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ERCC1-Positive Circulating Tumor Cells in the Blood of Ovarian Cancer Patients as a Predictive Biomarker for Platinum Resistance

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BACKGROUND: Platinum resistance constitutes one of the most recognized clinical challenges for ovarian cancer. Notably, the detection of the primary tumor-based excision repair cross-complementation group 1 (ERCC1) protein by immunohistochemistry was recently shown to be inaccurate for the prediction of platinum resistance. On the basis of the previous finding that circulating tumor cells (CTC) in the blood of ovarian cancer patients are prognostically significant, and given our hypothesis that the negative prognostic impact of CTC may arise from a cellular phenotype associated with platinum resistance, we asked whether expression of the excision repair cross-complementation group 1 (*ERCC1*) gene in the form of the *ERCC1* transcript in CTC may be a suitable blood-based biomarker for platinum resistance.

METHODS: The presence of CTC was analyzed by immunomagnetic CTC enrichment (n = 143 patients) targeting the epithelial epitopes epithelial cell adhesion molecule (*EPCAM*) (also known as *GA733-2*) and mucin 1, cell surface associated (*MUC1*), followed by multiplex reverse-transcription PCR to detect the transcripts *EPCAM*, *MUC1*, and mucin 16, cell surface associated (*MUC16*) (also known as *CA125*), including *ERCC1* transcripts in a separate approach. ERCC1 expression in primary tumors was comparatively assessed by immunohistochemistry, using the antibody 8F1.

RESULTS: At primary diagnosis, the presence of CTC was observed in 14% of patients and constituted an independent predictor of overall survival (OS) ($P = 0.041$). *ERCC1*-positive CTC (*ERCC1*$^+$CTC) were observed in 8% of patients and constituted an independent predictor, not only for OS but also for progression-free survival (PFS) ($P = 0.026$ and $P = 0.009$, respectively). More interestingly, we discovered the presence of *ERCC1*$^+$CTC at primary diagnosis to be likewise an independent predictor of platinum resistance ($P = 0.010$), whereas ERCC1 expression in corresponding primary tumor tissue predicted neither platinum resistance nor prognosis.

CONCLUSIONS: The presence of *ERCC1*$^+$CTC can serve as a blood-based diagnostic biomarker for predicting platinum resistance at primary diagnosis of ovarian cancer.

Epithelial ovarian cancer accounts for the majority of tumor-related deaths among female malignancies. At primary diagnosis, approximately 70% of all ovarian cancer patients already present with tumors at advanced stages *(1)*. Standard treatment of advanced ovarian cancer constitutes primary surgery aiming at macroscopic complete tumor resection and subsequent platinum- and paclitaxel-based chemotherapy *(2)*. So far, residual postoperative tumor load is one of the most important prognostic factors for the outcome of ovarian cancer. However, despite recent advances in treatment, more than 50% of all patients suffer from recurrent disease, resulting in worse overall prognosis *(1)*. Importantly, resistance to platinum-based chemotherapy constitutes one of the most recognized clinical challenges for ovarian cancer. Resistance occurs in about 20% of patients and can be assessed only retrospectively within the follow-up period after adjuvant chemotherapy *(3)*.

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Functionally, platinum resistance can be caused by either increased tolerance toward DNA–platinum adducts or enhanced DNA repair capacity of tumor cells *(4, 5)*. In this regard, the nucleotide excision repair (NER)[7] pathway is believed to be an essential mechanism for the repair of DNA–platinum adducts *(5)*. In terms of the cellular NER, excision repair cross-complementation group 1 (ERCC1) nuclease forms a heterodimer with exicision repair cross-complemenentation group 4 (ERCC4, also known as xeroderma pigmentosum, complementation group F) and accomplishes repair of bulky DNA–platinum adducts *(6–8)*. Expression of both the ERCC1 protein or the *ERCC1* transcript has been extensively studied in primary tumor tissue of several cancer entities and has been proposed as a potential predictor for response to platinum-based chemotherapy. However, this concept has been controversial, particularly in the context of immunohistochemical ERCC1 protein detection, and has not yet been translated into clinical practice *(9–19)*. A recent key publication in the *New England Journal of Medicine* reported on a comprehensive reevaluation study on 494 lung cancer patients and concluded that immunohistochemical ERCC1 detection in the primary tumor with all currently available antibodies is principally inappropriate for clinicians in terms of predicting platinum sensitivity and guiding therapy decisions *(20)*. Given this discouraging finding, and considering that primary tumor tissue is uniquely available only by resection, it would be of clinical interest to establish a noninvasive blood-based biomarker for stratifying response to platinum-based chemotherapy at primary diagnosis and for guiding individualized therapy decisions.

Following our previous observation that circulating tumor cells (CTC) in the blood of ovarian cancer patients are predictive for a poor overall survival (OS) *(21)*, and given our hypothesis that the negative prognostic impact of CTC may arise from a cellular phenotype, being associated with platinum resistance, we asked whether CTC expressing the excision repair cross-complementation group 1 (*ERCC1*)[8] gene in the form of the *ERCC1* transcript may be superior to commonly studied primary tumor-based ERCC1 protein detection in predicting response to platinum-based chemotherapy.

Materials and Methods

PATIENT CHARACTERISTICS

The present study was conducted at the Department of Gynecology and Obstetrics at the University Hospital of Essen, Germany. In this study, a total of 143 patients with histologically confirmed epithelial ovarian cancer were enrolled. Informed written consent was obtained from all patients and the study was approved by the Local Research Ethics Committee (05-2870). The patients' clinical data are summarized in Table 1. Tumors were classified according to the WHO classification of tumors of the female genital tract, grading was conducted using the grading system proposed by Silverberg *(22)*, and tumor staging was classified according to the Fédération Internationale de Gynécologie et d'Obstétrique *(23)*. The whole study population received primary radical surgery aiming at macroscopic complete tumor resection. Total abdominal hysterectomy, bilateral salpingo-oophorectomy, infragastric omentectomy, peritoneal stripping, and pelvic as well as paraaortic lymphadenectomy were performed, where feasible. All patients received platinum-based chemotherapy. Tumors were clinically defined as platinum resistant if they recurred within 6 months after the completion of platinum-based chemotherapy.

ENRICHMENT AND MOLECULAR CHARACTERIZATION OF CTC

Peripheral blood was collected for CTC isolation with an S-Monovette® (Sarstedt AG & Co.) and was processed within 4 hours after withdrawal. Blood samples were subjected to immunomagnetic tumor cell enrichment using the AdnaTest OvarianCancerSelect (AdnaGen AG). All experimental steps were performed, according to the manufacturer's instructions. Briefly, epithelial cell adhesion molecule (*EPCAM*; also known as GA733–2)-positive and mucin 1, cell surface associated (*MUC1*)-positive cells were targeted, followed by RNA isolation and subsequent gene expression analysis by reverse-transcription (RT) and multiplex PCR, detecting the tumor-associated transcripts *EPCAM*, *MUC1*, and mucin 16, cell surface associated (*MUC16*; also known as *CA125*) (AdnaTest OvarianCancerDetect; AdnaGen AG). *ERCC1* transcripts were assessed in a separate singleplex RT-PCR. PCR reactions were performed with the HotStarTaq Master Mix (Qiagen), using actin, beta (*ACTB*) as an internal positive control. PCR composition and cycling conditions of *ERCC1* transcript detection exactly corresponded to the detection of the already established AdnaTest OvarianCancerDetect marker panel

[7] Nonstandard abbreviations: NER, nucleotide-excision repair; ERCC1, excision-repair cross-complementation group 1; CTC, circulating tumor cells; OS, overall survival; RT, reverse transcription; TMA tissue microarray; FFPE, formalin-fixed and paraffin-embedded; PS, proportion score; IS, intensity score; TS, total Allred score; PFS, progression-free survival; FIGO, International Federation of Gynecology and Obstetrics; HR, hazard ratio; OR, odds ratio.

[8] Human genes: *ERCC1*, excision repair cross-complementation group 1; *EPCAM*, epithelial cell adhesion molecule (also known as *GA733–2*); *MUC1*, mucin 1, cell surface associated; *MUC16*, mucin 16, cell surface associated (also known as *CA125*); *ACTB*, actin, beta; *PPIC*, peptidylprolyl isomerase C (cyclophilin C).

Table 1. Patient characteristics at the time of primary diagnosis of ovarian cancer.

Total no. of patients	143
Age	Median, 59 years (range, 21–89 years)
FIGO stage	
FIGO I–II	26 (18%)
FIGO III	87 (61%)
FIGO IV	30 (21%)
Lymph node metastasis	
N_0	46 (32%)
N_1	62 (43%)
N_x	35 (24%)
Tumor grading	
I–II	68 (48%)
III	75 (52%)
Histologic subtype	
Serous papillary subtype	109 (76%)
Any other subtype	34 (24%)
Residual tumor	
Macroscopic	
Complete resection	83 (58%)
Any residual tumor	60 (42%)
Survival	
OS	Median, 23 months (range, 1–71 months)
Alive	71 (50%)
Dead	68 (48%)
Unknown	4 (3%)
PFS	Median, 17 months (range, 2–69 months)
Recurrence	
No relapse	54 (38%)
Relapse	74 (52%)
Unknown	15 (10%)
Platinum resistance	
Platinum sensitive	85 (59%)
Platinum resistant	42 (29%)
Unknown	16 (11%)

(15 min at 95 °C for initial activation, followed by 35 cycles of 30 s at 94 °C for denaturation, 30 s at 60 °C for annealing, 60 s at 72 °C for extension, and 10 min at 72 °C for termination). The primers generated amplicons with the following sizes: *EPCAM*, 395 bp; *MUC1*, 293 bp; *MUC16*, 432 bp; *ERCC1*, 366 bp; and *ACTB*, 114 bp.

EVALUATION OF CTC POSITIVITY ACCORDING TO ADNATEST OVARIAN CANCER

A given blood sample, processed with the AdnaTest OvarianCancer, was considered CTC positive if at least one of the tumor-associated transcripts *EPCAM*, *MUC1*, or *MUC16* was detectable with an amplicon concentration above the indicated diagnostic threshold (>0.15 ng/μL). Analysis of the PCR fragments was carried out with the 2100 Bioanalyzer using the DNA 1000 LabChip kit and the Expert Software Package (version B.02.03.SI307; Agilent Technologies).

IMMUNOHISTOCHEMICAL STAINING FOR ERCC1

ERCC1 expression in primary tumor tissues was analyzed by immunohistochemistry, using tissue microarrays (TMA). Routinely formalin-fixed and paraffin-embedded (FFPE) tissue blocks were retrieved from the Institute of Pathology and Neuropathology of the University Hospital of Essen, Germany. Hematoxylin-and-eosin–stained sections were prepared and reviewed by a pathologist. Tissue cores of 3 mm in size with the greatest possible intratissue tumor content (in most cases at least 60%) were punched from a predefined region of a given tumor block and assembled to a TMA block, each comprising 24 tumor samples. Subsequently, TMA-sections of 4-μm thickness were processed for ERCC1 immunohistochemistry.

Immunohistochemical staining was performed with an automated staining device (Dako Autostainer; Dako), using a monoclonal mouse antihuman ERCC1–IgG2b antibody (Clone 8F1; Laboratory Vision). After deparaffinization of TMA sections, antigen retrieval was performed in 0.01 mol/L sodium citrate buffer at pH 6.0 for 20 min in a hot water bath (95 °C). Incubation with the primary antibody was carried out for 30 min at room temperature, using a dilution of 1:200. Secondary and tertiary immunoreactions were performed using a commercially available anti-mouse IgG detection kit (En-Vision, DakoCytomation). Normal tonsil tissue was used as positive control; replacement of the primary antibody with mouse immunoglobulin and omission of the primary antibody served as negative controls. ERCC1 positivity was graded by the Allred score, which is based on the percentage of stained tumor cell nuclei [proportion score (PS) ranging from 0 to 5] and staining intensity [intensity score (IS) ranging from 0 to 3]. The total Allred score (TS) was calculated by the sum of both scores (TS = IS + PS), ranging from 0 to 8. Samples with a TS of >2 were considered ERCC1 positive, whereas a TS of >6 indicated ERCC1 high positivity.

STATISTICAL ANALYSIS

Statistical analysis was performed using SPSS Statistics software 20.0 (IBM). To evaluate the clinical significance

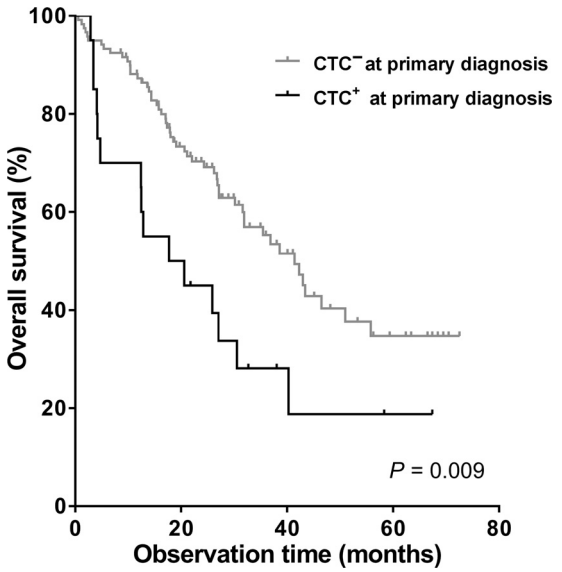

Fig. 1. Kaplan-Meier curves comparing overall survival of ovarian cancer patients bearing CTC in their blood at primary diagnosis (bottom curve) vs patients with no evidence of CTC (top curve).

of CTC or *ERCC1*-positive CTC (*ERCC1*$^+$CTC), univariable as well as multivariable regression analyses were performed. For univariable regression analysis, 3 independent analyses were performed in which progression-free survival (PFS), OS, or platinum resistance were individually regressed by International Federation of Gynecology and Obstetrics (FIGO) stage, grading, residual tumor, CTC, or *ERCC1*$^+$CTC, respectively. In the case of multivariable regression analysis, 3 independent analyses for PFS, OS, or platinum resistance as the selected outcome variable in dependence on *ERCC1*$^+$CTC were performed and adjusted for the clinical parameters FIGO stage, tumor grading, and residual tumor. "Negativity" for *ERCC1*$^+$CTC events was defined as *ERCC1*$^-$CTC$^+$, *ERCC1*$^+$CTC$^-$, or *ERCC1*$^-$CTC$^-$. Complete models were used to report hazard ratios (HR), odds ratios (OR), and related (2-tailed) *P* values. In case of significant findings, Kaplan–Meier analyses were performed to create survival curves.

Results

CTC POSITIVITY IN PATIENT BLOOD ACCORDING TO ESTABLISHED ADNATEST OVARIANCANCER

From a cohort of 143 patients with epithelial ovarian cancer, preoperative blood samples obtained at primary diagnosis were subjected to immunomagnetic CTC enrichment and subsequent molecular CTC characterization, analyzing the transcripts of *EPCAM*, *MUC1*, and *MUC16* (Adnatest OvarianCancer). CTC were detected in 20/143 patients (14%) and the presence of CTC was significantly associated with reduced OS (HR, 2.16; 95% CI, 1.22–3.84; $P = 0.009$) (Fig. 1), but not with PFS (HR, 1.50; 95% CI, 0.81–2.79; $P = 0.199$). Moreover, according to multivariable analysis, the presence of CTC was an independent predictor of OS (HR, 1.85; 95% CI, 1.03–3.32; $P = 0.041$).

Table 2. Univariable analysis to evaluate the clinical relevance of *ERCC1* expression in CTC and in the primary tumor.

Univariable analysis	PFS	OS	Platinum resistance
FIGO stage	$P < 0.0005$	$P = 0.002$	$P = 0.01$
Grading	$P = 0.882$	$P = 0.170$	$P = 0.201$
Residual tumor	$P < 0.0005$	$P < 0.0005$	$P < 0.0005$
ERCC1$^+$CTC	$P = 0.079$	$P = 0.042$	$P = 0.015$

ESTABLISHING *ERCC1* TRANSCRIPT DETECTION AS A NOVEL CTC MARKER

To establish an appropriate threshold for CTC-based *ERCC1* detection, ROC curve plots were created to compare the CTC-derived *ERCC1* signal in our patient samples to the corresponding *ERCC1* signal in the blood samples of 21 healthy controls, which were previously subjected to the AdnaTest OvarianCancer-Select (see Fig. 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol60/issue10). An amplicon threshold concentration of >0.2 ng/μL was defined as *ERCC1* positive (*ERCC1*$^+$), whereas a value <0.2 ng/μL was considered *ERCC1* negative (*ERCC1*$^-$). Notably, the selection of this threshold ensured the highly specific performance of our CTC-based *ERCC1* detection by stratifying 100% of the evaluated healthy controls as *ERCC1*$^-$.

ERCC1$^+$CTC PREDICT POOR PROGNOSIS

For our patient cohort, univariable analysis revealed that FIGO stage and residual tumor after surgery significantly correlated with PFS, OS, and platinum resistance (Table 2). Moreover, following multivariable analysis, FIGO stage and residual tumor burden after surgery were independent predictors of PFS, OS, and platinum resistance, whereas tumor grading constituted an independent predictor of PFS and platinum resistance (Table 3).

Considering *ERCC1* transcripts as an additional CTC-associated biomarker, we intended to compile the presence of the established Adnatest Ovarian-

Table 3. Multivariable analysis to evaluate clinical relevance of *ERCC1*$^+$CTC with regard to the patient's survival and platinum resistance.

	Cox regression PFS			Cox regression OS			Log regression platinum resistance[a]		
Variable	Independent	HR	95% CI	Independent	HR	95% CI	Independent	OR	95% CI
FIGO stage	$P < 0.0005$	16.6	3.7–74	$P = 0.011$	14.1	1.9–108	$P = 0.021$	14.0	1.5–131
Grading	$P = 0.043$	0.57	0.33–0.98	$P = 0.561$	0.85	0.49–1.48	$P = 0.009$	0.27	0.10–0.72
Residual tumor	$P = 0.030$	1.8	1.1–3.0	$P < 0.0005$	3.2	1.9–5.7	$P = 0.021$	3.1	1.2–8.0
ERCC1$^+$CTC	$P = 0.009$	3.4	1.4–8.3	$P = 0.026$	2.5	1.1–5.5	$P = 0.010$	8.5	1.7–43.6

[a] Platinum resistance status for 9 patients was unknown.

Cancer CTC marker with the presence of *ERCC1* transcripts. In particular, we were interested in the incidence and clinical relevance of CTC being positive for at least one of the established Adnatest marker-transcripts (*EPCAM*, *MUC1*, or *MUC16*) and additionally for *ERCC1* transcripts. Those CTC were herein defined as *ERCC1*$^+$CTC.

From the 143 preoperative blood samples processed by Adnatest OvarianCancer, cDNA of 120 patients was available for additional *ERCC1* singleplex RT-PCR. *ERCC1*$^+$CTC were detected in 10/120 patients (8%). Following univariable analysis, the presence of *ERCC1*$^+$CTC correlated with decreased OS (HR, 2.18; 95% CI, 1.03–4.62; $P = 0.042$) (Table 2 and Fig. 2), but not with PFS (HR, 2.14; 95% CI, 0.92–4.98; $P = 0.079$). Moreover, multivariable analysis revealed the presence of *ERCC1*$^+$CTC at primary diagnosis to be an independent predictor of a poor PFS (HR, 3.4; 95% CI, 1.4–8.3; $P = 0.009$) and OS (HR, 2.5; 95% CI, 1.1–5.5; $P = 0.026$) (Table 3), whereas the presence of *ERCC1*$^-$CTC was noninformative for PFS according to univariable and multivariable analysis.

ERCC1$^+$CTC PREDICT PLATINUM RESISTANCE

The next step was to inquire whether the detection of *ERCC1*$^+$CTC could serve as a blood-based biomarker for stratifying response to platinum-based chemotherapy. Using the Adnatest OvarianCancer, we revealed that the presence of CTC at primary diagnosis significantly correlated with platinum resistance in univariable analysis (OR, 3.00; 95% CI, 1.13–7.94; $P = 0.027$). Notably, the presence of *ERCC1*$^+$CTC at primary diagnosis was likewise associated with platinum resistance, as determined by univariable analysis (OR, 5.79; 95% CI, 1.40–23.96; $P = 0.015$) (Table 2) and additionally constituted an independent predictor of platinum resistance, as revealed by multivariable analysis (OR, 8.5; 95% CI, 1.7–43.6; $P = 0.010$) (Table 3). Accuracy of *ERCC1*$^+$CTC detection for identifying platinum-resistant ovarian cancer patients was documented with a positive predictive value of 70% and a negative predictive value of 71%. Moreover, the presence of *ERCC1*$^+$CTC was still an independent predictor of platinum resistance when patients with serous histology were considered exclusively (OR, 11.3; 95% CI, 1.3–95.7; $P = 0.026$).

ERCC1$^+$CTC DETECTION IS SUPERIOR TO PRIMARY-TUMOR–BASED ERCC1 DETECTION IN PREDICTING PLATINUM RESISTANCE

We intended to relate clinical relevance of our CTC-based *ERCC1* assay to the commonly studied primary-tumor-based ERCC1 detection *(20)*. Out of the 120 patients studied for *ERCC1*$^+$CTC, corresponding primary tumor tissue (FFPE) was available in 77 cases and sub-

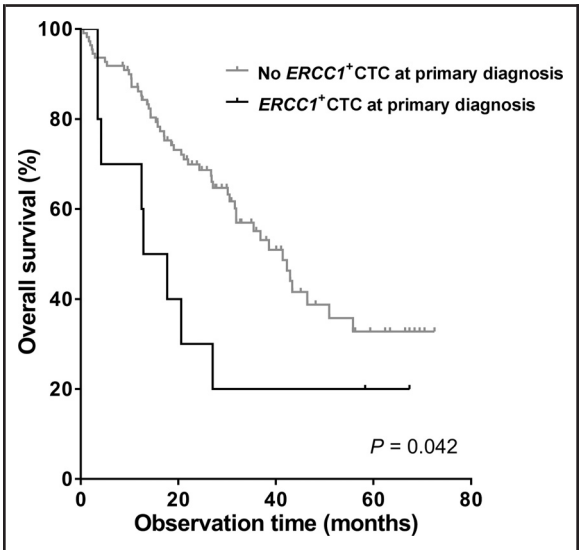

Fig. 2. Kaplan-Meier curves comparing overall survival of ovarian cancer patients bearing *ERCC1*$^+$CTC in their blood at primary diagnosis (bottom curve) vs patients with no evidence of *ERCC1*$^+$CTC (top curve), defined as *ERCC1*$^-$CTC$^+$, *ERCC1*$^+$CTC$^-$, or *ERCC1*$^-$CTC$^-$.

jected to comparative immunohistochemical ERCC1 detection. Interestingly, in 2/77 patients (3%), concordant positivity of both the *ERCC1* transcript in CTC and the ERCC1 protein in corresponding primary tumor was observed, whereas 44/57 patients (57%) were negative for *ERCC1* transcript expression in CTC and ERCC1 protein expression in the primary tumor. In 25/77 patients (33%), ERCC1 protein was detected in the primary tumor, whereas corresponding CTC were negative for *ERCC1* transcripts. Moreover, in 6/77 patients (8%) *ERCC1* transcripts were detected in CTC, whereas corresponding primary tumor tissue was negative for ERCC1 protein. The overall concordance rate of *ERCC1* transcript and ERCC1 protein expression in CTC and corresponding primary tumor, respectively, was 60% [resulting from concordant expression in 46/77 patients (see online Supplemental Table 1)], and the Cohen's κ coefficient was −0.055 (95% CI, −0.214 to 0.104).

Next, we investigated whether ERCC1 expression in primary ovarian cancer tissue is clinically significant and may predict prognosis or platinum resistance. However, considering that a statistically substantiated analysis in only 77 patients was not feasible, we extended the ERCC1 analysis with primary tumor tissues from a further 111 independent ovarian cancer patients from whom no blood samples for *ERCC1*$^+$CTC analyses were available. Hence, a total number of 188 patients were subsequently subjected to immunohistochemical ERCC1 protein detection. Clinicopathological characteristics in this extended group were comparable to those of our initial patient cohort for CTC-analysis and are separately listed in online Supplemental Table 2. ERCC1 positivity was observed in a total of 65/188 patients (35%), including 15/188 patients (8%) with highly ERCC1-positive tumors (TS >6). However, ERCC1 positivity, as well as ERCC1 high positivity in the primary tumor, did not correlate with PFS and OS or with platinum resistance (data not shown).

Discussion

Our investigation was based on the AdnaTest platform for CTC detection in the blood of ovarian cancer patients. We successfully established *ERCC1* transcript detection as a complementary CTC-based biomarker and reported the prognostic relevance of *ERCC1*$^+$CTC. Most interestingly, as our key finding, we revealed the presence of *ERCC1*$^+$CTC at primary diagnosis as an independent predictor of platinum resistance, whereas ERCC1 positivity in the corresponding primary tumor was clinically noninformative.

Recent studies of several cancer entities, including our own investigations of ovarian cancer, have demonstrated the clinical utility of CTC as promising blood-based biomarkers for diagnosing and monitoring disease in terms of a "real-time liquid biopsy" *(24)*. At present, there is no standard definition for the identification of CTC *(25)*. The CellSearch® system, based on immunomagnetic *EPCAM*-mediated cell-selection, is the only Food and Drug Administration-cleared system for CTC enumeration and is the most frequently used platform in clinical studies, showing a significant correlation between the presence of CTC and decreased PFS and OS *(26–30)*. Despite the prognostic impact of CTC counts, several molecular methods have been proposed to complement these studies by improving overall detection rates and sensitivity, thus permitting the assessment of molecular markers in CTC of cancer patients *(21, 31–35)*. By analyzing CTC in our patient cohort, according to Adnatest OvarianCancer, we demonstrated that positivity of at least one of the tumor-associated transcripts *EPCAM*, *MUC1*, or *MUC16* in the patient's blood was associated with decreased OS, consistent with our previous observation *(21)*. Moreover, this previous report could be complemented by the finding that the presence of CTC likewise constitutes an independent predictor of OS according to multivariable analysis.

Moreover, in the present study we successfully established a CTC-based RT-PCR assay for *ERCC1* expression analysis, intending to complement the previous marker panel and to extend clinical utility of molecular CTC characterization to the most recognized clinical challenge for ovarian cancer, the detection of platinum-resistant disease. Notably, *ERCC1*$^+$CTC at primary diagnosis were superior in predicting the patient's prognosis than *ERCC1*$^-$CTC, by additionally constituting an independent predictor of a poor PFS. The prognostic relevance of *ERCC1*$^+$CTC is principally in accordance with a recent very small pilot study analyzing 17 patients with metastatic non–small-cell lung cancer who were receiving platinum-based chemotherapy *(36)*. Increased ERCC1 protein expression in the patient's CTC was associated with reduced PFS. However, this pilot study did not resolve any association between CTC-derived ERCC1 expression and the patient's response to platinum-based chemotherapy *(36)*. In this context, our findings support the idea that the additional evaluation of CTC-based *ERCC1* transcripts in ovarian cancer patients significantly improves the prognostic impact and clinical utility of CTC as a blood-based biomarker for stratifying prognosis.

Most interestingly, we revealed the presence of *ERCC1*$^+$CTC at primary diagnosis as an independent predictor of platinum resistance, with a reasonable predictive value. In this context, a recent comprehensive study of the OVCAD (OVarian CAncer Diagnosis) consortium, analyzing a cohort of 216 patients, found that peptidylprolyl isomerase C (cyclophilin C) (*PPIC*)-positive CTC were significantly more abundant in

platinum-resistant than in platinum-sensitive patients *(32)*. Importantly, however, this observation exclusively referred to the follow-up situation after accomplished chemotherapy and not to primary diagnosis. To the best of our knowledge, we provide the first report suggesting a prospective CTC-based biomarker for the detection of platinum resistance at primary diagnosis of ovarian cancer. Moreover, given that *ERCC1*$^+$CTC retain this predictive capacity for platinum resistance, when only patients with serous tumors were considered, our findings may likewise be representative for the major histologic subtype of ovarian cancer. These findings are of high clinical significance because they show that the additional characterization of *ERCC1* transcripts renders CTC a liquid biomarker for the stratification of platinum-resistant patients before the initiation of chemotherapy. In this regard, the detection of *ERCC1*$^+$CTC could aid clinicians in guiding individual therapy decisions by minimally invasive blood collection. Once platinum resistance is predicted, an alternative therapeutic strategy could be scheduled, preventing the patient from unnecessary systemic toxicity and side effects of platinum-based chemotherapy.

We confirmed the superiority of CTC-based *ERCC1* evaluation in predicting response to cisplatinum therapy compared to commonly studied primary tumor-based ERCC1 detection. Notably, we observed that ERCC1 protein expression in primary tumor tissue predicted neither prognosis nor platinum resistance. These findings were not surprising because they agree with the results from a publication reporting on the discouraging finding that immunohistochemical ERCC1 detection in the primary tumor, with all currently available antibodies, constitutes an inappropriate diagnostic tool for predicting platinum sensitivity and for guiding therapy decisions *(20)*. Moreover, we observed virtually no concordance between positivity for *ERCC1* transcript expression in CTC and ERCC1 protein expression in corresponding primary tumor tissue. This finding is corroborated by a recent study on advanced breast cancer, in which poor correlation between ERCC1 protein expression in CTC vs primary tumor was reported *(37)*. These findings indicate that ERCC1 protein and *ERCC1* transcript detection in these 2 compartments may capture "orthogonal" snapshots due to the different biological behavior of primary tumor vs blood-derived tumor cells and may provide complementary and independent clinical information on prognosis and platinum sensitivity. However, this interpretation must be used with care, because ERCC1 protein and *ERCC1* transcript expression in primary tumor tissue and CTC, respectively, was analyzed with different methods in our study, and therefore the lack of concordant ERCC1 protein and *ERCC1* transcript expression could also be due to differing analytical sensitivities of the applied methodologies.

Given our present experimental framework, we cannot distinguish whether an *ERCC1*$^+$CTC event is derived from a coexisting population of "AdnaTest-positive" CTC and separately present *ERCC1*-positive CTC or whether it is derived from CTC with combined marker positivity in the same cell. However, we can hypothesize that *ERCC1*-(over)expressing CTC in the blood may be characterized by an enhanced capacity to resolve DNA-platinum adducts, consequently bypassing cisplatinum-mediated cytotoxicity and possibly converting to the well-known molecular phenotype of "on-target" platinum resistance *(5)*. Consecutively, these cells may survive multiple cycles of chemotherapy and, in line with the fact that metastasis-initiating cells are present among CTC in the blood *(38)*, *ERCC1*$^+$CTC may be responsible for platinum-resistant recurrence, metastasis, and poor prognosis. Importantly, our data also indicate the presence of a clinically informative subpopulation of CTC at primary diagnosis, possibly providing innate platinum resistance and expressing a certain *ERCC1* level without any previous contact with cisplatinum.

This is the first report proposing a noninvasive biomarker for stratifying response of ovarian cancer patients to platinum-based chemotherapy. This CTC-based biomarker constitutes a promising blood-based alternative to the commonly suggested primary tumor-based ERCC1 detection for guiding therapeutic decisions at primary diagnosis of ovarian cancer. Due to the limited number of patients, the present study should be considered explorative and our results need to be validated in larger patient cohorts.

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