and Preparation

The objective is to test if response of cancer patients to chemotherapy can be monitored using flow cytometric counting of iCTCs. This study was approved by the institutional review board overseeing human research at Stony Brook University. The study group consisted of 64 healthy donors, 49 patients with benign abdominal diseases and 123 EOC patients for establishing analytic performance of the assay. Among the 123 EOC patients, 31

EOC patients (9 stage I; 1 stage II; 13 stage III; 8 stage IV) seen between 2009 and 2013 who had greater than three serial measurements of iCTCs and CA125 were selected for the response monitoring study. Female patients at least 18 years of age were included. Blood samples were collected from patients at each regular visit to assess disease status through monitoring iCTC counts and CA125 levels. Time frame of the iCTC assay: prior to surgery; prior to chemotherapy; at each follow-up (1, 3, 6, 9, 12, 18, and 24 months). All patients in the 31 patients' cohort were treated with standard taxol / carboplatin chemotherapy; specific therapies of individual patients were indicated in Fig. 4 legend. There was no relation observed between iCTC changes and types of chemotherapy. A total of 129 samples from the 31 patients were analyzed for enumeration of iCTCs (CAM+TP+NA+HLcells) and Epis (CAM±TP+NA+HL- cells); a total of 169 plasma samples from 25 patients were analyzed for enumeration of CA125 (data abstracted from the medical record, Fig. 3A).

Two to twenty milliliters (mL) of blood was collected from patients using Vacutainer® tubes (Becton Dickinson; green top, lithium heparin as anticoagulant) and processed within 48 hours from collection. Blood was stored at 2–8°C when storage longer than 4 hours was needed. Stability of iCTCs in blood stored at 2–8°C over 5 days was examined (Fig. 2C), showing that >90% of CTCs remained viable up to 3 days of storage.

Statistical Analysis

Fisher's exact test and Pearson's Chi-square test for categorical variables were used to compare clinical status of patients between iCTC- or Epis-positive and negative groups [21]. The interval overall clinical status of the disease between visits was assessed and classified as progressive disease (PD) or no evidence of disease (NED) mainly by clinical examination and CT scan. The clinical cutoff was determined as 5 iCTCs/1-mL of blood or 52 Epis/1-mL of blood (mean±3×SD) as the baseline based on the study in normal donors [21].

Exploratory statistical analyses were conducted to assess the association between interval changes in iCTCs / CA125 and the corresponding clinical assessments. For each patient and interval, the changes in log10 transformation of biomarker values were calculated. Sensitivity and specificity were calculated to assess the concordance between the directional changes in biomarkers (increasing or decreasing) and the interval clinical status groups (PD or NED). Biomarker changes were further dichotomized as increasing or decreasing. Analysis of variance was conducted to compare the biomarker changes in the PD group with changes in the NED group. Logistic regression was conducted to assess the association between the biomarker changes and the probability of PD. These changes were also used in the logistic regression analysis. The odds ratio was analyzed using a Cohort study model [23]. Pearson correlation coefficients were calculated for the interval changes: CA125 level vs iCTC count.

RESULTS

Identification of Antibody Labels for iCTCs

Classification of CTCs depends upon specific subsets of surface biomarkers [24, 25]. However, it is not well-known which biomarkers would be useful to indicate their potential

role in tumor progression. Previously, we showed that the tumor cells captured by CAM from blood expressed Epi (EPCAM/*EPCAM* and CA125/*MUC16*) and TP (CD44/*CD44* and seprase/*FAP*) cell surface proteins/*genes*, as shown by gene expression and multiplex flow cytometric analyses on CAM-avid cells in blood of breast cancer patients [17]. EPCAM [26, 27]; CA125 [18, 28]; CD44 [29]; seprase [30–32] were potential biomarkers for subtypes of CTCs. To confirm these results, we used qRT-PCR assay to show that CAM-avid cells from blood samples of ovarian cancer (n=16) had significantly higher expression levels of the mRNAs of *FAP* (P-value<0.001), *CD44* (P-value <0.001), *MUC16* (P-value=0.001) and *EPCAM* (P-value=0.008) than that from healthy women (n=16) (Fig. 1A). Each biomarker was expressed relatively differently in individual EOC patients (Fig. 1B). EPCAM was high in 8/16; CA125 in 6/16; CD44 in 11/16; and seprase in 8/16 blood samples of EOC patients (Fig. 1B). A combination of seprase and CD44 (termed “TP”) detected 14/16 patients; combined EPCAM and CA125 (termed “Epi”) detected 13/16 patients (Fig. 1C), suggesting that anti-TP antibodies could be biomarkers specific for detecting CTCs in patients’ blood.

We further used functional CAM uptake by internalization (CAM+) [17–21] to recognize a population of CTCs with invasive capability, called iCTCs. To examine whether iCTCs could be imaged as invasive tumor cells, parallel aliquots of blood samples from patients with advanced EOC were subjected to microscopic imaging using cytospin preparations of stained cells in suspension. In general, iCTCs tended to be heterogeneous in size, and exhibited either CAM+TP+NA+HL– solitary cells (Fig. 1D) and cluster (Fig. 1F) or CAM+Epi+NA+HL– (Fig. 1E) cytological features under microscopy. We found that CTCs consisted of TP+ cells (CD44/seprase+HL–) and Epi+ cells (EPCAM/CA125+HL–), in which 39% to 75% of the two populations overlapped with being CAM+, the overlapping cells representing iCTCs (Fig. 1D–1F). When parallel samples from EOC patients were analyzed using CAM+TP+NA+HL– and CAM+Epi+NA+HL– criteria, iCTCs resolved using the two labeling methods were correlated (n=34; $R^2=0.66$, Fig. 1G). Taken together, iCTCs show phenotypic characteristics of CAM+TP+NA+HL– or CAM+Epi+NA+HL–.

Standardization of Flow Cytometry Assay to Measure iCTCs

Having identified positive and negative biomarkers for identifying iCTCs (CAM+TP+NA+HL–), we standardized the CTC flow cytometry assay by using the experimental spiking model (Fig. 2A;2B) and blood samples donated by healthy donors, patients with benign abdominal diseases and EOC patients (Fig. 2C–2G). The experimental spiking model involved spiking 1 to 1,000 invasive SB-MET human metastatic cells, originally described as LOX cells [32, 33], into 1-mL blood from a healthy donor as described [17–19]. The model was used to determine the analytical performance. Following the pre-enrichment step using red blood cell lysis, two populations of CTCs, viable CAM±TP+NA+HL– circulating tumor cells (Epi) and CAM+TP+NA+HL– cells (iCTCs), were enriched and identified from the nucleated cell fraction by the CTC flow cytometry assay. Within detection ranges of 1 to 1,000 tumor cells per 1-mL of blood, high recovery rate and linearity of iCTCs/1-mL blood were observed (Fig. 2A, $97\% \pm 3\%$; $R^2=0.99$). Similarly, high recovery rate and linearity of Epi/1-mL blood were obtained (Fig. 2B, $96\% \pm 1\%$; $R^2=0.99$).

In general, iCTCs had nuclei that stained with a nucleic acid (NA) dye, i.e., 7AAD, and exhibited CAM+TP+NA+HL– whereas Epis had the cellular phenotype CAM±TP+NA+HL – as detected by flow cytometry. Detection sensitivity and specificity of iCTCs and Epis were examined using blood freshly collected from 64 healthy donors, 49 patients with benign abdominal diseases and 123 EOC patients (Fig. 2C–2G). Fig. 2C revealed that heparinized blood stored at 4°C for a period of three days preserve viability of iCTCs. Fig. 2D showed examples of detecting 0 iCTCs in a healthy donor and 59 iCTCs/1-mL blood in an EOC patient using the standardized assay. Fig. 2E showed examples comparing numbers of iCTCs and Epis in an EOC patient prior to and two weeks after treatment with taxol / carboplatin.

The assay had 97% specificity for detecting iCTCs, i.e., with 5 iCTCs/1-mL blood cutoff, 97% of patients who did not have a cancerous condition (benign diseases and normal) and who were iCTCs-negative, and 83% sensitivity, i.e., 50% stage I, 40% stage II, 89% stage III and 96% stage IV EOC who were iCTCs-positive (Fig. 2F). However, the assay had 92% specificity to detect Epis, i.e., with 52 Epis/1-mL blood cutoff, 92% of benign diseases and normal who were Epis-negative, and 55% sensitivity, i.e., 25% stage I, 0% stage II, 62% stage III and 62% stage IV EOC who were Epis-positive (Fig. 2G).

Serial Measurement of iCTCs in EOC Patients Treated with Chemotherapy

Serial fresh blood samples were collected prospectively from 31 women undergoing chemotherapy for EOC, whereas serial serum CA125, imaging findings and clinical progress notes of corresponding samples were collected retrospectively (Fig. 3A). The standardized assay to measure changes in iCTCs and Epis were initially tested for sensitivities in detecting treatment response of 31 patients who had greater than four serial measurements, a total of 129 serial time points (Fig. 3B). iCTCs were elevated (>5 iCTCs/mL cutoff) at one or more time points in 31 of the 31 women (100%) and in 110 of the 129 samples (85.3%). In contrast, Epis were only detected in 19 of 31 women (61.3%) and in 52 of 129 samples (40.3%). Of the 59 samples without elevated Epis, 18 (30.5%) had measurable iCTCs. Together with results from the analytical performance described above, the CTC flow cytometry assay for iCTCs appears appropriate to use in the intended treatment monitoring.

Among the 83 samples, samples from 17 women underwent comparison of iCTCs and CA125, a significant correlation between changes in iCTCs (cells per milliliter) and CA125 (U per milliliter) was identified (Fig. 3C, $r^2=0.47$; $p<0.0001$). Concordance in the directional changes (increasing vs decreasing) between iCTCs and CA125 (Fig. 3D) reflected the Pearson correlations. There was a stronger concordance between directional iCTC / CA125 changes for increasing values (sensitivity, 92.0%) than for decreasing values (specificity, 82.9%) (Fig. 3D). Among the 34 intervals associated with PD (progressive disease), 23 were found to have increasing CA125 (67.6% sensitivity) and 27 had increasing iCTCs (79.5% sensitivity) (Fig. 3E). The concordance between iCTC changes (specificity: 90.6%) or CA125 changes (specificity: 93.8%) and the intervals associated with NED (no evidence of disease) were markedly stronger than the intervals associated with PD (Fig. 3E). Overall, positive predictive values (PPV) were high (iCTCs 90%; CA125 92%) while the negative predictive values (NPV) moderate (iCTCs 80.6%; CA125 73.2%) (Fig. 3E). Variance of

changes in CA125 levels and iCTCs in the intervals between blood collections were analyzed as a function of the assessed disease status (Fig. 3F). In general, these two biomarkers trended upward in PD, whereas they reduced considerably in NED. Finally, the correlation between risk of disease progression and changes in biomarkers was assessed by using logistic regression, showing that increases in CA125 levels ($p<0.001$) and iCTCs ($p<0.00001$) were associated with increased risks for PD (Fig. 3G). While the odds ratio of CA125 was 14.4, that of iCTCs was 121.3. Together, this exploratory statistical analysis suggested that increases in iCTCs indicated higher risks for PD than increases in CA125.

Treatment monitoring of individual patients using iCTCs and serum CA125

Among the 25 patients who had been assayed prior to the first line of chemotherapy using taxol / carboplatin, 9 (36%) had CA125 levels lower than the clinical cutoff, 35U per mL [28, 34], and exhibited little changes during and after therapy. Among these cases, changing CA125 levels within a normal range (examples below – Figs. 4A, 4B, 4C, and 4E: red arrows with ?) did not correlate with changes of clinical status.

Six patients who had greater than 6 measurements (iCTC and CA125) were individually analyzed (Fig. 4). All six patients exhibited decreases in CA125 levels after surgery; however, four of the six that showed CA125 dropped to levels within a normal range, <35U per mL, that were not correlated with changes in clinical status in response to the first line of chemotherapy (Figs. 4A, 4B, 4C, and 4E: red arrows with ?). On the other hand, decreases in iCTCs in all six patients correlated with positive response from PD to NED during the primary therapy, and antedated 3 months (Fig. 4A), 2 months (Fig. 4C), 1 month (Fig. 4B) ahead of and at month 0 (Fig. 4D–4F) corresponding to clinical status from PD to NED during the primary therapy (Fig. 4A–4F, red arrows).

Furthermore, iCTCs successfully detected relapse of three out of three patients earlier than these were revealed with radiographic imaging (Figs. 4A, 4D, 4E; blue arrows), whereas CA125 could only identify one out of three (Figs. 4A, 4D, 4E; blue arrows with ?). In three patients who underwent two or three lines of therapy, iCTCs detected all three positive responses from PD to NED whereas CA125 only detected two positive responses from PD to NED (Figs. 4A, 4D, 4E; green arrows). Results of individual patients described here support further investigations examining if serial measurements of iCTCs are effective in predicting therapeutic responsiveness in EOC patients.

DISCUSSION

In this study, we identified seprase and CD44 as TP markers specific for CTCs and optimized the CTC flow cytometry assay to achieve 97% specificity and 83% sensitivity (Fig. 1–2) needed for serial measurements of iCTCs in cancer patients, raising the possibility of an application for treatment monitoring of ovarian cancer. We found that the CTC assay exhibited 79.5% sensitivity and 90.6% specificity in monitoring response of chemotherapy in a cohort of 25 EOC patients (Fig. 3). By comparison, the effectiveness of the standard CA125 assay yielded 67.6% sensitivity and 93.8% specificity (Fig. 3). The positive predictive values (PPV) are high (iCTCs 90%; CA125 92%) but the negative predictive

values (NPV) moderate (iCTCs 80.6%; CA125 73.2%) (Fig. 3). Significantly, increases in iCTCs showed higher risk for progressive disease than did increases in CA125 (Fig. 3).

We found that 9 of 25 EOC patients (36%) had CA125 levels below cutoff before chemotherapy and also found that the fluctuating CA125 levels during primary chemotherapy were not useful in evaluating treatment response. Furthermore, among the six patients who had greater than 6 measurements, iCTCs but not CA125 antedated a change in clinical status from PD to NED during and after chemotherapy and predated relapse (Fig. 4).

Although exploratory in nature, these observations suggest that serial measurements of iCTCs may offer an improved monitoring tool in predicting therapeutic responsiveness. Therefore, a confirmatory study with a larger patient cohort is warranted to further examine the use of iCTCs as a treatment monitoring test. A preliminary power analysis was performed to determine the number of patients needed to generate solid, meaningful data using Cochran's formulas [35]. Based on this model under a 90% confident level, 0.5 Standard deviation and $\pm 10\%$ confidence interval, 68 EOC patients are needed to obtain confident results.

We have used a platform to isolate viable, live circulating tumor cells for various downstream applications. These include: (a) enumeration using flow cytometry and microscopy to indicate prognostic significance [17–21]; (b) proliferation of tumor cells in culture [17–19] to enable *ex vivo* drug sensitivity testing and characterization (unpublished results); (c) propagation of tumor cells in mice for establishing metastasis models (unpublished results) to investigate mechanism of metastasis and drug resistance; and (d) single cell sorting using FACS [17, 20] or novel cell sorting devices to provide pure cells for further molecular analyses by RT-PCR or next generation sequencing thus enabling the characterization of cancer mutations and acquisition of drug resistance information for example. The rationale behind our assay is that the CAM captures CTCs of different sizes and phenotypes without bias to particular capture antibody biomarkers or physical properties of the tumor cell. Thus, the CAM enrichment / identification platform presents the CTC population with a minimal loss of viable tumor cells. However, analytic performance of our iCTC assay is based on a clinical cutoff value, in which iCTCs detected with low frequency (2 out of 64) in blood samples of healthy women (Fig. 2F) were used to determine the cutoff. Although currently un-known, it is believed that rare HL cells in healthy blood may express the CAM+ and TP+ phenotype of true iCTCs in cancer patients.

Most antibody-based enrichment methods including CellSearch CTC analysis using EPCAM antibody lose some tumor cells since EPCAM is down-regulated in the tumor cells undergoing Epithelial Mesenchymal Transition (EMT) [36]. Furthermore, it is acknowledged that CTC counts derived from anti-EPCAM antibody-captured methods were useful solely as a prognostic biomarker in patients with metastatic diseases but did not seem to consistently correlate with standard measures of response to therapy [37].

A blood test useful in monitoring response to therapy requires assay standardization enabling the detection of CTCs with high sensitivity and reproducibility. In this paper, we identified seprase and CD44 as cell surface TP markers specific for CTCs using molecular

and microscopic analyses. TP markers facilitated optimization of the CTC assay using the experimental spiking model (Fig. 2A;2B) and blood samples of healthy donors, patients with benign abdominal diseases and EOC patients (Fig. 2C–2G). The optimized assay had 97% specificity for detecting iCTCs, i.e., with 5 iCTCs/1-mL blood cutoff, 97% of patients who did not have a cancerous condition (benign diseases and normal) and who were iCTC-negative, and 83% sensitivity, i.e., 50% stage I, 40% stage II, 89% stage III and 96% stage IV EOC who were iCTC-positive (Fig. 2F), suggesting that the iCTC assay appears suitable to serve the intended treatment monitoring. However, detection sensitivities of other platforms in the field are very low [12–15], i.e., 30–50% in advanced or recurrent ovarian cancer. Essential steps that enable the iCTC assay to achieve the high sensitivity and specificity include to: (1) collect anti-coagulated blood using heparin tubes and store tubes in 4°C if blood processing occurs after 4 hours, (2) label tumor cells using the functional CAM uptake (CAM+), tumor progenitor (TP: seprase and CD44) positive staining, and nucleic acid (NA) positive staining but negative staining with hematopoietic lineage (HL: mix of anti-CD45 antibodies), and (3) detect tumor cells with an automated, four channel flow cytometer.

A noteworthy finding in this study is the predictive ability of a decrease in iCTCs but not in CA125 for chemotherapeutic responsiveness in 9 of 25 EOC patients (36%) with low levels of CA125 at the beginning of chemotherapy, and increases in iCTCs predated relapse earlier than CA125 (see Fig. 4). This observation suggests that serial measurement of iCTCs offers a better reflection of tumor progression than that of CA125 in the 9 EOC patients studied. One potential reason why iCTCs seem to be better than CA125 in predicting tumor progression may be related to the biologic factors that impact variations in these biomarkers: release of invasive tumor cells and shedding CA125 into the circulation, respectively. Considering cell invasion and metastasis as the hallmark of tumor progression, a small fraction of tumor cells would have acquired the propensity to invade tissue, penetrate blood vessels, and evade the immune system in the circulation [24, 38, 39]. Tumor cells surviving through the metastatic cascade would effectively be captured by the CAM enrichment method used in this study. The circulating tumor cells with the invasive phenotype, called iCTCs, would likely contribute to metastatic growth and cancer progression. On the other hand, serum CA125 might be shed from both normal and tumor tissue. Although monitoring serum CA125 levels is useful for determining how ovarian cancer is responding to treatment [2], the specificity of CA125 is particularly low in premenopausal women because many benign conditions, such as menstruation, pregnancy, and pelvic inflammatory disease, cause fluctuations in CA125 levels [40]. We postulate that because it involves the uncertainty of its biological activity, serial measurement of serum CA125 might not reflect therapeutic responsiveness and cancer progression in patients as nimbly as that of iCTCs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Research Highlights

- Molecular and microscopic analyses described here identified novel tumor progenitor (TP) markers, seprase and CD44, to recognize CTCs
- The optimized flow cytometry assay described here enabled serial measurement of iCTCs in ovarian cancer patients who underwent standard chemotherapy.
- Increases in iCTCs (79.5%) were more sensitive than increases in CA125 (67.6%) to predict progressive disease or relapse.

Fig. 1. CTC biomarkers include functional CAM uptake and TP antibody labels
(A) CTC associated genes identified by qRT-PCR. Individual TP (*seprase/FAP* and *CD44*) and Epi (*EPCAM* and *CA125/MUC16*) genes were upregulated in blood of EOC patients compared with that of healthy women. Bar graph plot is used to demonstrate the typical gene expression patterns of different cell groups as well as fluctuations of expression levels within and between cell groups. (B) Relative expression of individual TP and Epi genes in blood of individual ovarian cancer patients. Expression levels of *FAP* and *CD44* were multiplied by 2 and 4, respectively, to facilitate graphing them with *MUC16* and *EPCAM*.

(C). Sensitive detection of CTCs in EOC using the expression of combined TP (*FAP* and *CD44*) and Epi (*EPCAM* and *MUC16*) genes in individual patients. (D). Microscopic examination of iCTCs using CAM uptake and anti-CTC antibody labels TP(+) / NA(+) (nuclear) fluorescence where TP = seprase and CD44; NA (+) (nuclear) = DAPI nuclear markers. Bar=40µm. (E). Microscopic examination of iCTCs using CAM uptake and anti-CTC antibody labels Epi(+) / NA(+) (nuclear) fluorescence where Epi = EPCAM and MUC16; NA(+) (nuclear) = DAPI nuclear markers. Note that Epis include CAM+Epi+NA+HL– cells (iCTCs, yellow arrows and yellow labels), and CAM-Epi+NA+HL– cells. False positives = CAM-Epi+NA+HL+ cells (white arrows and white Epi+HL+labels). Bar=40µm. (F). Microscopic examination of a cellular cluster of CAM+TP+NA+HL– iCTCs. Bar=40µm. (G). Scatter plot of CAM+TP+NA+HL– cells versus CAM+Epi+NA+HL– cells in the study samples (n=34), as measured under a microscope for iCTCs/1.0-mL blood.

Fig. 2. Standardization of the flow cytometry assay to measure iCTCs

(A). Analytical performance of the CTC flow cytometry assay for iCTCs using the experimental spiking model. (B). Analytical performance of the CTC flow cytometry assay for Epis using the experimental spiking model. (C). Stability of iCTCs in blood stored over time. Blood samples were stored at 4°C for period of time indicated. (D). An example of detection of iCTCs in blood of a normal donor and an EOC patient using the standardized assay. Numbers of iCTCs were measured in 1-mL of blood. In the first two panels, G1 and G2 gateings are illustrated by the curved lines in the lower right corners. The last panel

(G1+G2) is a plot of the gated cells with TP positive on the X axis and CAM positive on the Y axis. **(E)** An example of detection of iCTCs and Epis in blood of an EOC patient one day prior to and two weeks post to treatment with taxol / carboplatin using the standardized assay. Numbers of iCTCs and Epis were measured in 1-mL of blood. **(F)** High detection sensitivity and specificity of iCTCs in normal, benign and patients within a stage. Error bars indicate SEM; horizontal lines within boxes are median; dots show outliers. **(G)**. Detection of Epis in normal, benign and patients within a stage. Error bars indicate SEM; horizontal lines within boxes are median; dots show outliers.

Fig. 3. Clinical correlates of iCTCs and serum CA125 to monitor treatment response
(A) Enrollment of patients and collection of clinical samples for monitoring treatment response using iCTC and CA125 assays. 86 samples from 8 women who underwent measurement of CA125 did not have matching iCTC enumeration data. iCTCs denotes invasive circulating tumor cells; and CA125 denotes cancer antigen 125. (B) Comparison of detection sensitivity to monitor treatment response in EOC using the standardized CTC flow cytometry assay for changes in iCTCs and Epis. Changes in iCTCs were more sensitive in detecting treatment response in EOC than changes in Epis. (C) Correlation between changes

in iCTCs (cells per milliliter) and CA125 (U per milliliter) in patients underwent therapy. Numbers represent changes in $\log_{10}$ transformed values of iCTCs or CA125. **(D)** Concordance between CA125 and iCTCs directional changes for increasing values (sensitivity) and for decreasing values (specificity). **(E)** Concordance between CA125 levels and iCTCs with interval disease status. **(F)** Analysis of the variance of CA125 and iCTC changes on interval disease status. **(G)** Logistic regression for risk of disease progression.

Fig. 4. Treatment monitoring of individual patients using iCTCs and serum CA125

Patients were designated using specific numbers and numeric stages. iCTCs (upper plot, data shown in red) and CA125 (lower plot, data shown in red) were plotted against same clinical status (both upper and lower plots, data shown in blue) that was expressed as either progressive disease (PD) or no evidence of disease (NED). Treatments were shown by specific patterned boxes on the top line of plots – red boxes indicate surgery; black boxes the first line treatment (Taxol/Carbo and/or Avastin); blank boxes the second line treatment (Topotecan, Taxol/Carbo or Avastin); patterned boxes the third line treatment (Taxol/

Pazopanb). Red arrows indicate changes in iCTCs or CA125 relative to the positive response during the primary therapy (from PD to NED), while red arrows with marks (?) indicate failure of detecting response. Blue arrows indicate changes in iCTCs or CA125 relative to relapse (from NED to PD), while blue arrows with marks (?) indicate failure of detecting relapse. Green arrows indicate changes in iCTCs or CA125 relative to the positive response during the second and/or third lines of therapy (from PD to NED), while green arrows with marks (?) indicate failure of detecting response.

(A) Changes in iCTCs (upper plot) and CA125 (lower plot) of SB407 patient with stage III EOC treated with two lines of therapy as indicated. iCTCs at visit prior to the second cycle of the first line of therapy (75 days prior to the clinical status NED; red arrow) detected positive response from PD to NED, whereas CA125 failed detecting response. Similarly, iCTCs, but not CA125, detected relapse approximately 100 days earlier than it was revealed with radiographic imaging (blue arrows). During the second line of chemotherapy, both iCTCs (upper plot, green arrows) and CA125 (lower plot, green arrows) detected positive response from PD to NED. **(B)** Changes in iCTCs (upper plot) and CA125 (lower plot) of SB406 patient with stage III EOC treated with therapy as indicated. iCTCs, but not CA125, at visit prior to the second cycle of therapy (red arrow) detected positive response from PD to NED. **(C)** Changes in iCTCs (upper plot) and CA125 (lower plot) of SB405 patient with stage IC EOC treated with a therapy as indicated. iCTCs, but not CA125, at visit prior to the second cycle of therapy (red arrow) detected positive response from PD to NED. **(D)** Changes in iCTCs (upper plot) and CA125 (lower plot) of SB414 patient with stage IIIC EOC treated with two lines of therapy as indicated. In this case, both iCTCs and CA125 detected positive response in the first line of therapy (red arrows), relapse (blue arrows), and positive response from PD to NED in the second line of chemotherapy (green arrows). **(E)** Changes in iCTCs (upper plot) and CA125 (lower plot) of SB419 patient with stage IV EOC treated with three lines of therapy as indicated. In this case, decrease in iCTCs, but not CA125, detected positive response in the first line of therapy (red arrows); increase in iCTCs, but not CA125, identified relapse and non-responsiveness of the second line of chemotherapy (blue arrows) and the third line of chemotherapy (green arrows). **(F)** Changes in iCTCs (upper plot) and CA125 (lower plot) of SB445 patient with stage IV EOC treated with therapy as indicated. Decrease in both iCTCs and CA125 detected positive response from PD to NED during therapy (red arrows).