Excellent prognosis in a subset of patients with Ewing sarcoma identified at diagnosis by CD56 using flow cytometry

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Running title: CD56 as a prognostic marker in localized Ewing sarcoma

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Statement of Translational Relevance.

We have shown that multi-parametric flow cytometry, using CD99, CD90, and a negative hematopoietic panel, provides a possible strategy for detecting circulating Ewing sarcoma cells. There was a highly significant correlation between CD56 expression on these Ewing sarcoma cells in BM samples at diagnosis and progression free survival for the whole group, for patients with localized disease and for patient with localized non-pelvic disease.

This is the first report in Ewing sarcoma patients, reporting CD56 as a prognostic marker for relapse in BM samples at diagnosis. CD56 expression in BM samples at diagnosis could be used to identify ES patients predisposed to relapse, thus applying treatment intensification and implementation of personalized therapy. Furthermore, CD56 evaluation can identify a subgroup of patients with excellent prognosis, in whom treatment reduction could carefully be considered.
Abstract

Purpose

Ewing sarcoma (ES) is considered a systemic disease with the majority of patients harboring micrometastases at diagnosis. Multi-parameter 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 bone marrow 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 using a cut off 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– expression. 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 non pelvic disease, those expressing low/negative CD56 had 100% PFS versus 40% in the high expression 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.

Keywords: Ewing sarcoma, Multi-parameter flow cytometry, CD99, CD56, Progression free survival
Introduction

Ewing sarcoma (ES) is the second most common primary bone tumor in children and adolescents, belonging to the neuroectodermal tumor group known as ES family tumors (EFT). Approximately 25% of patients have metastases at diagnosis, which is a major adverse prognostic factor. Additional clinical adverse prognostic features include pelvic primary site and poor response, defined as less than 90% necrosis, at definitive surgery [1].

Despite aggressive multimodal therapy, one third of patients with a localized tumor still may develop a recurrence or progress. Thus, ES should be considered a systemic disease with the majority of patients harboring micrometastases already at diagnosis [1, 2]. ES tumors are characterized by specific translocations that result in the fusion of the EWS gene on chromosome 22q12 with different ETS-related genes, including FLI-1, ERG, ETV1, E1AF and FEV [1, 3]. These chimeric transcripts have been detected by reverse-transcriptase polymerase chain reaction (RT-PCR) for the diagnosis of ES. Due to the high sensitivity of the RT-PCR assay, it can also be used for detecting circulating tumor cells in peripheral blood (PBL) and bone marrow (BM) samples [4-8].

Flow cytometry (FC) is an alternative sensitive method used routinely for the detection of Minimal Residual Disease (MRD) in hematopoietic neoplasm [9, 10]. It is also used for diagnosis and follow up evaluation in solid tumors including breast and prostate cancer [11, 12] in adult patients and rhabdomyosarcoma and neuroblastoma in pediatric patients [13-15].

The surface antigen CD99 (MIC2) is a marker of ES cells. CD99 is involved in cell adhesion, migration, apoptosis and differentiation of T cells and thymocytes. CD99 is also highly expressed on T-cell lymphoblastic leukemia/lymphoma and other
hematopoietic progenitors [16, 17] which, in contrast to ES cells, also express CD45, the pan leukocyte common antigen. Thus the combination of both antigens CD99+ and CD45- can be used to evaluate ES tumor samples. Indeed, DuBouis et al.[18] have recently demonstrated 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 25–37 kDa heavily N-glycosylated, glycoprophosphatidylinositol (GPI) 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/PNET tumors 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 primitive neuroectodermal tumor (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 Multi-parameter flow cytometry (MPFC) to evaluate the presence of circulating ES cells in BM samples of ES patients at diagnosis using 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 SCMCI from 1990 to 2008. In 6 of these patients, tumor aspirates were also available. Mononuclear Cells (MNCs) 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 19/46 (41.3%), pelvis-sacrum in 8/46 (17.4%), Askin tumor in 6/46 (13.1%), other axial 10/46 (21.7%) and multifocal 3/46 (6.5%). Thirty five (76%) of patients presented a localized disease (Table 1).

All patients were treated by an in house protocol which included 6 courses of chemotherapy (Vincristine, Actinomycin-D, Cyclophosphamide, Doxorubicin Ifosfamide and Etoposide), high radiation dose of 4500-6000cGy and surgery. Histopathological diagnosis was based on the appearance of small round cells; immunohistochemical staining included CD99, Synaptophysin, Chromogranin, 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, CT scans, MRI and Tc99 bone scans. Median follow up was 60.9 months. Overall survival (OS) and PFS for the whole group was 67.6 % and 50.5 % in 5 years, respectively.

Ten control BM samples were obtained from children that 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 five-color combination of eight different antibodies: CD99-FITC (clone TU12; Becton Dickinson [BD] San Jose, CA), CD90-PE (clone 5E10, BD), CD45 Per-CP (clone 2D1, BD), CD3,14,16,19-PE-Cy7 (clones: UCHT1, RMO52, 3G8, J3-119 respectively, Beckman Coulter [BC], Hialeah, FL), CD56-APC (clone NCAM 16.2 BD).

At least one million defrosted MNCs were stained and analyzed on a FACSCanto II flow cytometer BD Biosciences using the Diva software for acquisition and analysis. For sensitivity analysis serial dilutions of pre labeled and fixed ES cell line (MHHES5) were performed in PBS. Desired amounts were spiked into control BM which were then labeled with the five-color combination and fixed with CellFIX (BD) [29].

MPFC analysis

MPFC analysis was performed using the gating strategy based on sequential removing of non relevant cells as described in Figure 1. A debris exclusion gate was set on the forward and side scatter (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–/CD3–/CD14–/CD16–/CD19–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 cell 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 which were then analyzed for CD56 positivity on
CD56 versus CD99 and CD56 versus CD90 plots (Figure 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 (Leibizu, Germany), and cultured according to DSMZ recommendations.

Statistical analysis

ROC analysis was used for the determination of the cut-off 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 as or lower than the cut-off obtained. PFS by Kaplan Meier analysis was assessed by high versus low CD56 expression levels. The correlations between CD56 expression and clinical parameters as age, metastasis at diagnosis were evaluated by Fisher's exact test. Multivariate analysis using Cox regression was performed for patients with localized disease by the parameters age, primary site and CD56 expression. 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, 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**

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 which 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 one 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 cut-off of 22% that was defined by ROC analysis. Sixty percent of the samples (27/45) harbored high CD56 expression. No significant correlation was found between CD56 expression and clinical parameters 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 for the whole cohort. For the group of patients harboring low/negative CD56 expression PFS at 10 years was 69% versus 30% in the group with high expression (p=0.024)(Figure 2A). When analyzing only the group with localized disease (n=35), those expressing low/negative CD56 had 81% 10 years PFS versus 38% in the high expression group (p=0.02) (Figure2B). 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% 10 years PFS for the low expression versus 40% for the high expression (p=0.007) (Figure 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. A 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 that will eventually relapse and offer them intensified treatment or to reduce 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 ES cases were reported using 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 et al [18]). They also showed that six color panel was more sensitive than the two color assay in detecting residual ES cells with a background rate of 0.0001%, 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 using a gated strategy of CD45–/CD3–/CD14–/CD16–/CD19– in addition to the positivity of CD90 and CD99 on ES cells. Using this approach we could reach the same range of sensitivity of 1 in 100,000 as described by Dubois et al [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 et al. [32] have demonstrated that CD99 is expressed on mesenchymal cells, which is considered the origin of ES tumor. They have shown that CD99 is required for ES oncogenic phenotype by preventing terminal neural differentiation. Chang et al. [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 using 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] and also shown by de Alava et al. [35]. However, two 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 to 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, Wilm's tumor and hematopoietic malignancies [37-40]. Its expression in ES is controversial. Using 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 out 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, those expressing low/negative CD56 had even a better PFS at 10 years compared to patients with high expression. When excluding the patients with the pelvic primary site, we observed a more pronounced significant PFS: 100% 10 years PFS for the low expressing group versus 40% for the high expressing group at 10 years (p=0.007).

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 using an aggressive treatment protocol of chemotherapy, surgery and radiotherapy that have proved to be effective in delaying relapse in resected non metastatic 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, 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, 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 naïve humanized monoclonal antibodies such as huN901, and eight other monoclonal anti-NCAM Abs using radioimmunotargeting with iodine. [37, 39, 43-45]

In conclusion, MPFC using CD99, CD90, and a negative hematopoietic panel provides a possible alternative strategy for detecting micrometastatic Ewing sarcoma 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 personalized therapy. Furthermore, CD56 evaluation can identify a subgroup of patients with excellent prognosis, in whom treatment reduction could carefully be considered.
Acknowledgement

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Legends to figures

FIGURE 1: MPFC strategy:

A. Debris exclusion in gate P1 on SSC/FSC dot plot.

B. Exclusion by CD45: CD45 negative cells are shown in P2 on CD45/SSC dot plot.

C. Exclusion by lineage specific stem cells out of CD45- cells: Selection of the CD3, CD14, CD16, CD19 negative cells by gate P3.

D. Cells fulfilling characteristics defined in gates P1, P2 and P3 are measured for CD99+CD90 positivity in gate P4 on CD99/CD90 dot plot.

E, F. Evaluation of CD56 on ES cells gated on CD56/99 and CD56/90 dot plots, shown in P5 and P6, respectively.

FIGURE 2: Kaplan Meier analysis for progression free survival by CD56 expression

A: The whole cohort of ES patients (n=45).

B: Localized ES patients (n=35).

High: above or equal to the cut-off of 22%; Low: under the cut-off of 22%

FIGURE 3: Kaplan Meier analysis for progression free survival by CD56 expression in the non-pelvic localized ES patients (n=30). High: above or equal to the cut-off of 22%; Low: under the cut-off of 22%
References


Fig 1
Fig 2

A

Low/negative expression 69%
High expression 30%
p=0.024

B

Low/negative expression 81%
High expression 38%
p=0.02

Probability

Months

Probability

Months

Fig 3

Low/negative expression 100%

High expression 40%

p=0.007
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Age: at diagnosis; Metas: metastasis at diagnosis; CD56: expression on the CD90/99 positive cells only; Follow up: months from diagnosis until relapse.
apse or until last follow up.
TABLE 2: Univariate and Multivariate analyses of the Clinical Parameters and CD56 expression results for localized ES patients (n=35).

<table>
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P: p value, RR: relative risk to relapse; CI: confidence interval
Excellent prognosis in a subset of patients with Ewing sarcoma identified at diagnosis by CD56 using flow cytometry


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