Impact of Two Measures of Micrometastatic Disease on Clinical Outcomes in Patients with Newly Diagnosed Ewing Sarcoma: A Report from the Children’s Oncology Group


Abstract

Purpose: Flow cytometry and RT-PCR can detect occult Ewing sarcoma cells in the blood and bone marrow. These techniques were used to evaluate the prognostic significance of micrometastatic disease in Ewing sarcoma.

Experimental Design: Newly diagnosed patients with Ewing sarcoma were enrolled on two prospective multicenter studies. In the flow cytometry cohort, patients were defined as “positive” for bone marrow micrometastatic disease if their CD99+/CD45− values were above the upper limit in 22 control patients. In the PCR cohort, RT-PCR on blood or bone marrow samples classified the patients as “positive” or “negative” for EWSR1/FLI1 translocations. The association between micrometastatic disease burden with clinical features and outcome was assessed. Coexpression of insulin-like growth factor-1 receptor (IGF-1R) on detected tumor cells was performed in a subset of flow cytometry samples.

Results: The median total bone marrow CD99+/CD45− percent was 0.0012% (range 0%–1.10%) in the flow cytometry cohort, with 14 of 109 (12.8%) of Ewing sarcoma patients defined as “positive.” In the PCR cohort, 19.6% (44/225) patients were “positive” for any EWSR1/FLI1 translocation in blood or bone marrow. There were no differences in baseline clinical features or event-free or overall survival between patients classified as “positive” versus “negative” by either method. CD99+/CD45− cells had significantly higher IGF-1R expression compared with CD45− hematopoietic cells (mean geometric mean fluorescence intensity 982.7 vs. 190.9; P < 0.001).

Conclusions: The detection of micrometastatic disease at initial diagnosis by flow cytometry or RT-PCR is not associated with outcome in newly diagnosed patients with Ewing sarcoma. Flow cytometry provides a tool to characterize occult micrometastatic tumor cells for proteins of interest. Clin Cancer Res; 1–8. ©2016 AACR.

Introduction

Ewing sarcoma is the second most common primary bone cancer in children and young adults (1). A majority of patients have localized disease, which is typically treated with a uniform chemotherapy regimen. This practice does not account for individual differences in risk of relapse that may be mediated by disease burden, extent of micrometastatic disease, or chemosensitivity.

Occult Ewing sarcoma cells can be detected in the peripheral blood and in bone marrow using a PCR assay to detect EWSR1 fusion transcripts characteristic of this tumor (2–9). PCR studies have shown that approximately 20% to 43% of patients with newly diagnosed Ewing sarcoma have detectable EWSR1 fusion transcripts in their peripheral blood and/or bone marrow (2–9). PCR benefits from high sensitivity but requires knowledge of the subtype of EWSR1 translocation. Moreover, tumor cells cannot be
Ewing sarcoma is considered a systemic disease, and patients are presumed to have micrometastatic disease at diagnosis. In two large prospective studies using complementary techniques (flow cytometry and PCR) to detect micrometastatic disease, we show that the detection of micrometastatic disease by either method is not associated with outcome in newly diagnosed Ewing sarcoma. Instead, the value of these approaches, if any, may be in assessment of occult tumor cells during therapy, at the end of therapy, or during long-term follow-up. Furthermore, as proof of principle of the utility of flow cytometry to characterize defined Ewing sarcoma tumor cells, we evaluated coexpression of insulin-like growth factor I receptor (IGF-1R). We show that flow cytometry provides a potential way to quantify the expression of Ewing sarcoma tumor cell IGF1R. This assay has been incorporated as a new pharmacodynamic tool in an ongoing trial of an IGF-1R inhibitor in patients with newly diagnosed metastatic Ewing sarcoma (NCT02306161).

The prognostic impact of micrometastatic disease burden at initial diagnosis is not clear. Some PCR-based studies have shown that detectable EWSR1 fusion transcripts in the peripheral blood and/or bone marrow at diagnosis is an adverse prognostic factor (3, 6). Other PCR-based studies have not confirmed this finding (2, 8), nor did a small retrospective study that utilized flow cytometry to quantify bone marrow micrometastatic disease burden (13). Beyond initial diagnosis, the persistence of peripheral blood and/or bone marrow fusion transcripts after chemotherapy has been shown to confer an adverse prognosis (2).

Ewing sarcoma cells commonly overexpress the insulin-like growth factor 1 receptor (IGF-1R) (14). Monoclonal antibodies against IGF-1R have resulted in objective clinical responses in patients with Ewing sarcoma (16–19) and this class of agents is being evaluated in newly diagnosed patients with metastatic Ewing sarcoma. The ability to quantify tumor IGF-1R expression by flow cytometry may provide a tool to assess pharmacodynamic effects of IGF-1R monoclonal antibodies in patients over time and also to provide a proof of principle of the utility of flow cytometry to characterize disseminated tumor cells.

We therefore conducted two prospective multicenter cohort studies using samples from patients participating in Children’s Oncology Group (COG) studies with the following objectives. First, we sought to quantify the burden of occult micrometastatic disease in bone marrow and/or peripheral blood using two complementary approaches, RT-PCR and flow cytometry, in patients with newly diagnosed Ewing sarcoma. Second, we aimed to assess the association between micrometastatic disease burden and clinical features and outcomes in both cohorts. Finally, we sought to evaluate the ability of flow cytometry to quantify IGF-1R coexpression on occult bone marrow Ewing sarcoma cells.

Materials and Methods

Patients and collection of bone marrow and/or peripheral blood samples

Flow cytometry cohort Patients greater than 12 months of age with pathologically confirmed Ewing sarcoma diagnosed between 2008 and 2014 comprise this prospective "flow cytometry cohort". All enrolled patients or their guardians provided informed consent and were enrolled onto one of two biology studies: a multicenter study focused on flow cytometry detection of bone marrow Ewing sarcoma cells [University of California, San Francisco (UCSF); San Francisco, CA); Seattle Children’s Hospital (Seattle, WA); Primary Children’s Hospital (Salt Lake City, UT); and Dana-Farber Cancer Institute (Boston, MA)]; and the national COG Ewing sarcoma biology study (AEWS07B1). Each treating institution obtained Institutional Review Board (IRB) approval. In addition to providing bone marrow samples for analysis, participating sites also provided follow-up data and clinical characteristics at the time of diagnosis, including age, sex, tumor site, tumor stage (localized vs. metastatic), and sites of metastases, if applicable. Patients were included in the analytic cohort if they had no morphologic evidence of bone marrow metastatic disease and had an evaluable bone marrow sample from diagnosis (as defined below). Patients with clinical evidence of bone marrow metastatic disease or recurrent disease were also eligible to submit samples for flow cytometry analysis, but results from these patients were not included in the primary flow cytometry analytic cohort.

Bone marrow samples from adult and pediatric subjects at UCSF (San Francisco, CA) without cancer were used as control samples. These subjects underwent bone marrow aspiration for evaluation of a nonmalignant hematologic disorder, with consent to obtain additional material or for allogeneic bone marrow transplantation. Samples were shipped fresh overnight on ice to the UCSF Flow Cytometry Core Laboratory for processing and flow cytometry analysis.

PCR cohort. The prospective “PCR cohort” included patients less than 50 years of age with pathologically confirmed localized Ewing sarcoma who were enrolled on the COG randomized trial of interval compressed chemotherapy (AEWS0031, NCT00006734) between 2001 and 2005 (20). Each treating institution obtained IRB approval and informed consent. Patients submitted a baseline sample of 10 mL peripheral blood and/or 2 to 4 mL bone marrow aspirate in EDTA tubes for analysis. Peripheral blood and bone marrow samples were shipped fresh overnight on ice to the Biopathology Center (Columbus, OH). A patient was included in the PCR analytic cohort if they had a RT-PCR–evaluable blood or bone marrow sample before enrollment or within 30 days of enrollment on the AEWS0031 study.

Flow cytometry to quantify IGF-1R
Method of micrometastatic disease detection

**Flow cytometry cohort: antibodies and flow cytometry.** CD99-PE and CD34-FITC were obtained from BD Biosciences/Pharmingen. CD45-PB, CD14-ECBD, and CD221-APC were obtained from Thermo Fisher, Beckman Coulter, and eBioscience, respectively. LIVE/DEAD Fixable Dead Cell Stain Kit, aqua-fluorescent amine reactive dye (AARD), was obtained from Invitrogen. Human γ-globulin (HGG) was obtained from BioDesign International. CD221-APC (IGF-1R) was only added to the panel in the last two years of the study.

Bone marrow mononuclear cells (BMMC) were isolated on a Ficoll density gradient. Isolated cells were counted and the viability assessed on a Guava PCA using the ViaCount procedure (Guava Technologies). Cells (1 × 10^6) were washed in wash buffer, labeled for 20 minutes at room temperature with four times the recommended volume of AARD, blocked with HGG buffer, and stained with CD99-PE, CD45-PB, CD14-ECBD, CD34-FITC, and CD221-APC. Cells were collected on a customized LSRII Flow Cytometer (BD Biosciences) within 18 hours of staining. Data were compensated and analyzed using FlowJo software (Tree Star).

The gating strategy for the 6-color panel was as follows: a debris exclusion gate was set on a forward and side scatter plot. A CD45<sup>+</sup> gate was then drawn on all CD45<sup>+</sup> cells to define total BMMCs on a CD45 versus side scatter plot. Following debris exclusion on the forward- and side-scatter plot, AARD-positive dead cells, CD14<sup>+</sup> monocytes, and CD34<sup>+</sup> progenitor cells were sequentially removed. As described previously, Ewing sarcoma cells derived from established A673 cell lines (ATCC) were “spiked” into control peripheral blood mononuclear cell (PBMC) samples to identify an appropriate CD99<sup>+</sup> “positive” gate (10). A673 cells were not authenticated upon receipt from ATCC, but their CD99<sup>+</sup> CD45<sup>+</sup> immunophenotype remained consistent throughout the study. “Unspiked” control PBMC samples were used as the negative controls in these flow cytometry assays. A CD99<sup>+</sup> CD45<sup>+</sup> gate was set to include Ewing sarcoma cells but exclude CD45<sup>+</sup> cells on a CD99 versus CD45 plot. In subanalyses, the proportion of CD99<sup>+</sup> CD45<sup>+</sup> cells was further divided into those with bright CD99 expression (CD99<sup>+</sup> CD45<sup>+</sup>-bright). Results in a given gate were expressed as a percentage of the total CD45<sup>+</sup> BMMCs. Only samples with >750,000 total CD45<sup>+</sup> BMMCs were considered evaluable.

**PCR cohort: molecular analysis.** RNA was extracted from samples in RLT buffer using the RNeasy Kit (Qiagen) according to the manufacturer’s instructions, and 1 µg of RNA was used for cDNA synthesis (First Strand Synthesis Kit, Roche). Each cDNA sample was analyzed for quality using 6GPD-specific primers (G6PD1: 5'-CCGGATGATGCGACACCACTGCTGGGAGCAAG-3'; G6PD2: 5'-GGTTCCCCAGGTACGTTGCCCGAGCAACA-3') and probes (G6PDH1P: 5'-GGTTCCCAAGATGCGCCGCGAGATCCTGTCCG-3'; G6PDPH2: 5'-GAAATCTCAGACCACTGAGGTTCCCTGAGC-3').

Samples negative for type 1 were probed for type 2. This assay was not capable of detecting translocations with less common breakpoints or the EWSR1/ERG translocation. Appropriate positive and negative controls were run with each experiment. For samples wherein both bone marrow and peripheral blood were available, the patient was classified as positive if the RT-PCR was positive in either sample. If only one of the two samples was available, that result (positive or negative) determined the classification of the patient.

**Statistical analysis**

Micrometastatic disease burden by flow cytometry (CD99<sup>+</sup> CD45<sup>+</sup> percent) or by RT-PCR was the primary predictor variable of interest. In the flow cytometry cohort, patients with Ewing sarcoma were classified as “positive” or “negative” for bone marrow micrometastatic disease if the CD99<sup>+</sup> CD45<sup>+</sup> percent was respectively above or below the upper limit observed in control subjects. In addition, the continuous data were used in subanalyses.

For both cohorts, the clinical outcome variables available at diagnosis are listed in Table 1. Age, tumor site, and tumor size were further dichotomized as listed in Table 2. Clinical features were compared between groups defined as positive or negative for micrometastatic disease using the exact conditional test of equality of proportions.

**Event-free survival (EFS) was defined as the time from diagnosis for the flow cytometry cohort or time from enrollment onto AEWS0031 for the PCR cohort to relapse, progression, diagnosis of second malignancy, death, or date of last patient contact, whichever occurred first. Patients who experienced a relapse, disease status at diagnosis were considered to have “died.”**

**Table 1. Clinical characteristics of the primary analytic cohorts**

<table>
<thead>
<tr>
<th></th>
<th>Flow cytometry cohort (N = 109)</th>
<th>PCR Cohort (N = 225)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>64 (58.7)</td>
<td>115 (51.1)</td>
</tr>
<tr>
<td>Female</td>
<td>45 (41.3)</td>
<td>110 (48.9)</td>
</tr>
<tr>
<td><strong>Age at diagnosis, years (median; range)</strong></td>
<td>13 (1-27)</td>
<td>12 (0-40)</td>
</tr>
<tr>
<td><strong>Primary tumor site</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bone</td>
<td>92 (85.2)</td>
<td>180 (80.0)</td>
</tr>
<tr>
<td>Soft tissue</td>
<td>16 (14.8)</td>
<td>45 (20.0)</td>
</tr>
<tr>
<td>Distal extremity</td>
<td>20 (18.5)</td>
<td>47 (20.1)</td>
</tr>
<tr>
<td>Proximal extremity</td>
<td>25 (23.1)</td>
<td>46 (20.4)</td>
</tr>
<tr>
<td>Pelvis</td>
<td>22 (20.4)</td>
<td>40 (17.8)</td>
</tr>
<tr>
<td>Spinal/parspinal</td>
<td>6 (5.6)</td>
<td>12 (5.3)</td>
</tr>
<tr>
<td>Thorax/abdomen</td>
<td>17 (15.7)</td>
<td>60 (26.7)</td>
</tr>
<tr>
<td>Head and neck</td>
<td>2 (1.9)</td>
<td>20 (8.9)</td>
</tr>
<tr>
<td>Other</td>
<td>16 (14.8)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td><strong>Longest tumor diameter, cm (median; range)</strong></td>
<td>9.7 (4.1-19.3)</td>
<td>N/A&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Disease status at diagnosis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Localized</td>
<td>74 (67.9)</td>
<td>225 (100.0)</td>
</tr>
<tr>
<td>Metastatic</td>
<td>35 (32.1)</td>
<td>0 (0.0)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Primary tumor site known for 108 patients in the flow cytometry cohort and for 225 patients in the PCR cohort.

<sup>b</sup>Longest tumor diameter known for 36 patients.

<sup>c</sup>Tumor size was not available on AEWS0031/AEWS02B1.
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Table 2. Association of clinical features with presence of detectable micrometastatic in flow cytometry and PCR cohorts

<table>
<thead>
<tr>
<th></th>
<th>Flow cytometry cohort</th>
<th>PCR Cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive* N (%)</td>
<td>Negative N (%)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>9 (14.1)</td>
<td>55 (85.9)</td>
</tr>
<tr>
<td>Female</td>
<td>5 (11.1)</td>
<td>40 (88.9)</td>
</tr>
<tr>
<td>Age at diagnosis</td>
<td>&lt;18 years</td>
<td>11 (12.9)</td>
</tr>
<tr>
<td></td>
<td>≥18 years</td>
<td>3 (12.5)</td>
</tr>
<tr>
<td>Primary tumor site</td>
<td>Bone</td>
<td>13 (14.1)</td>
</tr>
<tr>
<td></td>
<td>Soft tissue</td>
<td>1 (6.3)</td>
</tr>
<tr>
<td></td>
<td>Appendicular</td>
<td>5 (11.1)</td>
</tr>
<tr>
<td></td>
<td>Axial</td>
<td>7 (14.9)</td>
</tr>
<tr>
<td></td>
<td>Pelvic</td>
<td>5 (22.7)</td>
</tr>
<tr>
<td></td>
<td>Non-pelvic</td>
<td>9 (10.5)</td>
</tr>
<tr>
<td>Longest tumor diameter</td>
<td>&lt;8 cm</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>≥8 cm</td>
<td>4 (17.4)</td>
</tr>
<tr>
<td>Disease status at diagnosis</td>
<td>Localized</td>
<td>7 (9.5)</td>
</tr>
<tr>
<td></td>
<td>Metastatic</td>
<td>7 (20.0)</td>
</tr>
</tbody>
</table>

aCD99⁺CD45⁻ (bright + dim) percent burden classified as "positive" or "negative" for bone marrow micrometastatic disease if the CD99⁺CD45⁻ percent was respectively above or below the upper limit observed in control subjects.

bP refers to the exact conditional test of the quality of proportions across groups.

cTumor size was not available on AEWS0031/AEWS02B1.

dAll patients in the PCR cohort had localized (nonmetastatic) disease.

Results

Patient characteristics

The clinical characteristics at diagnosis in the two primary analytic cohorts (flow cytometry cohort, N = 109; and PCR cohort, N = 225) are listed in Table 1. Features were typical of this disease.

Burden of micrometastatic disease in patients with Ewing sarcoma

In the flow cytometry cohort, the 109 patients with Ewing sarcoma had a median total bone marrow CD99⁺CD45⁻ (bright + dim) percent of 0.0012% (range 0%–1.10%; Fig. 1). The 22 control subjects had a median CD99⁺CD45⁻ (bright + dim) percent of 0.00038% (range 0%–0.0082%; P = 0.015). Only 14 (12.8%) Ewing sarcoma patients had CD99⁺CD45⁻ (bright + dim) percent values above the upper limit seen in controls and were defined as "positive."

We repeated these analyses, focusing exclusively on events in the CD99⁺CD45⁻ (bright) gate in the flow cytometry cohort. The Ewing sarcoma patients had a median total CD99⁺CD45⁻ (bright) percent of 0.00012% (range 0%–0.36%). The 22 control subjects had a median CD99⁺CD45⁻ (bright) percent of 0.00004% (range 0%–0.0017%; P < 0.001). Again, only 14 (12.8%) Ewing sarcoma
patients had CD99⁺CD45⁻ (bright) percent values above the upper limit in controls.

To confirm that the CD99⁺CD45⁻ cells represent Ewing sarcoma cells in the flow cytometry cohort, we used FACS to collect cells from two bone marrow samples. We then tested the cells for the presence of the characteristic EWSR1 translocation using FISH in a commercial laboratory (Genzyme Corp) and demonstrated the presence of this translocation in ≥88% of the isolated cells in both samples.

In the PCR cohort, 19.6% (44/225) of patients were “positive” for any EWSR1/FLI1 translocation by RT-PCR in either blood and/or bone marrow. The rate of PCR positivity was similar between patients evaluated in peripheral blood (25/122; 20.5%) versus bone marrow (19/103; 18.5%).

Detection of micrometastatic disease is not associated with clinical features or outcome

We next evaluated whether detectable micrometastatic disease was associated with clinical features (Table 2). There were no differences in the distribution of clinical features between patients classified as ‘positive’ versus ‘negative’ for micrometastatic disease by either flow cytometry or RT-PCR (Table 2). For the flow cytometry cohort, sensitivity analyses focused on results using the CD99⁺CD45⁻ (bright) gate yielded similar results. We next compared CD99⁺CD45⁻ cell burdens among groups defined by clinical features of interest (Supplementary Table S1). The only statistically significant difference was that male patients had higher CD99⁺CD45⁻ cell burdens than female patients.

We next evaluated the impact of micrometastatic disease on EFS and OS. EFS and OS were similar between flow cytometry cohort patients classified by flow cytometry as “positive” or “negative” for bone marrow micrometastatic disease according to the upper limit from control subjects (Fig. 2A and B). We performed a series of sensitivity analyses to confirm this lack of association in the flow cytometry cohort. We repeated our survival analyses using data from the CD99⁺CD45⁻ (bright) gate and again saw no difference in EFS or OS between groups. We also repeated our primary survival analyses using only data from patients with localized disease and again saw no difference in EFS or OS between groups. We utilized Cox proportional hazard methods to evaluate bone marrow micrometastatic disease burden as a continuous variable, without a statistically significant association with either EFS or OS.

This analysis was repeated using results in the PCR cohort. EFS and OS were similar between patients classified as “positive” or
did not observe an association between detectable micrometastatic disease and clinical outcome. Finally, we were able to utilize flow cytometry to quantify IGF-1R coexpression on occult bone marrow Ewing sarcoma cells and observed a wide range of IGF-1R expression using flow cytometry between patients, but overall much higher expression than normal bone marrow hematopoietic cells.

Our findings of the lack of association between micrometastatic disease burden and clinical characteristics and outcome in patients with newly diagnosed Ewing sarcoma confirm and extend previous observations (2, 8, 13). Of particular interest, Ash and colleagues retrospectively analyzed 46 archival frozen bone marrow samples from newly diagnosed Ewing sarcoma patients (of which 35 bone marrow samples were from patients with localized disease) using a similar multiparametric flow cytometry method. They observed no significant differences in outcome and clinical parameters according to the level of occult bone marrow involvement (13). Another PCR-based study also observed that among patients with metastases without morphologic evidence of bone marrow involvement, the frequency of bone marrow PCR positivity was similar to that observed in patients with clinically localized disease (6). In contrast, in the largest retrospective PCR-based study to date, Schleiermacher and colleagues reported a decrease in 2-year disease-free survival from 80% to 53% when bone marrow micrometastasis testing was positive (6). Bone marrow PCR positivity, but not micrometastatic disease detection in the peripheral blood, was also reported as a strong prognostic marker in another smaller retrospective study (3). Consistent with our results, another group showed that detection of PCR fusion transcripts at diagnosis was not prognostic (2). However, this group showed that the persistence of peripheral blood and/or bone marrow transcripts after initiation of therapy may identify relapse in patients before it is clinically apparent by conventional imaging studies (2).

Interestingly, Ash and colleagues reported that flow cytometry identified micrometastatic disease in all diagnostic bone marrow samples from patients with localized disease (13). This incidence of 100% is in sharp contrast to our study where <20% of patients had detectable micrometastastic disease by either method. Our incidence of micrometastatic disease in the flow cytometry and PCR cohorts is more consistent with results from prior PCR-based studies (2–9). There are several important differences between the study by Ash and colleagues and our study. The report from Ash and colleagues does not provide data on the upper limit of cell detection in their 10 normal controls, although they report using a cutoff of 0.001% to define a sample as having a positive signal. Our study used a larger number of controls, and it is therefore possible that the study of only 10 control samples by Ash and colleagues did not provide the full range of normal cell burden in the gate of interest. We note that our cutoff for positivity (>0.0082%) is more stringent than that of Ash and colleagues (>0.001%); theirs is similar to the median CD99$^+$ CD45$^-$ cell burden of positive patient samples in our study. Also, the study by Ash and colleagues used CD90 as an Ewing sarcoma marker. CD90 (Thy-1) is expressed on human hematopoietic progenitor cells as well as on Ewing sarcoma cells (23–25), and its use may have increased their positivity rate. Moreover, all samples from that study were obtained from a single institution and therefore were not subject to any tumor cell degradation that might occur during overnight shipping. Despite these differences, the overall conclusion that micrometastatic cell burden is not prognostic is shared between these studies.

**Flow cytometry enables quantification of IGF-1R coexpression on disseminated Ewing sarcoma cells**

Finally, we evaluated the ability of flow cytometry to quantify IGF-1R coexpression on occult bone marrow Ewing sarcoma cells from 20 patients (Fig. 3). We observed significantly higher IGF-1R expression on CD99$^+$ CD45$^-$ (bright) cells (mean gMFI, 982.7; SD, 867.8) compared with CD45$^+$ hematopoietic cells (mean gMFI, 190.9; SD, 99.0; P < 0.001).

**Discussion**

In these two large prospective studies of micrometastatic disease burden in newly diagnosed Ewing sarcoma patients, we observed that 12.8% and 19.6% had detectable micrometastatic disease by flow cytometry and RT-PCR methods, respectively. The presence of detectable micrometastatic disease by flow cytometry or RT-PCR was not associated with conventional prognostic factors in this disease, such as age, stage, and tumor site. We also did not observe an association between detectable micrometa-

**Figure 3.** Box plots of IGF-1R (CD221) coexpression expressed as gMFI on occult bone marrow Ewing sarcoma cells (CD99$^+$ CD45$^-$ bright) versus normal bone marrow hematopoietic cells (CD45$^+$).

“negative” for the presence of fusion transcript in blood and/or bone marrow (Fig. 2C and D).

CD99$^+$ CD45$^-$ cell burden by flow cytometry in patients with bone marrow metastasis and in patients with relapsed Ewing sarcoma

The preceding analyses focused on patients with newly diagnosed Ewing sarcoma without clinical evidence of bone marrow metastasis. In the course of the flow cytometry study, six samples were obtained from patients with morphologically evident bone marrow metastatic disease at initial presentation (Supplementary Table S2), and five samples were obtained from patients with relapsed disease. The median CD99$^+$ CD45$^-$ cell burden for patients with clinically evident bone marrow metastasis was 0.76% (range 0.013%–27.3%) compared with 0.0012% (range 0%–1.1%) for patients without morphologic bone marrow metastasis (P < 0.001). The median CD99$^+$ CD45$^-$ cell burden from samples obtained at the time of disease recurrence (0.0011%, range 0.00008%–1.6%) was not significantly different compared with patients with newly diagnosed disease (0.0012%, range 0%–1.1%; P = 0.92).

Flow cytometry enables quantification of IGF-1R coexpression on disseminated Ewing sarcoma cells

Finally, we evaluated the ability of flow cytometry to quantify IGF-1R coexpression on occult bone marrow Ewing sarcoma cells from 20 patients (Fig. 3). We observed significantly higher IGF-1R expression on CD99$^+$ CD45$^-$ (bright) cells (mean gMFI, 982.7; SD, 867.8) compared with CD45$^+$ hematopoietic cells (mean gMFI, 190.9; SD, 99.0; P < 0.001).
Although the flow cytometry cohort included patients with localized and metastatic disease, the rate of detection of micrometastatic disease was lower compared with the PCR cohort that included only localized patients. Our lower rates of bone marrow micrometastases by flow cytometry compared with PCR-based studies may be reflective of lower assay sensitivity compared with PCR, bone marrow sampling heterogeneity, and potential dilution with peripheral blood during the bone marrow collection. Indeed, these same factors may partly explain why 2 of 6 patients with clinically evident bone marrow metastases had CD99⁺CD45⁻ percent value of <0.1%. Although all 6 samples had CD99⁺CD45⁻ cell burdens above our upper limit of normal, some values were lower than expected. It is common for morphology assessments of solid tumor disease burdens to differ between sides and between the aspirates and core biopsies (26, 27). Therefore, bilateral bone marrow aspirations were routinely performed for Ewing sarcoma patients due to the potential for patchy tumor involvement. Although bone marrow and peripheral blood collection were standardized in both cohorts, our studies did not specify whether both sides are pooled together or sent separately. We also acknowledge that we have only binary clinical data on the presence or absence of bone marrow metastatic disease and do not have more specific clinical details, such as percent of bone marrow involvement reported in aspirate material, biopsy material, or laterality of involvement.

Given the negative conclusion that micrometastatic tumor burden was not associated with survival, it is important to note that the two techniques available at the start of these two prospective cohort studies (RT-PCR and flow cytometry) may have had inadequate sensitivity to quantify fully the extent of micrometastatic disease in patients. Newer measures of micrometastatic disease, such as circulating tumor DNA (ctDNA), may be of interest to evaluate as a potential prognostic marker, as DNA provides a more stable source of material for analysis compared with whole cells (flow cytometry cohort) or mRNA (RT-PCR cohort).

A key advantage of our study is our ability to prospectively study and analyze micrometastatic disease burden using two complementary methods of detection in two separate Ewing sarcoma cohorts. Collectively, this is the largest study of the prognostic value of micrometastatic disease at diagnosis in Ewing sarcoma patients. Although several studies have used the PCR-based method to study micrometastatic disease burden, we introduced an alternative method of detection in this investigation that exploits the near universal expression of CD99 and lack of expression of CD45 by Ewing sarcoma cells, allowing evaluation of micrometastatic disease burden in nearly all patients with Ewing sarcoma.

Given our primary finding of lack of association of CD99⁺CD45⁻ cell burden with prognosis, our investigation of IGF-1R expression by flow cytometry provides important proof of principle of the utility of this assay to characterize (rather than just quantify) detected Ewing sarcoma tumor cells. Indeed, we show that flow cytometry provides a potential way to quantify the expression of a clinically relevant target on Ewing sarcoma tumor cells and provides a new pharmacodynamic tool that can be applied to clinical trials of inhibitors of IGF-1R in Ewing sarcoma. As a result of our work, this novel pharmacodynamic assay has been incorporated in an ongoing COG clinical trial using an IGF-1R inhibitor plus standard chemotherapy in patients with newly diagnosed metastatic Ewing sarcoma (NCT02306161). However, we acknowledge that it is not known whether the IGF-1R expression on occult Ewing sarcoma bone marrow cells as detected by flow cytometry correlates with overall IGF-1R expression in other primary or metastatic tumor samples.

In summary, the key strengths in our study of this rare pediatric malignancy include (i) use of two complementary approaches for the detection of micrometastatic disease burden; (ii) evaluation of peripheral blood and bone marrow in the RT-PCR cohort; (iii) use of prospective samples from patients participating in COG cooperative group studies; and (iv) our large sample size relative to the number of patients diagnosed with Ewing sarcoma each year. On the basis of our findings, we conclude that the detection of micrometastatic disease by either flow cytometry or PCR does not impact outcomes in newly diagnosed Ewing sarcoma. Although the prognostic potential of micrometastasis detection at diagnosis was not seen, there is evidence of the correlation between the presence of micrometastases in the bone marrow during follow-up and disease progression in a small retrospective PCR-based study (2). Therefore, assessment of occult tumor cells during therapy, at the end of the therapy, and during long-term follow-up as a marker of treatment resistance may be a more appropriate application of these approaches. In addition, novel approaches to detecting micrometastatic disease, such as quantification of ctDNA, may be of value in future studies.

Disclosure of Potential Conflicts of Interest
S.L. Lessnick is listed as a co-inventor on patents on the diagnosis and treatment of drug-resistant Ewing sarcoma and the methods and compositions for the diagnosis and treatment of Ewing sarcoma, which are both owned by The University of Utah Research Foundation, and has ownership interest (including patents) and is a consultant/advisory board member for Salarius Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

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Acknowledgments
The authors thank Shelly Allen for her administrative assistance as clinical research coordinator and assistance with maintaining the patient database for the flow cytometry cohort and Damon Jacobson at Seattle Children’s Hospital.

www.aacrjournals.org Clin Cancer Res; 2016 0F7

Published OnlineFirst February 9, 2016; DOI: 10.1158/1078-0432.CCR-15-2516

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for his assistance in obtaining samples for the flow cytometry study. The authors also thank Heather Hartig and Alice Tan in the UCSF Core Immunology Lab for their technical assistance in flow cytometry techniques.

Grant Support
This work was supported in part by the NIH grant K23 CA154530 (to S.G. DuBois); NIH Intramural Research Program (to C.L. Mackall); NIH grant P30AI027763 to the UCSF-GEI Center for AIDS Research (UCSF Flow Cytometry Core Laboratory); Alex's Lemonade Stand Foundation (to K.T. Vo, K.K. Matthay, and S.G. DuBois); Frank A. Campbell Foundation (to K.K. Matthay and S.G. DuBois); Hope Street Kids (to S.G. DuBois); Sarcoma Foundation of America (to S.G. DuBois); CureSearch for Children's Cancer (to S.G. DuBois); John M. Gilbertson Foundation (to D.S. Hawkins); Daniel P. Sullivan Fund (to R.B. Womer); WWWW (QuadW) Foundation (to M.D. Krailo and D.A. Bar- kauskas); Children's Oncology Group grants (U10CA180886, U10CA180899, U10CA098543, and U10CA098413). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received October 19, 2015; revised January 6, 2016; accepted January 29, 2016; published OnlineFirst February 9, 2016.

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Impact of Two Measures of Micrometastatic Disease on Clinical Outcomes in Patients with Newly Diagnosed Ewing Sarcoma: A Report from the Children's Oncology Group


Clin Cancer Res Published OnlineFirst February 9, 2016.

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