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Circulating tumor cell telomerase activity as a prognostic marker for overall survival in SWOG 0421: A phase III metastatic castration resistant prostate cancer trial

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Early Detection and Diagnosis

Circulating tumor cells (CTC) are promising biomarkers in metastatic castration resistant prostate cancer (mCRPC), and telomerase activity (TA) is a recognized cancer marker. Therefore, we hypothesized that CTC TA may be prognostic of overall survival (OS) in mCRPC. To test this, we used a novel Parylene-C slot microfilter to measure live CTC TA in S0421, a phase III SWOG-led therapeutic trial. Blood samples underwent CTC capture and TA measurement by microfilter, as well as parallel enumeration by CellSearch (Janssen/J&J). Cox regression was used to assess baseline (pre-treatment) TA *versus* OS, and recursive

Key words: circulating tumor cells, telomerase activity, prostate cancer, prognosis, biomarker

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partitioning was used to explore potential prognostic subgroups and to generate Kaplan-Meier (KM) OS curves. Samples were obtained from 263 patients and generated 215 TA measures. In patients with baseline CTC count ≥5 (47% of patients), higher CTC TA was associated with hazard ratio 1.14 ($p = 0.001$) for OS after adjusting for other clinical covariates including CTC counts and serum PSA at study entry. Recursive partitioning identified new candidate risk groups with KM OS curve separation based on CTC counts and TA. Notably, in men with an intermediate range baseline CTC count (6–54 CTCs/7.5 ml), low *versus* high CTC TA was associated with median survival of 19 *versus* 12 months, respectively ($p = 0.009$). Baseline telomerase activity from CTCs live-captured on a new slot microfilter is the first CTC-derived candidate biomarker prognostic of OS in a large patient subgroup in a prospective clinical trial. CTC telomerase activity thus merits further study and validation as a step

What's new?

Circulating tumor cells (CTC) are emerging as prognostic markers in castration-resistant prostate cancer. Here the authors measured telomerase activity of live-captured CTCs in a Phase III prostate cancer trial. They found that telomerase activity independently predicted overall survival in men with CTC counts ≥5 (hazard ratio 1.14; p=0.001). Although these results require confirmation, they underscore the clinical value of the molecular characterization of CTCs in the quest for personalized cancer care.

Metastatic prostate cancer (PC) is the second most common cause of cancer death in U.S. men.[1] Although androgen deprivation therapy is initially effective in this disease state, response duration is highly variable. Eventually, most men progress to metastatic castration resistant disease (mCRPC), which is associated with overall survival of approximately 16 to 25 months.[2,3] Individualized therapy of mCRPC has been slow to develop due to a paucity of molecular biomarkers associated with distinct prognostic subgroups as well as scant available tumor tissue in advanced disease. Recently, these unmet prognostic and predictive needs have been thrust into high relief by a succession of new drugs that have emerged for mCRPC, further emphasizing the need for improved patient selection. As a result, novel PC biomarkers have been urgently sought for disease detection, prognostication, prediction of response to therapy, and for patient monitoring while on therapy (reviewed in Ref. 4).

In recent years, analysis of circulating tumor cells (CTCs) has garnered increasing attention as a potentially ready source of cancer tissue with prognostic and predictive value in metastatic prostate cancer.[5–7] CTCs are cancer cells shed by solid tumors into the peripheral blood, and CTC capture and analysis allows repeated, minimally-invasive disease sampling, thus offering the possibility of improved patient selection and real-time assessment of response to therapy. Enumeration of CTCs has been prognostic in several large prostate cancer trials,[8–10] most recently in a phase III study that we conducted in men with mCRPC treated with first-line chemotherapy.[11] Additional efforts have been directed at molecular characterization of CTCs for cancer-specific phenotypes such as the TMPRSS2-ERG fusion product or androgen receptor mutations in mPC.[6,12,13] Our own group also has explored this approach, specifically focusing on CTC telomerase activity. Telomerase is an enzyme which lengthens and protects telomeres, the tandem repetitive DNA sequences that cap the ends of human chromosomes.[14] Whereas benign, terminally differentiated tissues have extremely low telomerase levels, malignant cells from a variety of cancers have significantly elevated telomerase expression and telomerase activity.[15] The robust presence of telomerase in cancer cells and its relative absence from benign tissues has led to a profusion of studies to assess its value as a biomarker, and telomerase activity has been shown to yield significant diagnostic and prognostic utility in prostate cancer[16] and in a broad spectrum of other malignancies.[17–19]

Reasoning that a telomerase-based biomarker strategy would be applicable to nearly all solid malignancies, we set out to analyze telomerase activity from live CTCs. This could not be accomplished with the commercially available CellSearch CTC enrichment platform (J&J), nor with our own prior microfiltration capture strategies,[20,21] because all of these required sample fixation and did not yield live cells. Therefore, we developed a new Parylene-C slot microfilter coupled to a low constant pressure delivery apparatus designed to capture live CTCs for telomerase activity measurement.[22] To evaluate the prognostic utility of CTC telomerase analysis in a prospective clinical cohort, we used the new slot microfilter platform to capture live CTCs for telomerase activity measurement in S0421, a SWOG Phase III Clinical Trial in mCRPC.[11,23,24]

Material and Methods

Study population

The "parent" trial for this CTC correlatives study was SWOG Trial S0421 (Participants: SWOG, ECOG, CALGB), a North American Intergroup Phase III trial for patients with mCRPC involving bone who were randomized in a "double blind" manner to docetaxel given every three weeks at a dose of 75 mg/m^2 intravenously with oral daily prednisone in combination with placebo or atrasentan, a novel endothelin 1 receptor antagonist that inhibits osteoblast activity.[23] S0421 had dual primary endpoints of overall survival (OS) and progression-free survival (PFS), where progression was defined as the confirmed development of new bone lesions, soft tissue or visceral progression by RECIST criteria or symptomatic pain progression. Overall

and progression-free survival were not significantly different between the placebo and atrasentan arms of SWOG S0421.[23] Secondary endpoints related to evaluation of pain, patient reported outcomes (quality of life), serum PSA kinetics (including PSA response and progression as predictive and prognostic factors), serum bone markers, and other translational endpoints.

CTC analysis in S0421 was proposed and funded after the parent trial had been initiated. Specifically, CTC collection was initiated in 7/2009, at which point approximately 630 of planned 930 patients had already been accrued to S0421. CTC collection ended with the closing of the parent trial in 7/2010, at which point 1,038 total patients had been registered to S0421. Therefore, there were approximately 400 eligible patients registered to S0421 during the period of active CTC collection (7/2009–7/2010). Of these 400 eligible patients, 263 (66%) submitted specimens for CTC telomerase activity measurement. Of these 263 patients 215 (82%) had telomerase activity (TA) assay results included in the analysis.

Specimen collection

Blood samples for CTC analysis were collected in accordance with a protocol amendment to S0421 that was reviewed and approved by CTEP Central IRB as well as by each treating institution's IRB, and each patient gave informed consent. Institutions registered to participate in S0421 were sent specimen collection kits containing blood collection tubes as well as packing materials and return mailing labels. For each patient, 7.5 ml of blood was drawn into an EDTA tube prior to cycle 1 and 2 of chemotherapy (days 0 and 21) and shipped overnight at room temperature to the University of Southern California (USC) for CTC capture and telomerase activity measurement. Each patient had an additional 7.5 ml of blood drawn in parallel into a CellSave tube (Immunicon) and shipped overnight at room temperature for CellSearch CTC enumeration.

Sample processing

Upon arrival at USC, the peripheral blood sample was withdrawn from the EDTA tube and subjected to Ficol-Paque centrifugation to isolate the "buffy coat" containing peripheral blood mononuclear cells (PBMCs); these were collected and the rest of the sample discarded. The PBMCs were passed through two microfilter devices in series (Fig. 1) using a low pressure delivery system as described previously (filter 1 captures CTC + background white blood cells; filter 2 captures only background white blood cells).[22] After filtration, the microfilters containing the trapped live cells were washed 1× with PBS. To lyse the cells, 50 µl of TRAPeze® 1X CHAPS Lysis Buffer (Millipore) was added directly to the filters for 30 min on ice. The resulting cell lysates were collected into a 1.7 ml eppendorf tube and centrifuged at 4°C for 20 min to remove cell debris. Telomerase activity was measured using standard Telomeric Repeat Amplification Protocol (TRAP).[25,26] Standard control samples were generated from serially-diluted lysates of DU145 prostate cancer cell line and were run with every qPCR-TRAP reaction to generate a standard curve which served both as a control for the efficiency of the real-time PCR reaction and for normalization and comparison of the unknown samples.

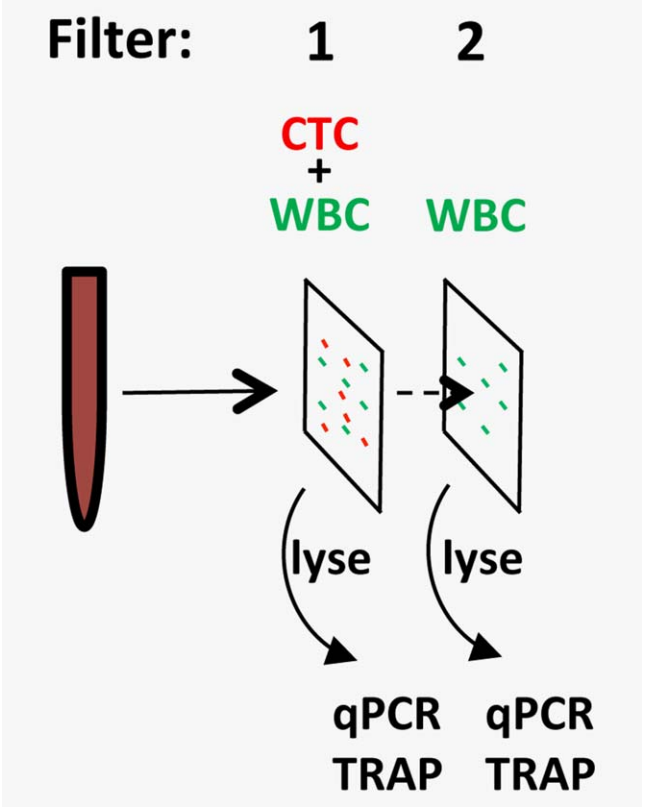


Figure 1. Scheme of CTC telomerase activity (TA) measurement by slot microfilter. Each specimen was passed through two filters in series. Filter-captured cells were lysed on filter, and the lysates were analyzed for telomerase activity by qPCR-TRAP. CTC TA equaled the difference between filter 1 and filter 2 (correction for background telomerase activity signal from WBCs).

Data analysis and statistical considerations

Analyses were performed on the patients participating in the CTC analysis with usable CTC lysate. Clinical characteristics were tested between patients with high CTC TA (expressed as C_t) *versus* low CTC TA, at randomization, using a cut-point at the median TA measured from filter 1. Continuous clinical covariates were tested by two-sided *t*-tests of unequal variances; categorical covariates were tested by χ^2 tests.

We evaluated the association between CTC TA and OS using Cox regression. We fit models for baseline TA and a number of derived variables (*e.g.*, C_t filter 2 – C_t filter 1 for filters run sequentially on the same specimen). Among the models, we considered analyses adjusting for CTC counts, and we considered analyses in subgroups defined by CTC counts (*e.g.*, d0 CTC counts $\geq$5). Statistical significance was based on two-sided tests, and in this hypothesis generating

Table 1. Patient and disease characteristics for patients with valid baseline filter 1 telomerase activity split at the median

	Filter 1 C_t <33 (higher TA)	Filter 1 $C_t \geq 33$ (lower TA)	*p*
Total patients	103	112	
Race: *N* (%)			
Black	20 (0.19)	10 (0.09)	0.05
White	78 (0.76)	99 (0.88)	
Other	5 (0.05)	3 (0.03)	
Type of progression: *N* (%)			0.93
Measureable/Eval	86 (0.83)	94 (0.84)	
PSA Only	17 (0.17)	18 (0.16)	
Bisphosphonate usage: *N* (%)			
No	37 (0.36)	36 (0.32)	0.56
Yes	66 (0.64)	76 (0.68)	
Worst pain: *N* (%)			
<4	57 (0.55)	63 (0.56)	0.89
≥4	46 (0.45)	49 (0.44)	
Extraskeletal metastases: *N* (%)			
No	39 (0.38)	58 (0.52)	0.04
Yes	64 (0.62)	54 (0.48)	
Performance status: *N* (%)			
0	50 (0.49)	54 (0.48)	1.00
1	45 (0.44)	49 (0.44)	
2	7 (0.07)	8 (0.07)	
3	1 (0.01)	1 (0.01)	
Gleason's: *N* (%)			
<7	11 (0.11)	12 (0.11)	0.73
7	27 (0.26)	34 (0.30)	
>7	62 (0.60)	61 (0.54)	
Missing	3 (0.03)	5 (0.04)	
Age at registration			
Mean (SD; *N*)	68 (9; 103)	70 (9; 112)	0.08
Median (IQ range)	68 (62–74)	70 (63–76)	
Baseline PSA			
Mean (SD; N)	216 (401; 103)	239 (710; 112)	0.77
Median (IQ range)	79 (23–223)	76 (22–175)	

Table 2. Multivariate proportional hazards model evaluating the association between CTC telomerase activity and overall survival (OS) in patients with ≥5 CTC by CellSearch ($n = 104$) at baseline (d0)

Variable	HR for OS	95% CI	p
d0 TA filter 2 − 1	1.14	(1.06–1.23)	0.001
d0 log2(CTC counts)	1.13	(1.02–1.26)	0.023
d0 log2(PSA)	1.09	(0.98–1.22)	0.11
Age	0.98	(0.95–1.00)	0.052
Black *vs*. Other race	0.60	(0.26–1.38)	0.22
Performance Status (0 *vs*. 1)	0.73	(0.46–1.17)	0.19
PSA only progression	1.95	(1.11, 3.44)	0.02
Atrasentan (*vs*. placebo)	0.84	(0.52–1.37)	0.49

Pain status, bisphosphonate usage and extraskeletal disease at study entry were evaluated and removed from the model since they were non-significant risk factors ($p > 0.50$). Interaction of treatment arm (atrasentan) with telomerase activity (TA filter 2 − 1) was not significant ($p > 0.05$).
Abbreviation: HR = hazard ratio.

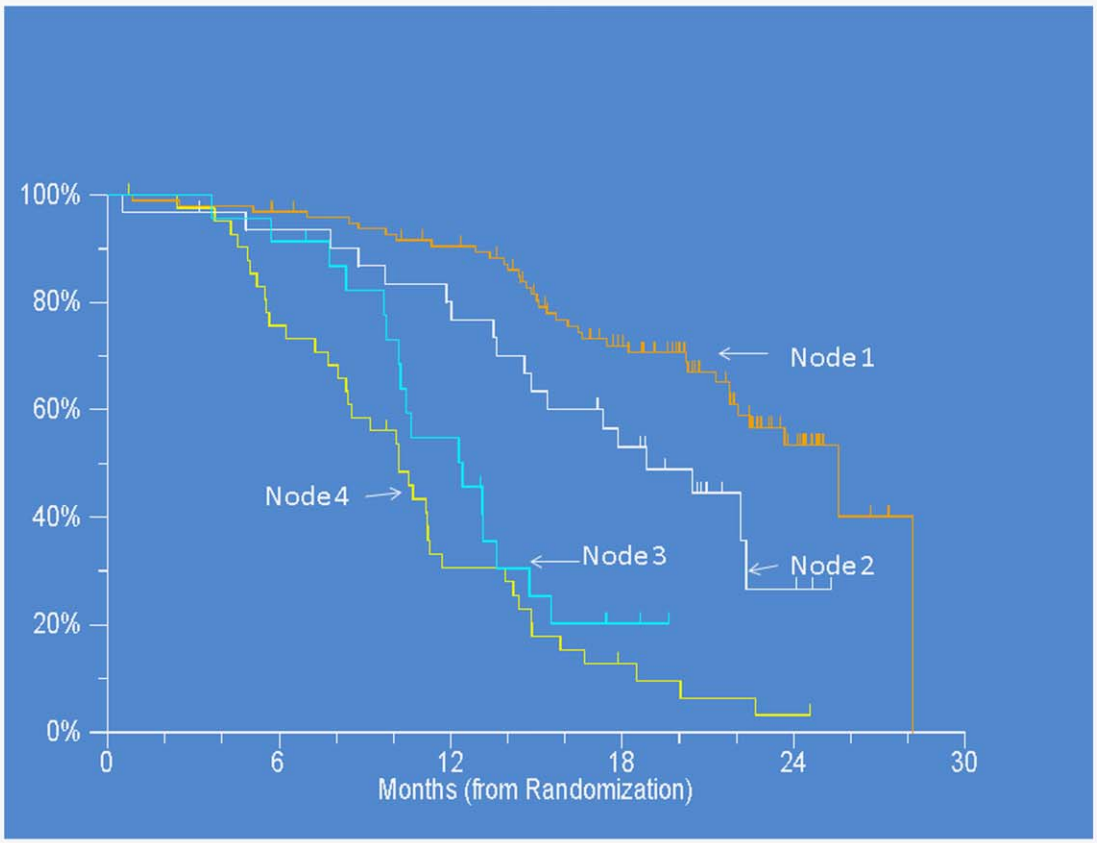
analysis we considered statistical significance to be $p < 0.01$. All covariates were measured at baseline (d0).

All models were adjusted for a priori prognostic covariates: treatment arm, (log 2) baseline CTC counts, ($\log_2$) baseline PSA, age, race (African American *vs*. all other), performance status, PSA-only progression *versus* radiologic progression at study entry, worst bone pain ≥4, and extraskeletal mets: yes *versus* no. Factors with a *p* value >0.50 were not included in the multivariate models. In a further exploratory analysis, prognostic subgroups were identified by recursive partitioning analysis to find the optimal cutpoint(s) when considering the variables baseline CTC counts, TA, and PSA.[27] Kaplan-Meier survival curves were constructed for each prognostic group. All analyses were done in R 2.15.1 (http://www.r-project.org/). Recursive partitioning was implemented using R package 'rpart'.

Results

The baseline demographic characteristics of patients participating in CTC correlatives were similar to those of the overall population of S0421 with respect to age, race, bisphosphonate use, pain assessment, extraskeletal metastases, Gleason score, and baseline PSA (Supporting Information Table 1); there was a suggestion that patients submitting specimens had better performance status compared to those that did not participate ($p = 0.05$) and may have had more measureable disease ($p = 0.08$). In Table 1, patients with high TA tended to have more extensive disease ($p = 0.04$) and a higher percentage with race designated as black ($p = 0.05$). High *versus* low TA was not significantly associated with baseline PSA only *versus* clinical progression, with bone pain, with bisphosphonate use, or with treatment response (by PSA or RECIST). The TA values (expressed as C_t) for the two in-series filters used to process each sample had similar distributions. Both filters had a median $C_t \sim 33$ with an interquartile range of 31 to 34. Calculation of rank based correlation between the filters yielded a correlation coefficient of 0.53.

Absolute TA measured on either of the two filters was not statistically significantly associated with OS over the entire cohort after adjusting for covariates, nor was the difference in TA (Filter 2 – Filter 1) between filters and OS over the entire cohort (Supporting Information Table 2). Exclusion of likely technical outliers (C_t <20) or analysis only of high telomerase activity specimens (C_t <32) or TA from d0 to



Node	Group	At Risk	Deaths	Median Survival (in months	HR (95% CI) (relative to node I)	HR p-value (relative to Node 1)
1	CTC 0-5, any CTC TA	96	36	26	1.0	N/A
2	CTC 6-54, Low CTC TA	31	18	19	2.0 (1.1, 3.5)	0.02
3	CTC 6-54, High CTC TA	33	17	12	4.9 (2.7, 8.9)	< 0.0001
4	CTC > 54, any CTC TA	42	38	10	6.8 (4.2, 11.0)	< 0.0001

Figure 2. KM overall survival curves by CART nodes baseline CTC counts and TA. Low *versus* high CTC TA connotes TA below or above the median, respectively. *p* Value for comparison between nodes 2 and 3 (low *vs*. high TA) = 0.009 using 1 degree of freedom χ^2.

d21 also did not yield a statistically significant association between OS and C_t (data not shown).

Based on our prior published data,[22] we reasoned that the signal-to-noise capability of microfilter telomerase analysis may not be sufficiently sensitive to detect the signal from very few CTC superimposed on a background of residual WBCs. Therefore, we examined the patient subgroup with ≥5 CTC counted by CellSearch and estimated hazard ratios for an association between OS and TA. While no significant association was observed between CTC TA and OS on Filter 1 alone, the difference in TA between filter 1 and 2—corresponding to the TA attributable to CTC alone after correction for WBC background (Fig. 1 and Ref. 22)—was significantly associated with OS in men with ≥5 CTC counted by CellSearch. Specifically, $TA_{Filter\ 2} - TA_{Filter\ 1}$ had a statistically significant OS HR of 1.14 (95% CI 1.06–1.23, $p = 0.001$) after adjusting for covariates including baseline CTC counts (Table 2). The interaction of TA with treatment arm was evaluated and found to be non-significant ($p > 0.05$), and so all analyses reported are for the pooled treatment arms.

To further explore the prognostic roles of CTC counts and telomerase activity in OS and also to evaluate the role of PSA, we conducted a regression tree analysis for OS based on d0 CTC counts, d0 PSA, and d0 telomerase activity ($TA_{filter\ 2} - TA_{filter\ 1}$) using all patients with valid baseline data ($n = 202$). This analysis showed that lower *versus* higher CTC telomerase activity served as a prognostic cutpoint to further stratify patients into distinct survival groups (Fig. 2): Specifically, in

men with intermediate range CTC counts between 6 and 54, lower CTC TA was associated with a median OS of 19 months whereas higher CTC TA was associated with a median OS of only 12 months ($p = 0.009$). CTC TA at d0 was identified as a significant factor only in the subset of patients with d0 CTC counts of 6 to 54 but not in the low or high CTC count groups.

Discussion

The high incidence and marked biological heterogeneity of prostate cancer present a pressing need for improved prognostic and predictive biomarkers, and CTC analysis may offer valuable real-time insights that enhance personalized management and facilitate disease monitoring. Telomerase is a tumor marker with demonstrated diagnostic and prognostic utility in multiple cancer types,[15–19] and telomerase activity is not only cancer-specific (relative to most benign tissues) and cancer-universal (high in >90% of malignancies), but also constitutes a uniquely "functional" assay that reflects the presence of live cancer cells. Moreover, telomerase activity can be readily amplified from as little as one cell using qPCR-TRAP to yield quantitative data. Therefore, our goal in this study was to test whether CTC telomerase activity measurement could serve as a surrogate for CTC enumeration or perhaps even enhance the prognostic utility of CTC counts. To address this question, we collected and analyzed specimens from 263 patients in S0421. While this cohort size was not as large as initially expected (due to unexpected acceleration of accrual to the parent trial prior to the inclusion of this biomarker substudy), this nonetheless stands as the largest prospective cohort to date with CTC biomarker correlatives in the first-line docetaxel setting for mCRPC.

We assessed CTC TA parameterized in a variety of models in a hypothesis-generating approach, and most did not yield a statistically significant association with OS across the entire participating cohort. However, we found that in the subset of men with baseline (d0) CTC counts ≥5, higher CTC telomerase activity was associated with worse overall survival (OS) even after adjustment for other risks such as baseline CTC counts, PSA level, and other clinical factors. Furthermore, an exploratory regression tree analysis using telomerase activity, CTC counts, and PSA level yielded prognostic cutpoints based on CTC counts and telomerase activity only, suggesting that in certain patient subgroups the prognostic utility of CTC telomerase activity can potentially exceed that of PSA. Such subgroups may be sizeable in this advanced disease setting; for example, men with ≥5 CTC at baseline—the group for which telomerase activity was prognostic of OS—accounted for nearly half (47%) of patients for whom telomerase measures were obtained in this study.

Telomerase activity was prognostic of OS only when expressed as (TA Filter 2 – TA Filter 1), a necessary normalization step proposed and tested previously to account for inter- and intra-patient variations in WBC number and telomerase activity over time and with therapy.[22] Moreover, CTC telomerase activity was significantly associated with OS only when measured in men with ≥5 CTC by CellSearch, a finding that may reflect the inherent limits of detection of this assay. In early experiments leading to the development of the slot microfilter, the presence of residual background WBCs with low telomerase activity on the filter generated a "signal to noise" phenomenon: Specifically, whereas even single cancer cells spiked into buffer with no background WBCs were detectable by qPCR-TRAP after capture on the microfilter, the telomerase activity of cancer cells spiked into 7.5 ml whole blood was detectable down to ~25 cancer cells due to residual background WBCs remaining on filter (10^3 range, similar to other enrichment platforms).[22] Thus, in S0421 CTC telomerase activity was prognostic only in the 47% of patients with ≥5 CTC by CellSearch because perhaps only these patients had sufficient CTCs (relative to background WBCs) for detection by this assay.

Another interesting consideration is the possibility that telomerase activity may not simply function as a surrogate marker for CTC numbers (*i.e.* the more CTCs, the more telomerase); rather, high telomerase levels within CTCs may itself drive dissemination and aggressive disease. In the recursive partitioning experiment (Fig. 2), low *versus* high telomerase activity discriminated the KM survival curves for patients with similar CTC counts (6–54 CTCs/7.5 ml), suggesting that telomerase indeed may be discriminating a particular phenotype of aggressive CTCs rather than simply reflecting the presence or absence of CTCs. Hence, telomerase activity may represent more than a static surrogate for CTC counts, but also a biologically relevant cancer property over time and with exposure to therapy. The methods used in this study may have not been as well suited to comparisons of TA over time (*e.g.* d0 *vs.* d21) because of the residual WBCs remaining on filter. Exposure to chemotherapy may impact WBC numbers and WBC TA differently than CTCs, resulting in observed changes in CTC TA that may not be due to disease response or progression alone but also due to changes in background WBCs. Hence, the CTC TA methods used in this study were best suited to determining CTC TA at one point in time (*e.g.* baseline). Current work ongoing in our group aims to address these limitations using new techniques to isolate ultra-pure CTC populations (no background WBCs) for high precision TA comparison at multiple time points.

Telomerase activity assayed from live-captured CTCs was prognostic of overall survival in a substantial subgroup of patients in S0421, a large Phase III Intergroup trial of men with mCRPC prospectively treated with first-line docetaxel-based chemotherapy. These results illustrate the prognostic potential of CTC-derived biomarkers, and they are hypothesis generating with regard to the use of telomerase as a CTC biomarker in this disease setting. The near-ubiquity of telomerase elevation in cancer, coupled with the ever-widening role of CTC collection in multiple malignancies, makes this a promising biomarker strategy that merits additional investigation and further validation in future studies.

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