Pooled Analysis of the Prognostic Relevance of Disseminated Tumor Cells in the Bone Marrow of Patients With Ovarian Cancer

Tanja Fehm, MD, PhD, Malgorzata Banys, MD,†‡ Brigitte Rack, MD, PhD,§ Wolfgang Janni, MD, PhD,|| Christian Marth, MD, PhD,¶ Christina Blassl,† Andreas Hartkopf, MD,† Claes Trope, MD, PhD,# Rainer Kimmig, MD, PhD,** Natalia Krawczyk, MD,* Diethelm Wallwiener, MD, PhD,† Pauline Wimberger, MD, PhD,†† and Sabine Kasimir-Bauer, PhD***

Objective: Detection of disseminated tumor cells (DTCs) in the bone marrow (BM) of patients with breast cancer is associated with poor outcomes. Recent studies demonstrated that DTCs may serve as a prognostic factor in ovarian cancer. The aim of this 3-center study was to evaluate the impact of BM status on survival in a large cohort of patients with ovarian cancer.
Materials and Methods: Four hundred ninety-five patients with primary ovarian cancer were included in this 3-center prospective study. Bone marrow aspirates were collected intraoperatively from the iliac crest. Disseminated tumor cells were identified by antibody staining and by cytomorphology. Clinical outcome was correlated with the presence of DTCs.
Results: Disseminated tumor cells were detected in 27% of all BM aspirates. The number of cytokeratin-positive cells ranged from 1 to 42 per 2×10^6 mononuclear cells. Disseminated tumor cell status did correlate with histologic subtype but not with any of the other established clinicopathologic factors. The overall survival was significantly shorter among DTC-positive patients compared to DTC-negative patients (51 months; 95% confidence interval, 37–65 months vs 33 months; 95% confidence interval, 23–43 months; $P = 0.023$). In the multivariate analysis, BM status, International Federation of Gynecology and Obstetrics stage, nodal status, resection status, and age were independent predictors of reduced overall survival, whereas only BM status, International Federation of Gynecology and Obstetrics stage, and resection status independently predicted progression-free survival.
Conclusions: Tumor cell dissemination into the BM is a common phenomenon in ovarian cancer. Disseminated tumor cell detection has the potential to become an important biomarker for prognostication and disease monitoring in patients with ovarian cancer.

Key Words: Ovarian cancer, Disseminated tumor cells, Overall survival, Progression-free survival, Bone marrow

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*Department of Gynecology and Obstetrics, University Hospital Düsseldorf, University of Düsseldorf, Germany; †Department of Obstetrics and Gynecology, University of Tuebingen, Germany; ‡Department of Gynecology and Obstetrics, Marienkrankenhaus Hamburg, Germany; §Department of Gynecology and Obstetrics, University Hospital Munich, Ludwig Maximilian University of Munich, Germany; ||Department of Gynecology and Obstetrics, University Hospital Ulm, University of Ulm, Germany; ¶Department of Obstetrics and Gynecology, Innsbruck Medical University, Innsbruck, Austria; #Gynecologic Department, The Norwegian Radium Hospital, Oslo, Norway; **Department of Gynecology and Obstetrics, University Hospital Essen, University of Duisburg-Essen, Germany; and ††Department of Gynecology and Obstetrics, University Hospital Dresden, University of Dresden, Germany.
Address correspondence and reprint requests to Prof. Tanja Fehm, Head of Department of Obstetrics and Gynecology, University of Düsseldorf, Moorenstr. 5, 40225 Düsseldorf, Germany. E-mail: tanja.fehm@med.uni-tuebingen.de

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Ovarian cancer is currently the fifth leading cause of cancer death of women in Europe and the United States.[1] The prognosis of patients with ovarian cancer is limited owing to the lack of early symptoms and a high rate of recurrence. The disease is diagnosed in most patients at an advanced stage, and although the initial response to chemotherapy is generally good, a significant proportion of patients will have a relapse despite optimal treatment based on current guidelines.[2] The identification of new biomarkers, reflecting current disease status and tumor activity, could optimize prediction and monitoring of oncologic treatment and provide insights into the biology of ovarian cancer. In this regard, recent studies have increasingly focused on disseminated tumor cells (DTCs) in the bone marrow (BM).

The presence of DTCs is a common phenomenon in solid tumors of epithelial origin. For breast cancer, DTC detection has been demonstrated to be a strong independent prognostic factor (level I evidence).[3] Available data support the notion that hematogenous tumor cell dissemination may be clinically relevant in ovarian cancer as well.[4–8] Detection rates of DTC, as a surrogate parameter for occult hematogenous spread, vary between 30% and 50% of patients with primary ovarian cancer. Interestingly, ovarian metastases to the bone are only rarely observed.[5,9,10] Possibly, BM acts as a secondary "homing site" in these patients where tumor cells are able to persist and may subsequently cause a relapse.

The aim of the present 3-center study was to prospectively evaluate the impact of BM status on clinical outcome in a group of 495 patients treated for ovarian cancer at 3 comprehensive cancer centers (University Hospital Tuebingen, Germany; University Hospital Essen, Germany; and University Hospital Munich, Germany).

MATERIALS AND METHODS

This 3-center analysis was performed at the Department of Obstetrics and Gynecology, University Hospital Tuebingen, Germany; the Department of Gynecology and Obstetrics, University Hospital Essen, Germany; and the Department of Gynecology and Obstetrics, University Hospital Munich, Germany. A total of 495 patients with ovarian cancer patients (International Federation of Gynecology and Obstetrics [FIGO] stages I–IV) treated between January 1994 to November 2010 in these centers were included in the analyses (Tuebingen, 229 patients [46%]; Essen, 148 patients [30%]; and Munich, 118 patients [24%]). The patients' characteristics at the time of diagnosis are shown in Table 1. All specimens were obtained after written informed consent. Tissue sampling and analysis of data were approved by the local ethic committee (114/2006A, 05-2870). Treatment of ovarian cancer and follow-up examinations were performed according to current treatment guidelines. Relapse was confirmed by physical examination, computed tomographic scan, x-ray, ultrasound, tumor marker, and/or relaparotomy depending on localization of recurrence.

Preparation of Slides

Detection of DTCs was performed as described in detail previously for all 3 centers.[4] Ten to 20-mL BM was aspirated intraoperatively from the iliac crest of both sides into syringes containing heparin anticoagulant under general anesthesia using the Jamshidi technique. Bone marrow samples were processed within 24 hours. Tumor cell isolation and detection were performed based on the recommendations for standardized tumor cell detection.[11] Briefly, samples were separated by density centrifugation using Ficoll (density, 1077 g/mL; Biochrom, Germany). Mononuclear cells (MNCs) were collected from the interphase layer, spun down onto a glass slide (10^6 MNCs per spot; Hettich cytocentrifuge, Tuttlingen, Germany) and air-dried overnight at room temperature.

TABLE 1. Incidence of DTC in patients with ovarian cancer based on diagnosis and clinical-pathological factors

	No. Patients (N = 495)	DTC Positive, n (%)	*P*
Total	495	134 (27)	
FIGO stage			0.459
I	87	27 (31)	
II	33	12 (36)	
III–IV	342	93 (27)	
Nodal status			0.061
N0	211	70 (33)	
N1	209	52 (25)	
Grading			0.107
G1	41	6 (15)	
G2	184	57 (31)	
G3	223	65 (29)	
Histologic type			0.026
Serous high-grade	313	88 (28)	
Serous low-grade	30	2 (7)	
Mucinous	19	9 (47)	
Endometrioid	29	8 (28)	
Clear cell	10	5 (50)	
Other	11	3 (27)	
Resection status			0.632
No remaining tumor	239	73 (30)	
Tumor rest	190	54 (28)	
Age, yrs			0.116
0–29	12	2 (17)	
30–49	108	27 (25)	
50–69	273	68 (25)	
70–89	102	37 (36)	

Staining of Slides and DTC Identification

For detection of cytokeratin (CK)-positive tumor cells, slides were fixed in 4% neutral buffered formalin for 10 minutes and rinsed in phosphate-buffered saline. Automatic immunostaining was performed on the Dako Autostainer

TABLE 2. Survival analysis of 456 patients depending on BM status

	No. Patients (N = 456)	Deaths, n (%)	Relapses, n (%)
Total		196 (43)	198 (43)
DTC status			
DTC positive	125	64 (51)	60 (48)
DTC negative	331	132 (40)	138 (42)

Relapse is defined as either local recurrence or distant metastasis (median follow-up: 46 months).

using the monoclonal mouse A45-B/B3 antibody (Micromet, Munich, Germany) and the DAKO-APAAP detection kit (DakoCytomation, Glostrup, Denmark) according to the manufacturer's instructions. The A45-B/B3 antibody is directed against a common epitope of CK polypeptides, including the CK heterodimers 8/18 and 8/19. The A45-B/B3 antibody is the most extensively studied antibody for DTC detection in ovarian cancer to date.[4,5,9,12] Reproducibility of DTC detection by A45-B/B3 antibody has been tested by using ovarian cancer cell line SKOV3.[12] In our analysis, for reasons of feasibility in the clinical laboratory routine the malignant breast cancer cell line MCF-7 was used as a positive control. For each patient, 2×10^6 cells were analyzed on 2 slides. All slides were evaluated by 2 independent cytologists. In case nonconcordant results were obtained, the slides were evaluated by a third investigator to obtain consensus. Identification of tumor cells was performed according to the ISHAGE evaluation criteria and the DTC consensus statements.

Statistical Analysis

The $\chi 2$ test and the Fisher exact test were used to evaluate the relationship between circulating tumor cells (CTCs) and clinicopathological factors. For the survival analysis, we considered in separate analyses the following primary end points: (1) death and (2) relapse, defined as distant or local disease recurrence, or both. Survival intervals were measured from the time of BM aspiration to the time of death or of the first clinical, histological, or radiographic diagnosis of relapse. We constructed Kaplan-Meier curves and used the log-rank test to assess the univariate significance of the parameters. The effects of multiple variables on survival were evaluated by a Cox proportional-hazards regression model. All reported P values are 2-sided. The initial model included BM status, age at diagnosis, FIGO stage, nodal status, resection status, and grading. Subjects with missing values were excluded from modeling. Statistical analysis was performed by SPSS version 17.0 (SPSS Inc, Chicago, IL). $P < 0.05$ was considered statistically significant.

RESULTS

Incidence of DTC

Bone marrow aspirates were obtained intraoperatively from 495 patients with primary ovarian cancer. Most (74%) of the patients were at FIGO stages III to IV, 19% at FIGO stage I, and 7% at FIGO stage II. The mean age was 58 years (range, 18–88 years). Disseminated tumor cells were detected in 134 (27%) of 495 BM aspirates. The number of CK-positive cells ranged from 1 to 42 per 2×10^6 mononuclear cells. Bone marrow status did not correlate with age ($P = 0.116$), FIGO stage ($P = 0.459$), lymph node status ($P = 0.061$), histopathologic grading ($P = 0.107$), or resection status ($P = 0.632$; Table 1). The lowest prevalence of positive BM status was observed in patients with low-grade serous cancer (7%), whereas 28% of patients with high-grade serous carcinoma presented with DTC ($P = 0.011$). The prevalence of DTC presence in other histologic subtypes is presented in Table 1.

BM Status and Progression-Free Survival

Patients were not considered for survival analysis if the follow-up period was less than 4 months. Thus, follow-up data were available in 456 cases. Only these patients were included

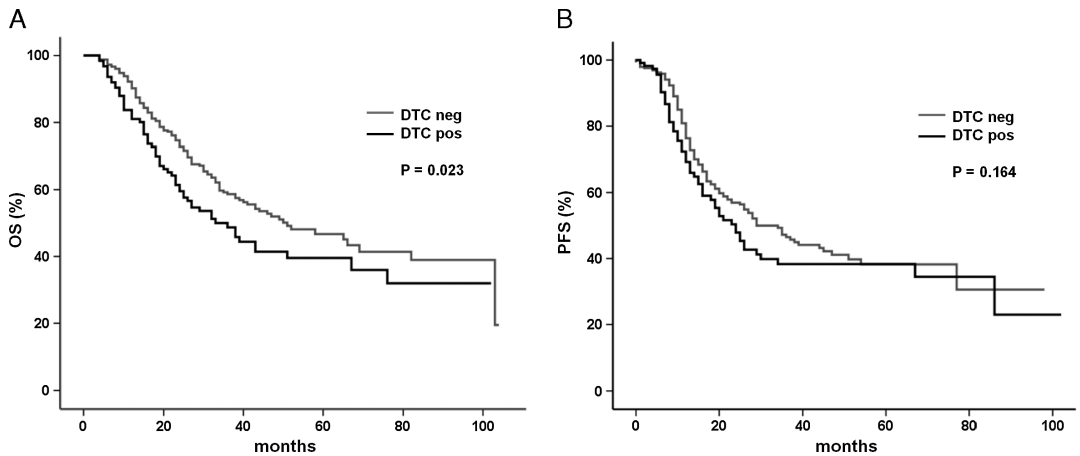

FIGURE 1. Kaplan-Meier survival analysis depending on BM status, P values calculated by the log-rank test (a, OS; b, PFS).

in the survival analysis. Median follow-up was 46 months (range, 4–104 months). Data on clinical outcome are summarized in Table 2. One hundred ninety-eight patients (43%) had recurrence during follow-up. Relapse was diagnosed in 48% of DTC-positive and 42% of DTC-negative patients ($P = 0.225$). Median progression-free survival (PFS) was shorter in patients with DTC in the BM (29 months; 95% CI, 22–36 months vs 24 months; 95% CI, 18–30 months; $P = 0.164$, determined by log-rank test; Fig. 1). In the multivariate regression analysis, BM status ($P = 0.030$), FIGO stage ($P < 0.001$), and resection status ($P = 0.003$) were the only independent predictors of PFS (Table 3).

BM Status and Overall Survival

One hundred ninety-six patients (43%) died during follow-up. Patients with DTC were more likely to die than BM-negative patients (51% vs 40%; $P = 0.029$). The median overall survival (OS) was significantly shorter among DTC-positive patients compared with DTC-negative patients (51 months; 95% CI, 37–65 months vs 33 months; 95% CI, 23–43 months; $P = 0.023$, determined by log-rank test). Survival curves are presented in Figure 1. Multivariate Cox regression model confirmed BM status as a strong independent predictor of shorter OS ($P = 0.001$). Age at diagnosis ($P = 0.004$), FIGO stage ($P < 0.001$), nodal status ($P = 0.022$), and resection status ($P < 0.001$), but not the grading, also predicted reduced OS in the multivariate analysis (Table 3). In the subgroup analysis, BM status was a strong predictor of OS in patients with high-grade serous carcinoma (295 patients; $P = 0.001$; Fig. 2). The subgroup analysis was not performed for other histologic types owing to small sample sizes. The prognostic effect of BM status was assessed within FIGO stages; for FIGO stage III to stage IV tumors (n = 299), a significant association with OS was confirmed ($P = 0.001$; Fig. 3). No significant correlation with OS was observed in FIGO I (n = 84) and FIGO II (n = 31) tumors when analyzed separately.

TABLE 3. Multivariate hazard ratios for death and relapse

Parameter	*P*	Hazard Ratio	95% CI for Hazard Ratio	
			Lower	Upper
PFS				
DTC status (positive vs negative)	0.030	1.490	1.038	2.137
FIGO stage (III–IV vs II vs I)	<0.001	2.966	1.992	4.416
Nodal status (positive vs negative)	0.386	0.855	0.600	1.218
Grading (G3 vs G2 vs G1)	0.844	1.030	0.765	1.388
Resection status (R2 vs R1 vs R0)	0.003	1.396	1.122	1.738
Age (70–89 vs 50–69 vs 30–49 vs 0–29	0.553	0.930	0.731	1.183
OS				
DTC status (positive vs negative)	0.001	1.892	1.321	2.712
FIGO stage (stage 3–4 vs 2 vs 1)	<0.001	4.055	2.103	7.819
Nodal status (positive vs negative)	0.022	1.585	1.068	2.354
Grading (G3 vs G2 vs G1)	0.765	1.049	0.767	1.434
Resection status (R2 vs R1 vs R0)	<0.001	1.741	1.401	2.163
Age (70–89 vs 50–69 vs 30–49 vs 0–29)	0.004	1.486	1.134	1.948

BM status and established prognostic factors, such as FIGO stage, nodal status, resection status, age at diagnosis, and grading were included in this statistic model. a) progression-free survival; b) overall survival.

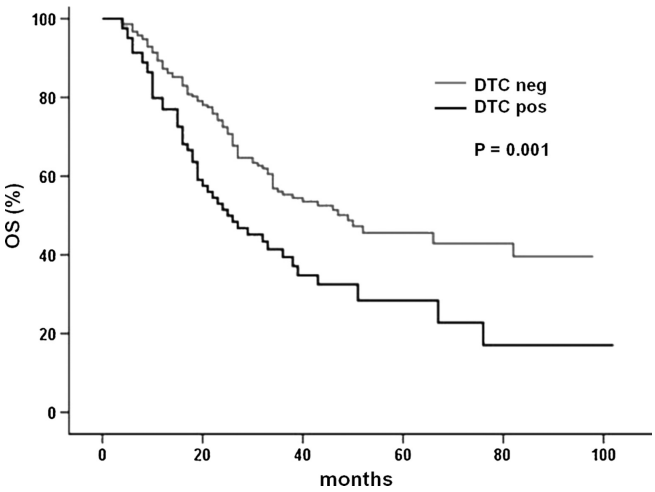

FIGURE 2. Subgroup analysis: high-grade serous carcinoma (n = 295); Kaplan-Meier OS analysis.

DISCUSSION

This is the largest pooled analysis so far on the prognostic relevance of DTC in primary ovarian cancer. A total of 495 patients were included in this prospective 3-center analysis. Disseminated tumor cells, as a surrogate parameter for minimal residual disease, could be detected in 27% of the patients, irrespective of the stage of the disease.

Other studies reported DTC incidence ranging from 20% to 60% in primary ovarian cancer, depending on the methodology and patients' collective.[4–6,8,13–16] Interestingly, ovarian malignancies rarely cause bone metastases.[10] This suggests that BM serves in these patients rather as a temporary "homing site" for isolated tumor cells, from where they are able to migrate and subsequently cause metastasis or locoregional recurrence. Because hematogenous dissemination of isolated tumor cells may already be observed in FIGO stage I, it may be hypothesized that contrary to the assumed natural history of ovarian cancer, single tumor cells acquire the potential to

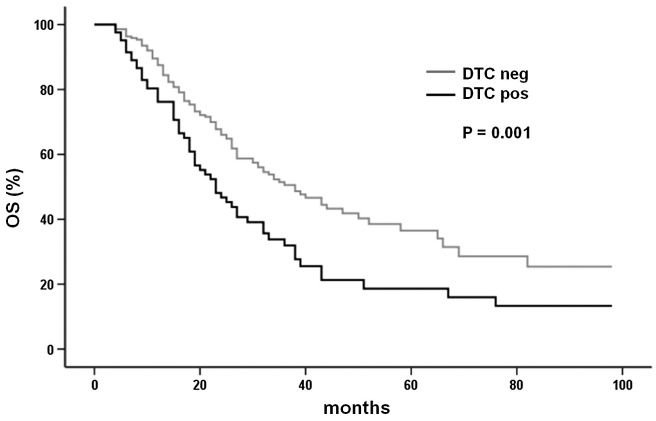

FIGURE 3. Subgroup analysis: patients with FIGO stages III to IV (n = 299); Kaplan-Meier OS analysis.

disseminate to extraperitoneal sites very early in the process of the disease.[5] Because DTCs may spread by means of the blood stream, we cannot exclude that those might also be able to repopulate the peritoneal cavity, an environment that easily supports ovarian cancer growth.

Influence of DTC Detection on Clinical Outcome

For ovarian cancer, data on the prognostic value of DTCs are so far limited (Table 4). In the present study, presence of DTCs predicted significantly shortened OS ($P = 0.001$ in the multivariate survival analysis). This is in accordance with several smaller studies. Braun et al[5] demonstrated impaired prognosis with regard to distant disease-free survival (DFS) in BM-positive patients at the time of diagnosis; in a subset of 64 optimally debulked patients, DTC presence remained a strong prognostic factor ($P = 0.002$), which highlights the role of DTC detection especially in patients who received successful surgical cytoreduction. We previously reported a significant correlation of positive BM status with reduced DFS in a group of 112 patients with FIGO stage I to stage III ovarian cancer.[4] Interestingly, in some studies, the presence of isolated tumor cells in secondary sites such as BM and blood was also associated with higher risk for local recurrence as well.[4,7] Therefore, it might be speculated that hematogenous tumor cell dissemination may serve as an indicator of a more aggressive phenotype of the primary disease that is likely to cause local relapse. In contrast, other authors reported no significant correlation between DTC detection and clinical outcome in ovarian cancer.[13,17] This discrepancy might be due to differences in study protocols, for example, time point of BM sample collection (preoperative vs postoperative aspiration). Hypothetically, a transient increase in cancer cell dissemination from the primary tumor due to intraoperative manipulation could contribute to false-positive results and therefore affect further analysis.[18]

Interestingly, in the current analysis, BM status did not influence PFS. This may be due to inconsistent relapse diagnosis. Most patients were treated within a clinical trial;

TABLE 4. Prognostic relevance of DTC and CTC in ovarian cancer

Author	No. Patients	Method	Median Follow-up (Months)	Positivity Rate (%)	Prognostic Significance
Our study	456	DTC (ICC)	46	27	OS, PFS*
Banys et al[4]	112	DTC (ICC)	12	25	DFS
Braun et al[5]	108	DTC (ICC)	45	30	DFS
Aktas et al[6]	95	DTC (ICC)	28	35	ns
Schindlbeck et al[8]	90	DTC (ICC)	28	23	DDFS
Marth et al[13]	73	DTC (immunobeads)	25	21	ns
Wimberger et al[14]	62	DTC (ICC)	18	54	DFS†
Poveda et al[7]	216	CTC (ICC: CellSearch)‡		14§	PFS, OS
Marth et al[13]	90	CTC (immunomagnetic beads)	25	12	ns
Aktas et al[6]	86	CTC (multiplex-RT-PCR: AdnaTest)	28	19	OS‖
Heubner et al[16]	68	Circulating 20S-proteasomes	19	—	OS
Fan et al[15]	66	CTC (immunofluorescence and cell invasion assay)	18	61	DFS
Wimberger et al[14]	62	Circulating nucleosomes, DNA, protease and caspase activity	18	—	DFS, OS

*Determined by multivariate Cox regression analysis.
†Disseminated tumor cells detected after chemotherapy.
‡Relapsed ovarian cancer.
§Two or more CTCs.
‖Both before and after chemotherapy.
DDFS, Distant disease-free survival; ICC, immunocytochemistry; ns, not significant; RT-PCR, reverse-transcriptase polymerase chain reaction

these patients received intensified follow-up with computed tomographic scans at regular intervals. In the group of patients treated outside clinical trials, follow-up care was carried out according to national guidelines and was based on clinical examination and patients' symptoms. Possibly, relapse was diagnosed later in the group treated outside clinical trials.

Disease Monitoring

Beyond the prognostic value of DTC detection, monitoring of minimal residual disease during and after treatment offers the opportunity to assess the residual risk of relapse. The presence of DTCs and/or CTCs may indicate persistent occult tumor load after treatment and thus an insufficient therapy response. Wimberger et al[12] assessed changes in DTC levels before and after first-line chemotherapy; marked increase in DTC numbers predicted significantly shorter PFS. Whether the reevaluation of the DTC status after completion of therapy may contribute to selection of patients who might benefit from extended or intensified treatment remains to be investigated.

Stem Cell Hypothesis

A provocative hypothesis has been introduced recently with respect to natural history and progression of ovarian cancer. While the "classical" stochastic model of cancer development holds that any cell may become a source of malignant transformation, emerging evidence supports the view that only a minor subpopulation of cancer cells has the potential to initiate cancer growth. These cells, called cancer stem cells (CSCs), have the ability to self-renew, propagate tumorigenesis, and are usually drug-resistant.[19] Experimental studies on stem cell biology have given new impetus to the cancer stem cell theory. Cancer stem cells are assumed to play an important role in the development of various tumor entities, such as breast and gastrointestinal cancer, retinoblastoma, and ovarian cancer.[20,21] Interestingly, ovarian cancer cell lines feature "side population" cells with potential to differentiate into cancers with different histologies, suggesting the pluripotent character of stem cells.[22]

One currently debated hypothesis is the theory that DTC/CTC, the surrogate marker for minimal residual disease and possibly precursor of systemic metastasis, represent in fact cancer stem cells. Most DTCs in breast cancer seem to express a putative CSC phenotype, such as ALDH1 positivity or presence of CD44 and absence of CD24, whereas CTCs were reported to exhibit stem cell and epithelial-mesenchymal transition markers.[23,24] Several characteristics of ovarian cancer (eg, high recurrence rates and multidrug resistance) suggest that the disease might be initiated and maintained by a unique population of cells with stem cell–like properties.[21] Advanced ovarian cancer generally responds to platinum-based combination therapy; however, this initial regression is often followed by emergence of therapy-resistant cell clones. One possible explanation for this phenomenon is the CSC-induced drug-resistance: standard therapies fail to target tumor-initiating cells.[21]

CONCLUSIONS

Despite advances in surgical and systemic therapy, ovarian cancer leads to relapse in 60% of patients within 5 years, resulting in poor OS. Currently, therapy efficacy is assessed by physical examinations, imaging, and evaluation of CA125 levels. New biomarkers are thus necessary for better prediction and prognostication.

Early hematogenous tumor cell dissemination is a common phenomenon observed in most solid tumors. Recent data support the clinical relevance of these cells; in the present report, we demonstrate significant impact of the presence of DTCs in the BM on survival. Whether these patients might benefit from extended or more aggressive therapy remains to be evaluated in future trials.

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