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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

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Abstract

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

Experimental Design—Newly diagnosed patients with ES were enrolled on two prospective multi-center studies. In the flow cytometry cohort, patients were defined as "positive" for BM 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 BM 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. Co-expression of IGF-1R on detected tumor cells was performed in a subset of flow cytometry samples.

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

Conclusion—The detection of micrometastatic disease at initial diagnosis by flow cytometry or RT-PCR is not associated with outcome in newly diagnosed patients with ES. Flow cytometry provides a tool to characterize occult micrometastatic tumor cells for proteins of interest.

Keywords

Ewing sarcoma; micrometastatic disease; survival; flow cytometry; polymerase chain reaction

INTRODUCTION

Ewing sarcoma (ES) is the second most common primary bone cancer in children and young adults.(1) The 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 ES cells can be detected in the peripheral blood (PB) and in bone marrow (BM) using a polymerase chain reaction (PCR) assay to detect *EWSR1* fusion transcripts characteristic of this tumor.(2–9) PCR studies have shown that approximately 20–43% of patients with newly diagnosed Ewing sarcoma have detectable *EWSR1* fusion transcripts in their PB and/or BM.(2–9) PCR benefits from high sensitivity, but requires knowledge of the subtype of *EWSR1* translocation. Moreover, tumor cells cannot be further characterized by this approach. To circumvent these concerns, we developed a flow cytometry assay for detecting ES cells in a background of hematopoietic cells.(10) This technique relies on the characteristic CD99-positive/CD45-negative (CD99+CD45−) immunophenotype of Ewing sarcoma cells.(11, 12) While this approach does not require knowledge of the specific *EWSR1* translocation, the flow cytometry approach is less sensitive than PCR, with a sensitivity of approximately 1 tumor cell in 500,000 hematopoietic cells.(10)

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 PB and/or BM 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 BM micrometastatic disease burden.(13) Beyond initial diagnosis, the persistence of PB and/or BM fusion transcripts after chemotherapy has been shown to confer an adverse prognosis.(2)

ES cells commonly overexpress the insulin-like growth factor-1 receptor (IGF-1R).(14, 15) Monoclonal antibodies against IGF-1R have resulted in objective clinical responses in patients with ES(16–19) and this class of agents is being evaluated in newly diagnosed patients with metastatic ES. 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 multi-center 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 BM and/or PB using two complementary approaches, RT-PCR and flow cytometry, in patients with newly diagnosed ES. 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 co-expression on occult BM ES 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 ES 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 multi-center study focused on flow cytometry detection of BM ES cells (UCSF; Seattle Children's Hospital; Primary Children's Hospital; and Dana-Farber Cancer Institute); and the national COG ES biology study (AEWS07B1). Each treating institution obtained institutional review board approval. In addition to providing BM samples for analysis, participating sites also provided follow-up data and clinical characteristics at the time of diagnosis, including: age; sex; tumor site; tumor size; 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 BM metastatic disease and had an evaluable BM sample from diagnosis (as defined below). Patients with clinical evidence of BM 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.

BM samples from adult and pediatric subjects at UCSF without cancer were used as control samples. These subjects underwent BM aspiration for evaluation of a non-malignant

hematologic disorder with consent to obtain additional material or for allogeneic BM donation with anonymized leftover material used for this study. Samples from allogeneic bone marrow donors (n = 16) were de-identified and did not have clinical features available. Patients who provided bone marrow samples during bone marrow evaluation for non-malignant conditions (n = 6) had a median age of 6 years (range 1–18) and 33% were males.

Each subject provided 5–10 mL of fresh aspirated BM collected into ethylene diamine tetra-acetic acid (EDTA) tubes prior to the initiation of chemotherapy. Samples were shipped fresh overnight 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 ES who were enrolled on the COG randomized trial of interval compressed chemotherapy (AEWS0031, NCT00006734) between 2001 and 2005.(20) Each treating institution obtained institutional review board approval and informed consent. Patients submitted a baseline sample of 10 ml PB and/or 2–4 mL BM aspirate in EDTA tubes for analysis. PB and BM samples were shipped fresh overnight on ice to the Biopathology Center in Columbus, Ohio. A patient was included in the PCR analytic cohort if they had a RT-PCR evaluable blood or BM sample before enrollment or within 30 days of enrollment on the AEWS0031 study.

Method of Micrometastatic Disease Detection

Flow cytometry cohort: antibodies and flow cytometry—CD99-PE and CD34-FITC were obtained from BD Bioscience/Pharmigen (San Jose, CA). CD45-PB, CD14-ECD, and CD221-APC were obtained from Thermo Fisher (Waltham, MA), Beckman Coulter (Miami, FL), and eBioscience (San Diego, CA), respectively. LIVE/DEAD Fixable Dead Cell Stain Kit, aqua-fluorescent amine reactive dye (AARD), was obtained from Invitrogen (Carlsbad, CA). Human Gamma Globulin (HGG) was obtained from BioDesign International (Saco, ME). CD221-APC (IGF-1R) was only added to the panel in the last two years of the study.

Bone marrow mononuclear cells (BMMCs) 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, Hayward, CA). One to eight million cells were washed in wash buffer, labeled for 20 min at room temperature with four times the recommended volume of AARD, blocked with HGG buffer, stained with CD99-PE, CD45-PB, CD14-ECD, 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, Ashland, OR).

The gating strategy for the six-color panel was as follows. A debris exclusion gate was set on a forward and side scatter plot. A CD45+ gate was then drawn on all CD45+ 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+ monocytes, and CD34+ progenitor cells were sequentially removed. As previously described, ES cells derived from established A673 cell lines (ATCC; Manassas, VA) were "spiked" into control peripheral

blood mononuclear cell (PBMC) samples to identify an appropriate CD99 "positive" gate. (10) A673 cells were not authenticated upon receipt from ATCC, but their CD99+CD45− immunophenotype remained consistent throughout the study. "Unspiked" control PBMC samples were used as the negative controls in these flow cytometry assays. A CD99+CD45− gate was set to include ES cells, but exclude CD45− cells on a CD99 versus CD45 plot. In sub-analyses, the proportion of CD99+CD45− cells was further divided into those with bright CD99 expression (CD99+CD45− bright). Results in a given gate were expressed as a percentage of the total CD45+ BMMCs. Only samples with > 750,000 total CD45+ BMMCs were considered evaluable.

PCR cohort: molecular analysis—RNA was extracted from samples in RLT buffer using the RNAeasy kit (Qiagen) according to the manufacturer's instructions, and 1 μg of RNA was used for cDNA synthesis (1st Strand Synthesis Kit, Roche, Indianapolis, IN). Each cDNA sample was analyzed for quality using G6PD-specific primers (G6PD1: 5'-CCGGATCGACCACTACCTGGGCAAG-3'; G6PD2: 5'-GTTCCCCACGTACTGGCCCAGGACCA-3') and probes (G6PDHP1: 5'-GTTCCAGATGGG-GCCGAAGATCCTGTTG; G6PDHP2: 5'-CAAATCTCAGCACCATGAGGTTCTGCAC-3') and only samples with a crossing time less than 30 cycles were considered adequate for analysis of *EWSR1/FLI1*. Five μL of cDNA was used for the first PCR reaction (20 cycles) performed using ESBP1 (5'-CGACTAGTTATGATCAGAGCA-3') and ESBP2 (5'-CCGTTGCTCTGT-ATTCTTACTGA-3') primers, then 2 μL of amplified DNA was amplified in a second (nested) PCR reaction (40 cycles) (Lightcycler, Roche Molecular Biochemicals, Indianapolis, IN) using *EWSR1* 696 (5'-AGCAGCTATGGACAGCAG-3') and *FLI1* 1041 (5'-TTGAGGCCAGAA-TTCATGTT-3') primers. *EWSR1/FLI1* primer pairs amplify regions outside the breakpoint of the translocation and thus amplify Type 1 or Type 2 translocations. Hybridization probes spanning the *EWSR1/FLI1* type 1 (EWSHP1: 5'-TATAGCCAACAGAGCAGCAGC-3'; EWSHP2: 5'-GGCAGCAGAACCCTTCTT) or *EWSR1/FLI1* type 2 (5'-TCCTACAGCCAA-GCTCCAAGTCAATA-3'; 5'-AGCCAACAGAGCAGCAGCTACGGGCAGCAGA-3') breakpoint were used to detect target template. All samples were analyzed for the Type 1 translocation. 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 BM and PB 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+CD45− percent) or by RT-PCR was the primary predictor variable of interest. In the flow cytometry cohort, patients with ES were classified as "positive" or "negative" for BM micrometastatic disease if the CD99+CD45− 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, second malignancy, or died were considered to have an event. All other patients were censored for EFS at last contact. Overall survival (OS) was defined as the time from diagnosis for the flow cytometry cohort or time from enrollment onto AEWS0031 for the PCR cohort until last patient contact. Patients who died were considered to have experienced an OS event; otherwise the patient was considered censored. EFS and OS as a function of time since the start of follow-up were estimated using the method of Kaplan-Meier.(21) Risks for EFS-event or death were compared between patients with and without detectable micrometastatic disease using two-sided log-rank test.(22)

The flow cytometry cohort provided an opportunity to evaluate the burden of micrometastatic disease as a continuous variable. We used Cox proportional hazards regression models to evaluate the prognostic impact of CD99+CD45− percent as a continuous variable.

IGF-1R (CD221) co-expression was assessed using the geometric mean fluorescence intensity (gMFI) in the flow cytometry cohort. Populations of interest [CD99+CD45− (bright) cells and CD45+ hematopoietic cells] were compared using paired Student *t*-tests, matched per patient.

All statistical analyses were performed using STATA, version 13 (StataCorp, College Station, TX).

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 ES had a median total BM CD99+CD45− (bright+dim) percent of 0.0012% (range 0–1.10%; Figure 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%) of ES 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 ES 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%) ES patients had CD99+CD45− (bright) percent values above the upper limit in controls.

In order to confirm that the CD99+CD45− cells represent ES cells in the flow cytometry cohort, we used fluorescent activated cell sorting (FACS) to collect cells from two bone marrow samples. We then tested the cells for the presence of the characteristic *EWSR1* translocation using fluorescent in situ hybridization (FISH) in a commercial laboratory (Genzyme Corp, Cambridge, MA) 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 BM. The rate of PCR positivity was similar between patients evaluated in PB (25/122; 20.5%) vs. BM (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” vs. “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 between groups defined by clinical features of interest (Supplemental Table 1). 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 BM micrometastatic disease according to the upper limits from control subjects (Figures 2A and 2B). 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) gates 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 BM 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 “negative” for the presence of fusion transcript in blood and/or BM (Figures 2C and 2D).

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 ES without clinical evidence of BM metastasis. In the course of the flow cytometry study, six samples were obtained from patients with morphologically evident BM metastatic disease at initial presentation (Supplemental Table 2) and five samples were obtained from patients with relapsed disease. The median CD99+CD45− cell burden for patients with clinically evident BM metastasis was 0.76% (range 0.013–27.3%) compared to 0.0012% (range 0–1.1%) for patients without morphologic BM 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 to patients with newly diagnosed disease (0.0012%, range 0–1.1%; $p=0.92$).

Flow Cytometry Enables Quantification of IGF-1R Co-Expression on Disseminated Ewing Sarcoma Cells

Finally, we evaluated the ability of flow cytometry to quantify IGF-1R co-expression on occult BM ES cells from 20 patients (Figure 3). We observed significantly higher IGF-1R expression on CD99+CD45− (bright) cells (mean gMFI 982.7, SD 867.8) compared to 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 ES patients, we observed that 12.8% and 19.6% had detectable micrometastatic disease by flow cytometry and RT-PCR methods, respectively. 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 micrometastatic disease and clinical outcome. Lastly, we were able to utilize flow cytometry to quantify IGF-1R co-expression on occult BM ES cells and observed a wide range of IGF-1R expression using flow cytometry between patients, but overall much higher expression than normal BM hematopoietic cells.

Our findings of the lack of association between micrometastatic disease burden and clinical characteristics and outcome in patients with newly diagnosed ES confirm and extend previous observations.(2, 8, 13) Of particular interest, Ash and colleagues retrospectively analyzed 46 archival frozen BM samples from newly diagnosed ES patients (of which 35 BM samples were from patients with localized disease) using a similar multi-parametric flow cytometry method. They observed no significant differences in outcome and clinical parameters according to the level of occult BM involvement.(13) Another PCR-based study also observed that the among patients with metastases without morphologic evidence of BM involvement, the frequency of BM PCR positivity is similar to that observed in patients with clinically localized disease.(6) In contrast, in the largest retrospective PCR-based study to date, Schleiermacher et al. reported a decrease in 2-year disease free survival from 80% to 53% when BM micrometastasis testing was positive.(6) BM PCR positivity, but not micrometastatic disease detection in the PB, 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 PB and/or BM transcripts after initiation of therapy may identify relapse in patients before it is clinically apparent by conventional imaging studies.(2)

Interestingly, Ash et al. reported that flow cytometry identified micrometastatic disease in all diagnostic BM samples from patients with localized disease.(13) This incidence of 100% is in sharp contrast to our study where < 20% of patients had detectable micrometastatic 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 et al. does not provide data on the upper limit of cell detection in their 10 normal controls, though they report using a cut-off 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 study of only 10 control samples in the Ash experience did not provide the full range of normal cell burden in the gate of interest. We note that our cut-off for positivity (>0.0082%) is more stringent than that of Ash et al. (>0.001%); theirs is similar to the median CD99+CD45 cell burden of positive patient samples in our study. Also, the Ash study used CD90 as an Ewing sarcoma marker. CD90 (Thy-1) is expressed on human hematopoietic progenitor cells as well as on ES 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 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.

While the flow cytometry cohort included patients with localized and metastatic disease, the rate of detection of micrometastatic disease was lower compared to the PCR cohort that included only localized patients. Our lower rates of BM micrometastases by flow cytometry compared to PCR-based studies may be reflective of lower assay sensitivity compared to PCR, BM sampling heterogeneity, and potential dilution with PB during the BM collection. Indeed, these same factors may partly explain why 2/6 of the patients with clinically evident BM 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 BM aspirations were routinely performed for ES patients, due to the potential for patchy tumor involvement. While BM and PB 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 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 to 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 ES cohorts. Collectively, this is the largest study of the prognostic value of micrometastatic disease at diagnosis in ES patients. While 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 ES cells, allowing evaluation of micrometastatic disease burden in nearly all patients with ES.

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 ES tumor cells. Indeed, we show that flow cytometry provides a potential way to quantify the expression of a clinically relevant target on ES tumor cells and provides a new pharmacodynamic tool that can be applied to clinical trials of inhibitors of IGF-1R in ES. 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 ES (NCT02306161). However, we acknowledge that it is not known whether the IGF-1R expression on occult ES BM 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: 1) use of two complementary approaches for the detection of micrometastatic disease burden; 2) evaluation of PB and BM in the RT-PCR cohort; 3) use of prospective samples from patients participating in COG cooperative group studies; and 4) our large sample size relative to the number of patients diagnosed with ES each year. On the basis of our findings, we conclude that detection of micrometastatic disease by either flow cytometry or PCR does not impact outcomes in newly diagnosed ES. While the prognostic potential of micrometastasis detection at diagnosis was not seen, there is evidence of the correlation between the presence of micrometastases in the BM 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 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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STATEMENT OF TRANSLATIONAL RELEVANCE

Ewing sarcoma (ES) 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 detection of micrometastatic disease by either method is not associated with outcome in newly diagnosed ES. 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 detected ES tumor cells, we evaluated co-expression of IGF-1R. We show that flow cytometry provides a potential way to quantify the expression of ES tumor cell IGF-1R. 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 ES (NCT02306161).

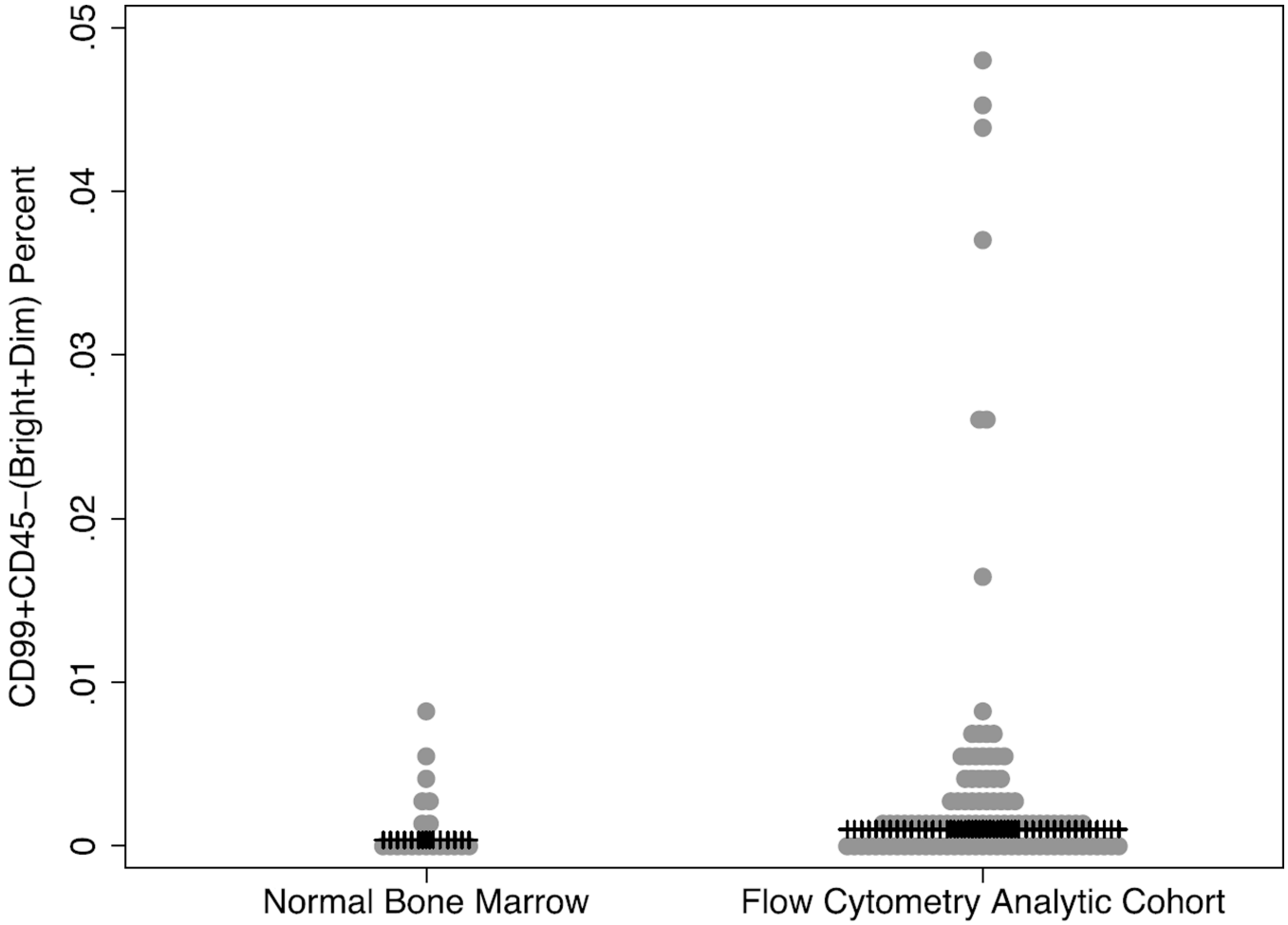

Figure 1.

Dot plot of CD99+CD45− (bright+dim) percent in primary flow cytometry analytic cohort (N=109) compared to normal BM controls (N=22). Values were truncated if CD99+CD45− (bright+dim) percent >0.05 [truncated in N=6 (range of values = 0.071 – 1.1) from analytic cohort vs. N=0 from normal BM controls]; median values for each group is shown by horizontal crosses.

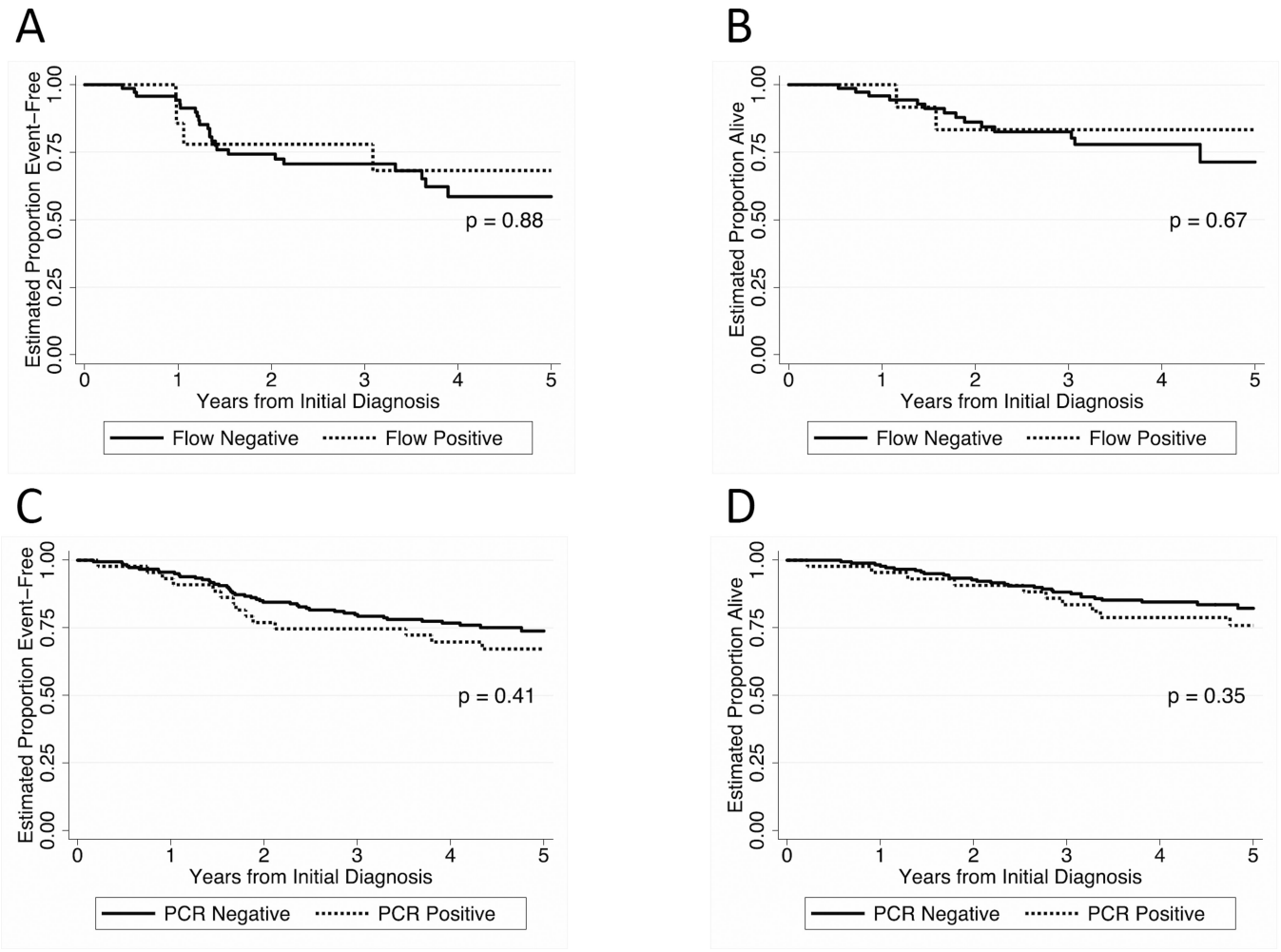

Figure 2.

Kaplan-Meier estimated (A) event-free survival and (B) overall survival of flow cytometry cohort by CD99+CD45− (bright+dim) percent classified as “positive” or “negative” for BM micrometastatic disease according to upper limit from control subjects. Kaplan-Meier estimated (C) event-free survival and (D) overall survival of PCR cohort according to presence (“positive”) or absence (“negative”) of detectable *EWSR1/FLI1* fusion transcript.

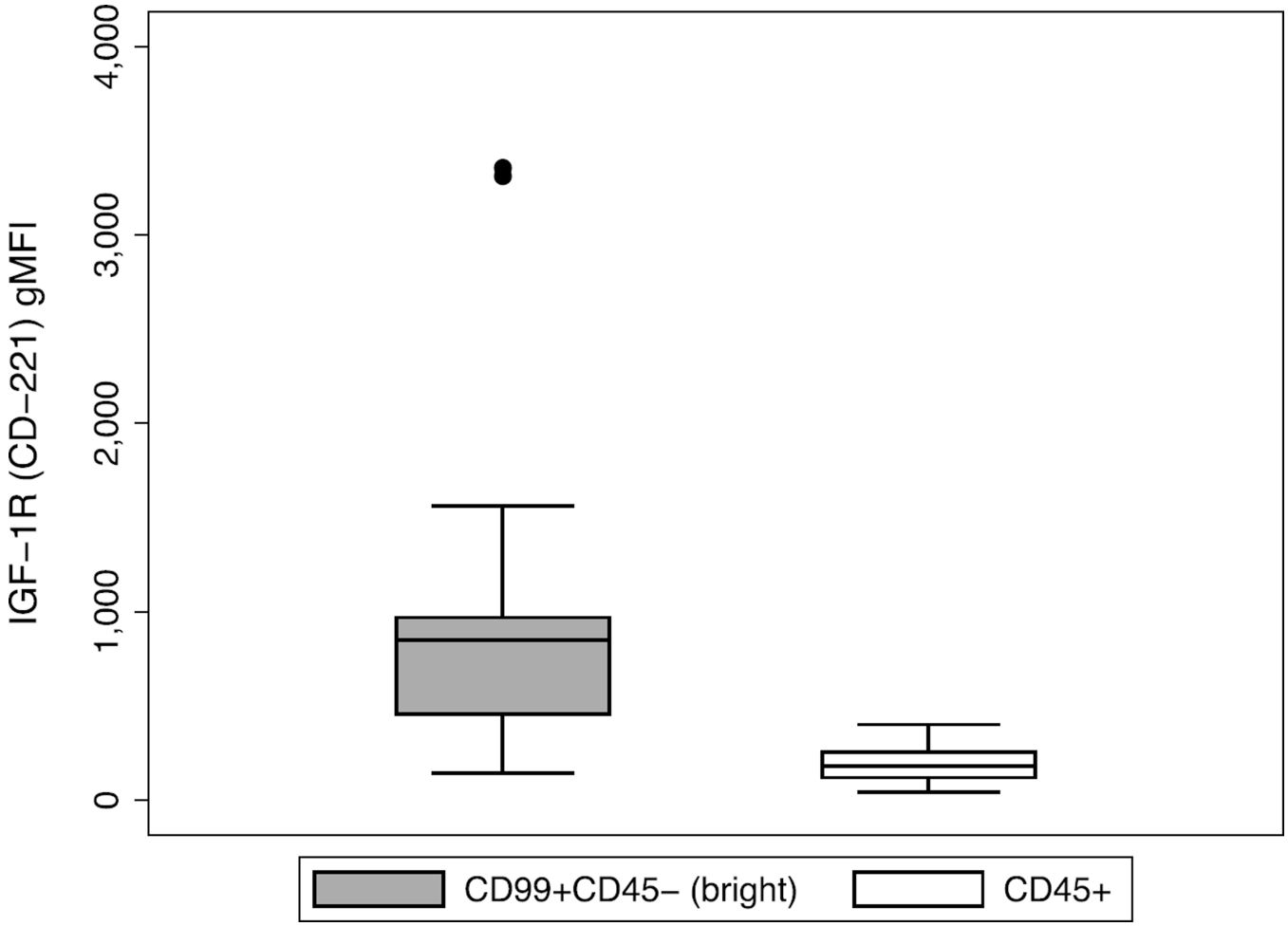

Figure 3.

Box plots of IGF-1R (CD221) co-expression expressed as geometric mean fluorescence intensity (gMFI) on occult BM Ewing sarcoma cells (CD99+CD45− bright) vs. normal BM hematopoietic cells (CD45+).

Table 1

Clinical characteristics of the primary analytic cohorts.

	Flow cytometry cohort (N=109)	PCR cohort (N=225)
	N (%)	
Sex		
Male	64 (58.7)	115 (51.1)
Female	45 (41.3)	110 (48.9)
Age at diagnosis, years (median; range)	13 (1 – 27)	12 (0 – 40)
Primary tumor site[1]		
Bone	92 (85.2)	180 (80.0)
Soft tissue	16 (14.8)	45 (20.0)
Distal Extremity	20 (18.5)	47 (20.1)
Proximal Extremity	25 (23.1)	46 (20.4)
Pelvis	22 (20.4)	40 (17.8)
Spinal/Paraspinal	6 (5.6)	12 (5.3)
Thorax/Abdomen	17 (15.7)	60 (26.7)
Head and Neck	2 (1.9)	20 (8.9)
Other	16 (14.8)	0 (0.0)
Longest tumor diameter, cm (median; range)[2]	9.7 (4.1–19.3)	N/A[3]
Disease Status at Diagnosis		
Localized	74 (67.9)	225 (100.0)
Metastatic	35 (32.1)	0 (0.0)

[1]Primary tumor site known for 108 patients in the flow cytometry cohort and for 225 patients in the PCR cohort.

[2]Longest tumor diameter known for 36 patients.

[3]Tumor size was not available on AEWS0031/AEWS02B1.

Table 2

Association of clinical features with presence of detectable micrometastatic in flow cytometry and PCR cohorts.

	Flow cytometry cohort			PCR cohort		
	Positive[1] N (%)	Negative N (%)	p-value[2]	Positive N (%)	Negative N (%)	p-value[2]
Sex						
Male	9 (14.1)	55 (85.9)	0.78	21 (18.3)	94 (81.4)	0.74
Female	5 (11.1)	40 (88.9)		23 (20.9)	87 (79.1)	
Age at diagnosis						
< 18 years	11 (12.9)	74 (87.1)	1.0	39 (19.6)	160 (80.4)	1.0
≥ 18 years	3 (12.5)	21 (87.5)		5 (19.2)	21 (80.8)	
Primary tumor site						
Bone	13 (14.1)	79 (85.9)	0.69	38 (21.1)	142 (78.9)	0.30
Soft tissue	1 (6.3)	15 (93.7)		6 (13.3)	39 (86.7)	
Appendicular	5 (11.1)	40 (88.9)	0.76	16 (17.2)	77 (82.8)	0.50
Axial	7 (14.9)	40 (85.1)		28 (21.2)	104 (78.8)	
Pelvic	5 (22.7)	17 (77.3)	0.16	9 (22.5)	31 (77.5)	0.66
Non-pelvic	9 (10.5)	78 (89.5)		35 (18.9)	150 (81.1)	
Longest tumor diameter						
< 8 cm	0 (0)	13 (100.0)	0.27	N/A[3]		
≥ 8 cm	4 (17.4)	19 (82.6)				
Disease Status at Diagnosis						
Localized	7 (9.5)	67 (90.5)	0.14	N/A[4]		
Metastatic	7 (20.0)	28 (80.0)				

[1] CD99+CD45− (bright+dim) percent burden classified as “positive” or “negative” for BM micrometastatic disease if the CD99+CD45− percent was respectively above or below the upper limit observed in control subjects.

[2] P-value refers to the exact conditional test of the quality of proportions across groups.

[3] Tumor size was not available on AEWS0031/AEWS02B1.

[4] All patients in the PCR cohort had localized (non-metastatic) disease.