Circulating tumor cells in ovarian cancer patients as a cellular marker for early detection of ovarian cancer in asymptomatic patients

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Research Article

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Abstract

Purpose

Ovarian cancer (OC) is the leading cause of death among gynecologic cancers worldwide. The aim of this study was to identify and enumerate circulating tumor cells (CTCs) in OC patients and correlate their numbers with the clinical outcomes.

Methods

we enrolled patients diagnosed with suspected OC based on history, ultrasound criteria, and tumor markers. Complete clinical examination, abdominal and pelvic ultrasonography, serum CA125, and risk of malignancy index (RMI) were recorded. The percentage of CTCs was analyzed using flow cytometry based on the following phenotypes CD105+, CD24+, CD117+, and Epcam+.

Results

CTCs were found in 100% of patients with primary OC and no CTCs were found in secondary or borderline OC. The mean of CTC numbers in all patients was 0.12 ± 0.11 cells/µl. The highest number of CTCs was observed among the malignant patients; A highly statistically significant (p-value < 0.001) positive correlation (r = 0.55) was found between CTCs and FIGO, between CTCs and RMI (r = 0.53; p-value < 0.001), and CTCs and CA-125 (r = 0.42; p-value < 0.001). The CTCs count allowed to distinguish between early and late FIGO stage at a cutoff level of > 0.82 cells/µl, with 66.7% sensitivity, 90.9% specificity, 88% PPV and 73.2% NPV (AUC = 0.65 & p-value = 0.076).

Conclusion

CTCs can be used as a cellular marker for the early detection of OC.

Introduction

Ovarian cancer is the leading cause of death among gynecologic cancers worldwide. Most cases are diagnosed in the late stages of the disease, resulting in poor survival [1]. The five-year survival rate of patients with late-stage ovarian cancer is only about 30% [2]. The reason for delayed diagnosis of ovarian cancer is partly due to the lack of sensitive signs, symptoms, and effective screening methods [3]. A recent study based on the parabiosis model, in which paired mice share blood rather than lymphatic vessels, has shown that hematogenous metastasis is an important form of ovarian cancer metastasis [4]. This experimental finding would explain why ovarian cancer is thought to spread directly. As such, the detection of ovarian circulating tumor cells (CTCs) might be useful after metastasis occurs.
The first observation of CTCs was made in 1867 by the Australian physician Thomas R. Ashworth who demonstrated that cells identical to those of original cancer could be found in the bloodstream and may explain the origin of multiple metastatic tumors [5]. Accordingly, the clinical significance of CTCs as a new target for early cancer detection, diagnosis, prognosis, and prediction has been widely studied in recent years [6], where CTCs were found to offer a minimally invasive 'liquid biopsy' [7]. In this setting, CTCs have been presented as a prognostic factor in the development of cancer and progress to metastases [8–11]. The role of CTCs in the spread, progression, and aggressiveness of cancer could be due to their acquisition of stem cell-like phenotype.

Indeed, a new type of cancer stem cell-like (CSC-like) cell has been established as a progenitor of metastases and relapses in cancer patients [12, 13]. In this regard, recent studies have found that CTCs have all the cellular hallmarks of CSC-like cells [14], hence, the role of CTCs in the spread, progression, and aggressiveness of cancer could be due to their acquisition of stem cell-like phenotype. Thus, CTCs in the peripheral blood of cancer patients have become a promising area of research in the field of biomarker development in contemporary cancer research [15] to determine metastasis and prognosis of cancer patients.

CTC detection technology has started to be widely used in clinical detection and clinical treatment of a variety of different tumors such as breast [8], prostate cancer, digestive tract cancer [16], small cell lung cancer [17], liver cancer [18], bladder cancer [19], and head and neck cancer [20]. Through current techniques of CTCs detection, different cancer types can be identified, this is usually done based on the physical, chemical, and biological properties of the tumor cells. For example, KRT7 and TTF-1 positive CTCs have been found to associate with lung cancer [21] PSA and PSMA positive CTCs are associated with prostate cancer, and KRT20 and CDX2 positive cells are associated with colorectal cancer [21]. Therefore, the tumor type can be identified through the characteristics of CTCs.

The gold standard for CTC testing is the CellSearch® system, which is the only US FDA-approved test platform for CTC isolation, primarily for breast, prostate, and colorectal cancer; it can detect ≥ 5 CTCs per 7.5 mL of peripheral blood [8, 16, 21]. The detection methods of CTCs are changing dramatically, and the clinical applications are becoming increasingly extensive [22]. CTCs can also be easily isolated by ScreenCell® system by utilizing a microporous membrane filter [23].

Clinically, commonly used imaging technologies include MRI (magnetic resonance imaging), PAT (Photoacoustic tomography), OCT (optical coherence tomography), CT (computed tomography) and PET (positron emission tomography) have also been proposed for real-time imaging of rapidly flowing cells in blood vessels, including cancer cells [4].

The clinical significance of CTC-related markers in patients with epithelial ovarian cancer has been evaluated by using quantitative real-time PCR, based on the expression of EPCAM, MUC1, CEA, HE4, and CA125 mRNAs, as putative markers of CTCs, in the blood of 51 epithelial ovarian cancer patients before and/or after adjuvant chemotherapy. As such, it is of paramount significance to explore new approaches for the identification of CTCs in ovarian cancer. In a previous study, the use of tumor-specific fluorescent
antibodies were much less efficient in quantitating CTCs than the use of tumor-specific fluorescent ligands like folate receptors to label CTCs in peripheral blood for determining the number of cancer cells circulating in the bloodstream [24]. Most recently, CTCs were identified in ovarian cancer patients by using a cocktail of antibodies including CD44, CD133, ALDH, EpCAM, and cytokeratin [25]. This study found that CD133 + ALDH + CTCs have the greatest prognostic potential in ovarian cancer among the phenotypes studied. As there is still no efficient and precious method to detect CTCs in the peripheral blood of ovarian cancer patients by flow cytometry, our study aimed to design fluorescent conjugates of four specific antibodies like CD105, CD24, CD117, and Epcam to identify CTCs in the peripheral blood of ovarian cancer patients by flow cytometry and to determine the value of their use in the diagnosis of suspected ovarian mass, as well as to differentiate between malignant and non-malignant ovarian masses. The overall results of this study reveal that CTCs can allow for the prediction of FIGO stage and optimum debulking, opening a new avenue for the use of CTCs in advancing diagnosis and prognosis of ovarian cancer as well as the precise decision for surgery and treatment.

Patients And Methods

Subjects

This prospective study was conducted on 66 patients with ovarian cancer recruited at Department of Obstetrics and Gynecology, Faculty of Medicine, Menoufia University between December 2018 and March 2021. The ethical and scientific committee of Menoufia University approved the study protocol, and the patients gave informed consent before participating in the study.

The patients enrolled in the study had a previous diagnosis of suspected ovarian cancer, depending on history, ultrasound criteria, and tumor markers, and were prepared for exploratory laparotomy for debulking surgery. The clinical data of all the patients studied were as follows: Regarding age, the mean age of all studied patients was 50.6 ± 16.6 years, with a minimum age of 20 years and a maximum age of 77 years. As for menopause, 11 patients (50%) were premenopausal, and 11 patients (50%) were postmenopausal. As for symptoms, 12 patients (54.5%) were symptomatic, and 10 patients (45.5%) were asymptomatic.

Clinical assessment

It included the history taking, clinical examination, abdomen and pelvic ultrasound, tumor markers according to the need, CA125 was done for all cases, routine preoperative investigations, RMI (Risk of malignancy index). RMI was calculated by CA-125× US index ×menopausal index (NICE; 2011). Ovarian masses were used for histopathology and surgical staging for malignant tumors was performed according to FIGO staging 2013.

Blood Samples Collection:
Peripheral blood samples were obtained from 66 women known to have suspected ovarian mass (risk of malignancy index ≥ 25) and prepared for debulking surgery. Thereafter, 2 ml of peripheral blood was collected from the patients in a tube with EDTA as anticoagulant.

**Detection Of Circulating Tumor Cell Using Flow Cytometry:**

Blood sample (100 µl) was stained with anti-human CD 105(Percp-cy5, Cat. 560819, BD BIOSCIENCE, CD24(APC, Cat. 555427, BD BIOSCIENCE), CD117(PE, Cat, 562407, BD BIOSCIENCE ) and Epcam (FITC, Cat. 551318, BD BIOSCIENCE) at the concentrations recommended by the manufacturers of each antibody. The stained samples were incubated in the dark for 20–30 minutes, then BD FACS lysis solution (1X) was added for 15 minutes to lyse the erythrocytes. The samples were then centrifuged at 1250 rpm for 5 minutes. Cells were washed twice with PBS and re-suspended in PBS. Negatively stained samples were used as internal controls. FACSCanto II (BD Biosciences) was used for cell acquisition and Flowjo software (BD Biosciences) was used for data analysis. The percentage of CTCs was determined using the following phenotypes CD 105+, CD24+, CD117+, and Epcam+.

**Statistical analysis:**

Numerical data expressed as mean ± SD and the statistical differences between patients and healthy groups were assessed using One-way Analysis of Variance (ANOVA). Graph Pad Prism (Graph Pad Software, Inc., San Diego, CA) was used to analyze P values. P values < 0.05 were considered statistically significant.

**Results**

**Histopathological feature of the patients:**

In our study, histopathological data were as follows in all patients studied: 11 patients (50%) were epithelial ovarian carcinoma, 2 patients (9.1%) were malignant germ cell tumor, 2 patients (9.1%) were malignant sex cord, 2 (9.1%) were secondary ovarian carcinoma, 3 patients (13.6) were benign carcinoma, and 2 patients (9.1%) were borderline cases(Table 1).
Table 1
Histopathological data, histopathological grade, and lymph node histopathology in all studied patients

<table>
<thead>
<tr>
<th>Histopathology results</th>
<th>2ry to ovary</th>
<th>Studied patients (N = 66)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benign</td>
<td>Epithelial ovarian tumor</td>
<td>3 4.5%</td>
</tr>
<tr>
<td></td>
<td>Dermoid</td>
<td>6 9.1%</td>
</tr>
<tr>
<td>Borderline</td>
<td>Borderline serous cyst-adenoma</td>
<td>3 4.5%</td>
</tr>
<tr>
<td></td>
<td>Borderline mucinous cystadenoma</td>
<td>3 4.5%</td>
</tr>
<tr>
<td>Malignant Sex cord stromal</td>
<td>Granulosa cell tumor</td>
<td>6 9.1%</td>
</tr>
<tr>
<td>Epithelial ovarian cancer</td>
<td>Endometroid carcinoma</td>
<td>3 4.5%</td>
</tr>
<tr>
<td></td>
<td>Serous cyst-adenocarcinoma</td>
<td>21 31.8%</td>
</tr>
<tr>
<td></td>
<td>Mucinous cyst-adenocarcinoma</td>
<td>9 13.6%</td>
</tr>
<tr>
<td>Malignant germ cell tumor</td>
<td>Immature teratoma</td>
<td>3 4.5%</td>
</tr>
<tr>
<td></td>
<td>Yolk sac tumor</td>
<td>3 4.5%</td>
</tr>
</tbody>
</table>

Evaluation Of Rmi In Patients:

Regarding RMI, in benign patients, the mean RMI was 35.5 ± 4.48 with a minimum RMI of 30 and a maximum RMI of 45. In borderline patients, the mean RMI was 40 ± 10.9 with a minimum RMI of 30 and a maximum RMI of 50. In patients with 1ry ovaries, the mean RMI was 3643.6 ± 4137.1 with a minimum RMI of 25 and a maximum RMI of 11700. In patients with 2 ovaries, the mean RMI was 1088.5 ± 472.7 with a minimum RMI of 657 and a maximum RMI of 1520.

Evaluation Of Ca-125 Marker In Patients

As for CA-125, the mean CA-125 in benign patients was 21.7 ± 6.6 µ/ml with a minimum CA-125 of 15 µ/ml and a maximum CA 125 of 30u/ml. In borderline patients, the mean CA-125 was 40 ± 10.9 u/ml with a minimum CA 125 of 30 u/ml and a maximum CA-125 of 50 µ/ml. In patients with 1ry ovaries, the mean CA-125 was 545.3 ± 616.2 µ/ml with a minimum CA 125 of 23 µ/ml and a maximum CA-125 of 1300 µ/ml. In patients with 2 ovaries, the mean CA 125 was 416.5 ± 376.3u/ml with a minimum CA 125 of 73 u/ml and a maximum CA-125 of 760 µ/ml. (Table 2)
Table 2
CTCs, risk of malignancy index and cancer antigen 125 regarding histopathological results.

<table>
<thead>
<tr>
<th>Histopathology</th>
<th>Benign (n = 9)</th>
<th>Borderline (n = 6)</th>
<th>1\textsuperscript{st} ovarian (n = 45)</th>
<th>2\textsuperscript{nd} ovarian (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTCs (cell/µl)</td>
<td>Mean ± SD</td>
<td>0.0 ± 0.0</td>
<td>0.12 ± 0.11</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>----</td>
<td>0.2–0.26</td>
<td>----</td>
</tr>
<tr>
<td>RMI</td>
<td>Mean ± SD</td>
<td>35.5 ± 4.48</td>
<td>40 ± 10.9</td>
<td>3643.6 ± 4137.1</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>30–45</td>
<td>30–50</td>
<td>25–11700</td>
</tr>
<tr>
<td>CA 125</td>
<td>Mean ± SD</td>
<td>21.7 ± 6.6</td>
<td>40 ± 10.9</td>
<td>545.3 ± 616.2</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>15–30</td>
<td>30–50</td>
<td>23–1300</td>
</tr>
</tbody>
</table>

The Numbers Of CTC Numbers In Patients:

The mean value of CTCs in all patients studied was 0.12 ± 0.11 cells/µl with a minimum of 0.02 cells/µl and a maximum of 0.26 cells/µl. (Table 2) (Fig. 1) (Fig. 2), also the result showed that the CD117 is not expressed in benign and borderline ovarian tumors. However, CTCs were present in the malignant ovarian tumors. Therefore, a combination of CD117 and CTCs may allow early detection of ovarian cancer even in asymptomatic patients they were well correlated with stage FIGO (Fig. 3). The description of CTCs, RMI and CA-125 in terms of histopathological results shows that regarding CTCs, the average CTC in malignant patients was 0.12 ± 0.11 cells/µl with a minimum CTC of 0.02 cells/µl and a maximum CTC of 0.26 cells/µl in primary ovarian malignancy and no CTCs were found in secondary ovarian malignancy, benign or borderline ovarian tumor.

Association Between CTCs, CA-125, And FIGO In Symptomatic And Asymptomatic Patients

There were 11 stage I patients & II among the patients studied, 5 patients (45.5%) were asymptomatic, and 6 patients (54.5%) were symptomatic. All asymptomatic patients (100%) were CTCs positive; 4 patients (80%) were CA 125 negative (less than 35 u/ml) and 1 patient (20%) was CA 125 positive (more than 35 u/ml). Among the patients studied, 6 patients were at stage IA & IB. Of these, 3 patients (50%) were symptomatic and 3 patients (50%) were asymptomatic. All asymptomatic patients (100%) were CTC positive; 2 patients (66.7%) were CA 125 negative and 1 patient (33.3%) was CA 125 positive. Pearson correlation between FIGO stage and CTCs and RMI showed that there was a highly statistically significant (p-value < 0.001) positive correlation (r = 0.55) between CTCs and FIGO stage in the studied patients and
that there was not statistically significant (p-value = 0.083) correlation (r = 0.24) between RMI and FIGO stage in the studied patients (Table 3).

### Table 3
Pearson correlation between circulating tumor cells and cancer antigen 125, risk of malignancy index and between International Federation of Gynaecology and Obstetrics.

<table>
<thead>
<tr>
<th>Clinical parameters</th>
<th>CTCs</th>
<th>FIGO staging</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>p-value</td>
</tr>
<tr>
<td>RMI</td>
<td>0.53</td>
<td>&lt; 0.001 HS</td>
</tr>
<tr>
<td>CA 125</td>
<td>0.42</td>
<td>&lt; 0.001 HS</td>
</tr>
<tr>
<td>Clinical &amp; biological parameter</td>
<td>FIGO staging</td>
<td>(r)</td>
</tr>
<tr>
<td>CTCs</td>
<td>0.55</td>
<td>&lt; 0.001 HS</td>
</tr>
<tr>
<td>RMI</td>
<td>0.24</td>
<td>0.083 NS</td>
</tr>
</tbody>
</table>

### Relationship Between CTCs And Npv/ppv

CTCs can be used to distinguish between early and late FIGO staging at a cutoff level of > 0.82 cells/µl, with a sensitivity of 66.7%, specificity of 90.9%, PPV of 88%, and NPV of 73.2% (AUC = 0.65 & p-value = 0.076). RMI can also be used to discriminate between early and late FIGO staging at a cutoff level of > 1510, with 66.7% sensitivity, 63.6% specificity, 64.7% PPV, and 65.6% NPV (AUC = 0.71 and p-value = 0.013) (Table 3).

CTCs can be used to discriminate between patients with optimal debulking and patients without optimal debulking at a cutoff level of < 1.9 cells/µl, with 100% sensitivity, 60% specificity, 71.4% PPV and 100% NPV (AUC = 0.65 & p-value = 0.063). In addition, at a cutoff level of < 213, the RMI can be used to discriminate between patients with optimal debulking and patients without optimal debulking with a sensitivity of 53.3%, specificity of 100%, PPV of 100%, and NPV of 68.2% (AUC = 0.76 and p-value = 0.002). (Table 4)

### Table 4
The diagnostic performance of CTCs & RMI in discrimination of early and late FIGO staging.

<table>
<thead>
<tr>
<th>Cut off</th>
<th>AUC</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTCs</td>
<td>&gt; 0.82</td>
<td>0.65</td>
<td>66.7%</td>
<td>90.9%</td>
<td>88%</td>
<td>73.2%</td>
</tr>
<tr>
<td>RMI</td>
<td>&gt; 1510</td>
<td>0.71</td>
<td>66.7%</td>
<td>63.6%</td>
<td>64.7%</td>
<td>65.6%</td>
</tr>
</tbody>
</table>
Discussion

Ovarian cancer is considered as the leading cause of death among gynaecologic cancers. This could be due to the lack of sensitive markers, precise symptoms, and effective screening methods that can help efficient treatment protocol. Therefore, this prospective study aimed to identify and enumerate CTCs in the bloodstream of ovarian cancer patients and correlate it with the clinical outcomes. To this end, we enrolled patients diagnosed with suspected ovarian malignancy who underwent exploratory laparotomy for debulking surgery. CTCs was analyzed using flow cytometry as CD105+CD24+CD117+Epcam+ phenotype. We found CTCs in 100% of patients with primary ovarian cancer while no CTCs were found in secondary ovarian cancer or in patients with either benign or borderline ovarian tumor. The highest number of CTCs was observed among malignant patients. Moreover, significant positive correlation was found between CTCs and FIGO, and RMI, and CA-125. Taken together, our data indicate that CTCs can allow to distinguish between early and late FIGO stage, presenting it as a new cellular marker for the early detection of ovarian cancer even in asymptomatic patients.

Ovarian cancer is thought to spread directly, so detection of CTCs is useful for early detection. A recent study based on the parabiosis model, in which paired mice share blood rather than lymphatic vessels, has shown that hematogenous metastasis is an important form of ovarian cancer metastasis [26].

Due to the lack of sensitive markers, precise symptoms and effective screening methods for ovarian cancer, quantitation of circulating tumor cells (CTCs) can provide information on the stage of malignancy, the onset of disease progression, and response to therapy. Previous studies tried to detect CTCs in ovarian cancer patients by using Cell Search® technology [8] and PCR analysis [27]. although these methods may identify CTCs, they are not precise and laborious. Flow cytometry-based analysis using cell antibodies against ovarian cancer can be an important alternative. So far, however, there is no efficient method has been established to detect CTCs in ovarian cancer patients. In a previous study, the use of tumor-specific fluorescent antibodies were much less efficient in quantitating CTCs than the use of tumor-specific fluorescent ligands like folate receptors to label CTCs in peripheral blood for determining the number of cancer cells circulating in the bloodstream [24]. Most recently, CTCs were identified in ovarian cancer patients by using a cocktail of antibodies including CD44, CD133, ALDH, EpCAM, and cytokeratin [25]. This study found that CD133 + ALDH + CTCs have the greatest prognostic potential in ovarian cancer among the phenotypes studied. With our aim to better quantitate CTCs more accurately, we have designed fluorescent conjugates of four specific antibodies including CD105, CD24, CD117, and Epcam by flow cytometry. We used CD117 to be able to identify CTCs with stem cell-like phenotype. To the best of our knowledge, this is the first study to analyze CTCs in ovarian cancer patients with primary and secondary and with debulking tumor.

Our ovarian cancer patients included 17 cancer patients, where 15 patients had primary ovarian cancer and 2 patients had secondary ovarian cancer. We found CTCs with CD105+CD24+CD117+Epcam+ phenotype in 100% of patients with primary, but not secondary, ovarian cancer or in patients with benign or borderline ovarian tumors. The most common marker used for CTCs is EpCAM, a epithelial marker of
cancers [28]. EpCAM expression varies among different cancer types [29], and EpCAM-based CTC detection technologies are widely applied for cancers that strongly express EpCAM, such as breast and prostate cancer. Many studies have shown that CTCs in breast and prostate cancer are EpCAM-positive, and have validated their prognostic value in either early or metastatic stage cases [30, 31]. Other epithelial-derived cancer types, such as pancreatic [32], colorectal [33], and hepatocellular cancers [34], also have a considerable detection rate of EpCAM-positive CTCs. Similarly, the presence of these EpCAM-positive CTCs predicts early distant metastasis and poorer survival of patients [33, 35, 36].

CD24 is frequently overexpressed in various human cancers and is correlated with a poor prognosis. Previous study examined the functions of CD24 in human ovarian cancer cell lines and evaluated how it contributes to the molecular mechanism underlying the regeneration of cancer stem-like cells (CSCs) through the EMT mechanism in ovarian carcinoma and they demonstrated that CD24 was expressed in 70.1% of primary ovarian carcinoma tissues, which were obtained from 174 patients, and that the expression of CD24 was an independent predictor of survival in patients with ovarian cancer, also they found the expression of CD24 has been correlated with the FIGO stage, presence of peritoneal and lymph node metastasis [37].

CD105 (endoglin) antibody binds preferentially to activated endothelial cells that are induced by tumoral factors for neoangiogenesis [38] and [39]. It stains blood vessels in or around the tumor tissues [40]. Therefore, it is accepted as a more specific and sensitive marker than others to detect tumoral angiogenesis [41, 42]. Previous study determined the intratumoral neoangiogenesis of ovarian cancer patients using endoglin (CD105) and investigate the relation with prognostic factors and the found that the mean microvessel density (MVD) with CD105 was 28.78 ± 22.20, with respect to prognostic factors, CD105 was significant for both advanced stage and suboptimal cytoreduction [43].

CD117 plays an important role in cell differentiation and survival, particularly in its impact on CSCs. In a study on non-small cell lung cancer patients, tumor cells positively expressing CD117 exhibited CSC characteristics, such as self-renewal and chemoresistance [44]. Similar characteristics are seen in CD117 positive ovarian tumor cells in which CD117 expression is related to the “stemness” of particular cancer cells [45, 46].

The consistent expression of CD117 on all analyzed CTCs indicate to the stem-like phenotype of these cells and accordingly their aggressiveness. They would allow us in future study to sort these cells for more molecular analysis using gene array to compare its molecular signature with the primary tumor. This would confirm the stemness of these cells and its molecular features in correlation to the disease progression.

We found that the mean number of CTCs in all patients was 0.12 ± 0.11 cells/µL, where the highest number was observed among the malignant patients. Of great interest, we found a significant positive correlation between CTCs and FIGO, RMI, and CA-125. The mean value of CTCs in all patients studied was 0.12 ± 0.11 cells/µL with a minimum of 0 cells/µL and a maximum of 0.26 cells/µL. However, in the
malignant patients, the minimum CTCs count was 0.02 cells/µL and the maximum CTCs count was 0.26 cells/µL. Our results agree with the findings of a previous report in which tumor-specific fluorescent ligands were used to count CTCs by flow cytometry. In this study, CTCs were detected in 18 cases out of 20 ovarian cancer patients, with an average number of CTCs of 0.2 cells/µL and a maximum number of 3.1 cells/µL [24]. While we were not able to detect CTCs in patients with secondary ovarian cancer, another study that used Cell Search® technology to detect CTCs in ovarian cancer patients, found that no CTCs in benign disease (14 patients), 17% (5/29) of patients with ovarian cancer, and 80% (4/5) of patients with secondary ovarian cancer [47], Another study used PCR to detect CTCs in ovarian cancer patients and found CTCs in 98 out of 109 ovarian cancer patients (benign or metastatic), and the average number of CTCs was 264 (range 0-1929) per 5 ml, i.e. 1.9 cells/µL [48].

To understand the role of CTCs in the early detection of ovarian cancer, we compared CTCs of early-stage patients. We found CTCs in all early-stage FIGO patients, i.e., stages I and II. We then compared CTC count with CA-125, which is the typical tumor marker. Interestingly, we found that CA-125 was negative (less than 35 U/mL) in 5 asymptomatic patients (45.5%) and was positive (more than 35 U/mL) in 6 symptomatic patients (54.5%). In contrast CA-125, we found CTCs in all asymptomatic patients (100%); 4 patients (80%) were CA-125 negative (less than 35 u/ml) and 1 patient (20%) was CA-125 positive (more than 35 U/mL). In addition, among the patients studied, there were 6 patients with stage IA or IB. Of these, 3 patients (50%) were symptomatic and 3 patients (50%) were asymptomatic. All asymptomatic patients (100%) were CTC positive; 2 patients (66.7%) were CA-125 negative, and 1 patient (33.3%) was CA 125 positive. These results are consistent with the findings of [48], who found that of 36 early-stage patients (1 and 2), 14 patients were occult, i.e., asymptomatic. In these 14 occult patients (93%; 13/14) were CTCs positive, while 86% (12/14) were CA-125 positive. A greater difference was noted in stages 1a and 1b, where the CTC-positive rate was 100% in the 7 occult patients at these stages, whereas only 57% were CA-125 positive. Taken together, our data provide a proof of concept that CTCs count in a small sample of peripheral blood is more accurate than the use the tumor marker. This concept, however, needs to be tested in a larger population of ovarian cancer patients.

Our study shows a statistically highly significant (p-value < 0.001) positive correlation (r = 0.55) between CTCs and FIGO staging in the patients studied, while no statistically significant (p-value = 0.083) positive correlation (r = 0.24) was found between RMI and FIGO staging. These results are consistent with the finding of [47, 48].

Our results show that CTCs can be used to discriminate between early and late FIGO staging at a cutoff level of > 0.82 cells/µL, with 66.7% sensitivity, 90.9% specificity, 88% PPV and 73.2% NPV (AUC = 0.65 and p-value = 0.076). Our results also show that RMI can be used to discriminate between early and late FIGO staging at a cutoff level of > 1510, with 66.7% sensitivity, 63.6% specificity, 64.7% PPV and 65.6% NPV (AUC = 0.71 & p-value = 0.0130).

Indeed, the most important factor in determining the prognosis of patients with EOC is tumor stage. The prognosis for patients with low-grade tumors at stage IA is excellent: > 90% are disease-free after 5 years.
At stage II, the 5-year survival rate is 60–80% with pelvic extension of the ovarian tumor. The 5-year survival rate is significantly lower in stages III and IV. 85% of ovarian cancer cases are diagnosed at stage III, which is associated with a 5-year survival rate of 20%. Stage IV is associated with a survival rate of less than 10% and includes distant metastases. Patients with advanced FIGO stage may benefit from 3 cycles of chemotherapy before surgery [49]. Therefore, the use of CTCs alone or in combination with CA-125 can help in offering the suitable treatment protocol during the early stage of tumor before its progression into the more aggressive stage.

In a Cochrane Database Review of 11 retrospective studies, near optimal cytoreduction with < 1 cm residual disease had better overall outcomes than suboptimal cytoreduction with > 1 cm residual disease, as optimal debulking is the most important prognostic factor [50]. Our results show that there is no statistically significant difference (p-value > 0.05) between patients with optimal debulking and patients without optimal debulking in terms of the number of CTCs. This result may be due to the absence of CTCs in patients with secondary ovarian cancer (6 patients), all of whom are very advanced and in whom optimal debulking is not possible. The absence of a statistically significant relationship between CTCs and optimal debulking was also noted previously [47]. We then compared the diagnostic performance of CTCs and RMI in predicting optimal debulking. We found that CTCs can be used to discriminate between patients with optimal debulking and patients without optimal debulking at a cutoff level of < 1.9 cells/μL, with 100% sensitivity, 60% specificity, 71.4% PPV, and 100% NPV (AUC = 0.65 & p-value = 0.063). When using RMI to discriminate between patients with optimal debulking and patients without optimal debulking at a cutoff level of < 213, the results were 53.3% sensitivity, 100% specificity, 100% PPV and 68.2% NPV (AUC = 0.76 & p-value = 0.002). Taken together, our results confirm the possibility of using CTC count as a maker to distinguish between ovarian cancer patients with or without optimal debulking, providing a reliable, easy, and fast approach for evaluation of prognosis and the possibility of surgery.

In summary, our study demonstrates that flow cytometry coupled with tumor specific fluorescent antibodies can be used to quantitate CTCs in the bloodstream of ovarian cancer patients. This approach can further be validated in future prospective studies focused on detection of cancer recurrence, monitoring of disease progression, and assessment of response to therapy. Our data indicate that CTCs allow early detection of ovarian cancer even in asymptomatic patients. These cells can aid for in diagnosis of suspected ovarian mass as they differentiate between malignant and non-malignant ovarian masses and allow prediction of FIGO stage and optimum debulking, therefore prediction of prognosis. Most importantly, CTCs count allowed to distinguish between the early and late FIGO stage. Overall, our results conclude that CTCs can be used as a cellular biomarker for the early detection of ovarian cancer even in asymptomatic patients.

Abbreviations

CTCs
Circulating tumor cells
CA125
Cancer antigen-125
CD24
Cluster of differentiation 24
CD105
Cluster of differentiation 105
CD117
Cluster of differentiation 117
EpCAM
Epithelial cell adhesion molecule
EOC
Epithelial ovarian cancer
FIGO
The Fédération Internationale de Gynécologie et d’Obstétrique
RMI
Risk of malignancy index
MVD
Microvessel density
NPV
Negative Predictive Value
OC
Ovarian cancer
PPV
Positive predictive value

**Declarations**

**Conflict of interest**

The authors have no conflicts of interest to declare. All co-authors have seen and agree with the contents of the manuscript and there is no financial interest to report.

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**Contributions**

All authors contributed to the study conception and design as well as the analysis of data. Mohammed Hussein Sheleby: Samples collection, statistical analysis, and manuscript preparation and editing.
Noura E. Sanoh: Performing the flow cytometry analysis and interpretation, manuscript writing, publication

Mohammed Salama Gad: Supervision, samples collection and monitoring all clinical data recruitment, assessment, and analysis

Alaaeldein Fathallah Alhalaby: Supervision clinical data analysis

Mohamed Labib Salem: Supervision, suggesting the idea, hypothesis, monitoring flow cytometry data for data interpretation, and revising the manuscript. writing and publication

References


5. Ashworth, TR. A case of cancer in which cells similar to those in the tumours were seen in the blood after death. Australian Medical Journal, 1869. 14, pp146-147.


Flow cytometric analysis for detection CTCs in blood of a healthy normal (A), benign OC patient (B) and malignant OC (C) using CD24, CD105, CD117 and EPCam antibodies. Numbers of CTCs were measured in 1-mL of blood. The gating strategy is CD24^+, CD105^+, CD117^+ and EPCam^+. 
Figure 2

(A) Number of circulating tumor cells per 1 ml of blood in malignant OC, borderline OC, benign OC cells were reported as mean standard deviation ± SD. Ns not significant, **** P ≤ 0.0001 statistically significant difference between healthy, borderline OC, benign OC and malignant OC. (B) Number of circulating tumor cells per 1 ml of blood in different grades of malignant OC according to FIGO staging, were reported as mean standard deviation ± SD, **** P ≤ 0.0001 statistically significant difference between healthy and different grades of malignant OC.
Flow cytometric analysis for CD117 subset as a malignancy marker in blood of a healthy normal (A), benign OC patient (B) and malignant OC (C). (D) Overlaid histogram for CD117 in Control, benign OC and malignant OC. (E) Percentage of CD117 subset as a malignancy marker in blood of a control, benign OC patient and malignant OC were reported as mean standard deviation ± SD. **** P ≤ 0.0003 and **** P ≤ 0.0001 statistically significant difference between Control, benign OC and malignant OC.