Detection of Circulating Tumor Cells in High-risk Endometrial Cancer

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Abstract. *Aim: To evaluate the presence of circulating tumor cells (CTCs) in patients with high-risk endometrial cancer (EC). Patients and Methods: We prospectively included 28 patients with a preoperative diagnosis of grade 3 EC undergoing surgery from June 2010 to December 2011. Their preoperative blood samples were tested for the presence of CTCs using an immunomagnetic and immunofluorescence assay technique. Results: Overall, 2 out of 28 patients (7%) were positive for CTCs. The presence of positive CTCs was significantly associated with myometrial invasion (MI) (33% vs. 0% for MI >50% vs. ≤50%; p=0.04) and lymph node positivity (40% vs. 0% for positive vs. negative nodes; p=0.03). Only patients with endometrioid histology had positive CTCs (29% in endometrioid vs. 0% in nonendometrioid; p=0.06). Conclusion: The presence of positive CTCs was associated with deep MI and lymph node positivity. The absence of CTCs in patients with type II histology suggests the need to find other markers in this sub-group of patients.*

Endometrial cancer (EC) is the most common gynecological malignancy in the United States with an estimated 52,630 new cases and 8,590 deaths for 2014 (1). For most patients, EC is detected at an early stage of disease. Generally, patients with EC have a good prognosis, with an estimated 5-year cause-specific survival of more than 95% (2). It is estimated that only 6% to 7% of patients with surgical stage I have distant failure (2, 3) but this percentage is much higher (21%) when considering patients with grade 3 cancer at preoperative biopsy (4). These recurrences are speculated to arise from occult disseminated tumor cells (*i.e.*, circulating tumor cells (CTCs)), which are not detected or removed surgically (5).

Growing evidence in different oncologic specialties suggests that the presence of CTCs correlates with prognosis and response to treatment in patients affected by various solid cancers (6, 7). Detecting CTCs in the blood may help determine the potential risk of recurrence and, thus, help assess the prognosis and possibly guide postoperative treatment. Several studies of patients with breast or prostate cancer have shown that CTCs are a promising prognostic tool for primary and metastatic tumors (6, 8). However, little is known about disseminated tumor cells (detected in the bone marrow) and CTCs in patients with EC (9-13), while no clear information is available about their potential diagnostic and therapeutic roles.

We designed this pilot study to identify the prevalence of CTCs in patients with high-risk EC and test its association with histological and postoperative findings. This would help identify a sub-group of patients at high risk of recurrence and possibly guide their surgical and postoperative treatment.

Patients and Methods

This study was approved by the Mayo Clinic Institutional Review Board.

Study population. We prospectively included patients who underwent surgery for EC at Mayo Clinic (Rochester, Minnesota) from June 1, 2010, through December 31, 2011. Because of the low probability of hematogenous spread in grade 1 and 2 EC (3), we only focused on patients with grade 3 disease. As grade 3 in the preoperative biopsy is highly correlated with poorly differentiated

Abbreviations: CK, Cytokeratin; CTC, circulating tumor cell; DAPI, 4',6-diamidino-2-phenylindole; EC, endometrial cancer; FDA, US Food and Drug Administration; FIGO, International Federation of Gynecology and Obstetrics; LVSI, lymphovascular space invasion; MI, myometrial invasion.

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cancer in the hysterectomy specimen (4), only patients with histologically proven International Federation of Gynecology and Obstetrics (FIGO) grade 3 EC at preoperative endometrial sampling were considered for inclusion in this study. Patients with preoperative grade 3 EC who were eligible for surgical treatment, did not have a history of previous or synchronous malignancies and were not undergoing neoadjuvant therapy, were asked to participate by consenting to provide a blood sample.

The taxonomy proposed by the World Health Organization was used for ascertaining histologic subtypes (14). Architectural grading was determined according to FIGO criteria (15). We used the 2009 FIGO staging stratum (16) to assign stage. Detailed descriptions of surgical guidelines are reported elsewhere (16). Briefly, all patients who had surgery for grade 3 tumors underwent total hysterectomy, bilateral salpingo-oophorectomy and lymph node dissection. Patients received postoperative treatment according to standard guidelines. External beam radiation was used in patients with positive lymph nodes, vaginal brachytherapy in patients with grade 3 tumor or with lymphovascular space invasion (LVSI). Chemotherapy was limited to patients with advanced disease or high risk of hematogenous dissemination (17). Clinical data, surgical and postoperative treatment, as well as time and site of recurrence were recorded.

Isolation and definition of CTC. CTC isolation and enumeration were performed by using the US Food and Drug Administration (FDA)–approved CellSearch technology (Veridex, LLC), which allows immunomagnetic selection, immunofluorescence staining, concentration and sample enrichment of CTCs. The CellSearch CTC test uses EpCAM for cell isolation and is the only diagnostic test currently approved for detection and enumeration of CTC (18).

The technical description is detailed elsewhere (19). Briefly, blood was drawn from patients before starting any treatment to a cell-save preservative tube (Veridex, LCC) that stabilizes CTCs. When the sample arrived at the laboratory, 7.5 ml of blood was transferred to a mechanical tube and centrifuged to separate packed cells from plasma. The sample was placed on the celltrack autoprep system (Veridex, LCC), which automated the remaining sample preparation steps. CTCs were then stained with monoclonal antibodies (Veridex – Janssen Diagnostic, Raritan NJ) against cytokeratin (CK) 8, 18 and 19, which are specific to epithelial cells. To distinguish contaminating leukocytes from CTCs, a panleukocyte antibody CD45 was also added. Finally, cells were stained with 4',6-diamidino-2-phenylindole (DAPI). CTCs were identified as those cells that were round or oval shaped, positive for cytokeratin and DAPI stain, and negative for CD45. Results were expressed as the number of CTCs per 7.5 ml of blood. Although a prior study of patients with breast cancer used a threshold of 5 CTCs to define positivity (6), specific guidelines for patients with EC are not available. Hence, in accordance with Allard *et al*. (19), we considered the presence of only 1 CTC as a negative result, to avoid false-positive outcomes. Detection of 2 or more CTCs was, thus, the minimum positive result.

Because nothing in the available literature correlated the presence of CTCs with EC characteristics, we were unable to perform a power calculation before commencing this prospective study. Hence, a sample of 30 patients was chosen as a pilot project. Statistical analysis was performed by using the SAS statistical package (version 9.2; SAS Institute Inc.). Associations between the presence of positive CTCs and patient or disease characteristics were evaluated using a 2-sided Fisher exact test. *p*-Values less than 0.05 were considered statistically significant.

Table I. *Patients' characteristics (n=28).*

Characteristics	Value
Age, mean (SD), y	69.1 (8.2)
Stage of disease, No. (%)	
I	18 (64)
II	1 (4)
III	6 (21)
IV	3 (11)
Histologic subtype, No. (%)	
Endometrioid	7 (25)
Mixed	11 (39)
Serous	8 (29)
Other type 2	2 (7)
Final FIGO grade, No. (%)[a]	
Grade 2	3 (11)
Grade 3	25 (89)
Myometrial invasion, No. (%)	
None	7 (25)
≤50%	15 (54)
>50%	6 (21)
Lymphovascular space invasion, No. (%)	5 (18)
Follow-up, median (IQR), mo[b]	34 (6.7-48)

FIGO, International Federation of Gynecology and Obstetrics; IQR, interquartile range; SD, standard deviation. [a]In the hysterectomy specimen.1.0. [b]Of censored patients.

Results

Among the 115 patients undergoing surgery for grade 3 EC during the study period, 30 (26%) were available to meet our study coordinator and consented to participate in this study. Blood samples of 30 consecutive patients with a preoperative diagnosis of FIGO grade 3 EC were collected. One patient had an intraoperative diagnosis of adenocarcinoma of the uterine cervix and one patient refused surgery after blood collection, thus leaving 28 patients for the final analysis. Mean and standard deviation (SD) patient age was 69.1 (8.2) years (range=53-90 years). Table I reports patients' characteristics. Six women (21%) had deep myometrial invasion (MI; >50%). LVSI was observed in 5 patients (18%). At the final pathologic examination, 7 (25%) had endometrioid histology; of these, 4 (57%) had MI >50% and 2 (29%) had LVSI.

CTCs were found in blood samples of 3 patients (11%) (Table II). One patient had only 1 CTC identified and, thus, was not considered "positive" (19). The other 2 patients (7%; 95% confidence interval (CI), 0.9%-23.5%) were positive for CTCs (with 2 and 23 CTCs identified); both had the diagnosis of stage IIIC EC and both had endometrioid histology. Neither patient had disease recurrence. One patient (with 23 CTCs) died of pulmonary embolism 4 months after surgery. The second patient was alive without evidence of disease after 21 months of follow-up.

Table II. *Characteristics of patients with CTCs (n=3)[a].*

Patient	Stage	Grade	Histologic subtype	Myometrial invasion	LVSI	CTC[b]	Treatment	Follow-up, months	Cause of death
1	IIIC1	3	Endometrioid	>50%	Present	23	Surgery	4	Pulmonary embolism
2	IIIC1	3	Endometrioid	>50%	Absent	2	Surgery, chemotherapy, radiotherapy	21	Alive, with no evidence of disease
3	IA	2	Clear cell and endometrioid	≤50%	Absent	1	Surgery	34	Alive, with no evidence of disease

CTC, Circulating tumor cell; LVSI, lymphovascular space invasion. [a]No patients had recurrence. [b]Detection of 2 or more CTCs in the blood was considered positive. Therefore, patient 3 was considered "negative" in our analysis.

Table III. *Characteristics of patients with recurrence (n=5)[a].*

Patient	Stage	Histologic subtype	Grade	First site of recurrence	Follow-up, months	Status
4	IVB	Clear cell and serous	3	Lungs, liver	21	Dead
5	IIIC2	Serous	3	Inguinal, pelvic and supraclavicular nodes	39	Alive
6	IVB	Endometrioid and serous	3	Peritoneal nodules, liver	28	Dead
7	IIIC	Clear cell	3	Peritoneal	15	Dead
8	IA	Endometrioid	2	Vaginal cuff	29	Alive
9	IIIA	Endometrioid, clear cell and serous	3	Peritoneal, pleura, paraaortic nodes	25	Dead

[a]No patients had circulating tumor cells. All had ≤50% myometrial invasion.

Six out of 28 patients (21%) had recurrence but none had CTCs detected in their peripheral blood. The characteristics of women with recurrent disease are listed in Table III. All 6 patients had tumors limited to the inner half of the myometrium and 5 had serous or clear cell histological features (or both). No cases of distant extrapelvic recurrence were recorded in patients with pure endometrioid histology.

The presence of positive CTCs was significantly associated with MI (2/6 (33%) *vs*. 0/22 (0%) for MI >50% *vs*. ≤50%; p=0.04) and lymph node positivity (2/5 (40%) *vs*. 0/20 (0%) for positive *vs*. negative nodes; p=0.03). Only patients with endometrioid histology had positive CTCs (2/7 (29%) *vs*. 0/21 (0%) for endometrioid *vs*. nonendometrioid; p=0.06). Among the 4 patients with endometrioid histology and MI exceeding 50%, positive CTCs were detected in 2 (50%). The presence of positive CTCs was not significantly associated with LVSI (1/5 (20%) *vs*. 1/23 (4%) for LVSI *vs*. no LVSI; p=0.33), stage (2/10 (20%) *vs*. 0/18 (0%) for stage II-IV *vs*. I; p=0.12) or older age (1/12 (8%) *vs*. 1/16 (6%) for age ≥70 *vs*. <70 years; p=0.99).

Discussion

The clinical relevance of CTCs in the blood has been already demonstrated for various solid cancers. Although the presence of CTCs has been correlated with prognosis and response to treatment in breast cancer (6, 7), no mature data about the significance of CTCs in EC are available. Our study suggests that the overall prevalence of positive (≥2) CTCs in patients undergoing surgery with a preoperative diagnosis of grade 3 EC is 7%. We showed that deep MI and lymph node positivity were associated with CTCs in the peripheral blood. We observed a relatively high rate of CTCs in patients with poorly differentiated endometrioid EC (29%), whereas no patients with type II histology had CTCs. Among the 7 patients with endometrioid histology, positive CTCs were detected in 2 of the 4 patients with deep MI and in 2 of the 2 patients with deep MI and lymph node metastases.

Interestingly, considering only patients with endometrioid histology, the rate of CTCs detected in the present study was similar to the rate of hematogenous dissemination reported

in another investigation from our group. In fact, Mariani *et al*. (3) reported that the rate of hematogenous dissemination was 19% and 23% in patients with grade 3 cancer and deep MI, respectively. Consistent with our current findings, that study also showed that deep MI (>50%) was an independent predictor of hematogenous dissemination.

The literature is devoid of adequate information regarding the value of CTCs in patients with EC. Klein *et al*. (11) and Ji *et al*. (12) previously evaluated the role of CTCs in EC, suggesting that CK-19 and CK-20 were potential biomarkers for CTC detection. Those studies examined preoperative blood samples of 20 (11) and 30 (12) patients with endometrial cancer, respectively, and observed CTC in 10% to 35% of patients. However, although all patients in our study had biopsy results showing a poorly differentiated cancer, only 25% to 35% of patients from the prior studies had high-grade tumors (11, 12). In contrast with our findings, the earlier studies did not show any association between presence of CTC and grade, depth of myometrial invasion or presence of metastatic disease. Differences in findings may be due to differences in population characteristics, in techniques for detecting CTC and to the relatively small number of patients studied.

Fehm *et al*. (9) evaluated the presence of disseminated tumor cells in the bone marrow of 201 patients with gynecological cancers (ovarian (n=69), cervical (n=54), and endometrial (n=78) malignancies) by immunohistochemistry using the pancytokeratin antibody A45B/B3 directed against the common CK epitopes, including CK heterodimers 8/18 and 8/19. They found a positivity rate of 26%, 26% and 17% in ovarian, cervical and endometrial malignancies, respectively. However, they did not correlate the presence of disseminated tumor cells with clinical-pathologic variables or survival outcomes. Recently, the same study group corroborated their initial findings and suggested that the presence of tumor cells in the bone marrow did not affect clinical outcome in EC (10). However, it is important to emphasize that evaluating CTCs from peripheral blood (instead of bone marrow) has obvious advantages because the procedure is less painful and invasive.

We performed our analysis using the CellSearch circulating tumor cell test, which uses EpCAM for cell isolation. EpCAM is a glycoprotein (17-1A) that is present on most epithelial tissues, including endometrial tissue. Active proliferation in a number of epithelial tissues is associated with increased or *de novo* EpCAM expression. Malignant proliferation is often associated with EpCAM expression. Currently, CellSearch is the only FDA-approved technique for detecting CTCs (18). It uses monoclonal antibodies against CK 8, 18 and 19, which are specific to epithelial tissues. The heterogeneity of EpCAM expression on the surface of CTCs can cause variation in the ability to detect and recover these cells from blood samples (20, 21). Our results suggest that CTCs are present only in endometrioid tumors. However, owing to the high rate of hematogenous spread in type II EC cancer (15%) (3), we speculate that other markers may be required to detect CTCs in patients with type II EC. We hypothesize that during the process of de-differentiation, serous and clear cell tumors lose their epithelial antigens, making their CTCs undetectable by conventional epithelial markers.

Several studies have emphasized that EpCAM might not be a universal marker for detection of CTCs in all cancer types (13, 20, 21). Of note, a 6-gene panel technique (using *CCNE2, MAL2, EMP2, SLC6A8, HJURP*, and *PPIC*) was superior to EpCAM for CTC detection in recurrent breast cancer and potentially in gynecological cancer (13). Recently, Obermayr *et al*. (21) suggested that molecular characterization of CTCs in patients with epithelial ovarian cancer is superior to CTC enumeration. The authors identified a panel of 11 novel gene markers for CTC detection. Among these, *PPIC* (the more representative gene) correlated with poor outcomes in terms of chemoresistance and survival, independent from traditional prognostic factors (21).

Our study has several strengths: (i) the prospective nature of the study minimizes possible selection bias, (ii) to our knowledge, this is the first study to report the rate of CTCs in EC patients using the FDA-approved detection technique and (iii) this study is the first to evaluate the association between CTCs and clinical-pathologic features in high-grade malignancies. This study was limited by its small sample size (although this now contributes a relatively large number of patients to the literature). Probably because of the small sample size (or the inability to detect CTCs in patients with serous EC), it was not possible to identify associations between the presence of CTCs and survival. Moreover, in our series, no patients with endometrioid histology had hematogenous recurrence, thus making it impossible to analyze this outcome. Additionally, we need to take in account that other non-FDA approved CTC-detection techniques (22) have shown higher sensitivity and specificity in CTC detection compared with the technique used by CellSearch, owing to their ability to change laminar flow or to stack different antibodies. Hence, we stress that our data are preliminary and need to be corroborated by further research.

In conclusion, our study describes a 7% prevalence of positive (≥2) CTCs in patients with grade 3 EC. Also, it allows the identification of associations between the presence of CTCs and characteristics of uterine disease. Although our results are preliminary, we observed an association between positive CTCs and both deep MI and positive lymph nodes in patients with endometrioid histologic subtype. Moreover, our study emphasizes on the need to find different markers for the detection of CTCs in type II EC. Potentially, CTC detection may facilitate identification of patients with occult extrauterine dissemination and may, therefore, help guide postoperative adjuvant treatment. Moreover, understanding the mechanism of interaction between CTCs and the microenvironment of secondary homing sites may provide

insight into inhibition of hematogenous tumor seeding. Further investigation is needed to better clarify the prognostic and potentially therapeutic aspects of CTC identification.

Conflicts of Interest

The Authors declare that there are no conflicts of interest. Giorgio Bogani is a research fellow supported by University of Insubria, Varese, Italy, and Fondo Miglierina, Provincia di Varese, Italy.

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