Clinical Cancer Research

Change in Topoisomerase 1–Positive Circulating Tumor Cells Affects Overall Survival in Patients with Advanced Breast Cancer after Treatment with Etirinotecan Pegol

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Abstract

Purpose: Preplanned exploratory analyses were performed to identify biomarkers in circulating tumor cells (CTC) predictive of response to the topoisomerase 1 inhibitor etirinotecan pegol (EP).

Experimental Design: The BEACON trial treated patients with metastatic breast cancer (MBC) with EP or treatment of physician's choice (TPC). Blood from 656 of 852 patients (77%) was processed with ApoStream to enrich for CTCs. A multiplex immunofluorescence assay measured expression of candidate response biomarkers [topoisomerase 1 (Top1), topoisomerase 2 (Top2), Ki67, RAD51, ABCG2, γH2AX, and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL)] in CTCs. Patients were classified as Top1 low (Top1Lo) or Top1 high (Top1Hi) based on median CTC Top1 expression. Correlation of CTC biomarker expression at baseline, cycle 2 day 1 (C2D1), and cycle 4 day 1 with overall survival (OS) was investigated using Cox regression and Kaplan–Meier analyses.

Results: Overall, 98% of samples were successfully processed, of which 97% had detectable CTCs (median, 47–63 CTCs/mL; range, 0–2,020 CTCs/mL). Top1, Top2, and TUNEL expression was detected in 52% to 90% of samples; no significant associations with OS were observed in pretreatment samples for either group. EP-treated patients with low C2D1Top1$^+$ CTCs had improved OS compared with those with higher positivity (14.1 months vs. 11.0 months, respectively; HR, 0.7; $P = 0.02$); this difference was not seen in TPC-treated patients (HR, 1.12; $P = 0.48$). Patients whose CTCs decreased from Top1Hi to Top1Lo at C2D1 had the greatest OS benefit from EP (HR, 0.57; $P = 0.01$).

Conclusions: CTC Top1 expression following EP treatment may identify patients with MBC most likely to have an OS benefit.
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Introduction

Although early diagnosis and treatment advances have markedly reduced the number of breast cancer–related deaths over the past two decades, it is estimated that in 2012, 522,000 people died from this disease worldwide (1). Systemic treatment options include hormone therapy, targeted agents, and single or combination sequential chemotherapy. The choice of chemotherapy is based on many factors. With the exception of CEP17 duplication or Top2A aberration, which may identify patients who benefit from adjuvant anthracycline chemotherapy (2), other predictive markers have yet to be identified that can select specific agents based on tumor biology.

Topoisomerase 1 (Top1) inhibitors, such as irinotecan, have limited clinical activity in patients with metastatic breast cancer (MBC; refs. 3, 4). To improve the efficacy and tolerability of Top1 inhibitors, a pegylated derivative of irinotecan (etirinotecan pegol; EP) was developed to improve pharmacokinetic properties by reducing peak concentrations and maintaining a constant concentration of SN38, the active metabolite (5). Subsequently, the Phase 3 BEACON trial randomized 852 patients with advanced breast cancer previously treated with an anthracycline, taxane, and capecitabine to EP or treatment of physician's choice, and found a nonsignificant 2.1 month difference in overall survival (OS; $P = 0.083$) favoring EP [median, 12.4 months; 95% confidence interval (CI), 11.0–13.6 months vs. 10.3 months; 95% CI, 9.0–11.3 months for the control arm; ref. 6]. One of only two preplanned, formal subgroup analyses suggested that patients with stable, previously treated brain metastases ($n = 67$) may gain particular benefit in OS from EP compared with TPC (10.0 months vs. 4.8 months; HR, 0.511; log-rank $P = 0.0099$).

Circulating tumor cells (CTC) have been detected in the blood of patients with MBC, and the number of CTCs correlates

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Translational Relevance

A validated, biomarker-directed approach for the selection of cytotoxic drugs, including topoisomerase 1 (Top1) inhibitors irinotecan and etirinotecan pegol (EP), would have clinical value in determining outcome and avoiding unnecessary toxicities from ineffective therapies. Repeat tumor biopsy is a challenge in the metastatic breast cancer (MBC) setting; hence, circulating tumor cells (CTC) isolated from blood samples provide an attractive option for biomarker assessment. Results of this preplanned exploratory substudy within a phase III trial of EP versus treatment of physician's choice single-agent chemotherapy care suggest that patients with MBC whose percentage of Top1-expressing CTCs is lower following EP treatment may have the best survival outcomes. Prospective clinical studies are needed to confirm the predictive value of this biomarker.

with progression-free survival (PFS) and OS (7, 8). Although prognostic significance of CTCs in patients with MBC has been confirmed, for patients with a persistent increase in CTCs after first-line chemotherapy, early switching to an alternate cytotoxic therapy was not effective in prolonging OS (9). Although assessment of the number of CTCs has not yet shown clinical utility based on American Society of Clinical Oncology guidelines, it is possible that characterization of biomarker expression in CTCs may improve the selection of patients for targeted therapies.

To identify patients most likely to benefit from EP treatment, a preplanned exploratory analysis of the BEACON trial aimed to evaluate key biomarkers implicated in the mechanism of action and resistance to Top1 inhibitors in CTCs. SN38, the active metabolite of EP, binds Top1 to form a ternary complex with the enzyme and DNA, stabilizing the single-stranded DNA cleavage complex, interfering with ligation, and promoting the generation of DNA double-strand breaks as well as phosphorylation of H2AX causing apoptotic cell death (10, 11). Although not a validated tumor marker, high levels of Top1 enzyme are correlated with increased irinotecan-induced cell death in tumor model systems (12) and are correlated with increased efficacy in retrospective studies in patients with colorectal cancer (13, 14). Therefore, Top1, topoisomerase 2 (Top2; ref. 15), ATP-binding cassette (ABC) family transporter breast cancer resistance protein, and ABC Subfamily G Member 2 (ABCG2) protein expression may have utility as biomarkers for predicting response to EP (16, 17).

As part of this prospective analysis in BEACON, candidate biomarkers were measured in CTCs as an alternative to tumor biopsies. To improve the low numbers of recovered CTCs found with techniques relying on epithelial cell adhesion molecule (EpCAM) enrichment, an antibody-independent methodology was used, ApoStream, by exploiting the morphologic and biophysical differences between malignant and normal blood cells (18–21). Qualified multiplex immunofluorescence assays were performed on enriched CTCs to measure putative pharmacodynamic biomarkers of EP activity in CTCs obtained serially during treatment. We report results of these biomarker analyses and association with the primary trial endpoint, OS.

Materials and Methods

Antibodies

The primary and secondary antibody reagents (and sources) for the multiplex detection of Top1, γH2AX, RAD51, Ki67, Top2, and ABCG2 are listed in Supplementary Table S1. Cytokeratin (CK)-FITC (#130-080-101) and CD45-PE (#130-080-201) conjugated antibodies used for CTC phenotyping, and their corresponding isotype controls IgG1-FITC (#130-092-213) and IgG2a-PE (#130-091-835) were obtained from Miltenyi Biotec (Bergisch Gladbach).

Patients and clinical specimens

Participation in the CTC substudy of BEACON was voluntary. All participating patients provided informed consent and were enrolled under Institutional Review Board–approved protocols. Whole blood samples (8 mL) were collected at baseline or screening, cycle 2 day 1 (C2D1), and cycle 4 day 1 (C4D1) between December 19, 2011, and August 20, 2013, shipped at controlled, ambient temperature to ApoCell, and processed to enrich for CTCs within 96 hours of collection.

CTC enrichment

Peripheral blood mononuclear cells (PBMC) were harvested using the Ficoll–Paque gradient separation method (19). Isolated PBMC were suspended in ApoStream running buffer and processed on the ApoStream device as previously described (19, 20). The cell suspension was injected into the device, an AC voltage was applied to initiate the dielectrophoresis (DEP) field, and CTC-enriched isolates were collected into a microcentrifuge tube, cytospun onto a glass slide in three spots using a Shandon Cytospin 4 (Thermo Electron Corporation) at 1,000x RPM for 10 minutes, and fixed using 2% paraformaldehyde for 20 minutes. After each run, the flow chamber was cleaned with ApoStream cleaning solution.

Assay development and qualification

The conditions for antibody staining and immunofluorescence detection of the biomarkers in this multiplex analysis were optimized using cultured cancer cell lines (Supplementary Table S1 and Supplementary Fig. S1). Tumor cell–PBMC spiking experiments demonstrated that each of the biomarkers could be readily detected in the presence of a high number of PBMCs (Supplementary Fig. S2). Qualification experiments measured the interassay, interday, and interoperator variability using results obtained by three laboratory technicians, who performed the antibody staining in triplicate on each of 3 days. The variability observed for each biomarker was less than the prespecified acceptable assay variance of 25% (Supplementary Table S2), confirming the suitability of the assay for evaluation of clinical samples.

Immunofluorescence staining and analysis

For CTC phenotyping and biomarker staining of ApoStream-enriched CTCs, fixed cells were washed with phosphate buffer saline (PBS), permeabilized (0.2% Triton-X100 for 5 minutes), and blocked [1% Human AB Serum (HABS; VWR, #45001-062)/2% normal donkey serum (NDS; Jackson ImmunoResearch, #017-000-121)] for 10 minutes. Endogenous biotin/avidin binding was blocked using a biotin/avidin blocking kit (Life Technologies; #00-4303). All antibodies were diluted in 1% HABS/2% NDS. After

Table 1. Baseline demographic and clinical characteristics of patients in the CTC substudy

	TPC ($N = 302$)	EP ($N = 309$)
Age, years, median (range)	56 (33–80)	55 (28–84)
Race		
White	224 (74.2%)	236 (76.4%)
Black/African American	25 (8.3%)	28 (9.1%)
Asian	22 (7.3%)	22 (7.1%)
Native Hawaiian/Pacific Islander	0	1 (0.3%)
Other/not reported	31 (10.3%)	22 (7.1%)
Geographic region		
Asia/Latin America/South Africa	12 (4.0%)	16 (5.2%)
Eastern Europe	7 (2.3%)	9 (2.9%)
North America/Western Europe	283 (93.7%)	284 (91.9%)
ECOG PS		
0	94 (31.1%)	121 (39.2%)
1	205 (67.9%)	186 (60.2%)
≥2	3 (1.0%)	2 (0.6%)
Time since initial breast cancer diagnosis (years)	7.1	7.8
Time since diagnosis of locally recurrent or metastatic disease (years)	3.0	3.3
Stage IV disease at initial diagnosis (*n*, %)	55 (18.2%)	44 (14.2%)
Visceral disease at enrollment (*n*, %)	229 (75.8%)	228 (73.8%)
Brain metastasis (history or stable)	20 (6.6%)	23 (7.4%)
Metastatic site at enrollment		
Liver metastasis	160 (51.8%)	162 (53.6%)
Lung metastasis	110 (35.6%)	118 (39.1%)
Bone	179 (59.3%)	172 (55.7%)
Receptor status (*n*, %)		
HR-positive	208 (68.9%)	210 (68.0%)
Triple negative	84 (27.8%)	90 (29.1%)
HER2 positive	20 (6.6%)	22 (7.1%)
Prior regimens for metastatic disease (median, range)	4.0 (1–7)	4.0 (1–7)
Prior chemotherapy exposure (*n*, %)		
Prior anthracycline	286 (94.7%)	297 (96.1%)
Prior taxane	302 (100.0%)	309 (100.0%)
Prior capecitabine	302 (100.0%)	309 (100.0%)
Prior eribulin	54 (17.9%)	58 (18.8%)

another PBS wash, primary antibodies were added to each spot (i.e., Top1 and γH2AX on spot 1, Ki67 and RAD51 on spot 2, and Top2 and ABCG2 on spot 3) at dilutions indicated in Supplementary Table S1 and incubated at 4°C overnight. Cells were then washed twice with 1X PBS. Spot two was stained with the biotin-linked anti-rabbit secondary antibody (1 hour); spots 1 and 3 were incubated in 1% HABS/2% NDS for the same period of time. After two additional washes of 1X PBS, cytokeratin-FITC (1:10), CD45-PE (1:10), and Brilliant Violet 570-streptavidin (1:25) were added to all spots, Alexa Fluor 647 anti-rabbit to spots 1 and 3, Alexa Fluor 647–conjugated Ki67 antibody to spot 2, and the slides were incubated for 2 hours. Positive controls were SN38-treated HCT116 for detection of Top1/γH2AX/RAD51 and A549 cells for Top2/ABCG2. PBMC from each patient served as negative controls on separate slides. Negative controls were IgG-FITC (1:20), IgG-PE (1:10), and mouse IgG1-AF647 (Cell Signaling Technology; #4843, 1:25). After washing, nuclear staining was visualized using 4', 6-diamidino-2-phenylindole (DAPI) and slides cover-slipped using 50% glycerol. The apoptosis assay was performed according to the manufacturer's instructions using the Promega Terminal Deoxynucleotidyl Transferase, Recombinant Enzyme (Promega, M1875) with Cy5-dUTP (GE-PA55022; refs. 22, 23). The positive control cell line was preincubated with DNAase I for 10 minutes.

Laser scanning cytometry image analysis

An iCys laser scanning cytometer (CompuCyte) equipped with 405 (blue/orange emission filters), 488 (green/orange), and 633 (red) lasers and iCys 3.4.12 software were used to enumerate $CK^+CD45^-DAPI^+$ cells using an automated analysis process as previously described (21). Gates were established using control slides of tumor cell lines (CK^+) and patient PBMCs ($CD45^+$) to define cutoffs for CK and CD45 positivity. Gating for background immunofluorescence was established for each patient using their PBMC sample and signals obtained using only secondary antibodies in the protocol. After these gates had been established, the enriched CTC samples from patients were processed, contouring on DAPI to define nucleated cells, and gated using the CK and CD45 gates. Individual CTCs were subsequently confirmed by visual confirmation of each immunofluorescent antibody. The settings used for these scans and gates were similar over the period of this study. Calibration controls were run daily to ensure consistency in the scanning and gating analysis. Biomarker (633-red and 405-orange filters) expression and percent positive cells were reported in confirmed $CK^+CD45^-DAPI^+$ cells. Biomarker expression in CTCs from patients treated with EP or TPC was compared in CK^+CD45^- CTCs.

Statistical analyses

SAS software version 9.4 was used for all statistical analysis. Summary statistics were used to determine the percentage of biomarker expression. OS was calculated as the time from the date of randomization to death from any cause or last contact date. Patients who were lost to follow-up or were not known to have died at the time of data cutoff for analysis were censored at last date shown to be alive. The impact of biomarker expression on CTCs on OS was investigated with Cox regression analyses,

using percent positive biomarker expression on CTCs as a continuous variable and graphically using Kaplan–Meier curves. HRs were reported with their *P* value and 95% CIs. Results were considered statistically significant if the *P* value was ≤ 0.05. Analysis and reporting of biomarkers adhered to REMARK guidelines (24).

The study was conducted according to the provisions of the Declaration of Helsinki and in accordance with International Conference on Harmonisation Good Clinical Practice standards, FDA regulations, as well as any and all applicable federal, state, and/or local laws and regulations. All patients provided written informed consent, and study approval was obtained by the relevant Institutional Review Board or independent ethics committee at each site.

Results

Biomarker expression in CTCs isolated from MBC patients in the BEACON study

Baseline demographic and disease characteristics for the patients with evaluable blood samples for this analysis ($n = 611$) were comparable with those reported for the intention-to-treat (ITT) population in the BEACON study ($n = 852$; ref. 6) and were relatively well balanced between treatment groups (Table 1). Most patients in the CTC substudy were treated in North America or Europe (95%). In all, 68% had hormone receptor–positive [HR$^+$; including either estrogen receptor (ER)– or progesterone receptor (PR)–positive BC) disease, whereas 28% had triple-negative (TN; negative for ER, PR, and HER2) and 7% were HER2-positive. The most common metastatic sites were bone (57%), followed by liver (53%), and then lung (37%). The median number of prior therapies for metastatic disease in both groups was 4 (range, 1–7), and almost all patients had received prior anthracyclines (95%), taxanes (100%), and capecitabine (100%). In addition, median OS (EP, 12.7 mo; TPC, 10.2 mo) and PFS (EP, 2.3 mo; TPC, 2.9 mo) in the CTC subgroup were similar to OS (EP, 12.4 mo; TPC, 10.3 mo) and PFS (EP, 2.4 mo; TPC, 2.8 mo) reported in BEACON (6).

Blood samples for CTC analysis were collected at baseline, cycle 2 day 1 (C2D1), and cycle 4 day 1 (C4D1). Evaluable blood samples represented 72% (611/852), 69% (519/755), and 72% (268/373) of patients on treatment in the BEACON study at baseline, C2D1, and C4D1, respectively. Most blood samples (1,398/1,431 samples; 98%) were successfully processed and enriched for CTCs (Table 2); 2% of samples could not be processed due to insufficient volume, lack of separation at Ficoll, and processing error. CK$^+$CD45$^-$ CTCs were detected in >97% of the samples. The median number of CTCs/mL for all 3 collection times was high (median, 47, 51, and 63 CTCs/mL; range, 0–2,020 CTCs/mL) and comparable across collection times (Table 2).

Representative images for CTCs isolated at baseline and positively stained for the mechanistic biomarkers are shown in Fig. 1. Enumeration of biomarker-positive CTCs is shown in Fig. 3. Top1 (67%–79%), Top2 (52%–74%), and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL; 81%–90%) were detected in the majority of CTC samples (Fig. 2A). There were no statistically significant differences in the mean or median Top1, Top2, or TUNEL expression between the EP- and TPC-treated groups at any sampling time. The percentage of TUNEL-positive cells detected in each patient sample increased significantly from C2D1 to C4D1 in the EP-treated group (median, 26%→48%; $P < 0.01$), whereas there was no significant change for the patients in the TPC arm (median, 21%→28%; $P = 0.26$). In contrast to Top1, Top2, and TUNEL, less than half of the patient samples contained CTCs that stained positive for Ki67 (142/294 samples), and even fewer CTCs (<30%) had detectable expression of RAD51, γH2AX, and ABCG2 (80/294, 34/291, and 50/295, respectively; Fig. 2B). Further analysis of these candidate biomarkers was, therefore, not pursued.

Correlation of Top1 expression with OS for patients treated with EP

Cox regression analyses were performed to investigate potential correlations between candidate biomarkers and OS, the primary endpoint of the BEACON study. There was no significant association with OS for expression of Top1, Top2, or TUNEL in CTCs from baseline blood samples ($P > 0.05$). However, the expression of Top1 in CTCs isolated at C2D1 and C4D1 following treatment with EP, but not TPC, was significantly correlated with OS (i.e., C2D1 EP: $n = 257$, $P = 0.015$ vs. TPC: $n = 229$, $P = 0.802$; C4D1 EP: $n = 128$, $P = 0.001$ vs. TPC: $n = 128$, $P = 0.803$). Because each of the breast cancer subtypes has known biological and molecular characteristics that might influence responsiveness to cytotoxic chemotherapy, this analysis was also performed separately for HR$^+$ and TN breast cancer patients. The number of HER2$^+$ patients enrolled in BEACON who participated in the CTC substudy was insufficient to perform a meaningful subgroup analysis (for EP, $n = 22/30$, and TPC, $n = 20/32$). An association of Top1 expression at C2D1 and C4D1 with OS was observed in EP-treated patients with HR$^+$ breast cancer (C2D1: $n = 174$, $P = 0.038$; C4D1: $n = 91$, $P = 0.002$) but not in those with TNBC (C2D1: $n = 75$, $P = 0.136$; C4D1: $n = 35$, $P = 0.366$). Unlike Top1, expression of Top2 or TUNEL was not significantly associated with OS with either treatment.

To further explore the impact of Top1 expression on OS, patients were classified based on their percentage of Top1-positive

Table 2. Number of blood samples collected, CTC enumeration, and number of samples analyzed for biomarker expression

	Baseline	Cycle 2 day 1	Cycle 4 day 1
Number of subjects	625	534	272
Number of subjects with blood samples processed by ApoStream[a]	611 (97.8%)	519 (97.2%)	268 (98.5%)
CTC harvest (CTCs/mL)			
Mean	153	140	160
Median	63	47	51
Range	0–1,852	0–1,895	0–2,020
25th Quartile	15	13	16
75th Quartile	190	163	192
Number of samples without CTCs (%)	17 (2.8%)	11 (2.1%)	2 (0.7%)

[a]After exclusion of duplicate blood samples ($n = 2$) and samples that could not be processed ($n = 37$, 2.6%) due to insufficient blood volume, lack of separation on Ficoll, or mislabeled slides.

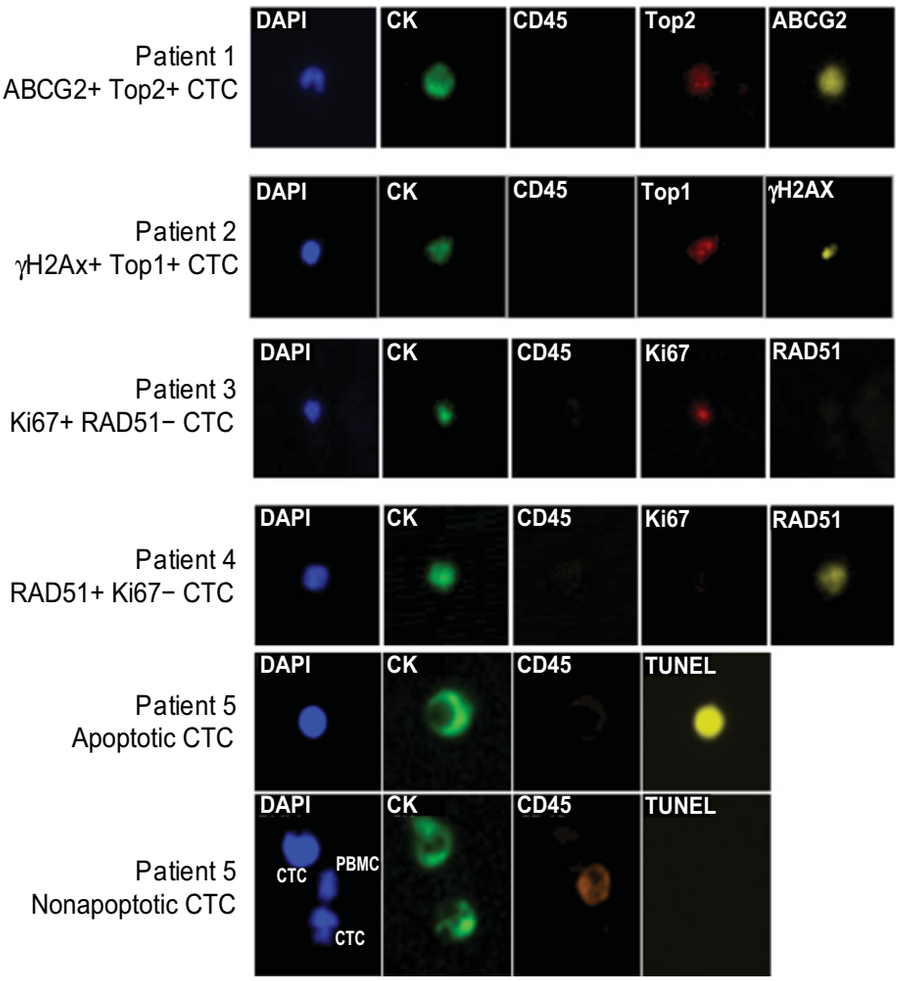

Figure 1.
Representative images of biomarker on predose CTCs isolated from BEACON patients.

CTCs at baseline. To establish a cutoff for Top1 expression on CTCs, we used the baseline samples independent of assignment to treatment arm. These samples provided the best representation of Top1 expression on CTCs in the BEACON patient population. Patients were classified based on the median number of CTCs that stained positive for Top1 (8%), such that half the patients at baseline were categorized as Top1 low (Top1Lo: < 8% of CTCs stained positively for Top1) and the other was categorized as Top1 high (Top1Hi: ≥ 8% of CTCs stained positively for Top1). Patients with Top1Lo CTCs at C2D1 for EP-treated patients ($n = 128/257$) had significantly longer OS than those with Top1Hi CTCs ($n = 129/257$; 14.1 vs. 11.0 months; HR = 0.7, $P = 0.02$; Fig. 3). This association was not observed in the TPC arm (11 months for Top1Lo vs. 12.5 months for Top1Hi; HR = 1.12, $P = 0.48$; Fig. 3). There was also a trend for improved OS in EP-treated patients with Top1Lo CTCs (63/128 patients) compared with those with Top1Hi CTCs (65/128 patients) at the EP C4D1 (20.6 vs. 14.7 months; HR = 0.68, $P = 0.09$).

The association between OS and Top1Lo CTCs at C2D1 for EP-treated patients was observed in patients with HR^+ disease (Fig. 3; 16.0 months for Top1Lo vs. 12.1 months for Top1Hi; HR, 0.7; $P = 0.05$). No significant associations were found for Top1 expression and OS for patients in the TPC arm overall or in patients with either HR^+ or TN disease.

Association of serial Top1 CTC expression and OS

To explore the potential relationship between change in CTC Top1 expression and OS over the course of treatment, patients were classified based on their CTC Top1 status (high or low) at baseline and at C2D1 (Fig. 4). Patients with matched baseline and C2D1 CTC samples represented 58% (436/755) of the patients on treatment in BEACON at C2D1. Patients in this subgroup were generally well balanced for baseline characteristics, except for the Top1Hi→Lo group, which had a higher number of patients with a history of brain metastases ($n = 7$; 14% vs. 2%–6%; Supplementary Table S3) and a smaller number of Black/African Americans ($n = 2$; 4% vs. 7.6%–12.3%). Among the patients with Top1Hi at baseline treated with EP, significantly improved OS was observed for those who converted to Top1Lo CTCs at C2D1 (Top1Hi→Lo) compared with those whose CTCs remained Top1Hi (Top1Hi→Hi; 14.7 months vs. 10.5 months; HR, 0.57; $P = 0.01$). OS in those patients treated with EP whose CTCs remained Top1Hi (i.e., Top1Hi→Hi) was similar to that observed in the ITT population treated with TPC (10.5 months vs. 10.2 months; ref. 6). There was no significant separation of OS in EP-treated patients with Top1Lo CTCs at baseline, and either remaining Top1Lo or converting to Top1Hi by C2D1, with median OS ranging between 12.6 and 14.0 months. No correlation with OS was

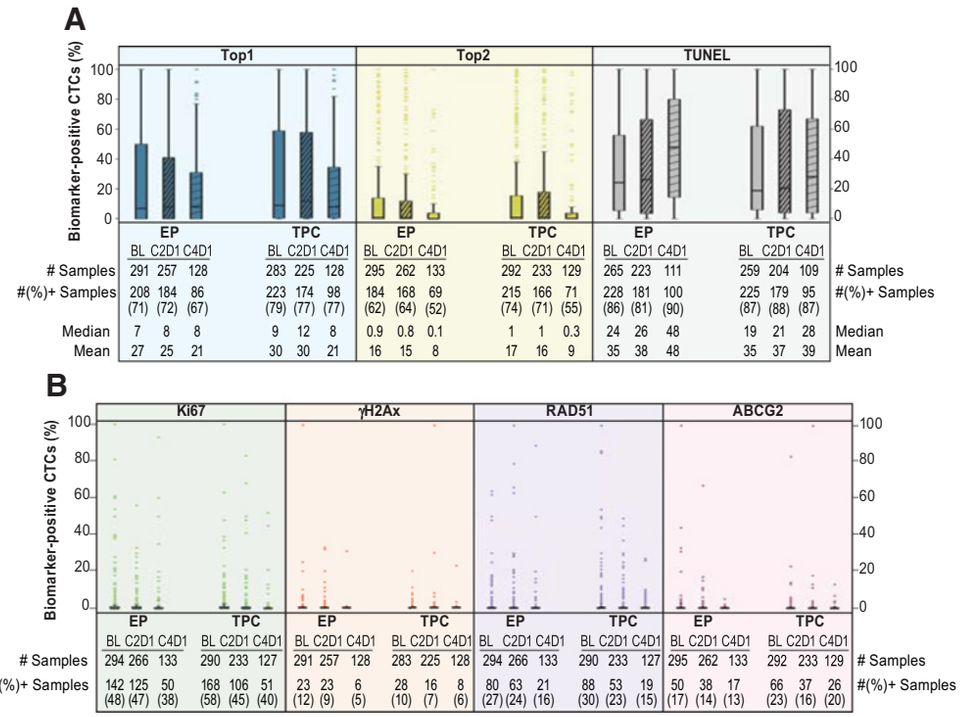

Figure 2.

Putative candidate biomarker expression in CTCs by treatment arm and collection time. Samples were analyzed for expression of (**A**) Top1, Top2, and TUNEL and (**B**) Ki67, γH2AX, RAD51, and ABCG2 using the multiplex immunofluorescence detection assay. The percentage of biomarker-positive cells detected in each patient sample is graphically represented for the EP- and TPC-treated patient groups. Boxplots: median (line), 75th to 25th percentiles (box), 3xSD (whiskers), and outliers (dots); # samples, number of samples analyzed; # (%)+ samples, number and percentage of samples with biomarker-positive cells; BL, baseline.

observed for the patients in the TPC arm. Patients treated with EP with HR^+ disease and Top1Hi CTCs at baseline going to Top1Lo CTCs at C2D1 had a median OS of 21.9 months, whereas patients with continuous Top1Hi CTCs upon treatment had a median OS of 10.8 months.

Discussion

In this preplanned but exploratory analysis of the Phase 3 BEACON clinical trial, CTCs were characterized to evaluate candidate predictive biomarkers for the Top1 inhibitor, EP. The results suggest patients with Top1Hi at baseline to Top1Lo CTC status at C2D1 obtain the most OS benefit from EP. The assay used in this study combined a high yield, antibody-independent DEP-based CTC-enrichment procedure with a multiplex immunofluorescence detection platform that identifies CTCs (i.e., CK^+CD45^-) and measures two additional biomarkers (Top1 and γH2Ax, Ki67 and Rad51, and Top2 and ABCG2) in each of three parallel analyses (19). This assay was used for the characterization of CTCs isolated from pretreatment blood samples in 72% of 852 enrolled in the BEACON trial, and in 69% and 72% of patients treated at C2D1 and C4D1, respectively.

In all, 98% of the patient samples collected were successfully processed, of which 97% had detectable CTCs, with a median of 47 to 63 CTCs/mL at the different collection times. This number of CTCs (376–504 CTCs/8 mL blood draw) is approximately 10-fold greater than typically isolated by the CellSearch procedure in patients with MBC (25). This increase in CTCs may be attributed to the enrichment of a more phenotypically diverse population of tumor cells by the ApoStream procedure (20, 26), which has no requirement for EpCAM expression that is needed for the CellSearch method. The high number and percentage of CTC-containing samples enabled correlative studies for each biomarker using pretreatment as well as posttreatment samples. The assay was developed to measure all 7 biomarkers, but only Top1, Top2, and TUNEL were detected in the majority of the samples.

Although Top1 and Top2 expressions have not been previously reported in CTCs, our results are consistent with the expression of Top1 detected by immunohistochemistry in 41% to 70% (26–28) and for Top2 in 40% to 65% of breast tumor samples (28, 29). The high percentage of samples positive for the apoptosis marker, TUNEL (23, 30–32), is consistent with the reported 1- to 2-hour half-life of CTCs (33) and with another study in MBC CTCs where 80% of the samples were positive for the M30 (from cleaved Cytokeratin 18) marker of apoptosis (34). That same study also reported Ki67 detection in CTC isolated from 44% of patients with MBC (34). This is similar to the 47% we observed, but differs from the 0% CTCs reported by Muller and colleagues (35), and the nearly universal detection (38/40 samples) in CTC isolated from patients with HR^+ MBC using the CellSearch platform (36). It is likely that detection methods, as well as CTC-isolation procedures and clinical factors, underlie this marked variation. γH2AX, ABCG2, and RAD51 were detected in only 5% to 30% of the samples, obviating meaningful correlative analyses with these markers. Of these, γH2AX has been evaluated as a potential pharmacodynamic marker for assessing drug-induced DNA

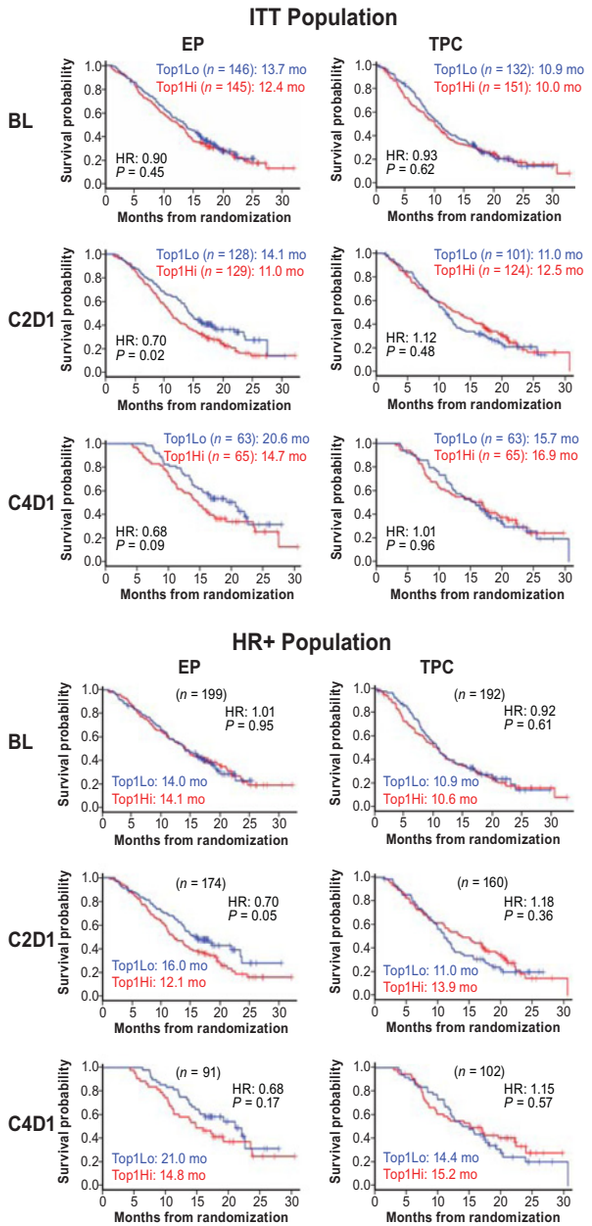

Figure 3.

Impact of Top1 expression at baseline, C2D1, and C4D1 on OS in ITT or HR$^+$ patients after treatment with EP or TPC. Kaplan–Meier OS analyses were performed for patients classified based on the median % of Top1-positive CTCs in baseline samples. Top1 low (Top1Lo): <8% of CTCs stained positively for Top1; Top1 high (Top1Hi): ≥8% of their CTCs stained positively for Top1. *n* = number of patients who had biomarker data and OS at the indicated sampling time.

damage response and as a surrogate for the efficacy of drugs, such as Top 1 inhibitors (37, 38). Because the induction of γH2AX following treatment with Top1 inhibitors can be dependent on treatment time (37, 38), it is possible that the timing of blood sample collection at the beginning of the second and fourth treatment cycles (21 days after the previous dose) may have been suboptimal for detection of this biomarker despite the long half-life of EP (4). This is the first study to detect Top1, Top2, or TUNEL in CTCs. The low expression levels of other markers may allude to the biology and Top1 association with apoptotic pathways in specific subtypes, i.e., cytokeratin-positive, of CTCs.

The percentage of Top1, Top2, or TUNEL-positive CTCs detected in the pretreatment blood samples revealed no significant associations with survival outcome for either EP- or TPC-treated patients. For the EP treatment arm, approximately 30% of baseline samples had no Top1 detected on their CTCs. Because Top1 expression on CTCs at baseline was not associated with OS based on Cox regression analysis, patients negative for Top1 at baseline are neither completely resistant nor particularly sensitive to EP treatment. Several nonclinical studies have demonstrated a positive association between the levels of Top1 enzyme and cytotoxic effects of Top1 inhibitors on tumor cells *in vitro* and in blocking tumor formation in animal models (12). Retrospective analyses from clinical trials in colorectal cancer patients receiving irinotecan-containing regimens have also suggested a correlation between efficacy and high levels of Top1 protein expression (13, 14). Two studies explored the relationship between Top1 levels and irinotecan response in a small number of MBC patients, and the results showed no statistically significant association with Top1 localization or expression (39, 40).

This preplanned exploratory analysis suggests that low Top1$^+$ expression in CTCs following start of therapy may predict benefit from EP compared with TPC as measured by OS and may be prognostic within the EP-treated cohort compared with those with high expression of Top1$^+$ CTCs. To better quantify the impact of Top1$^+$ expression on CTCs on OS, patients were categorized as Top1Hi and Top1Lo based on the median Top1 CTC expression. The BEACON study did not collect tumor tissue; hence, correlation between expressions of Top1 on CTCs compared with tumor tissue cannot be established. Expression of Top1 in breast cancer is described in several publications. In a cohort of 3,119 invasive breast cancer samples, Top1 was overexpressed (2+ by IHC) in 63.4% of specimens, close to the 50% of patients categorized as Top1Hi in our study based on Top1 expression on CTCs (41). In a cohort of 6,341 breast cancer samples, approximately 70% of specimens stained positive for Top1 (42). In our study, 70% to 80% of patients expressed Top1 on CTCs. Although these cross-study comparisons between expression of Top1 on CTCs and tumor tissue must be viewed with caution, these data suggest that Top1 expression on CTCs could correlate with Top1 expression in tumor, and that our categorization based on median expression of Top1 on CTCs might correlate with overexpression of Top1 by IHC.

When patients were split into two groups using the median Top1 CTC expression, a greater OS benefit from EP treatment at C2D1 was observed for patients classified as Top1Lo compared with Top1Hi (14.1 months vs. 11.0 months, $P = 0.02$). This observation is consistent with the decrease in Top1 levels that occurred after topotecan treatment in drug-responsive tumor xenograft models (38) but appears incongruent with conclusions from other studies, suggesting that Top1 inhibitor (i.e., camptothecin) effectiveness is correlated with the stability of the inhibitor–Top1–DNA cleavage complex (43). Timely proteasomal degradation of Top1 and subsequent removal of the remaining DNA-linked Top1 peptide by tyrosyl-DNA phosphodiesterase 1 are thought to enable DNA repair (11) and promote Top1 inhibitor resistance (44, 45). Multiple factors, including p53 status, antiapoptotic factors like bcl-2, cell-cycle checkpoint controls, and DNA damage repair functionality, are also known to

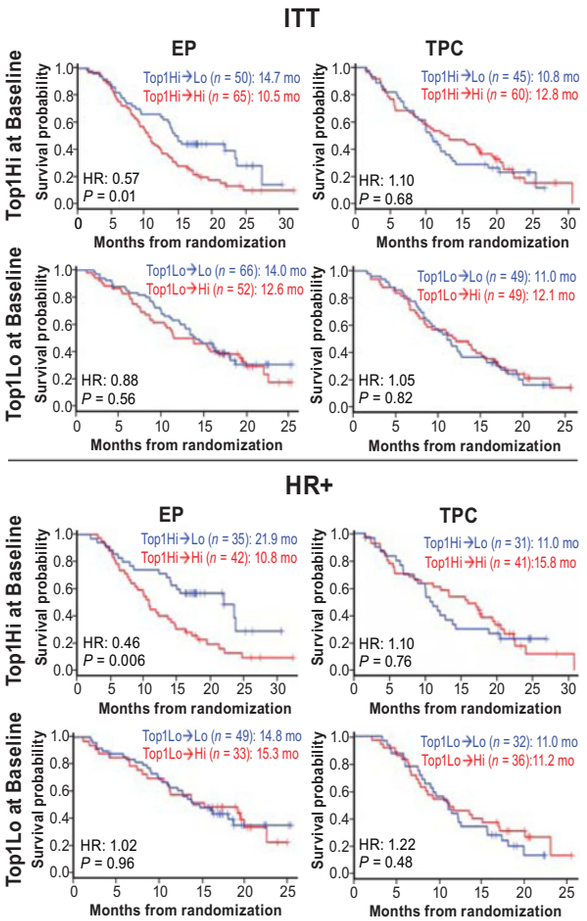

Figure 4.

Impact of Top1 expression at baseline compared with C2D1 on survival after treatment with EP and TPC in ITT and HR$^+$ patients. Kaplan–Meier OS analyses were performed for patients classified based on the median % of Top1-positive CTCs in baseline samples. Top1 low (Top1Lo): <8% of CTCs stained positively for Top1; Top1 high (Top1Hi): ≥8% of their CTCs stained positively for Top1.

influence the final cellular response to drug treatment (44, 46). Our data indicate that the efficacy of EP is correlated with a decreased proportion of Top1$^+$ CTCs in the blood. Those CTCs may have undetectable Top1 levels as a result of increased proteolytic degradation of total cellular Top1 as part of the response to induction of apoptosis. Therefore, it is intriguing that we observed a higher percentage of apoptotic CTCs, as measured by TUNEL, in the EP-treated patients than in those treated with TPC at C4D1, although there was no apparent correlation between increased apoptosis and EP response. Although this study assessed cytokeratin-positive, epithelial subtypes of CTCs, recent studies have characterized the more resistant mesenchymal-like CTC phenotypes (20). Top1-positive mesenchymal CTCs were not investigated in this analysis. Interestingly, a trend for prolonged OS in EP-treated patients based on Top1$^+$ CTCs at C4D1 was not observed until after about 4 months of treatment, which may suggest efficacy of treatment possibly by a mechanism targeting Top1 in mesenchymal CTCs (20). However, it is also possible that the Top1 expression dynamics in blood CTCs may not mirror what is observed in the solid tumor mass or in cell culture models.

Increased OS with EP treatment was significantly correlated with reduced Top1$^+$ CTCs at C2D1 in patients with HR$^+$ but not those with TNBC. In the TPC arm, there was no association between Top1 expression and OS for the overall population or in patients with either HR$^+$ or triple-negative disease, suggesting reduced Top1-positive CTCs as a biomarker of response for HR$^+$ disease may be specifically associated with EP treatment. Survival benefit for EP was greatest (22 months) for HR$^+$ patients who were classified as Top1Hi and converted to Top1Lo upon completion of the first-treatment cycle. The OS difference in these patients was twice as long as attained for the patients who were Top1Hi after treatment (11 months). Therefore, it is interesting that the HR$^+$ subgroup in the ITT analysis of the BEACON trial had an OS of 13.6 months with EP treatment compared with 11 months with TPC; however, HR$^+$ status was not significantly associated with EP response (6). Our results suggest that the decreased Top1$^+$ CTC phenotype is a marker for EP activity that is specific for some HR$^+$ patients. Given the similar expression of Top1 in TNBC and HR$^+$ breast cancer, factors other than Top1 expression may be more important for EP responses in patients with TNBC (42).

A previous study of CTCs in patients with breast cancer demonstrated that although CTCs from HR$^+$ population were predominantly epithelial, those from triple-negative were predominantly mesenchymal-like. Further investigation of Top1$^+$ mesenchymal CTCs may provide more insight into the effect of EP in this population (47). HR+ tumors and TNBC have different molecular characteristics that may affect the cellular response to Top1 inhibitors. Assuming concordance between the relevant genomic aberrations in recurrent tumors and the primary specimens used in most of the research studies that have characterized the breast cancer subtypes (48), the endocrine-resistant HR$^+$ breast cancers in this study are likely to be related to the luminal B type (49). Frequent amplification of cyclin D1 and CDK4 genes (49) in luminal B–type drives growth factor and estrogen-independent cell-cycle progression from G_1 to S. In contrast, most TNBCs have mutations in the cell-cycle checkpoint and apoptosis regulator TP53. Some TNBCs also have defects in the BRCA1/2 DNA repair pathway components, resulting in more genomically unstable tumors than those in HR$^+$ breast cancer. EP treatment in the context of functional p53 and high S-phase activity may cause a synchronized proteolytic and apoptotic response in HR$^+$ breast cancer cells that results in the reduced Top1$^+$ CTC fractions seen in responsive patients.

Additional studies are required to determine the molecular characteristics of the tumors from those patients who received the most OS benefit from EP. In addition to the measurement of other biomarkers, such studies could include examining CTCs that are cytokeratin-negative or mesenchymal-like phenotypes, since the current study was limited to the CK$^+$ population. Other study limitations included not obtaining blood samples for all patients in the BEACON trial, lack of collection of tumors to establish a correlation between Top1 expression on CTC and in tumors, and patients discontinuing study treatment for a variety of reasons, which could amplify the population with better prognosis and response at subsequent analyses. In addition, laboratory correlates using breast cancer models with high and low expression of Top1, and characterization of growth kinetics and metastatic

potential due to Top1 expression levels could further help to validate Top1 as biomarker of response to EP treatment.

The selection of targeted cancer therapy is increasingly informed by tumor molecular profiles. The clinical value of such a biomarker-directed approach for the selection of cytotoxic drugs, such as irinotecan or EP, has not been shown in prospective clinical trials, but, if validated, could improve therapeutic outcomes and reduce the toxicity associated with ineffective treatments. The potential to use CTCs isolated from blood samples provides an added benefit, as the collection of tumor biopsies remains challenging in the metastatic setting. This study is hypothesis-generating and suggests that the detection of lower percentage of $Top1^{+}$ CTCs following treatment with EP may identify patients with MBC who benefit most. Confirmation of the predictive value of this biomarker will require prospective clinical studies that are under consideration.

Disclosure of Potential Conflicts of Interest

J. Cortes is a consultant/advisory board member for AstraZeneca, Biothera, Celgene, Cellestia Biotech, Eisai, Merus, Novartis, Pfizer, and Roche. J. O'Shaughnessy reports receiving speakers bureau honoraria from AstraZeneca, and is a consultant/advisory board member for AstraZeneca, Novartis, Lilly, and Pfizer. C. Twelves reports receiving speakers bureau honoraria from Nektar, is a consultant/advisory board member for Nektar and Daiichi Sankyo, and reports receiving commercial research support from Nektar. A. Hannah and D.A. Zajchowski are consultant/advisory board members for Nektar. D.W. Davis has ownership interests (including patents) at ApoCell and reports receiving commercial research support from Nektar. E.A. Perez is an employee of and has ownership interests (including patents) at Genentech and Roche. No potential conflicts of interest were disclosed by the other authors.

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