Ewing Tumors That Do Not Overexpress BMI-1 Are a Distinct Molecular Subclass with Variant Biology: A Report from the Children’s Oncology Group

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Abstract

Purpose: Ewing sarcoma family tumors (ESFT) are aggressive tumors of putative stem cell origin for which prognostic biomarkers and novel treatments are needed. In several human cancers, high expression of the polycomb protein BMI-1 is associated with poor outcome. We have assessed the potential clinical significance of BMI-1 expression level in ESFT.

Experimental Design: BMI-1 expression was assessed in 130 tumors by immunostaining and associations with clinical features and outcome determined. The molecular signatures of BMI-1–low and BMI-1–high tumors were compared using microarrays and differentially activated canonical pathways identified by gene-specific enrichment analysis. Automated quantitative analysis of phosphoproteins was used to assess relative levels of pathway activation. Sensitivity to IGF1-R inhibition was determined using MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assays.

Results: BMI-1 is overexpressed by the vast majority of ESFTs. However, in 20% of cases, BMI-1 levels are low to undetectable. Significantly, although clinical presentation and outcome were similar between BMI-1–high and BMI-1–low tumors, whole genome expression array analysis showed marked differences in their respective gene expression profiles. Gene-specific enrichment analysis identified that several cancer-associated canonical biological pathways, including IGF1, mTOR, and WNT, are significantly down-regulated in BMI-1–low compared with BMI-1–high tumors. Consistent with these in vivo data, the response to IGF1-R inhibition in vitro was diminished in BMI-1–low compared with BMI-1–high ESFT cells.

Conclusion: ESFT that do not overexpress BMI-1 represent a novel subclass with a distinct molecular profile and altered activation of and dependence on cancer-associated biological pathways. Clin Cancer Res; 17(1); 56–66. ©2010 AACR.

Introduction

The Ewing sarcoma family of tumors (ESFT) are malignant neoplasms of bone and soft tissue that primarily affect children and young adults (1). Genetically, they are identified by expression of EWS-FLI1 or another related gene fusion (1). Although their histogenesis remains elusive, recent studies suggest that ESFT may arise from malignant transformation of mesenchymal and/or neural crest stem cells (2–6). Clinically, they are highly aggressive malignancies with a high propensity for relapse and metastasis. Unfortunately, despite aggressive systemic cytotoxic therapy and local control measures, relapse after initial clinical remission is not uncommon and overall survival for patients with relapsed or metastatic ESFT remains less than 20% (7). Given the profound need for novel and less toxic treatments for these patients, great hope is being placed on the development of targeted agents that will successfully inhibit biological pathways known to contribute to ESFT growth (8). However, knowledge about which tumors are likely to respond to these agents, such as the IGF1-R inhibitors that are currently being tested in clinical trials, is crucial if their efficacy is to be optimized. Therefore, coincident development of biomarkers that can predict response to therapy is essential.

BMI-1 is a member of the polycomb group gene family that promotes self-renewal of normal adult stem cells including neural crest stem cells through epigenetic repression of...
BMI-1 Expression in Ewing Tumors

Translational Relevance

The development of biologically targeted therapies holds tremendous promise for cancer patients. However, optimal efficacy of these approaches will be realized only if it can be determined which patients are most likely to respond and which tumors are likely to be resistant. The development of biomarkers that predict treatment response is, therefore, absolutely critical if novel biological agents are to be successfully translated into clinical practice. In this study, we have identified a subset of Ewing sarcoma family tumors (ESFT) that do not overexpress BMI-1. These tumors display relative downregulation of the IGF1 pathway and are less sensitive to the growth inhibitory effects of IGF1-R inhibition. Together, these data show that within ESFTs there exists a molecular subclass, defined by absence of BMI-1 overexpression, that are less likely to respond to IGF1-R inhibitors. Evaluation of BMI-1 as a potential predictive biomarker of response to IGF1-R-targeted agents is warranted.

Developmental and senescence pathways (reviewed in ref. 9). In addition, BMI-1 functions as an oncogene in many human cancers and has been implicated in the self-renewal of tumor-initiating cancer stem cells (10–12). Importantly, in at least some human cancers, overexpression of the BMI-1 protein is associated with a worse clinical outcome, suggesting that in these tumor types BMI-1 might be useful as a biomarker of aggressive disease (13–18). We previously reported that BMI-1 is overexpressed by ESFT and functions as a growth-promoting oncogene in these tumors (19). However, our data also revealed that the absolute level of BMI-1 expression is variable among both primary tumors and cell lines (19). For this study, we have assessed whether BMI-1 might be useful as a predictive biomarker in ESFT. We characterized BMI-1 protein expression in a large cohort of primary ESFT samples and evaluated whether BMI-1 expression levels were correlated with clinical outcome in a group of clinically annotated tumors obtained from patients treated on recent Children’s Oncology Group (COG) clinical trials. In addition, we assessed whether differences in the molecular phenotype of BMI-1–high and BMI-1–low tumors might also be used in the future to predict response to pathway-targeted therapies such as IGF1-R inhibition.

Materials and Methods

Sample accrual

Formalin-fixed, paraffin-embedded (FFPE) and fresh-frozen sections were acquired from tumor banks at the COG Biorepository in Columbus, Ohio (Cooperative Human Tissue Network—CHTN), Childrens Hospital Los Angeles (CHLA), and Memorial Sloan Kettering Cancer Center. Diagnosis of ESFT was confirmed for all specimens by pathologic review at each site. Samples were obtained as single tumor slides or as tissue microarrays (TMA). Serial sections of an ESFT TMA created at the Department of Pathology, University of Michigan, were used for validation studies. Clinical outcomes data were obtained from chart review and through the Biostatistical Office of the COG. All human specimens and correlative data were obtained in compliance with HIPAA regulations and following protocol review by institutional review boards in accordance with an assurance filed with and approved by the Department of Health and Human Services. Informed consent for use of tumor samples for research purposes was obtained from each subject or subject’s guardian.

Immunohistochemical analysis

Four-micron tissue sections were deparaffinized, pretreated with CCI (Tris/Borate/EDTA buffer pH 8, Ventana Medical Systems, Inc.), and incubated with anti-BMI-1 antibody (1:50; Millipore) according to the BenchMark UHC/ISH Staining Module BMI-1-T 1/50 protocol (32 minutes at 42°C). All sections were then treated with iView detection kit (Ventana Medical Systems, Inc.). Adjacent sections were stained with hematoxylin and eosin (H&E) to verify the presence of viable tumor tissue. Individually stained tumor slides were directly visualized and scored by direct light microscopy and photomicrographs acquired via digital camera (DP-11; Olympus). TMA sections were digitally imaged using Aperio ScanScopeT2 software (Aperio) at Columbus Children’s Research Institute Biopathology Center.

Stained sections were assigned a BMI-1 score, using published criteria (18). In brief, the percentage of positive cells (PP%) value (ranging from 0 to 3 for ≤5% to >50% positive cells, respectively) was multiplied by the signal intensity (SI) value (which ranges from 0 for absent to 3 for strongly positive) to generate a composite PPxSI value ranging from 0 to 9. Composite values of 0–1 are negative; 2–3 are 1+; 4–6 are 2+, and values ≥6 are scored as 3+.

For automated quantitative analysis (AQUA) of total and phosphorylated protein expression in tumor cells, double immunofluorescence staining was done as previously described (20). Deparaffinized and rehydrated TMA slides were subjected to microwave epitope retrieval in 7.5 mmol/L sodium citrate buffer, pH 6 (mTOR and P-mTOR), or 1 mmol/L EDTA buffer, pH 8 (IGF1R and P-IGF1R). Slides were washed in TBS (10 mmol/L Tris HCl, pH 8, containing 0.154 mol/L NaCl (TBS), 0.05% Tween-20) and then incubated with 1 of 5 antibodies, BMI-1 (clone 229F6, 1:400; Millipore), mTOR (Ab-51089, 1:200; Abcam), P-mTOR (Ab-51044, 1:100; Abcam), IGF1-R (clone JBW902, 1:1,000; Millipore), or P-IGF1R (07-841, 1:400; Millipore).
for 4°C overnight in a humid chamber). Double-stained slides were washed and then incubated with a combination of goat anti-rabbit or mouse IgG conjugated to AF555 (A21424/A, 1:200; Molecular Probes) in goat anti-mouse or anti-rabbit Envision+ (DAKO) for 60 minutes at room temperature in a dark humidity tray. Slides were washed in TBST and the target image was developed by a CSA reaction of Cy5-labeled tyramide (1:50; PerkinElmer). Finally, slides were stained with 4',6-diamidino-2-phenylindole (DAPI) in a nonfading mounting media (ProLong Gold; Molecular Probes) and allowed to dry overnight in a dark dry chamber.

The AQUA system (Software v2.2, HistoRx) was used for automated image acquisition and analysis (20). Within each slide, the area of tumor was distinguished from stromal and necrotic areas by creating a tumor-specific mask from the CD99 stain, which was visualized from AlexaFluor 555 signal. Fluorescence pixel intensity of the target protein/antibody complex was obtained from the Cy5 signal and reported as pixel intensity.

Clinical correlate analysis

The association between BMI-1 and age was analyzed via ANOVA and associations between BMI-1 and other clinicopathologic characteristics were analyzed via Fisher’s exact test. For survival analysis, event-free survival (EFS) was defined as the minimum interval from the date of diagnosis to the date of tumor recurrence, progression, occurrence of a second malignancy, death, or last follow-up. Overall survival (OS) was defined as the interval from the date of diagnosis to the date of death or last follow-up. Estimates of EFS and OS percent were based on the product-limit (Kaplan–Meier) estimate with Greenwood standard errors (21). The association of EFS and OS with BMI-1 protein expression was tested using the log-rank test, either univariately or with stratification based on stage at presentation, age, site, treatment era, and EWS-FLI1 transcript type. Survival analyses were done using STATA software, Version 9.2. All reported P values were 2-sided and a P < 0.05 was considered significant.

Expression microarrays

Total RNA was isolated from ESFT biopsies, using Qiagen miRNA kit (Qiagen), and processed for whole genome expression profiling, using Affymetrix GeneChip Human Exon 1.0 ST oligonucleotide microarrays according to Affymetrix protocols. Affymetrix CEL files from similarly processed ESFT cell line RNA were kindly provided by Dr. T. Triche (CHLA). Signal intensities from core probe sets were quantile normalized by robust multichip averaging and transcript expression determined by median summarization using Partek Genomics Suite software (Partek). Hierarchical clustering using both Ward’s and average linkage methods was done in Partek. To compare relative levels of pathway activation between BMI-1–high and BMI-1–low tumors, 639 gene sets representing canonical pathways were downloaded from the Molecular Signatures Database and gene set enrichment analysis (GSEA) done as described (22, 23). In brief, the mean of the t statistics for the transcripts of each gene set was calculated, and a P value was computed for each gene set by randomly permuting the gene labels 10,000 times. The expected false discovery rate (FDR) for multiple testing was controlled using the Benjamini and Hochberg procedure (24). All reported P values were 2-sided. Statistical computations were done using STATA software, Version 9.2 and R version 2.8.1 (http://www.R-project.org).

In vitro assays of ESFT cell lines

ESFT cells were obtained from CHLA (TC-71, TC-248, A673, A4573) and COG (CHLA-9) cell line repositories (Dr. T. Triche and Dr. C.P. Reynolds, www.cogcell.org, respectively). Cell lines were confirmed to be ESFT by RT-PCR amplification of a type 1 EWS-FLI1 fusion and by analysis of short tandem repeats (kindly done by Dr. C.P. Reynolds). Cells were maintained in 10% RPMI supplemented with 10% FBS, pen/strep, and β-glutamine and RT-PCR and Western blot analysis of logarithmic phase cells were done using standard protocols. PCR primer sequences for BMI-1 and EWS-FLI1 are available on request. To assess response to IGF1-R inhibition, subconfluent cells growing in 96-well plates in 0.5% FBS-supplemented RPMI were treated with increasing concentrations of picrophophyllin (PPP; Enzo Life Sciences), IMC-A12 or DMSO vehicle. IMC-A12 was provided by ImClone systems through the Cancer Therapy Evaluation Program (CTEP, NCI). Cell viability was assessed 72 hours posttreatment using MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assays (CellTiter 96 AQueous One Solution Cell Proliferation Assay; Promega). All experiments were repeated at least 3 times with a minimum of 6 wells counted/condition.

Results

BMI-1 is overexpressed by most ESFTs

BMI-1 expression was evaluated in 130 FFPE ESFT sections by immunohistochemistry (Table 1). Consistent with our prior studies of ESFT cell lines (19), BMI-1 protein was robustly expressed by most tumors (BMI-1-high; Fig. 1A and B). Comparison with 16 normal tissues confirmed that nonmalignant cells only rarely express such high levels of BMI-1 (not shown). Of note, however, in 20% of ESFTs, only weak or no BMI-1 was detected (BMI-1-low; Fig. 1C and D).

BMI-1 has been implicated as a marker of minority populations of tumor-initiating cancer stem cells (12, 25). As shown (Fig. 1A and B), we found that in a majority of ESFTs, all tumor cells are BMI-1 positive. Moreover, comparison of diagnostic biopsies with samples obtained postneoadjuvant chemotherapy revealed no difference in BMI-1 expression between these 2 groups (P = 0.49). Thus, if a subpopulation of chemoresistant, tumor-initiating cells exists in ESFT, BMI-1 will not be useful as a distinguishing marker.
Clinical presentation and outcome of BMI-1–high and BMI-1–low tumors are similar

In some cancers, BMI-1 is a marker of aggressive disease and worse outcome (13–18, 25). Although patients with metastatic ESFT have the worst outcome, prognostic stratification of patients with localized disease remains a challenge. Previous studies have reported that pelvic disease, older age at presentation, and variant EWS-FLI1 fusion type are bad prognostic features (26–28). As shown (Table 1), there was no association between any of these features and BMI-1 expression. In addition, BMI-1–low tumors were equally represented among tumors of bone or soft tissue origin. We next assessed 72 patients with localized disease for whom follow-up data were available. No significant difference in either EFS or OS was observed between BMI-1–high and BMI-1–low tumors (Fig. 2). Of the 24 events, 20 were disease progression or relapse, 2 were second malignant neoplasm, and 2 were death as a first event. EFS analyses that censor the nonprogression events yield identical conclusions to those presented. Thus, although the small number of BMI-1–low tumors in this cohort limits the statistical power of the analysis, our studies suggest that there is little or no association between BMI-1 expression level and either clinical presentation or outcome in patients with localized disease who are treated on current clinical protocols.

Gene expression signatures differ between BMI-1–low and BMI-1–high tumors

Silencing of innate p16/RB or p53 tumor suppressor pathways is necessary for tolerance of the EWS-FLI1 oncoprotein in normal cells (29, 30). BMI-1 is known

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**Table 1. Clinical and molecular features of primary ESFT biopsies**

<table>
<thead>
<tr>
<th>BMI-1 score</th>
<th>All patients</th>
<th>BMI-1-low</th>
<th>BMI-1-high</th>
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<tr>
<td></td>
<td>Total number (%)</td>
<td>130 (100)</td>
<td>18 (13.8)</td>
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<td></td>
<td>Median age (range), y</td>
<td>13 (0–47)</td>
<td>13 (5–47)</td>
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<tr>
<td>Gender</td>
<td>Male (%)</td>
<td>84 (65)</td>
<td>13</td>
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<tr>
<td></td>
<td>Female (%)</td>
<td>46 (35)</td>
<td>5</td>
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<td>Stage</td>
<td>Localized (%)</td>
<td>99 (85)</td>
<td>11</td>
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<tr>
<td></td>
<td>Metastatic (%)</td>
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</tr>
<tr>
<td></td>
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<td>3</td>
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<tr>
<td>Region</td>
<td>Pelvis (%)</td>
<td>20 (15)</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Nonpelvis (%)</td>
<td>110 (85)</td>
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</tr>
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<td>5</td>
</tr>
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<td>1</td>
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<tr>
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<td>2</td>
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<td>5</td>
</tr>
<tr>
<td></td>
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<td>2</td>
</tr>
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<td>Unknown</td>
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<td>11</td>
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<td></td>
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<td>0</td>
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<tr>
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<tr>
<td></td>
<td>Mutant</td>
<td>8</td>
<td>0</td>
</tr>
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</table>

\( ^{a}P \) value from ANOVA, \(^{b} \times 2 \) Fisher’s exact test (unknowns excluded), \(^{c} \times 2 \) Fisher’s exact test.
to epigenetically repress p16 and p53 pathways in both normal and tumor cells via transcriptional silencing the CDKN2A locus (31). We therefore reasoned that if BMI-1–high tumors silence p16 and p53 pathways epigenetically, BMI-1–low tumors may be more dependent on secondary genetic mutations. Homozygous deletion of p16 and/or mutation in p53 occurs in 20% to 25% of primary ESFT (32). To begin to address whether such mutations are more common in BMI-1–low tumors, we evaluated 24 tumors for which p53 and p16 status had been previously documented (32). As shown (Table 1), tumors harboring secondary genetic mutations were equally distributed between BMI-1–high (N = 18) and BMI-1–low (N = 6) groups. Thus, in this small cohort of tumors, we found no association between BMI-1 expression and mutations in p53 or p16. Studies with larger numbers of tumors are necessary, but these initial findings suggest that other mechanisms of tumor suppressor inactivation are likely to exist in BMI-1–low tumors.

To more fully define molecular differences between BMI-1–high and BMI-1–low ESFT, we conducted whole genome expression profiling of 5 age- and stage-matched tumors from each subclass (five 3+ tumors vs. 4 negative and one 1+; GEO accession number GSE16016). An EWS-FLI1 fusion was detected by RT-PCR in 9 of 10 tumors (not shown) and bone and extraosseous tumors were included in each group. Signal intensity data confirmed significantly lower expression of the BMI-1 transcript in the BMI-1–low tumor cohort (Fig. 3A). Unexpectedly, unsupervised principal components analysis of the 10 microarrays using all 17,881 core probe set-interrogated transcripts clearly segregated the tumors into 2 nonoverlapping groups (Fig. 3B). Thus, despite their common histologic appearance and expression of EWS-ETS fusions, BMI-1–high and BMI-1–low tumors displayed remarkably dissimilar transcriptional profiles. Consistent with this, more than 4,000 transcripts were identified as being differentially expressed between the 2 groups (FDR < 0.05). To better assess the potential biological and clinical significance of these genes, we conducted GSEA and identified differential activation of 100 different canonical pathways (see Materials and Methods; FDR < 0.05). The most highly statistically significant of these pathways are shown in Table 2. Importantly, the IGF1, mTOR, ubiquitin-mediated proteolysis, and WNT pathways were all identified as being significantly down-regulated in BMI-1–low tumors.
Levels of IGF1R and mTOR phosphorylation are significantly reduced in BMI-1–low tumors

Next, we sought to confirm that differences in gene expression were truly reflective of differential levels of pathway activation. To achieve this, we used quantitative immunohistochemical tools (20) to analyze levels of BMI-1 as well as total and phosphorylated IGF1-R and mTOR proteins in an independent cohort of ESFT samples obtained from newly diagnosed patients at the University of Michigan. Consistent with nonfluorescent staining protocols (Fig. 1), AQUA analysis of 58 ESFTs revealed a wide range of BMI-1 expression (range = 15–860 fluorescent intensity units, median signal = 142). To quantify relative levels of kinase pathway activation in BMI-1–low and BMI-1–high tumors, we compared tumors with the lowest BMI-1 signal (bottom 15%, median BMI-1 signal = 24) with tumors with the highest signal (top 50%, median BMI-1 signal = 416). As shown (Fig. 3D), consistent with gene expression data, levels of IGF1-R protein were equivalent between BMI-1–high and BMI-1–low tumors. However, the relative levels of IGF1-R phosphorylation as well as mTOR phosphorylation were significantly reduced in BMI-1–low tumors (Fig. 3D). Thus, both gene expression and protein array data suggest that BMI-1–low tumors represent a distinct molecular subclass of ESFT that may be less dependent on IGF1-R, mTOR, and other canonical cancer-associated pathways for growth and survival. As such, they may also be less responsive to pathway-targeted agents currently in development and in early-phase clinical trials.

ESFT cells with low expression of BMI-1 are less sensitive to IGF1-R inhibition

IGF1-R inhibitors are currently being evaluated as novel therapeutic agents in ESFT (8). As discussed earlier, molecular profiling studies revealed that the IGF1 pathway is one of the most significantly downregulated pathways in BMI-1–low ESFT and levels of IGF1-R phosphorylation and its downstream effector mTOR are reduced in these tumors (Fig. 3C and D). In light of these data, we hypothesized that BMI-1–low ESFT might be less sensitive to the effects of IGF1-R inhibition. To test this hypothesis, we first needed to identify representative cell lines for in vitro assays. We previously showed that BMI-1 is overexpressed in ESFT cell lines in vitro (19). To identify candidate BMI-1–low cell lines, we conducted hierarchical clustering of whole genome expression profiles obtained from the 10 aforementioned primary tumors and 19 ESFT cell lines. Interestingly, only the TC-248 cell line clustered with the BMI-1–low tumors (Fig. 4A). Although no other BMI-1–low cell lines were identified, among BMI-1–high cell lines, transcript expression was found to vary from very high (e.g., TC-32, TC-71, A673) to more moderate (e.g., CHLA-9, TC-466, TTC-487) levels. Reduced expression of BMI-1 in TC-248 cells was confirmed by Western blot (Fig. 4B) and expression of a type 1 EWS-FLI1 fusion confirmed by RT-PCR (Fig. 4C). Consistent with our hypothesis, TC-248 was significantly less sensitive than BMI-1–high cell lines to the growth inhibitory effect of picropodophyllin (PPP), a selective small molecule inhibitor of IGF1-R (ref. 33; Fig. 4D). Similarly, TC-248 was relatively insensitive to the IGF1-R inhibitory antibody IMC-A12 (Fig. 4E). These in vitro studies of ESFT cell lines support our studies of primary human tumors in vivo and suggest that patients whose tumors express low levels of BMI-1 may be less sensitive to IGF1-R–targeted therapy. Prospective testing of patient samples is now required to clinically test this novel hypothesis.

Discussion

New therapies for ESFT are needed to both overcome resistant disease and to reduce the short- and long-term side effects associated with current cytotoxic regimens (34). Recent preclinical data have generated great enthusiasm for biological pathway–targeted agents, including IGF1-R antagonists (8). In this study, we have identified a distinct subclass of ESFT that is characterized by low expression of
BMI-1. These tumors represent up to 20% of cases and are molecularly distinct from the more common BMI-1–high tumors. Importantly, genetic profiling studies revealed that numerous cancer-associated pathways, including the IGF1 and mTOR pathways, are relatively less active in this subset of ESFTs. In support of this observation, we showed, in an independent cohort of ESFTs, that the levels of IGF1-R and mTOR phosphorylation are significantly reduced in BMI-1–low compared with BMI-1–high tumors. Moreover, preliminary in vitro studies confirmed that the growth inhibitory effects of IGF1-R inhibition are significantly diminished in BMI-1–low cells. These studies suggest that BMI-1 might be useful as a predictive biomarker of response to IGF1-R–targeted agents in vivo.

Recent work from the Pediatric Preclinical Testing Program (PPTP) found that the in vivo efficacy of IMC-A12 therapy in ESFT xenografts was not predicted by the extent of in vitro response (35). Moreover, only 1 of 5 ESFT xenografts displayed a significant response to IMC-A12 as a single agent (35). Analysis of Affymetrix gene expression array data provided by PPTP (https://sharedoc.nchri.org/PPTP/default.aspx) shows that the lone ESFT xenograft that displayed an in vivo response to IMC-A12, EW-5, also expressed the highest level of BMI-1 (absolute as well as normalized signal intensities, not shown). Moreover, EW-5 xenografts showed complete regression when treated with combined therapy targeting IGF1-R and mTOR (36). Finally, we have found that the level of BMI-1 in TC-71 cells grown in vitro is significantly higher than that expressed by TC-71 xenografts (J.H. Hsu, E.R. Lawlor, unpublished data). We speculate that this difference in BMI-1 expression may contribute to the unexpected and disappointing differences in response that have been observed between IMC-A12–treated TC-71 cells in vitro and in vivo (35). Together, these data lend preclinical in vivo support to our hypothesis that targeted therapies against IGF1-R and mTOR will be most effective in ESFTs with the highest levels of BMI-1.

In some tumors, BMI-1 overexpression is a feature of tumor-initiating cells and because normal somatic stem cells also express high levels of BMI-1, it has been proposed that BMI-1 might be a universal marker of tumor-initiating
cancer stem cells (10–12). Our studies show that in BMI-1–high ESFTs, all tumor cells robustly express the protein. Thus, if a discrete population of tumorigenic stem cells exists in ESFT, BMI-1 will not be useful as discriminating marker. Alternately, if BMI-1 is a marker of cells with tumor-initiating potential, then all cells within BMI-1–high tumors would be predicted to possess tumor-initiating capability. This latter possibility is supported by the observation that in the absence of systemic chemotherapy, ESFTs are destined to recur at distant sites indicating that circulating tumor cells with tumor-initiating potential are universally present in ESFT patients at the time of diagnosis. Further studies with large numbers of prospectively acquired patient samples are required to definitively address this question and to determine if there is a relationship between BMI-1 and CD133, a recently reported marker of putative cancer stem cells in ESFT (37).

Given the role of BMI-1 in p16-RB and p53 pathway suppression in other tumor types, we hypothesized that mutations in these pathways might substitute for BMI-1 overexpression in the BMI-1–low tumors. Analysis of a limited subset of cases did not reveal any significant increase in p16 deletion or p53 mutation among BMI-1–low tumors. Although these are the 2 most common secondary mutations in ESFT multiple alternate mechanisms of p16-RB and p53 pathway inactivation exist, including p16 promoter hypermethylation and MDM2 amplification (1). However, the absence of BMI-1–mediated repression of CDKN2A in ESFT cell lines (19) suggests that other p16-independent functions of BMI-1 also contribute to its function as an oncogene in this family of tumors. Indeed, we have found that BMI-1 promotes ESFT tumorigenicity through modulation of cell adhesion (19). Significantly, in this study, GSEA analysis of biological pathways identified differential upregulation of cell adhesion molecules in BMI-1–low tumors (Table 2B). Moreover, recent studies of neural stem cells and brain tumors also link BMI-1 to regulation of cell:cell and cell:matrix adhesion pathways (38–40). These data suggest that deregulation of cell adhesion pathways, either through BMI-1–dependent (in BMI-1–high tumors) or BMI-1–independent (in BMI-1–low tumors) mechanisms is essential for ESFT tumorigenesis. Further mechanistic studies are now required to test this hypothesis and to fully elucidate the relationship between BMI-1, cell adhesion, and ESFT pathogenesis.

With the exception of metastatic disease, no clinical features allow prognostic stratification of ESFT patients. High expression of BMI-1 has been associated with an unfavorable prognosis in numerous malignancies (13, 16–18, 25). In contrast, in malignant melanoma, loss of BMI-1 expression is associated with disease progression (41), and in breast cancer conflicting reports have showed associations of BMI-1 with either aggressive (14, 15) or more favorable disease (42, 43). We identified no clinical or pathologic features that could distinguish BMI-1–high from BMI–low ESFT and patients treated on current

### Table 2. Gene-specific enrichment analysis identifies differentially expressed canonical pathways in BMI-1–low ESFT (FDR < 0.01 for all listed pathways)

#### A. Downregulated in BMI-1-low

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Description</th>
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<td>HSAG01430</td>
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#### B. Upregulated in BMI-1-Low

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BMI-1 Expression in Ewing Tumors

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clinical protocols fared equally well irrespective of BMI-1 expression level. Despite their clinical similarities, however, gene expression profiling studies showed that BMI-1–high and BMI-1–low tumors are markedly different at a molecular level. Significantly, many of these differences in gene expression are reflected in marked disparities in biological pathway activation, including several pathways that are the focus of novel, targeted agents currently in development. Whether these inherent biological differences are reflective of alternate cellular origins and/or alternate paths to malignant transformation remains to be determined and will require study in in vitro and in vivo models of ESFT initiation. Nevertheless, these findings have profound implications for the design and interpretation of preclinical and clinical trials designed to test biological pathway–targeted agents in ESFT. The cell lines used in most preclinical studies of ESFT are representative of the more common BMI-1–high tumors (e.g., TC-71, A673, TC-32, A4573, 6647, CHLA-9). In contrast, the sole BMI-1–low cell line that we have identified, TC-248, is rarely studied (44). Our findings suggest that current preclinical models of drugs that target canonical biological pathways are likely to be most informative for patients with BMI-1–high tumors and less predictive of response in the subset of patients who present with BMI-1–low ESFT. In addition, we propose that the existence of this novel molecular subclass will be worthy of consideration when evaluating treatment responses in clinical trials that test these agents.

In summary, we have identified a distinct subgroup of ESFTs that do not express high levels of BMI-1. These tumors represent up to 20% of cases and have markedly different molecular profiles compared with the more common BMI-1–overexpressing tumors. Importantly, the relative activation of numerous cancer-associated biological pathways is diminished in BMI-1–low tumors, indicating that patients with these tumors may be less likely to respond to novel pathway–targeted agents.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References
Ewing Tumors That Do Not Overexpress BMI-1 Are a Distinct Molecular Subclass with Variant Biology: A Report from the Children’s Oncology Group

Aaron Cooper, John van Doorninck, Lingyun Ji, et al.


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