Excellent Prognosis in a Subset of Patients with Ewing Sarcoma Identified at Diagnosis by CD56 Using Flow Cytometry

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Abstract

Purpose: Ewing sarcoma (ES) is considered a systemic disease with the majority of patients harboring micrometastases at diagnosis. Multiparameter flow cytometry (MPFC) was used to detect ES cells in bone marrow (BM) of ES patients at diagnosis and to evaluate the prognostic significance of CD56 expression in BM samples.

Experimental Design: BM samples from 46 ES patients, 6 tumor aspirates, 2 ES cell lines, and 10 control BM samples were analyzed by MPFC. ES cells were identified by the combination of CD45+/CD90+/CD99+. CD56 was evaluated on these cells by a cutoff of 22%.

Results: BM samples obtained from all patients at diagnosis were found to be positive for micrometastatic tumor cells assessed by CD99+/CD90+/CD45−. A total of 60% of the BM samples harbored high CD56 expression. There was a highly significant correlation between CD56 expression and progression-free survival (PFS; 69% in low/negative expression versus 30% in high expression groups, \( P = 0.024 \)). In patients with localized nonpelvic disease, those expressing low/negative CD56 had 100% PFS versus 40% in the high expressing group (\( P = 0.02 \)). By Cox regression analysis, CD56 was found to be an independent prognostic marker with an 11-fold increased risk for relapse in patients with localized disease (\( P = 0.006 \)).

Conclusion: All samples contained cells that are positive for the CD99+/CD90+/CD45− combination at diagnosis, indicating that ES is a systemic disease. CD56 expression could be used to reveal ES patients with excellent prognosis or patients predisposed to relapse, thus improving treatment stratification and implementation of personalized therapy.

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common antigen. Thus, the combination of both antigens CD99+ and CD45− can be used to evaluate ES tumor samples. Indeed, Dubois and colleagues (18) have recently shown that ES tumor cells can be accurately detected in a very low frequency of 1 in 100,000 normal cells in BM and PBL by FC using the combination of CD99 positive and CD45 negative cells. CD90 or Thy-1 is a 25 to 37 kDa heavily N-glycosylated, glycophosphatidylinositol-anchored conserved cell surface protein with a single V-like immunoglobulin domain, originally discovered as a thymocyte antigen in mice. CD90 (a stem cell–associated antigen) is expressed on subsets of hematopoietic (19) and nonhematopoietic stem cells (20, 21) and was found to be expressed in ES/primitive neuroectodermal tumors (PNET) and cell lines (22).

CD56, an isoform of neural adhesion molecule (NCAM), is an integral membrane protein expressed by a variety of normal cell types, including a range of neuroectodermal derivatives and natural killer cells. CD56 is expressed in a wide spectrum of neoplasms including epithelial neoplasms, some cases of ES and PNET (23–26). FC using CD99, CD56, and CD45 has been used to evaluate ES tumor samples by applying the profile CD99+/CD45− and CD99+/CD56+/CD45− (24, 27). In addition, the coexpression of bright CD90 increases the accuracy of ES diagnosis (22).

In this study, we used multiparameter FC (MPFC) to evaluate the presence of circulating ES cells in BM samples of ES patients at diagnosis by the combination of CD99+/CD90+/CD45− and to assess the prognostic value of CD56 expression on these cells.

**Patients and Methods**

We retrospectively analyzed BM samples from 46 patients with ES, treated at Schneider Children’s Medical Center of Israel, Petach Tikva, Israel, from 1990 to 2008. In 6 of these patients, tumor aspirates were also available. Mononuclear cells (MNC) extracted at diagnosis from BM of ES patients, tumor aspirates, and control BM samples were cryopreserved in DMSO in liquid nitrogen. This study was approved by the local and national ethics committees.

The median age at diagnosis was 15.8 years (0.3–26). The primary site was extremity in 19 of 46 (41.3%), pelvis-sacrum in 8 of 46 (17.4%), Askin tumor in 6 of 46 (13.1%), other axial 10 of 46 (21.7%), and multifocal 3 of 46 (6.5%). Thirty five (76%) of patients presented a localized disease (Table 1).

All patients were treated by an in-house protocol that included 6 courses of chemotherapy (vincristine, actinomycin-D, cyclophosphamide, doxorubicin ifosfamide, and etoposide), and high radiation dose of 4,500 to 6,000 cGy and surgery. Histopathological diagnosis was based on the appearance of small round cells; immunohistochemical staining included CD99, synaptophysin, chromogranin, and NSE. All tumor samples were positive for EWS-FLI-1 transcript by molecular analysis. Clinical staging was based on diagnostic imaging studies including x-rays, computerized tomography scans, MRI, and TC99 bone scans. Median follow-up was 60.9 months. Overall survival (OS) and progression-free survival (PFS) for the whole group were 67.6% and 50.5% in 5 years, respectively.

Ten control BM samples were obtained from children who were evaluated for hematological investigation and were found to be with no malignancy.

**Sample preparation and staining**

We implemented the MPFC methodology for MRD analysis (28) to evaluate ES cells in patient’s BM obtained at diagnosis. All samples were coded and evaluated blindly. Cells were stained as described previously, using 5-color combination of 8 different antibodies: CD99-FITC [clone TU12; Becton Dickinson (BD)], CD90-PE (clone 5E10, BD), CD45 Per-CP (clone 2D1, BD), CD3,14,16,19-PE-TU12; Becton Dickinson (BD), CD56-APC (clone NCAM-TU12; Becton Dickinson), CD45 Per-CP (clone 2D1, BD), CD3,14,16,19-PE-Cy7 [clones: UCHT1, RM052, 3G8, and J3-119, respectively, Beckman Coulter (BC)], CD56-APC (clone NCAM 16.2, BD).

At least 10⁸ defrosted MNCs were stained and analyzed on a FACSCanto II flow cytometer (BD Biosciences) by the Diva software for acquisition and analysis. For sensitivity analysis, serial dilutions of prelabeled and fixed ES cell line (MHHESS) were performed in PBS. Desired amounts were spiked into control BM that were then labeled with the 5-color combination and fixed with CellFix (BD; ref. 29).

**MPFC analysis**

MPFC analysis was performed by the gating strategy on the basis of sequential removing of nonrelevant cells, as described in Figure 1. A debris exclusion gate was set on the forward and side scatter (FSC/SSC) plot to define the total relevant MNCs. CD45− gate was then drawn on a CD45 versus SSC plot to define the suspected ES cells. The CD45− cells were gated again on a CD (3/14/16/19)−/dim versus...
SSC plot to exclude lineage-specific stem cells that are present in the BM. Cells defined as CD45⁻/C0⁻/CD3⁻/C0⁻/CD14⁻/C0⁻/CD16⁻/C0⁻ were analyzed for the ES-specific markers on a CD99 versus CD90 plot. We defined ES cells as CD45⁻/CD3⁻/CD14⁻/CD16⁻/CD19⁻ and positive for both CD99/CD90 (ES combination). ES positive cells

Table 1. Clinical parameters and expression results of CD90/99 and CD56

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NOTE: Age, at diagnosis; metas, metastasis at diagnosis; CD56, expression on the CD90/99 positive cells only; follow-up, months from diagnosis until relapse or until last follow-up.
were defined when a clear population of more than 10 events was captured by the gating strategy (30). The proportion of ES cells was expressed as a percent of the total MNCs. A gate was drawn on the double positive cells that were then analyzed for CD56 positivity on CD56 versus CD99 and CD56 versus CD90 plots (Fig. 1). The proportion of CD56+ was expressed as a percent of the gated ES cells.

Cell lines
ES cell lines: SK-ES1 and MHHES5 were obtained from DSMZ and cultured according to DSMZ recommendations.

Statistical analysis
Receiver operating characteristic (ROC) analysis was used for the determination of the cutoff values for the CD56 expression levels with relapse as the dependent variable and CD56 as the independent variable. High or low expression levels were determined as higher or lower than the cutoff obtained. PFS by the Kaplan–Meier analysis was used for PFS and OS of the whole cohort (PASW Statistics 18).

Results
Both cell lines were characterized as expressing CD99 and CD90 and were negative for the hematopoietic panel, consistent with our definition. All 6 tumor aspirates also expressed both CD99 and CD90. All control BM samples were negative for the CD99 and CD90 combination. By the spiking experiments, we reached the sensitivity of 1 in 100,000 normal cells (data not shown).

ES micrometastatic cells in BM samples
A total of 46 BM samples obtained at diagnosis were evaluated for the ES combination and all were found to be positive (≥0.001%), indicating that all BM samples harbor micrometastatic ES tumor cells (range 0.001%–0.4%; Table 1). No statistical differences could be shown in the OS or PFS of patients according to the level of BM involvement, nor could any correlation with clinical parameters be identified.
CD56 expression on ES cells

ES cell lines were also analyzed for CD56 that was found to be differentially expressed as follows: SKES-100% and MHHES-43%.

CD56 was evaluated on ES cells in BM samples obtained at diagnosis from 45 patients. In 1 patient (#25) the quality of the sample and low number of cells impeded the evaluation of CD56. High or low expression was defined as higher or lower than the cutoff of 22% that was defined by ROC analysis. Sixty percent of the samples (27 of 45) harbored high CD56 expression. No significant correlation was found between CD56 expression and clinical parameters such as age, primary site and metastasis at diagnosis (Table 1). A highly significant correlation between disease progression and CD56 expression was detected by Kaplan–Meier analysis for the whole cohort. For the group of patients, harboring low/negative CD56 expression PFS at 10 years was 69% vs. 30% in the group with high expression ($P = 0.024$; Fig. 2A). When analyzing only the group with localized disease ($n = 35$), those expressing low/negative CD56 had 81% PFS at 10 years versus 38% in the high expressing group ($P = 0.02$; Fig. 2B). Furthermore, when excluding patients with pelvic disease out of the localized group ($n = 30$), the difference between the high to low expressing groups was even more pronounced: 100% PFS at 10 years for the low expression versus 40% for the high expression ($P = 0.007$; Fig. 3).

Cox regression analysis in the localized group determined that CD56 expression is an independent prognostic marker with an 11-fold increased risk to relapse in patients with high expression (Table 2).

Discussion

ES is an aggressive malignant bone tumor. One third of patients with localized disease will progress. Currently, the clinical prognostic markers are inadequate to predict risk of relapse. An improved tool is needed to define those patients who will eventually relapse and offer them intensified treatment or to reduce the treatment for patients with good prognosis.

MPFC is a sensitive method for the quantitative detection of rare cell populations. It is used to detect MRD and accordingly make clinical decisions in childhood acute lymphoblastic leukemia (9, 10). This method has also been used to identify circulating and/or micrometastatic tumor cells in adult patients with carcinoma and was proved to be a significant prognostic factor in patients with prostate cancer and breast carcinoma (11, 12). In pediatric patients, micrometastatic cells were detected in neuroblastoma and rhabdomyosarcoma (13–15). Only a
few reports used FC for the diagnosis of ES tumors for the combination of CD45−/CD99+ (22, 27).

The feasibility of FC to detect circulating and micrometastatic ES cells was recently published by Dubois and colleagues (18). They also showed that 6-color panel was more sensitive than the 2-color assay in detecting residual ES cells with a background rate of 0.00019%, just below 1 in 500,000 cells.

Our strategy was based on the negativity of CD45 and positivity of CD99 on ES cells. In addition, we excluded hematopoietic stem cells and added another ES marker, CD90. The accuracy of our method was achieved by a gated strategy of CD45−/CD9−/CD14−/CD16−/CD19− in addition to the positivity of CD90 and CD99 on ES cells. By using this approach, we could reach the same range of sensitivity of 1 in 100,000 as described by Dubois and colleagues (18).

CD99 is expressed in the majority of cases of ES and is considered one of the best diagnostic markers to date (31). Interestingly, Rocchi and colleagues (32) have shown that CD99 is expressed on mesenchymal cells, which is considered as the origin of ES tumor. They have shown that CD99 is required for ES oncogenic phenotype by preventing terminal neural differentiation. Chang and colleagues (22) have shown that CD90 is highly expressed on ES cells and has also been shown to be expressed on mesenchymal stem cells (33). We have detected CD99 and CD90 expression on all cell lines and tumor aspirates studied.

All BM samples obtained from patients with localized and metastatic disease at diagnosis were found to be positive for circulating ES tumor cells. There was no correlation between the amount of ES cells in BM samples at diagnosis and survival.

In our previous study with RT-PCR, we could not show a correlation between the presence of ES tumor cells in BM at diagnosis and outcome, but detected a highly significant association between occult tumor cells in BM or PBL during follow-up and relapse (34), similarly shown by de Alava and colleagues (35). However, 2 reports from the same group have identified a significant correlation between BM micrometastases in localized patients and outcome (7, 36).

Although RT-PCR is the common technique for detecting circulating ES tumor cells, there are significant advantages for MPFC, including: rapid technique, no need to identify a transcript, less labor-intensive comparing with RT-PCR, no need for the extraction of RNA, and can be detected in other body fluids.

CD56 is expressed in a variety of tumors. It has been detected in pediatric malignancies like neuroblastoma, medulloblastoma, rhabdomyosarcoma, synovial sarcoma, Wilms’s tumor, and hematopoietic malignancies (37–40). Its expression in ES is controversial. By immunohistochemistry, ES tumors were found to be negative for CD56 expression (40) and a few case reports of ES tumors reported positivity by immunohistochemistry (41) or by FC (22, 24, 27). This controversy could be explained by the use of different monoclonal antibodies directed to different isoforms on the NCAM molecule (38, 42).

We analyzed CD56 expression only on the defined ES cells by the combination strategy. ES cell lines differentially expressed CD56 and 4 of the 6 tumor aspirates were positive for CD56. Twenty seven (60%) BM samples were CD56 positive with a large range of expression: 3%–96%.

We detected a highly significant correlation between CD56 expression and disease progression. Patients harboring low/negative expression had a significant better PFS. Furthermore, when reanalyzing only the group with localized disease, these expressing low/negative CD56 had even a better PFS at 10 years compared with patients with high expression. When excluding the patients with the pelvic primary site, we observed a more pronounced significant PFS: 100% PFS at 10 years for the low expressing group versus 40% for the high expressing group at 10 years ($P = 0.007$).

By using Cox regression analysis by age, primary site, and CD56 expression, high CD56 was found to be a significant independent prognostic marker for relapse in the localized group.

To our knowledge, this is the first study in ES patients at diagnosis, reporting CD56 as a prognostic marker for relapse in BM samples. CD56 expression analysis can identify prognostic groups and facilitate stratification of treatment. These results were found by an aggressive treatment protocol of chemotherapy, surgery, and radiotherapy that have proved to be effective in delaying relapse in resected nonmetastatic ES (unpublished data). It is important to analyze the significance of these findings in patients treated on other treatment protocols.

Increased CD56 expression has been associated with a more aggressive form of solid tumors, such as lung,

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<td>95% CI</td>
<td>2.0–68</td>
<td>2.0–68</td>
</tr>
</tbody>
</table>

Abbreviations: $P$, P value, RR, relative risk to relapse, CI, confidence interval.
ovarian, and renal cell, and in hematopoietic malignancies—lymphoblastic and myeloid leukemias (37, 43–45). CD56 over expression in ES may contribute to the aggressiveness of this childhood tumor.

It had been shown that high expression of NCAM (CD56) can be a target for therapy. Indeed, there are several trials targeting the NCAM in malignancies, such as small-cell lung cancer, neuroblastoma, and glioma. The huN90-DMI immunotoxin is the most advanced anti-NCAM therapeutic strategy and its current clinical development is intensively promoted by pharmaceutical companies. There are also reports on naive humanized monoclonal antibodies such as huN901, and 8 other monoclonal anti-NCAM antibodies by using radioimmunotargeting with iodine (37, 39, 43–45).

In conclusion, MPFC with CD99, CD90, and a negative hematopoietic panel provides a possible alternative strategy for detecting micrometastatic ES tumor cells. CD56 expression in BM samples at diagnosis could be used to identify ES patients predisposed to relapse, thus applying treatment intensification and implementation of personal-

ized therapy. Furthermore, CD56 evaluation can identify a subgroup of patients with excellent prognosis, in whom treatment reduction could carefully be considered.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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