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Prognostic analysis of invasive circulating tumor cells (iCTCs) in epithelial ovarian cancer

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Abstract

Goals: Circulating tumor cells (CTCs) have been introduced as a biomarker in detecting advanced Epithelial Ovarian Cancer (EOC). The goals are to examine the prevalence of the invasive subpopulation of CTCs (iCTCs) in patients at high risk of EOC and to compare this biomarker to serum CA125.

Methods: We used a unique Cell Adhesion Matrix (CAM)-based, functional cell enrichment and identification platform to isolate iCTCs from 129 preoperative patients. We confirmed the identity of iCTCs using positive epithelial (Epi+) markers and negative hematopoietic lineage (HL-)

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markers. Sensitivity and specificity of the assays were examined and iCTCs / CA125 were correlated with overall survival (OS), progression-free survival (PFS) and clinical parameters.

Results: We found a 41.2% sensitivity, 95.1% specificity and 77.8% positive predictive value (PPV) of the iCTC assay in detecting patients with stage I and II EOC malignancy, and a 83% sensitivity and 97.3% PPV in detecting all stages of EOC malignancy. However, a positive CA125 test provided weak evidence to detect stage I and II malignancy (61.6% PPV) and all EOC (92.1% PPV), because of its 76.2% specificity. A significantly stronger concordance in OS and PFS of clinical factors (tumor stage, debulking and platinum sensitivity) was noted for elevated iCTCs than for serum CA125.

Conclusion: The CAM-initiated CTC enrichment / identification method enabled the detection of early stage EOC. iCTCs were better correlated with worse OS and PFS, more specific and better PPV than CA125 in detecting EOC malignancy in patients at high risk of EOC.

Keywords

Cell invasion; CTC; metastasis detection; 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]. At initial diagnosis, over 75% of patients have detectable evidence of spread to extra-ovarian sites, primarily within the peritoneal cavity. Although the majority of EOC patients who undergo contemporary management with aggressive cytoreductive surgery and multi-agent chemotherapy will go into remission, most are destined to recur within three years and ultimately succumb to progressive, metastatic disease. Biomarkers, such as the rate of decline of serum cancer antigen CA125 (also known as mucin-16) level or the absolute CA125 nadir, can be predictors of progression free survival (PFS) and overall survival (OS) [2-5].

Recently, circulating tumor cells (CTCs) have been introduced as a biomarker for diagnosis and management of multiple types of cancers including EOC [6-14]. The detection of CTCs, using positive and negative antigen identification techniques, has been shown to be useful in distinguishing between metastatic cancer patients with favorable and unfavorable prognosis. The CellSearch system (VERIDEX), which depends on antigenic enrichment and identification of CTCs and has been approved by the US Food and Drug Administration (FDA) for prognostic use in metastatic breast, prostate and colon cancer, could only detect CTCs in patients with metastatic and recurrent ovarian cancer [6, 10, 13]. However, these CTC numbers do not significantly correlate with clinical characteristics or EOC patient outcomes. Investigations deciphering into sensitive detection of the CTC population with metastatic potential are needed.

From a practical standpoint however, the current unreliability and infrequent detection of CTCs in non-metastatic cancer, impede their clinical utility. Because CTCs are present at an extremely low frequency (one tumor cell in one-billion blood cells or one-million nucleated

blood cells), pre-enrichment methods are needed to remove the vast majority of red and white blood cells, prior to detection of the one rare CTC in a thousand nucleated blood cells. Accordingly, we have developed a functional cell enrichment / identification method capable of enriching viable CTCs one-million fold and identifying CTCs by their invasive phenotype. We then demonstrated the prevalence of CTCs in both metastatic and non-metastatic cancers of the breast, ovary and prostate [11, 15-17]. 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 +), indicating the invasive phenotype of cells isolated called iCTCs. Captured iCTCs are further distinguished by microscopy and flow cytometry using epithelial (Epi+) markers, i.e., any of antibodies against epithelial antigens (EPCAM, CA125, DPP4 and cytokeratins) to confirm the tumor cell, and negative hematopoietic lineage markers (HL-: leukocyte common antigen CD45 and neutrophil CD66b) to exclude hematopoietic cells [11, 15, 16].

In this study, we first assessed if the CAM enrichment / identification method could allow a robust measurement of iCTCs in early and advanced stage EOC. If so, we asked whether the sensitivity and specificity of the iCTC assay was adequate for a screening application in 129 preoperative patients at high risk of EOC. In addition, we asked whether iCTCs were comparable to the widely studied serum CA125 in prediction of recurrence and survival, and in screening this group of patients at high risk of EOC.

METHODS

Patients, blood collection and preparation

Between January 1, 2002 and February 7, 2013, 48 healthy women and 129 patients undergoing evaluation for suspected EOC malignancy (initial clinical diagnosis), which represented approximately 4% of all the cases on the Gynecologic Oncology service, were recruited to this project. We consecutively enrolled every patient who was eligible and consented, so there wasn't any selection bias. This study was approved by the Committees on Research Involving Human Subjects at Stony Brook University. Surgical diagnosis of EOC was defined using the FIGO classification system. Among 129 patients who underwent qualification of iCTCs, only 86 patients with benign and EOC underwent CA125 testing. Among the 88 patients diagnosed with EOC, 65 had both iCTC and CA125 data available for analysis (Fig. 1E); 63 were treated with debulking surgery and 61 were treated with platinum based chemotherapy . Clinical data was extracted from patient charts and retrospectively analyzed. Disease recurrence was confirmed either by clinical examination, CT scan and biopsy or by repeat surgery. The median follow-up time was 21.4 months (range: 0-140.9 months) for the patients included in this study. Patients that underwent debulking surgery were categorized into optimal and suboptimal status dependent upon whether tumors were effectively debulked. Platinum sensitivity, as a measure of treatment response, was defined as no disease progression or relapse during 6 months follow up after chemotherapy.

Two to twenty milliliters (mL) of blood were collected from healthy women volunteers and patients prior to surgery. Blood was collected in Vacutainer® tubes (Becton Dickinson;

green top, lithium heparin as anticoagulant) and processed within 72 hours from collection. Blood was stored at 2-8°C when storage longer than 4 hours was needed.

Flow cytometry analyses

CAM-avid cells from patient blood were identified as described [15, 17] by multi-parameter flow cytometry (FACSCalibur and ARIA, BD Biosciences) with minor modifications as detailed below. Specifically, the antibodies and reagents used for these experiments were: any of phycoerythrin (PE)-conjugated anti-Epi antibodies (ESA clone B29.1, Biomeda; EPCAM clone Ber-Ep4, DakoCytomation; CA125, DakoCytomation; DPP4, Vitatex), and allophycocyanin (APC)-conjugated anti-HL antibody cocktail (anti-CD45 clone 5B1 and CD66b clone REA306, Miltenyi Biotech). Events were analyzed using FlowJo software (version 7.6.5 for Windows).

Imaging of CTCs by microscopy

Microscopic characterization on CTCs was performed [11, 15-17] with minor modifications as described below. To prepare for staining, CAM-avid cells were suspended, fixed with 1.0% paraformaldehyde, and first stained for negative selection using an anti-HL antibody cocktail against CD45 and CD66b, followed by red or blue color alkaline-phosphatase–anti-alkaline-phosphatase (APAAP) conjugated secondary antibodies (DakoCytomation), and then FITC-conjugated anti-Epi antibodies. Nuclei were visualized using DAPI or Hoechst 33342 (NA) staining. Stained cells in suspension were mounted using a Cytospin device (StatSpin cytofuge and Filter Concentrators). Microscopic analyses were performed on a Nikon Eclipse E400 inverted fluorescence microscope equipped with Nikon 40× and 20× / NA objectives, a Microfire digital camera system and Image Pro Plus software.

Statistical Analysis

Fisher’s exact test or Pearson’s Chi-square test for categorical variables and Wilcoxon rank sum test for continuous variables were used to compare patient characteristics between iCTC - or CA125-positive and negative groups. Overall survival (OS) was defined from the date of blood sample collection to either the date of death or last follow-up as of Feb. 7, 2013. Progression-free survival (PFS) was defined from the date of blood sample collection to either the date of death, recurrence, progression or last follow-up as of Feb.7, 2013, whichever occurred first. Kaplan–Meier curves were used to estimate OS/PFS and log-rank test statistics were used for comparing OS/PFS between iCTC/CA125 positive and negative groups. Limited by the small sample size in this clinical data set, Kaplan-Meier and Log-rank tests were used to examine if there was a significant difference in OS/PFS between the iCTC/CA125 positive and negative groups combined with each of these three clinical factors, including disease stage (I+II vs III+IV), debulking (optimal vs suboptimal) or platinum sensitivity (yes versus no) [18]. OS/PFS rates at 5 years after the time of blood sample collection based on Kaplan-Meier curves were reported with their 95% confidence intervals [19]. Statistical significance was set at 0.05 and analysis was done using SAS 9.3 (SAS Institute, Inc.).

RESULTS

Measurement of iCTCs in Pre-op and Post-op Patients at High Risk of EOC

We tested the assay's capacity to measure iCTCs from pre-operative blood samples donated by 41 patients with benign abdominal diseases and 88 EOC patients, as well as post-operative blood samples from 21 EOC patients (Fig. 1; Table 1). iCTCs were quantified by automated flow cytometry using combined CAM uptake and Epi+ but HL- (Fig. 1A). We found that CTCs consisted of Epi+HL- cells; 50–95% of the Epi+HL- cells were CAM+Epi + HL- iCTCs (Fig. 1A).

To examine whether the cells identified as iCTCs by flow cytometry could be imaged as invasive tumor cells, parallel aliquots of blood samples from patients with advanced EOC, in which iCTCs were detected by flow cytometry, were subjected to microscopic imaging (Fig. 1B). In general, iCTCs tended to be heterogeneous in size, had nuclei stained with a nucleic acid (NA) dye, and exhibited CAM+Epi+NA+HL- (Fig. 1B), and had cytological features under microscopy that were consistent with the cellular phenotypes detected by flow cytometry.

To determine the prevalence of iCTCs in the blood of the 129 pre-operative patients at high risk of EOC at the time of abdominal surgery (Table 1), we found that 78 of 88 (88.6%) patients had >5 iCTCs/1.0-mL blood; iCTC counts ranged from 0 to 254 with 42±77 (median±IQR [interquartile range]) (Fig. 1C). iCTCs were detected more frequently in patients with higher stage disease. Specifically, 5/13 (38.5%) Stage I; 2/4 (50%) Stage II; 46/50 (92%) Stage III and 20/21 (95.2%) stage IV patients had more than 5 iCTCs/1-mL blood. There were significant differences between iCTC positive and negative ovarian cancer patients (P-value<0.0001, Table 1) in terms of tumor stage, recurrence and death, but not in age, tumor grade, histologic type, cancer type, debulking and platinum sensitivity (Table 1).

Comparison of iCTCs and Serum CA125 in Detecting Ovarian Cancer

Employing a clinical cutoff of >35U per mL [3, 5, 20, 21], serum CA125 levels were elevated in 86 of 88 patients diagnosed with EOC (P-value<0.0001, Fig. 1D; Table 1). However, although serum CA125 levels were significantly different across patient groups and showed a trend of increase in preoperative patients diagnosed with a more advanced disease stage, the serum CA125 assay had a high false-positive rate (23.8%) and large variation in women with benign disease (Fig. 1D, n=21) as previously described, whereas the iCTC assay had a low false-positive rate (4.9%) and small variation (Fig. 1C, n=41).

iCTCs and serum CA125 levels were both available for 65 patients with EOC (Fig. 1E; Table 1). CA125 levels were elevated (>35U per mL) in 57 of these 65 women (87.7%), and iCTCs were increased in 55 of 65 women (84.6%). Of the 10 women without elevated iCTCs, 8 (80%) had increased levels of serum CA125. Conversely, of the 8 women without elevated CA125, 6 (75%) had increased iCTCs (Fig. 1E).

Previously, a reduction in the level of serum CA125 in patients after surgery was shown to be a prognostic factor and an indicator of reduced tumor burden [3, 5, 20, 21]. To examine if

iCTCs could be an indicator of tumor burden, we compared changes in iCTCs and serum CA125 levels from 21 patients between pre-op and post-op to assess the sensitivity of detecting tumor burden (Fig. 1F). Reduction of CA125 levels and iCTCs were observed in 21 of 21 (100%) and 17 of 21 (81%) patients after surgery, respectively, suggesting that CA125 is more sensitive than iCTCs in detecting tumor burden.

Specificity and Sensitivity of the iCTC Assay in Screening Patients at High Risk of EOC

The specificity and sensitivity of the iCTC assay in screening the 129 pre-operative patients undergoing evaluation for suspected EOC malignancy at Stony Brook University Cancer Center were examined (Fig. 2A;2B). To explore the possibility of using the iCTC assay for early detection of EOC, we examined 58 patients with benign, stage I and II diseases. The iCTC assay had 41.2% sensitivity, 95.1% specificity, 77.8% positive predictive value, and 79.6% negative predictive value, resulting in 8.4 likelihood ratio (Fig. 2A) indicating that stage I and II EOC patients are 8.4 times more likely to have a positive iCTC test than benign patients. Importantly, among the 129 patients also include stage III and IV diseases, the assay had 83% sensitivity, 95.1% specificity, 97.3% positive predictive value, and 72.2% negative predictive value, resulting in 16.9 likelihood ratio (Fig. 2B) indicating that EOC patients are 16.9 times more likely to have a positive iCTC test than benign patients.

To compare the specificity and sensitivity of the CA125 assay with the iCTC assay, we examined the 86 among the 129 patients who were also tested for CA125 levels (Fig. 2C; 2D). Among the 33 patients with benign, stage I and II diseases, the CA125 assay had 66.7% sensitivity, 76.2% specificity, 61.6% positive predictive value, and 80% negative predictive value, resulting in 2.8 likelihood ratio (Fig. 2C), whereas, among the 83 patients also including stage III and IV diseases, the assay had 89.2% sensitivity, 76.2% specificity, 92.1% positive predictive value, and 69.6% negative predictive value, resulting in 3.7 likelihood ratio (Fig. 2D). Together, these results suggest that a minimum of 40% sensitivity and 95% specificity of an assay such as the iCTC test are necessary for providing strong evidence (based on the likelihood ratio) for the early detection of EOC, and at least 80% sensitivity and 95% specificity are necessary for the detection of EOC.

Prognostic value of iCTCs in Predicting Patient OS and PFS

To examine the prognostic significance of iCTCs as compared to CA125 in preoperative blood samples from EOC patients, we performed univariate analysis for these assays (Fig. 3A-3B; Table 1). Patients, with positive iCTCs (>5/mL; Fig. 3A) had significantly shorter overall survival (OS: p=0.0219) and progression-free survival (PFS: p=0.0024), as compared to patients with <5 iCTCs/mL. However, while patients with increased levels of CA125 (>35 units/mL; Fig. 3B) displayed shorter OS and PFS (averages) than negative patients, the data lacked statistical significance (p=0.1362 and p=0.5601, respectively). Thus, univariate analysis showed that measurement of iCTCs outperformed CA125 levels in EOC patients in predicting OS and PFS.

Consistent with previous studies, the clinical factors significantly associated with OS and PFS in this study were tumor stage, debulking and platinum sensitivity (P-values from univariate Cox proportional hazard models were all <0.001). We compared OS and PFS

after combining iCTC- or CA125-positive/negative status with each of these three variables (Fig. 3C;3D;4A-4D; Table 2). Because of a limited numbers of patients, Kaplan-Meier analysis of OS/PFS for tumor stage I+II vs III+IV subgroups were evaluated by iCTCs (Fig. 3C) and CA125 (Fig. 3D). The iCTC – stage I+II vs III+IV subgroups had significantly different OS and PFS risk (Fig. 3C: p=0.0136 and p=0.0010, respectively). The CA125 subgroups also had significantly different PFS risk (Fig. 3D: p=0.0095), and a trend for OS risk (Fig. 3D: p=0.0557).

Similarly, Kaplan-Meier analysis of OS/PFS for optimal vs suboptimal debulking subgroups were evaluated with iCTCs (Fig. 4A) and CA125 (Fig. 4B). The four iCTC – optimal vs suboptimal debulking subgroups had significantly different OS and PFS risk (Fig. 4A: p=0.0037 and p=0.0009, respectively); but the four CA125 subgroups did not have significantly different OS and PFS risk (Fig. 4B: p=0.0582 and p=0.1307, respectively). Interestingly, a stronger concordance in OS and PFS of platinum sensitivity was seen for iCTCs than for serum CA125 (Fig. 4C;4D; Table 2). Thus, a significantly stronger concordance in OS and PFS of clinical factors (tumor stage, debulking and platinum sensitivity) was noted for elevated iCTCs than for serum CA125.

DISCUSSION

Measurement of CTCs requires an initial enrichment step to enable effective downstream cell counting. We have used a functional (CAM adhesion and invasion) method to enrich tumor cells in blood 1 million-fold that allows a significantly sensitive detection of iCTCs in early stage EOC, raising the possibility of an application for screening. A noteworthy finding in this study is a 41.2% sensitivity and 95.1% specificity of the iCTC assay in detecting patients with stage I and II EOC malignancy (Fig. 2A), and a 83% sensitivity in detecting all stages of EOC malignancy, resulting in a 97.3% positive predictive value (Fig. 2B). The former had an 8.4 likelihood ratio and the latter a 16.9 likelihood ratio, indicating that a positive iCTC test provided strong evidence to detect stage I and II malignancy and all EOC, respectively. However, a positive CA125 test provided weak evidence to detect stage I and II malignancy and all EOC, because of its 76.2% specificity (Fig. 2C;2D). These observations suggest that measurement of iCTCs in a limited group of patients with a high risk of EOC, like in this study, offers a better detection of stage I and II malignancy and all EOC as compared to CA125. Therefore, a confirmatory study with a larger patient cohort at high risk of EOC is warranted to further examine the use of iCTCs as a screening test.

Furthermore, the lifetime risk is 1.4% for women in the US and the age-adjusted incidence is ~ 13/100,000. Because the prevalence of EOC is very low, the positive predictive value (PPV) of using iCTCs as a screening test for the diagnosis of stage I and II malignancy in a general population will also be low and may prevent the use of the iCTC assay as a widespread screening tool in asymptomatic women [22]. However, we are not using the iCTC assay this way. We are only testing patients who are at high risk, which raises the prevalence in the group being tested and thus the PPV. A similar example is Lyme disease, which has the prevalence of 7/100,000 in 2012 http://www.cdc.gov/lyme/stats. As a screening test without regard for clinical evaluation, the test only has a PPV of 6%. However, if patients who clinicians determine a possible Lyme disease are tested, the PPV

rises to well over 97% (co-author Marc Golightly, personal communication), as in the present study (Fig. 2B).

Previous studies have shown wide molecular and cellular heterogeneity of CTCs from the same types of cancer and even from the same patient. Current FDA-approved CellSearch CTC analysis using EPCAM enrichment and Epi+CD45- identification criteria showed low CTC detection numbers, i.e., only 31 of 216 patients with metastatic and recurrent diseases (14.4%) had 2 or more CTCs in 7.5-mL blood detected prior to the start of therapy (range 2–566), although patients with ≥2 CTCs prior to therapy had 1.89 and 2.06-fold higher risk for progression and death, respectively [10]. Furthermore, a recent study using CellSearch platform showed that CTC numbers did not significantly correlate with clinical characteristics or EOC patient outcomes [6].

In this emerging CTC diagnostic field, we have used a unique CAM-based, functional cell enrichment and identification platform which allows for the isolation of viable, live tumor cells from blood for downstream: (1) enumeration using flow cytometry and microscopy to indicate prognostic significance [11, 15-17]; (2) proliferation of tumor cells in culture [11, 15, 16] to enable *ex vivo* drug sensitivity testing and animal metastasis models (unpublished results) to investigate mechanism of metastasis; and (3) single cell sorting using FACS [15, 17] or novel cell sorting devices to provide pure cells for further molecular analyses by RT-PCR or next gen sequencing to characterize cancer mutations and drug resistance information for example. The rationale behind our assay is that 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 minimal cell loss. However, 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) [23]. In this study, however, the use of anti-EPCAM antibody allowed confirmatory identification of CAM+ cells as iCTCs because of the recognition of the low level of EPCAM protein on the cell surface by the anti-EPCAM antibody. This is different from using epithelial labels to enrich for CTCs that has the potential to miss cells that undergo EMT.

The present study shows that the iCTC assay effectively measures iCTCs across all stages of EOC. We also show its capability to better correlate the likelihood of disease progression and death in patients at all EOC stages as compared to the widely used serum CA125 assay. When iCTCs were correlated with each clinical factor (tumor stage, debulking and platinum sensitivity) used in current treatment scenarios, a statistically significant difference in the risk for disease progression and death exists. In particular, iCTC positive patients with early cancer stage had significantly worse clinical outcome than iCTC negative patients. Therefore, the iCTC technology presented here has the potential to provide better prognostic information than serum CA125 on the probability of metastasis in early stage cancer patients when used in conjunction with standard clinical measures such as tumor staging, debulking status and platinum sensitivity.

An issue that remains to be resolved is in the identification of CTCs in healthy and benign individuals. Circulating cells identified with both epithelial and hematopoietic lineage (Epi +CD45+) have been noted in healthy subjects and cancer patients [12, 24, 25], as well as patients with benign disease [26, 27]. It has been noted that patients with benign disease of the colon exhibited "tumor cells" as detected with the CellSearch system (11.3%), the CK19-EPISPOT assay (18.9%) [26], and multiplex qRT-PCR (12%) [27]. It is unclear, however, whether normal epithelial cells can be disseminated into the blood stream resulting in false-positives using the current assay techniques, which lack unambiguous criteria for the malignant nature of the marker-positive cells. Some CTCs representing cancer stem-like or tumor progenitor cells are suggested to have entered the blood early in metastatic progression [28, 29]. In experimental models [30], these blood-borne tumor cells have been proven to be of tissue origin, spread early in cancer progression, and have potential to seed new tissue sites. However, a ntigenic overlap between tumor cells and circulating hematopoietic cells and limited efficiency of cell enrichment techniques represent important roadblocks in the field.

In this paper, we have taken the above into consideration to include functional CAM uptake (cell adhesion and invasion) as the primary identification of iCTCs, and to confirm the tumor identity with Epi+ markers and to exclude hematopoietic lineage (HL) cells using HL markers (including CD45 and CD66b). We found that the optimized iCTC flow cytometric assay (using the positive Epi+ markers in addition to negative markers of HL-) detected a smaller number of iCTCs in healthy women (n=48); 25 women had no cells detected; 21 women had 1-2 cells detected; 2 women had 5-6 cells/mL blood). Hence a cutoff value of 5 cells/1.0-mL blood (mean±3xSD) was established as the upper limit for a healthy population. Importantly, similar low counts of iCTCs were detected in 2/41 patients (4.9%) operated on for benign ovarian disease (P-value <0.0001, Fig. 1C; Table 1). These cells from healthy and benign patients are CAM+Epi+HL-. Potential explanations for rare iCTCs in the peripheral blood of non-cancer patients are: 1) rare progenitor cells derived from fetal epithelial tissue have been found to circulate in maternal blood for many years [31-33], 2) circulating endothelial progenitor cells involved in vascular abnormality or repair [34, 35] might also express Epi+ antigens; and 3) non-malignant hematopoietic progenitor cells co-expressing Epi+ and CAM+ markers. A possible explanation on the finding of an extremely low number of “normal invasive epithelial cells” in healthy blood may be that the invasive migration through the tissue occurs in a developing phase of epithelial cells, which is under-investigated. These cells, when disseminated in blood, could be enriched and identified as CAM+Epi+HL- cells. Future investigation using single cell sorting and gene sequencing of these rare cells may be useful in defining biomarkers specific for circulating fetal, hematopoietic or endothelial progenitor cells. Resulting biomarkers could then be incorporated into a novel iCTC assay to exclude false-positive cells circulating in blood that might be confused with tumor cells.

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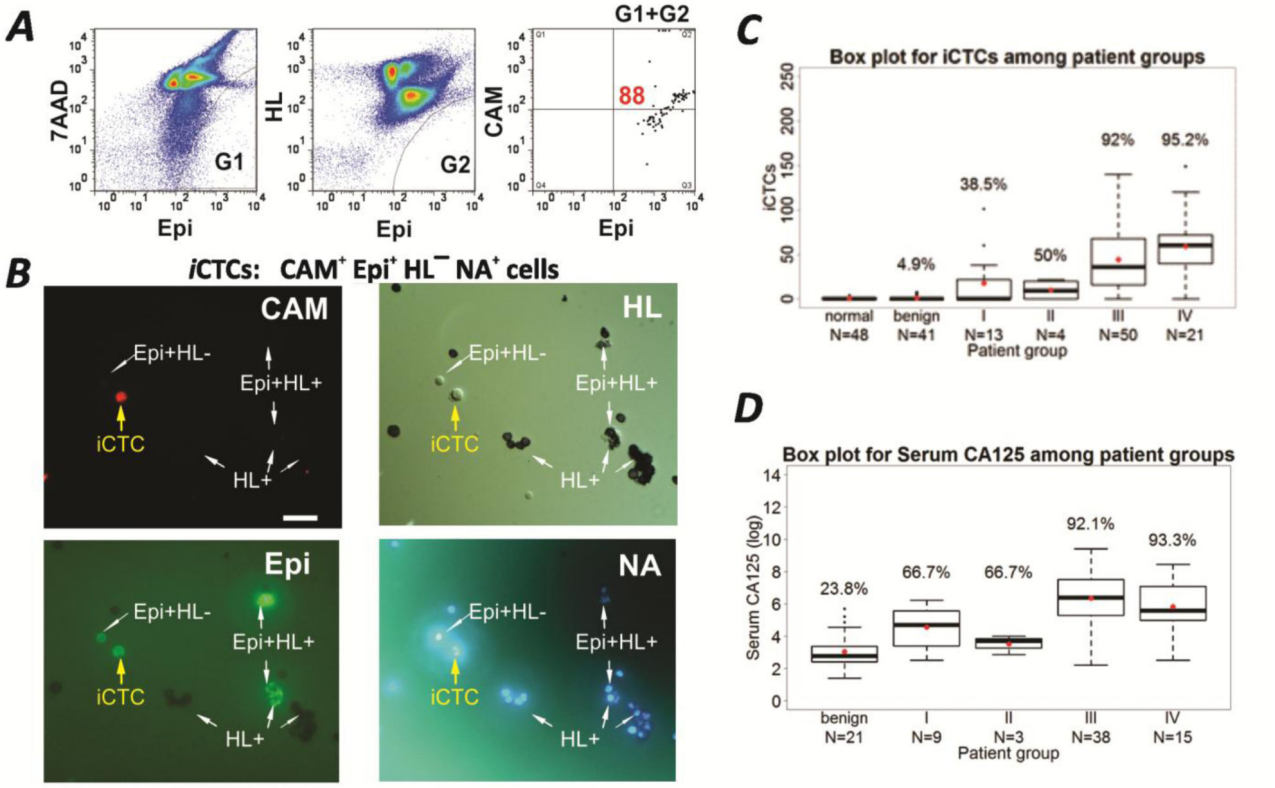
Research Highlights

- The functional CTC enrichment method described here enabled the early detection of ovarian cancer in a high risk group.
- iCTCs were better correlated with worse OS and PFS than CA125.
- iCTCs were more specific and had better PPV than CA125 in screening patients at high risk of ovarian cancer.



E

CA125	iCTCs: Elevated *	Not elevated *	Total
Patients			
Elevated *	49	8	57
Not elevated *	6	2	8
Total	55	10	65
iCTCs sensitivity, 55/65 (84.6%)			
CA125 sensitivity, 57/65 (87.7%)			

* iCTCs and CA125 levels were estimated as "Elevated" or "Not elevated" based on assay cutoffs, 5 iCTCs/mL and 35U CA125/mL, respectively.

F

CA125	iCTCs: Elevated *	Reduced *	Total
Patients			
Elevated *	0	0	0
Reduced *	4	17	21
Total	4	17	21
iCTCs sensitivity, 17/21 (81.0%)			
CA125 sensitivity, 21/21 (100.0%)			

* Changes in iCTCs and CA125 levels were estimated as "Elevated" or "Reduced" based on the difference between pre-op and post-op measurements.

Fig. 1. Measurement of iCTCs in pre-op and post-op patients with high risk of EOC

The number of iCTCs was measured in 1.0-mL of blood. ***(A)*** An example of flow cytometry enumeration of iCTCs by FITC-CAM(+) / PE-anti-Epi(+), APC-anti-HL(−), and 7AAD(+) (nuclear) fluorescence where CAM = functional uptake of matrix and Epi = tumor markers. In the first two panels, G1 and G2 gating is illustrated by the curved lines in the lower right corners. The last panel (G1+G2) is derived from sum of the G1 gated area in the first panel and the G2 gated area in the second panel; it is a plot of the gated cells with Epi positive on the X axis and CAM positive on the Y axis. 88 cells indicated in Q2 of the last panel are CAM+Epi+7AAD+HL-. ***(B)*** Validation of CAM+ flow cytometric isolation of iCTCs by microscopic imaging. An iCTC is detected as the CAM+Epi+NA+HL- cell in four fields (CAM, Epi, and NA in DAPI were detected by fluorescence, and HL was detected in DIC bright field; see yellow arrows and labels); Epis, on the other hand, were CAM-Epi+NA +HL- (white arrows and labels); HL cells were NA+HL+ (white arrows and labels); false positives were CAM-Epi+NA+HL+ (white arrows and white Epi+HL+ labels). Bar in CAM panel =40μm. ***(C;D)*** Measurement of iCTCs and serum CA125 as function of tumor stages.

Mean and median iCTC and serum CA125 counts in each group are indicated by red dots and thick bars, respectively. Black dots are outliers. % patients are those that show iCTCs and CA125 levels above cutoffs, >5 iCTCs/1.0-mL blood and >35U CA125/mL. ***(E)*** Comparison of iCTCs and serum CA125 levels as blood-based biomarkers in pre-op patients. ***(F)*** Comparison of changes in iCTCs and serum CA125 levels from 21 patients between pre-op and post-op to assess the sensitivity of detecting tumor burden.

A

iCTCs	Benign	Stage I	Stage II
Elevated *	2	5	2
Not elevated *	39	8	2
Total	41	13	4
Sensitivity,	7/17 (41.2%)		
Specificity,	39/41 (95.1%)		
Positive predictive value,	7/9 (77.8%)		
Negative predictive value,	39/49 (79.6%)		
Likelihood	8.4		

* iCTCs were estimated as "Elevated" or "Not elevated" based on assay cutoffs, 5 iCTCs/mL.

B

iCTCs	Benign	Stage I	Stage II	Stage III	Stage IV
Elevated *	2	5	2	46	20
Not elevated *	39	8	2	4	1
Total	41	13	4	50	21
Sensitivity,	73/88 (83.0%)				
Specificity,	39/41 (95.1%)				
Positive predictive value,	73/75 (97.3%)				
Negative predictive value,	39/54 (72.2%)				
Likelihood	16.9				

* iCTCs were estimated as "Elevated" or "Not elevated" based on assay cutoffs, 5 iCTCs/mL.

C

CA125	Benign	Stage I	Stage II
Elevated *	5	6	2
Not elevated *	16	3	1
Total	21	9	3
Sensitivity,	8/12 (66.7%)		
Specificity,	16/21 (76.2%)		
Positive predictive value,	8/13 (61.6%)		
Negative predictive value,	16/20 (80.0%)		
Likelihood	2.8		

* CA125 value was estimated as "Elevated" or "Not elevated" based on assay cutoffs, 35U CA125/mL.

D

CA125	Benign	Stage I	Stage II	Stage III	Stage IV
Elevated *	5	7	2	35	14
Not elevated *	16	2	1	3	1
Total	21	9	3	38	15
Sensitivity,	58/65 (89.2%)				
Specificity,	16/21 (76.2%)				
Positive predictive value,	58/63 (92.1%)				
Negative predictive value,	16/23 (69.6%)				
Likelihood	3.7				

* CA125 value was estimated as "Elevated" or "Not elevated" based on assay cutoffs, 35U CA125/mL.

Fig. 2. Specificity and Sensitivity of iCTC and CA125 assays in screening patients with high risk of EOC

(A) iCTCs as a biomarker for screening early stage I and II malignancy in the 58 high risk patients. ***(B)*** iCTCs as a biomarker for screening EOC malignancy in the 129 high risk patients. ***(C)*** CA125 as a biomarker for screening early stage I and II malignancy in the 33 high risk patients. ***(D)*** CA125 as a biomarker for screening EOC malignancy in the 86 high risk patients. The likelihood is the ratio between the sensitivity and (1.0 – the specificity). >10 likelihood ratio indicates strong evidence to rule in disease; 5-9 moderate evidence; 2-5 weak evidence.

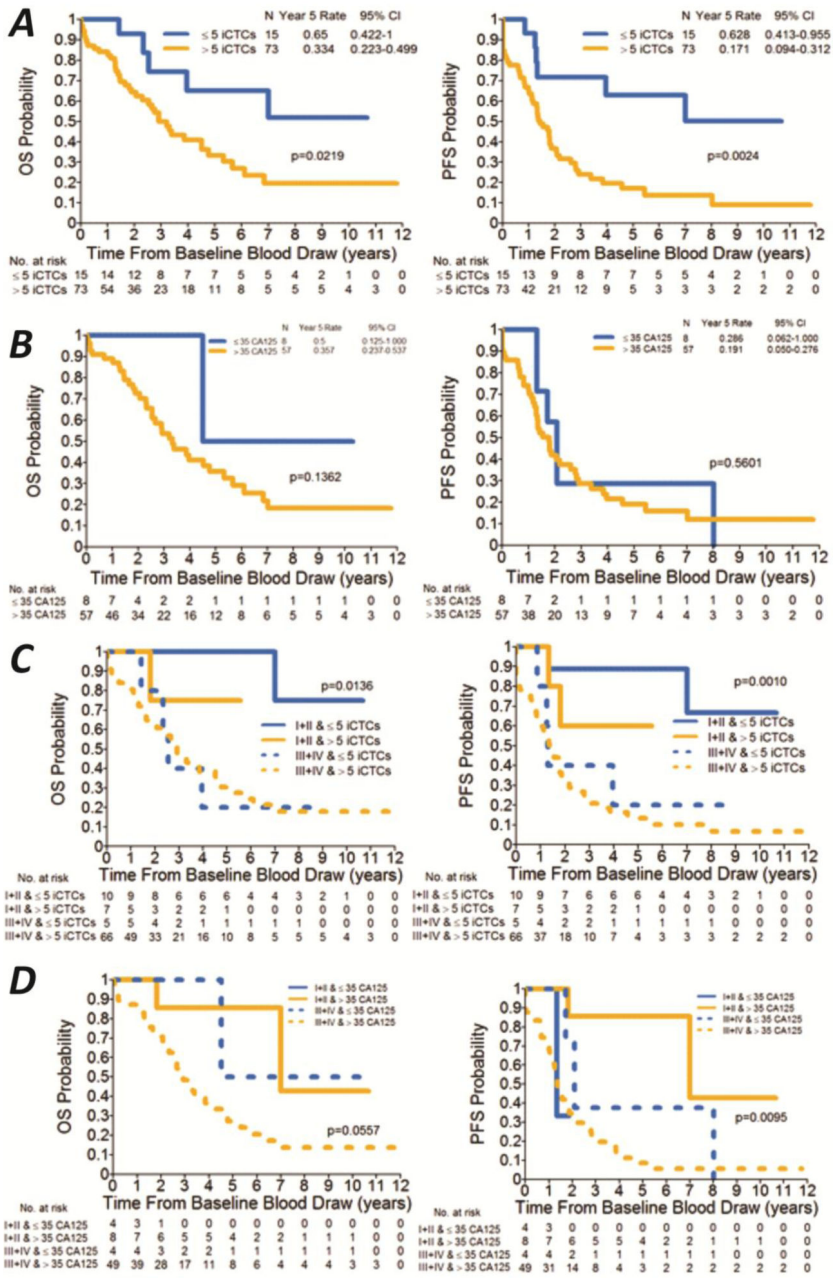


Fig. 3. Kaplan-Meier plots of OS and PFS by iCTCs (A), serum CA125 (B), iCTCs and tumor stage (C), and serum CA125 and tumor stage (D)

Negative samples are defined as ≤5 iCTCs/1-mL blood and ≤35U/mL serum CA125 levels. The difference between negative and positive groups is evaluated for significance based on the log-rank test. Relative numbers of patients with negative (top) and positive (bottom) iCTCs and serum CA125 are indicated below the year on x-axis.

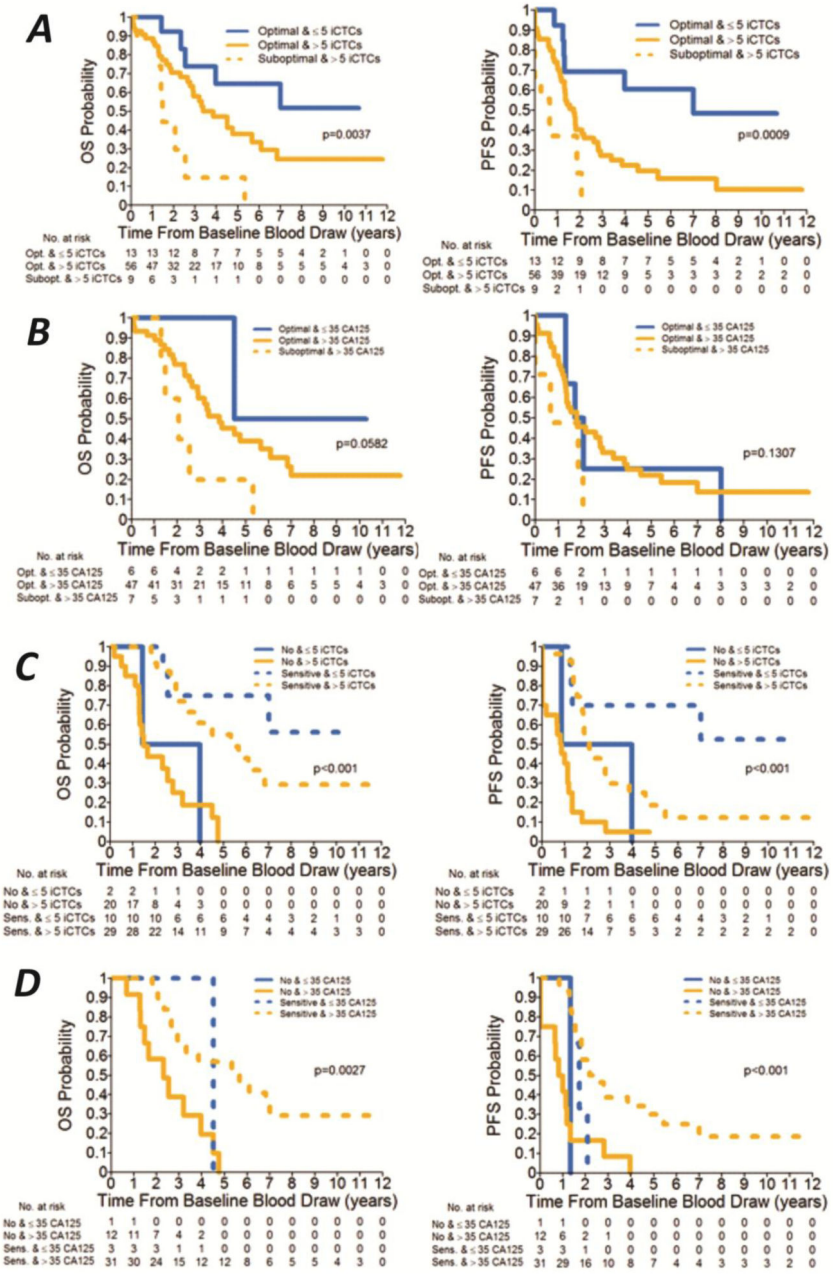


Fig. 4. Kaplan-Meier plots of OS and PFS by iCTC counts and debulking status (A), by serum CA125 counts and debulking status (optimal/suboptimal) (B), by iCTC counts and platinum sensitivity (C), and by serum CA125 counts and platinum sensitivity (D)

Negative samples are defined as ≤5 iCTCs/1-mL blood and ≤35U/mL serum CA125 levels. The difference between negative and positive groups is evaluated for significance based on the log-rank test. Relative numbers of patients with negative (top) and positive (bottom) iC TCs and serum CA125 are indicated below the year on x-axis.

Table 1

Baseline Characteristics of benign and EOC patients.

Characteristics (N=129)		iCTCs≤5[Δ] 54 (41.86%)	iCTCs>5[Δ] 75 (58.14%)	P-value[§]	CA125≤35[◇] 24 (27.9%)	CA125>35[◇] 62 (72.1%)	P-value[§]
Age (mean ± SD)		55.86 ± 11.24	59.94 ± 12.77	0.051	57.65 ± 12.00	57.80 ± 12.35	0.8404
Stage[*]	Benign (n=41)	39(72.22%)	2(2.67%)	<0.0001	16(66.67%)	5(8.06%)	<0.0001
	I (n=13)	8(14.81%)	5(6.67%)		3(12.5%)	6(9.68%)	
	II (n=4)	2(3.7%)	2(2.67%)		1(4.17%)	2(3.23%)	
	III (n=50)	4(7.41%)	46(61.33%)		3(12.5%)	35(56.45%)	
	IV (n=21)	1(1.85%)	20(26.67%)		1(4.17%)	14(22.58%)	
Grade[*]	1 (n=6)	2(18.18%)	4(6.35%)	0.2514	3(50%)	1(2.04%)	0.0033
	2 (n=3)	1(9.09%)	2(3.17%)		0(0%)	3(6.12%)	
	3 (n=65)	8(72.73%)	57(90.48%)		3(50%)	45(91.84%)	
Histology[*]	Serous (n=44)	6(40%)	38(52.05%)	0.1317	5(62.5%)	28(49.12%)	0.4943
	papillary serous (n=17)	3(20%)	14(19.18%)		0(0%)	12(21.05%)	
	endometrioid (n=2)	1(6.67%)	1(1.37%)		0(0%)	1(1.75%)	
	clear cell (n=6)	3(20%)	3(4.11%)		0(0%)	3(5.26%)	
	Other+Mix (n=19)	2(13.33%)	17(23.29%)		3(37.5%)	13(22.81%)	
Cancer type[*]	Ovary (n=76)	13(86.67%)	63(86.3%)	0.6746	6(75%)	48(84.21%)	0.7233
	Primary Peritoneal (n=9)	1(6.67%)	8(10.96%)		1(12.5%)	7(12.28%)	
	Fallopian Tube (n=3)	1(6.67%)	2(2.74%)		1(12.5%)	2(3.51%)	
Debulking[*]	Optimal (n=69)	13(100%)	56(86.15%)	0.3424	6(100%)	47(87.04%)	1.000
	Suboptimal (n=9)	0(0%)	9(13.85%)		0(0%)	7(12.96%)	
Platinum sensitivity[*]	Sensitive (n=39)	10(83.33%)	29(59.18%)	0.1821	3(75%)	31(72.09%)	1.000
	Resistant (n=22)	2(16.67%)	20(40.82%)		1(25%)	12(27.91%)	
Subsequent recurrence (as of 12/31/2012) (n=34)		4	30	<0.0001	5	25	0.1300
Subsequent death (as of 12/31/2012) (n=46)		5	41	<0.0001	1	34	<0.0001

[Δ] A baseline of >5 iCTCs/l.0-mL blood in 48 healthy women donors was chosen as the assay sensitivity cutoff (see text for discussion); a baseline of CA125>35 U / mL was chosen as cutoff

[◇] All 129 patients in the study group were analyzed for iCTCs; 86 patients of benign and EOC underwent comparison of iCTCs vs serum CA125.

[*] Benign samples were not included for these characteristics.

[§] P-values were given by Fisher's exact test for binary variables. Pearson's Chi-square test for multinomial variables and Wilcoxon rank sum test for continuous variables.

Table 2

Estimated survival rate at year 5 and 95% confidence interval

Characteristics		Overall Survival			Progression Free Survival		
		Estimate year 5 survival rate	95% Confidence Interval	P-value	Estimate year 5 survival rate	95% Confidence Interval	P-value
iCTCs	Negative	0.650	0.422-1.000	0.0219	0.628	0.413-0.955	0.0024
	Positive	0.334	0.223-0.499		0.171	0.094-0.312	
Stage* iCTCs	I+II & Negative	1.000	1.000-1.000	0.0136	0.889	0.706-1.000	0.0010
	I+II & Positive	0.750	0.426-1.000		0.600	0.293-1.000	
	III+IV & Negative	0.200	0.035-1.000		0.200	0.035-1.000	
	III+IV & Positive	0.306	0.197-0.476		0.136	0.066-0.279	
Debulking** iCTCs	Optimal Deb & Negative	0.646	0.418-0.998	0.0037	0.606	0.387-0.947	0.0009
	Optimal Deb & Positive	0.3777	0.2509-0.5686		0.1963	0.1081-0.3563	
	Suboptimal Deb & Positive	0.1481	0.0244-0.8999		0	NA	
Platinum Sensitivity*** iCTCs	Not Sensitive & Negative	0.000	NA	<0.0001	0.000	NA	<0.0001
	Not Sensitive & Positive	0.000	NA		§ 0.050	0.007-0.338	
	Sensitive & Negative	0.750	0.503-1.000		0.700	0.467-1.000	
	Sensitive & Positive	0.549	0.365-0.827		0.187	0.076-0.461	

* Stage - I+II and III+IV subgroups.

** Debulking - Optimal Debulking and Suboptimal Debulking subgroups.

*** Platinum Sensitivity - Not Sensitive and Sensitive subgroups.

§ The last censoring was before year 5.