Potential Applications for Circulating Tumor Cells Expressing the Insulin-Like Growth Factor-I Receptor

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Abstract

Purpose: To detect insulin-like growth factor-IR (IGF-IR) on circulating tumor cells (CTC) as a biomarker in the clinical development of a monoclonal human antibody, CP-751,871, targeting IGF-IR.

Experimental Design: An automated sample preparation and analysis system for enumerating CTCs (CellTracks) was adapted for detecting IGF-IR–positive CTCs with a diagnostic antibody targeting a different IGF-IR epitope to CP-751,871. This assay was used in three phase I trials of CP-751,871 as a single agent or with chemotherapy and was validated using cell lines and blood samples from healthy volunteers and patients with metastatic carcinoma.

Results: There was no interference between the analytic and therapeutic antibodies. Eighty patients were enrolled on phase I studies of CP-751,871, with 47 (59%) patients having CTCs detected during the study. Before treatment, 26 patients (33%) had CTCs, with 23 having detectable IGF-IR–positive CTCs. CP-751,871 alone, and CP-751,871 with cytotoxic chemotherapy, decreased CTCs and IGF-IR–positive CTCs; these increased toward the end of the 21-day cycle in some patients, falling again with retreatment. CTCs were commonest in advanced hormone refractory prostate cancer (11 of 20). Detectable IGF-IR expression on CTCs before treatment with CP-751,871 and docetaxel was associated with a higher frequency of prostate-specific antigen decline by >50% (6 of 10 versus 2 of 8 patients). A relationship was observed between sustained decreases in CTC counts and prostate-specific antigen declines by >50%.

Conclusions: IGF-IR expression is detectable by immunofluorescence on CTCs. These data support the further evaluation of CTCs in pharmacodynamic studies and patient selection, particularly in advanced prostate cancer.

A substantial body of evidence supports the development of insulin-like growth factor-IR (IGF-IR) inhibitors as a therapeutic approach for malignancy (1, 2). This receptor is implicated in proliferation, angiogenesis, apoptosis, and carcinogenesis (3–5), with IGF-IR inhibition in xenografts increasing the activity of cytotoxics (6, 7). CP-751,871 is a fully human IgG2 antibody with high affinity for the IGF-IR (8), inducing the down-regulation of IGF-IR by promoting receptor internalization and degradation.

Circulating tumor cell (CTC) isolation and characterization from the blood of patients with a wide range of malignancies is well described (9, 10). Evidence that CTCs have many of the molecular characteristics of the primary tumors and metastases (11–13), with the ability to characterize these cells by fluorescence *in situ* hybridization or immunofluorescence support the use of CTCs as a novel, noninvasive approach for patient selection and pharmacodynamic studies (14–18). The analysis of CTCs in clinical trials of CP-751,871 is particularly appealing because IGF-IR is postulated to play a key role in metastasis, regulating cell adhesion, motility, migration, and angiogenesis (19).

We generated an assay to evaluate the presence of the IGF-IR on CTCs and evaluated the frequency of these cells in patients with solid tumors, and their prognostic and pharmacodynamic potential, in patients treated with CP-751,871 either as a single agent or in combination with chemotherapy.

Patients and Methods

Patients. All procedures were institutional review board approved. All patients provided informed consent. For the training set of

experiments, 7.5 mL of blood were drawn into CellSave tubes (Immunicon) from 50 patients with metastatic carcinoma. The detection of IGF-IR on CTCs in patients treated with CP-751,871 was conducted using multiple 7.5-mL blood samples collected from 80 patients with advanced solid tumors enrolled in three phase I studies of CP-751,871: (*a*) an open-label dose-escalation phase I trial; (*b*) an open-label dose-escalation phase Ib trial in combination with docetaxel; (*c*) an open-label dose-escalation phase Ib trial in combination with paclitaxel and carboplatin. CP-751,871 was administered by i.v. infusion every 3 weeks at doses of 0.05 to 20 mg/kg. The dose of paclitaxel and carboplatin in the second study were 200 mg/m^2 and AUC 6, respectively. The dose of docetaxel in the third study was 75 mg/m^2. Blood samples were maintained at room temperature and processed within 72 h of collection. Response to treatment was by Response Evaluation Criteria in Solid Tumors (20) and Prostate Specific Antigen Working Group criteria (21).

Cell culture. Cell suspensions from the breast cancer cell line MCF-7, the prostate cell line PC3-9, the bladder cell line T-24, and the hematopoietic cell line CEM were obtained and were used only when their viability, as assessed by trypan blue exclusion, exceeded 90%. Cells for spiking studies were fixed with paraformaldehyde and diluted to a 10 cell/μL spiking stock. The cell count for spiking studies was generated using 10-well masked slides, where 10 μL aliquots of cell suspension and an equal volume of an acridine orange ethidium bromide staining solution were mixed and cells were counted under a fluorescent microscope. The resulting mean spot count was used to determine the expected spike number based on a fixed volume spike of 20 μL. The fixed spiking stock was used daily as a normal donor spike control. For IGF-IR detection, spiked cell numbers were between 130 and 220 in 7.5 mL of blood.

Isolation and enumeration of CTCs. CTCs were isolated and enumerated at each blood draw using the CellTracks system (Immunicon) as previously described (10, 22). For the detection of carcinoma cells, 7.5 mL of blood were mixed with ferrofluids coated with antibodies directed against EpCAM. After immunomagnetic enrichment, FITC-labeled antibodies recognizing cytokeratins 4, 5, 6, 8, 10, 13, 18, and 19; allophycocyanin-labeled antibodies recognizing CD45; phycoerythrin-labeled antibodies recognizing IGF-IR (1H7, BD Phar-Mingen); and the nucleic acid dye 4′,6-diamidino-2-phenylindole were added, together with a permeabilization buffer, to fluorescently label the enriched cells. After incubation on the system, the magnetic separation was repeated and excess staining reagents were aspirated. In the final processing step, the cells were resuspended in the MagNest Cell Presentation Device. The MagNest consists of a chamber and two magnets that orient the fluorescently labeled cells for analysis using the CellSpotter Analyzer, a four-color semiautomated fluorescent microscope. Image frames covering the entire surface of the cartridge for each of the four fluorescent filter cubes were captured. The captured images were presented to trained operators, blinded to patient outcomes, who selected objects that met the definition of CTCs, including round to oval morphology, a visible nucleus (4′,6-diamidino-2-phenylindole positive), positive staining for cytokeratin, and negative staining for CD45. Results were expressed as the number of cells per 7.5 mL of blood.

Results

IGF-IR CTCs assay development. The antigen density of the IGF-IR on tumor cells was initially assessed using flow cytometry. CEM, PC3-9, T-24, and MCF-7 were investigated. Figure 1A shows an overlay of the expression levels of IGF-IR on these cell lines. Antigen density was calculated using a known number of spectrally matched fluorescent beads. The IGF-IR density on PC3-9, T-24, and MCF-7 cells was estimated to be ∼10,000, 50,000, and ≥250,000 antigens per cell, respectively. Based on these results, the CellSpotter analyzer was configured
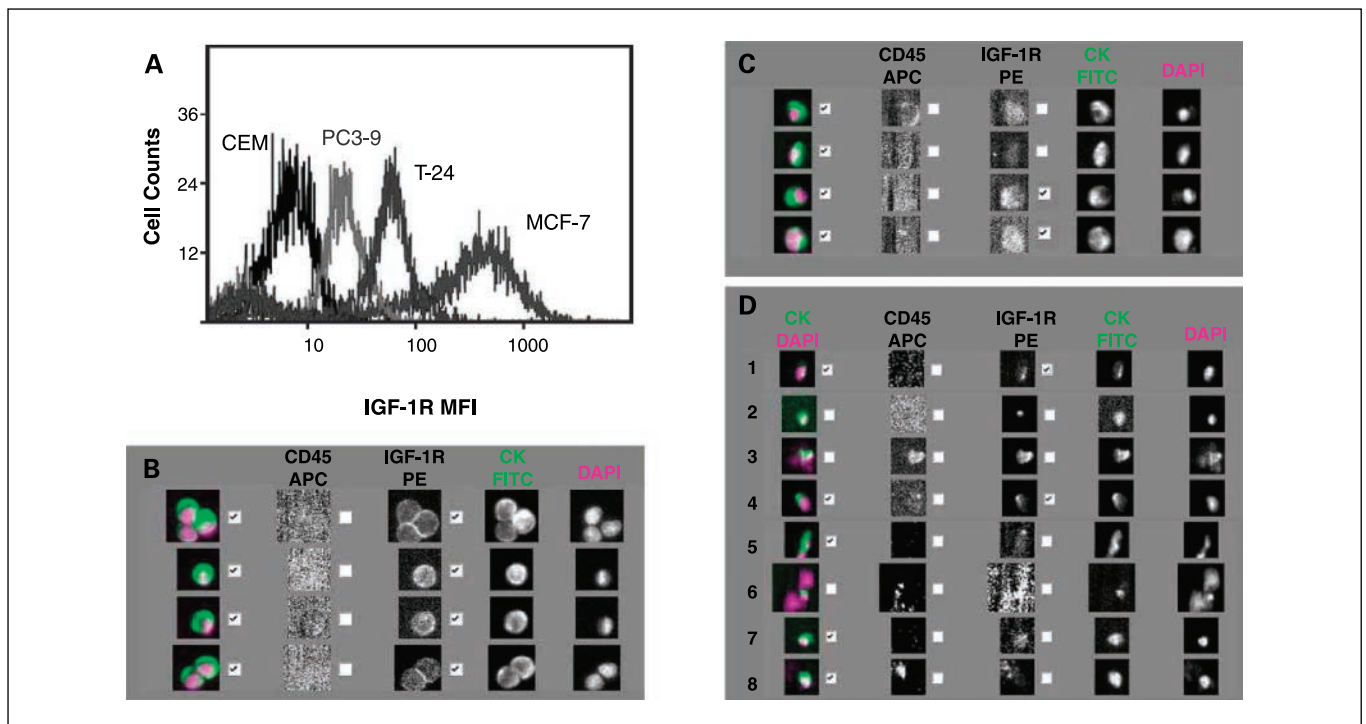
Fig. 1. CTCs expressing IGF-IR. *A,* overlay of flow cytometry histograms representing IGF-IR – phycoerythrin staining of cell lines CEM, PC3-9, T-24, and MCF-7. Median fluorescence intensity (*MFI*) was acquired for 10,000 events using a BD FACSCalibur device. *B* and *C,* detection of MCF-7 cells (*B*) and T-24 cells (*C*) spiked in blood of healthy volunteers using a CellSpotter device. APC, allophycocyanin; PE, phycoerythrin; DAPI, 4′,6-diamidino-2-phenylindole. *D,* analysis of eight rare cell events in a blood sample of a cancer patient using a CellSpotter device.

Table 1. CTCs and IGF-IR–positive CTCs in CP-751,871 trials

| CP-751,871 study	Patients providing blood samples	Patients with CTCs at anytime during study	Patients with CTCs at enrollment	Patients with IGF-IR (+) CTCs at enrollment
Single agent (Trial 1)	26	16	6	5
With carboplatin/paclitaxel (Trial 2)	27	17	6	5
NSCLC (subset of Trial 2)	21	14	4	3
With Taxotere (Trial 3)	27	19	14	13
HRPC (subset of Trial 3)	20	17	11	10

to a limit of detection that would correctly identify at least 80% of the IGF-IR–positive MCF7 cell population. The CellTracks reagent kit was modified to use FITC-labeled antibodies for the detection of cytokeratin that is expressed at high densities on epithelial cells, which permitted the use of the commercially available 1H7 phycoerythrin-labeled antibody for the detection of IGF-IR. Using the new settings, the average percentage of cells considered IGF-IR–positive using 130 to 200 cell spikes in 10-mL blood samples from healthy donors were $80.6 \pm 7.7\%$ ($n = 16$ spiked blood samples), for MCF7, 13.6 ± 3.9 9% ($n = 6$), and for T-24 $3.8 \pm 6.0\%$ ($n = 6$). The anti–IGF-IR staining of CEM cells was found to be similar to that of negative control cells (cells treated with excess CP-751,871) and considered below the detection limit (not shown). Negligible expression levels of the IGF-IR on B-lymphoblastoid cell lines have been previously reported (23). Recovery experiments showed that 95% of MCF-7 cells (25 and 250 cell spikes) were detected using the CellTracks system ($n = 6$). The analytic imprecision of these counts was ≤10%. Recovery rate remained ≥80% for test periods up to 96 h. Cross-blocking flow cytometry experiments showed lack of analytic interference between CP-751,871 and antibody 1H7 as reported previously (8).

Figure 1B shows a typical example of MCF-7 cells recovered from 7.5 mL of blood. The top row shows a cluster of three MCF-7 cells that clearly expressed IGF-IR. The check mark next to the composite image indicates that the operator classified the cells as CTCs and the check mark next to the IGF-IR staining image indicated that the operator selected these CTCs as expressing IGF-IR. All CTCs in Fig. 1B expressed IGF-IR. In Fig. 1C, typical examples of T-24 cells are shown. Staining for IGF-IR on the four T-24 cells was weaker than on MCF-7 cells. The operator classified only the bottom two cells as CTCs that expressed IGF-IR.

IGF-IR expression on CTCs from metastatic carcinoma patients. To test whether IGF-IR could be detected on CTCs in patients with metastatic carcinomas, blood samples from 50 patients were tested [18 breast cancer, 13 colorectal cancer, 12 non–small cell lung cancer, 4 ovarian cancer, and 3 hormone refractory prostate cancer (HRPC)]. CTCs were detected in 11 of 50 (22%) patients; in 8 of these 11 patients, IGF-IR–positive CTCs were also detected. The number of CTCs and IGF-IR–positive CTCs were in the range of 1 to 180 and 1 to 47 cells, respectively (Fig. 1D). The ratio of IGF-IR–positive and total CTC counts varied greatly; 8 CTC candidates are shown in Fig. 1D. Events 1, 4, 5, 7, and 8 were classified as CTCs; however, only CTCs in rows 1 and 4 were classified as expressing IGF-IR. Potential IGF-IR staining was observed in rows 5 and 7 but was not considered sufficient for classification as IGF-IR positive. The enumeration of CTCs and IGF-IR–

positive CTCs was then incorporated as a pharmacodynamic end point in three clinical trials of CP-751,871.

IGF-IR CTCs in a phase I study of single-agent CP-751,871. In the first clinical trial, blood samples were collected from 26 patients consented to a dose-finding phase I study of single-agent CP-751,871. The commonest tumor types enrolled were colorectal (6), lung (4), prostate (3), and bladder (2). Nonepithelial malignancies studied included soft tissue sarcomas and gastrointestinal stromal tumors (5).
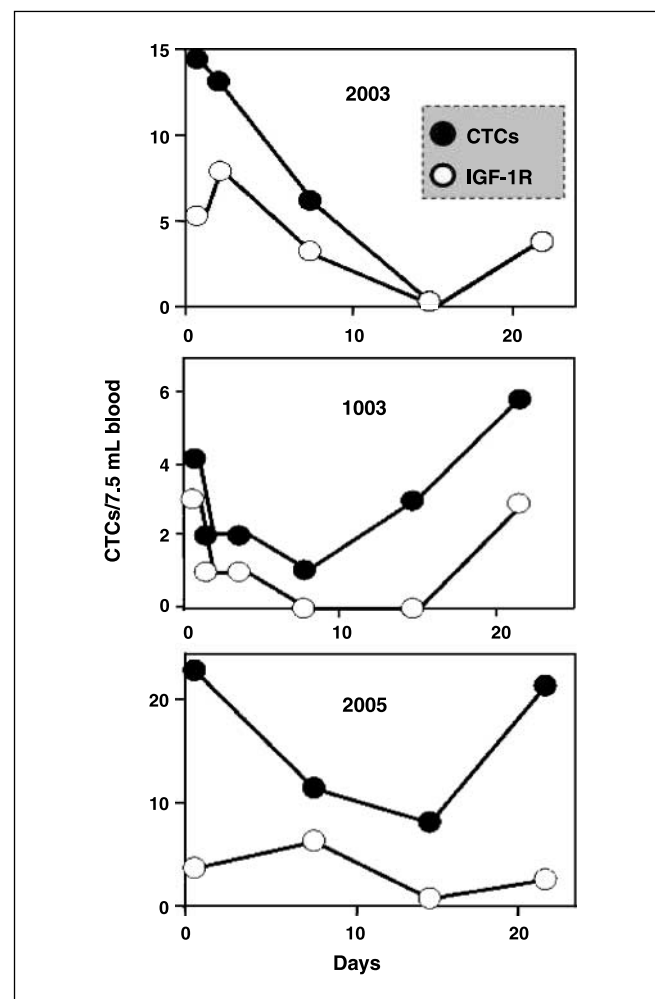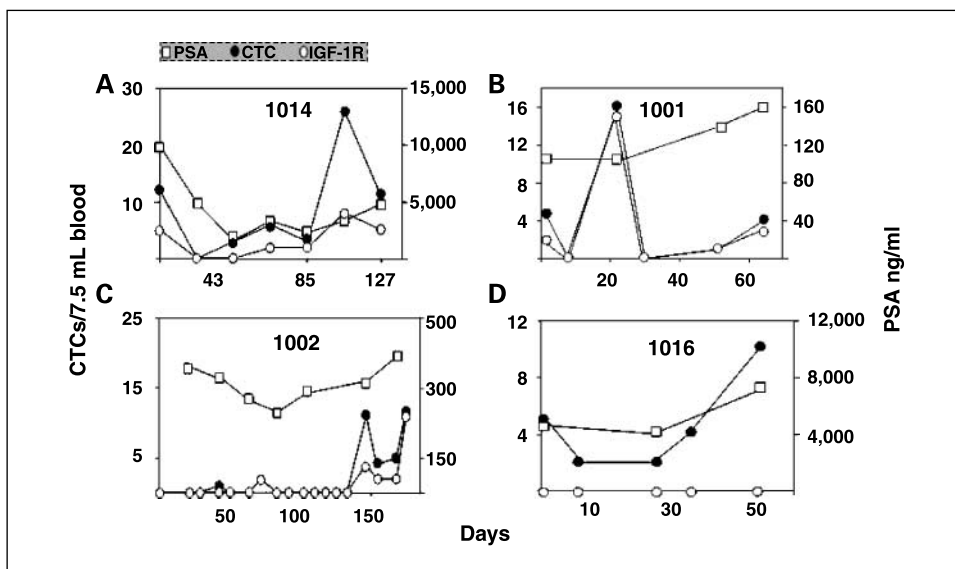

Fig. 2. Changes in the number of CTCs and IGF-IR – positive CTCs in patients enrolled in the phase I study of single-agent CP751,871. Patients 2003 (ovarian carcinoma), 1003 (HRPC), and 2005 (CRC) received doses of 6, 6, and 10 mg/kg of CP-751,871, respectively, on study day 1. The first sample was taken before CP-751,871 administration.

Samples were drawn immediately before the first dose of CP-751,871 and on days 1, 3, 7, 14, and 21 days after treatment. All patients had a minimum of 4 weeks washout period from previous therapy before study entry. Sixteen of 26 patients (61%) had detectable CTCs at some point during the study. Only 6 of 26 patients (26%) had one or more CTCs at study entry. IGF-IR expression on CTCs was detected in five of these six patients (Table 1). The number of CTCs and IGF-IR–positive CTCs detected was plotted in relation to time, with plots corresponding to four patients with the highest CTC counts being shown in Fig. 2. One additional patient had one CTC/IGF-IR CTC at study entry. A decrease in total and IGF-IR–positive CTCs after treatment with CP-751,871 was observed with a rebound increase in the number of CTCs at the end of the 21-day treatment cycle. Increases in total CTC and IGF-IR–positive CTC counts were also noted after discontinuing antibody therapy.

IGF-IR CTCs in phase Ib studies of CP-751,871 in combination with chemotherapy. Samples for the enumeration of CTCs were acquired from 27 patients on a phase Ib study of docetaxel and CP-751,871, including 20 HRPC patients. Overall, 14 of 27 patients, and 11 of 20 HRPC patients, had IGF-IR–positive CTCs at baseline (Table 1). All HRPC patients with detectable CTCs at baseline were also IGF-IR CTCs positive, except one patient in whom IGF-IR CTCs were not detected despite repeated testing. Figure 3 shows the prostate-specific antigen (PSA), total CTC, and IGF-IR CTC temporal profile in four patients during the 1st month of treatment. Decrements in CTC counts immediately after treatment were frequently observed.

The third clinical trial was a phase Ib study of CP-751,871 in combination with paclitaxel and carboplatin. Twenty-seven patients provided blood samples, including 21 NSCLC patients (Table 1). Seventeen patients had detectable CTCs at some point during the study. Only 1 to 2 cells were detected in most cases, with all NSCLC patients having ≤2 CTCs at study entry. One HRPC patient enrolled in the study had a large number of CTCs at study entry (71 CTCs and 15 IGF-IR CTCs) that decreased in response to treatment (21 CTCs and 2 IGF-IR CTCs by treatment day 21).

Detection of IGF-IR–positive CTCs and clinical responses. No objective responses to CP-751,871 alone were observed when CP-751,871 was given as a single agent. Patients with detectable CTCs at study entry experienced more rapid tumor progression. In the phase Ib trial of CP-751,871, with docetaxel, HRPC patients who had at least one detectable IGF-IR CTC at enrollment had higher PSA levels ($n = 10$, median PSA 475 ng/mL) than those who were IGF-IR CTC negative ($n = 8$, median PSA 92 ng/mL). No assessment was possible in two patients due to loss of samples. Patients with IGF-IR–positive CTCs at enrollment were more likely to have PSA declines by >50% (6 of 10), with these occurring more rapidly than those who were IGF-IR CTCs negative (2 of 8; Table 2). Changes in CTC counts correlated with PSA declines. Figure 3A shows a representative example of a patient responding to therapy. In this patient, who initially responded to treatment, his disease became refractory to therapy on cycles 5 to 6, with increasing numbers of CTCs and IGF-IR–positive CTCs increasing in parallel to PSA levels. Although most patients with IGF-IR–positive CTCs at study entry responded to CP-751,871 and docetaxel therapy, or experienced disease stabilization, progression of disease was observed in two patients. Figure 3B represents the profile of CTC counts and IGF-IR–positive CTC counts and PSA levels in one of these patients (low dose of CP-751,871; 0.1 mg/kg), in which the CTC count decrease immediately after treatment was short lived, creating a pattern of peaks and troughs, suggesting that only sustained decreases in CTC counts reflect response to treatment. Eight HRPC patients had no detectable CTCs at study entry, with six of these patients not responding to treatment by Prostate Specific Antigen Working Group criteria. In three of these patients, at disease progression, CTCs and IGF-IR–positive CTCs became detectable at progression. The profile of one of these patients is shown in Fig. 3C. Finally, the one HRPC patient treated with docetaxel and CP-751,871 with detectable CTCs, but no detectable IGF-IR CTCs, did not respond to the combination treatment. The decrease in his CTC count was short lived (one cycle; Fig. 3D).

Fig. 3. Changes in the number of CTCs and IGF-IR – positive CTCs and PSA levels in HRPC patients enrolled in the phase I study of CP751,871 and docetaxel. Patients 1014, 1001, 1002, and 1016 received, respectively, doses of 3, 0.1, 0.1, and 6 mg/kg of CP-751,871 in combination with 75 mg/m^2 of docetaxel on 3-wk treatment cycles.

Table 2. Baseline PSA levels and CTC counts in patients that responded to treatment with the combination of CP-751,871 and docetaxel

HRPC patients responding to treatment	Baseline PSA (ng/mL)	Baseline CTCs	Baseline IGF-IR (+) CTCs	Treatment cycle of initial PSA response
1006	238	2	1	3rd
1008	471	4	1	12th
1014	9,944	12	5	2nd
1019	617	44	19	3rd
1022	1,639	6	2	1st
1025	807	160	37	1st
1003	13	0	0	6th
1011	131	0	0	12th

Discussion

The study of CTCs in the development of rationally designed, molecularly targeted drugs could be critically important to predict patient outcome, response to treatment, patient selection, and dose optimization. These studies describe one of the first attempts to use CTCs in such a clinical trial. Our results indicate that the IGF-IR protein is frequently expressed on CTCs of patients with metastatic tumors, possibly supporting the postulated relationship between IGF-IR expression and more aggressive disease because patients with high CTC counts at baseline usually had high CTC IGF-IR expression and more rapid disease progression (24).

Treatment with CP-751,871 alone decreased both total CTC count and IGF-IR–positive CTC count, suggesting that treatment with CP-751,871 had a cytotoxic effect. However, because studies in preclinical models indicate that IGF-IR inhibition decreases tumor cell metastatic potential (25–27), it is possible that CP-751,871 exerted its effects by inhibiting CTC migration. CP-751,871 can down-regulate IGF-IR on WBC without affecting their viability (28). The number of patients with detectable CTCs in this single-agent trial was insufficient to establish a dose-effect relationship, this being an important limitation of our studies.

CTCs and IGF-IR–positive CTCs were most frequently detected in HRPC patients. A previous small study reported CTC counts as the most significant variable predictive of survival in HRPC patients (29). A larger study evaluating the utility of CTC counts to predict survival and treatment benefit has now finished accrual, and survival data indicate that CTC counts are independent predictors of outcome in multivariate analyses. The potential role of IGF-IR in prostate carcinogenesis (30–32), and its association with resistance to androgen deprivation (33), has been described although this is not well established (32, 34). In these studies, HRPC patients with IGF-IR–positive CTCs (at least one IGF-IR CTCs detected) had a 4-fold higher median serum PSA at enrollment than those patients with no detectable CTCs, in keeping with both genes being driven by androgen receptor signaling. The proportion of patients with PSA decrements by >50% to combined CP-751,871 and docetaxel therapy in this study was higher in patients with detectable IGF-IR–positive CTCs at study entry than in those in which these cells were not detected. Overall, these data suggest a potential utility for IGF-IR–positive CTC enumeration for the identification of HRPC patients that could benefit from anti–IGF-IR therapy.

Although we and others (10) have found that CTCs are most commonly present in HRPC, further evaluation of CTC enumeration and molecular characterization is ongoing in other solid epithelial tumor types, including breast, colorectal, and gastric carcinomas. The main limitation to the molecular characterization of CTCs is that the study of small numbers of CTCs can prove to be technologically challenging and complicated by the presence of contaminating leukocytes in isolated CTC preparations. This technology is therefore currently better suited to the study of patients with a large tumor burden and tumor phenotypes that result in shedding of CTCs. However, it is envisioned that, for the study of HRPC, in particular, the evaluation of CTCs and target expression on these cells could have an important effect on clinical trial design, particularly because the acquisition of tumor tissue from these patients remains difficult. Moreover, the reproducibility of the results obtained by different operators and centers using this technology (18) make it possible to evaluate this technology in randomized clinical studies that are now required to establish the clinical utility of this technology for the expedited development of targeted therapeutics. In particular, randomized therapeutic phase III trials of active antitumor agents incorporating CTC evaluation in patients with castration refractory prostate cancer are now urgently needed to evaluate whether decreases in CTC counts may serve as robust predictors of survival benefit from treatment in this disease.

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