Biomarkers in the Ewing sarcoma family of tumors

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Abstract: This review provides a concise analysis of the well-characterized phenotypic and biological biomarkers having diagnostic and prognostic significance in the Ewing sarcoma family of tumors. Of particular interest, and potentially high clinical relevance, are studies of cell cycle biomarkers, growth signaling pathway expression, biomarkers related with angiogenesis, epithelial mesenchymal transition, and tumor microenvironment. In addition, we discuss findings of specific interest from recent biomarker studies, including copy number alterations, minimal residual disease and predictive biomarkers, and biological targets such as CD133, insulin-like growth factor 1, and poly(adenosine diphosphate-ribose) polymerase 1. In conclusion, several diagnostic and prognostic biomarkers for the Ewing sarcoma family of tumors have been published in recent years; nevertheless, these all need to be validated in a series of prospective studies. The findings that only a small proportion of patients with relapsed Ewing sarcoma family of tumors respond to targeted therapies serve as an example of the critical need for predictive biomarkers in this disease.

Keywords: Ewing sarcoma, biomarkers, immunohistochemistry, molecular biology

Introduction

Ewing sarcoma (ES) is a solid tumor of bone and soft tissue that primarily affects adolescents and young adults.1 These tumors can arise in any bone of the body, although the most common sites of disease include the pelvis, ribs, and long bones of the extremities. Approximately 25% of patients have metastatic disease at diagnosis, most often in the lungs, bone, and bone marrow. The treatment of ES relies on a multidisciplinary approach, coupling highly intensive chemotherapy with surgery and/or radiotherapy to control the primary site of disease and metastatic lesions.2 Patients with advanced disease have less than 30% disease-free survival, whereas those with disease localized to a single site generally have more than 70% disease-free survival at 5 years after diagnosis.3 In addition, no improvements in outcomes have been experienced for patients with metastatic disease in more than 30 years.1,4 Apart from metastasis, other clinicopathological features have been considered as markers of high-risk disease. Although increasing tumor size, decreased tumor necrosis after neoadjuvant chemotherapy, central tumor site, and increasing patient age have all been implicated as independent prognostic factors of poor outcome, none of these are as significant as the presence of metastatic disease at diagnosis.5 Nowadays, we have no significant evidence about which patients with localized disease are at risk for recurrence or which of those with metastatic disease may be curable with conventional therapy. In addition, with the incorporation of new directed therapies within the clinical context,
it will also be important to introduce them rationally through the selection of optimal cohorts of patients who will be most likely to respond.

The aim of this review is to provide a detailed description of those biomarkers most recognized as having a clinical prognostic and predictive effect in ES.

**What do we expect from biomarkers?**

A National Health Institutes working group defines a biomarker as a characteristic that can be objectively measured and evaluated as an indicator of normal biologic or pathogenic processes or pharmacologic responses to a therapeutic intervention. To consider a biomarker as useful in the clinical context, it should provide a clear risk/benefit ratio to facilitate clinical decisions, be available in an efficient and cost-effective manner, and should be assessed on available technological platforms from easily obtainable samples.

Biomarkers can be divided into three types: diagnostic, prognostic, and predictive. Most of the biomarkers studied in ES are prognostic and provide information about the disease outcome after standard therapy.

**Diagnostic and prognostic biomarkers in ES**

**Immunohistochemical panel**

ESFT (Ewing sarcoma family of tumors) constitutes a group of small round cell sarcomas with specific molecular alterations showing a spectrum of neuroectodermal differentiation. The histopathology reveals a diffuse proliferation of small round cells fluctuating between undifferentiated patterns (conventional ES) and neoplasms that show neuroectodermal differentiation with Homer-Wright pseudorosettes (so-called primitive neuroectodermal tumors [PNET]; Figure 1A and B).

Geographic necrosis is frequent, apoptosis/mitosis varies from case to case, and commonly the tumor cells display abundant Periodic acid–Schiff-positive glycogen. Three larger categories appear according to their predominant morphological criteria: conventional/classical/typical ES, PNET with neuroectodermal features, and atypical ES comprising subtypes distinct from the other two types. Atypical ES includes large cell variant, tumor with spindle cells, hemangioendothelial ES, adamantinoma-like ES, and sclerotic ES (Figure 1B and C).

At present, some issues remain as to whether or not unusual patterns (atypical ES) may be seen in combination with the classical pattern and whether the morphological phenotype of recurrent tumors can be modified by treatment regimens.

The atypical variant of ESFT seems to be associated with less-favorable prognosis when compared with the conventional and PNET variant; however, these results should be confirmed in prospective multicenter studies. Tumor size greater than 8 cm, the presence of metastasis, and poor histological response to neoadjuvant therapy are potential predictors and are significantly related to a negative outcome.

Immunohistochemistry constitutes a useful tool in the diagnosis and identification of ESFT, especially in the differential diagnosis with other small round cell tumors (SRCT) of bone and soft tissue. CD99, friend leukemia integration 1 (FLI-1), and human natural killer 1 (HNK-1) represent the more traditional antibodies, and recently, caveolin 1 (CAV-1) has been described as an additional marker that can be useful in the diagnosis of ESFT (Figure 2A–D).

CD99 immunoexpression is usually membranous and/or cytoplasmic and is, as a rule, moderately (++) or intensely (+++) positive in ESFTs. However, the specificity of CD99 is not exclusive for ESFT because it can be expressed in other SRCTs, including small cell osteosarcoma, rhabdomyosarcomas, poorly differentiated synovial sarcoma, desmoplastic small round cell tumors, undifferentiated carcinomas, neuroendocrine carcinomas, and lymphomas.

CD99 immunoexpression in SRCT (non-ESFT) is generally cytoplasmic, weak, and focal, although there are cases in which the expression is membranous and intense.

At first, it was thought that the immunoreactivity with FLI1 and HNK-1 would be of great support in the diagnosis of ESFT; however, several authors agree that this marker lacks specificity, and its sensitivity is quite variable. In contrast, CAV-1 is overexpressed in a high proportion of ESFTs. When the four antibodies described earlier (CD99,
Other immunohistochemical markers considered of use in the diagnosis of ESFTs are NKX2.2 and ERG. NKX2.2 has revealed a sensitivity and specificity of 93% and 89%, respectively, suggesting this marker could be used in the differential diagnosis of SRCT.\(^\text{19}\) In contrast, ERG is expressed not only in ESTF bearing the ERG rearrangement\(^\text{20}\) but also in a wide variety of tumors, including angiosarcomas, prostate carcinoma, myeloid sarcomas, and epithelioid sarcomas.\(^\text{21–23}\) For this reason, specificity for ERG staining in sarcomas should be considered with caution, and the type of immunoexpression is undoubtedly influenced by antibody selection.

Morphological and immunohistochemical neural and neuroendocrine differentiation in ESFT has been widely described, but at present it does not provide additional data for the diagnosis, differential diagnosis, or prognosis of this family of tumors.\(^\text{10,11,13,24–27}\) Neural differentiation can be demonstrated in ESFTs by use of biomarkers such as neuron-specific enolase, S-100, neurofilaments, CD56, chromogranin A, and synaptophysin.\(^\text{10,11,13,24–27}\)

Epithelial differentiation in ESFT has been described in several studies and is particularly observed in the atypical variant characterized by extensive epithelial differentiation ( adamantinoma-like ES).\(^\text{9–11,17,18,28–30}\)

### Table 1

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<th>Diagnosis</th>
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| **Confirmed Ewing sarcoma** | • CD99, caveolin 1-positive  
• Fluorescent in situ hybridization and/or reverse-transcriptase polymerase chain reaction positive for EWSR1/ETS, EWSR1/non-ETS gene fusion or other less-frequent translocations without EWSR1 rearrangement (FUS/FEV, FUS/ERG)  
• Desmin- and myogenin-negative (absence of muscular differentiation)  
• Can reveal immunoreactivity for cytokeratin and/or carcinoembryonic antigen; epithelial membrane antigen can be positive (poor and focal)  
• Neural and/or neuroendocrine markers are positive  
• Conventional/primitive neuroectodermal or atypical variant |
| **Ewing-like sarcomas** | • Histopathology similar to atypical Ewing sarcoma  
• CD99, caveolin 1-positive  
• Fluorescent in situ hybridization and/or reverse-transcriptase polymerase chain reaction negative or noninformative for EWSR1 or FUS rearrangement  
• Presence of CIC/DUX4 or BCOR/CCNB3 gene fusion  
• Absence of specific translocations of other SRCT (synovial sarcoma, rhabdomyosarcoma, congenital fibrosarcoma, etc)  
• Desmin- and myogenin-negative (absence of muscular differentiation)  
• Can reveal immunoreactivity for cytokeratin; epithelial membrane antigen can be positive (poor and focal) |
| **Ewing sarcoma or Ewing-like sarcomas excluded** | • CD99, caveolin 1-negative  
• Fluorescent in situ hybridization and/or reverse-transcriptase polymerase chain reaction negative or noninformative for EWSR1 or FUS rearrangement  
• CIC/DUX4 and BCOR/CCNB3 are negatives  
• Muscular differentiation, strong epithelial membrane antigen immunoreactivity, or any other immunohistochemical profile or molecular findings specific for non-Ewing sarcoma family of tumors, even with CD99 and/or caveolin 1 positivity  
• Tumors corresponding to undifferentiated small round cell sarcomas or pediatric/adult small round cell tumors with non-Ewing sarcoma family of tumors profile (undifferentiated synovial sarcoma, small cell osteosarcoma, rhabdomyosarcoma, Wilms tumor, undifferentiated carcinoma, Merkel cell carcinoma, lymphomas/leukemia, rhabdoid tumors, neuroblastoma, epithelioid sarcoma, malignant peripheral nerve sheath tumors) |

**Abbreviations:** ESFT, Ewing sarcoma family of tumors; SRCT, small round cell tumors.
differentially has also been described in conventional and variants of PNET.\textsuperscript{17,18,28} Cytokeratin (AE/AE3), epithelial membrane antigen (EMA), and carcinoembryonic antigen (CEA) are the most widely used antibodies for detecting epithelial differentiation in ESFTs. In addition, many adhesion molecules such as desmoplakin, desmoglein, claudin, occludin ZO-9, and E-cadherin have been also explored.\textsuperscript{30} Our group reported (AE1/AE3) immunoexpression in 20\% of a series of 415 ESFT (Figure 3A and B), mainly in the atypical variant.\textsuperscript{17,18} Similarly, Elbashier et al,\textsuperscript{29} in a series of 43 cases, demonstrated cytokeratin expression in 39.5\% of tumors, finding no significant association between cytokeratin immunoreactivity and patient age, sex, and skeletal and extraskeletal primary location, as well as primary, metastatic, or recurrent tumors or chemotherapy treatment. Both studies support the evidence for epithelial differentiation in ESFTs and emphasize that the expression of cytokeratin does not exclude ESFT in the differential diagnosis of SRCT.\textsuperscript{9–11,17,18,28,30} Other epithelial biomarkers such as epithelial membrane antigen and carcinoembryonic antigen immunoexpression are not usually described in ESFT (Figure 3B). However, in the same recently published study, we also found that up to 6.6\% and 20.8\% of ESFT can show epithelial membrane antigen or carcinoembryonic antigen expression, respectively, a fact that complicates the differential diagnosis with other tumors (sarcomas, lymphomas, and carcinomas).\textsuperscript{17,18}

Unlike epithelial biomarkers, muscular differentiation markers such as desmin and myogenin are consistently negative in ESFT (Figure 3C). In fact, muscular differentiation in SRCT virtually excludes ESFT;\textsuperscript{14,15,31} nevertheless, biphrenotypic tumors (neural/neuroectodermal and muscular differentiation) have been reported in the literature.\textsuperscript{31–33} Molecular analysis has provided important diagnosis clues in such neoplasms (Table 1).

**Genetics**

From the genetic point of view, ESs are specifically characterized by the presence of recurrent chromosomal translocations. In more than 85\% of cases, the translocation occurs between chromosomes 11 and 22, resulting in the creation of a pathognomonic chimeric fusion gene, \textsc{EWSR1/FLI1}, that encodes the \textsc{EWS/FLI1} protein.\textsuperscript{34} Alternative chromosomal translocations between members of the \textsc{EWSR1} and \textsc{ETS} gene families such as t(21;22) and \textsc{EWSR1/ERG} have been identified in the majority of cases that lack the classic \textsc{EWSR1/FLI1} fusion.\textsuperscript{4} The most common fusion type joins exon 7 of \textsc{EWSR1} with exon 6 of \textsc{FLI1}, also known as the type 1 fusion. However, numerous, less-common breakpoints between both genes have been identified. Furthermore, about 10\% of cases involve alternate \textsc{ETS} family genes such as the 3′ translocation partner.\textsuperscript{35}

Associations between fusion type and prognosis were observed in the late 1990s, such that tumors harboring a type 1 fusion had a significantly better outcome compared with those with other fusion types.\textsuperscript{36} However, these results have not been further validated. Prospective sets of tumors from equivalently treated patients enrolled in the Children’s Oncology Group and Euro-Ewing studies were evaluated for fusion status and outcome and failed to reproduce the original findings.\textsuperscript{37} Hence, current evidence strongly suggests that among the more than 90\% of ES tumors that harbor EWSR1 rearrangements, the fusion type is no longer a reliable prognostic marker and should not be used to stratify therapy or instruct treatment decisions.\textsuperscript{8}

**Ewing-like sarcomas**

Those tumors make up a subgroup of sarcomas with a histopathology similar to ES, but with the \textsc{CIC/DUX4} or \textsc{BCOR/CCNB3} gene fusion instead of \textsc{EWSR1} or \textsc{FUS} rearrangement.\textsuperscript{38,39} Sarcomas with \textsc{CIC/DUX4} gene fusion are usually extraskeletal lesions that affect young adults with an aggressive clinical evolution and metastases (lung and lymph nodes). Morphologically, \textsc{CIC/DUX4} sarcoma resembles an atypical ES with variable expression of CD99 and cytokeratin.\textsuperscript{38} Sarcomas with the \textsc{BCOR/CCNB3} gene fusion also reveal a morphology very similar to atypical ES and frequently arise in bone. Strong nuclear immunoexpression of \textsc{CCNB3} characterizes these tumors.

![Figure 3](https://www.dovepress.com/)

**Figure 3** (A) Strong cytokeratin (AE1/AE3) immunoexpression in primitive neuroectodermal tumors, 20×. (B) Moderate epithelial membrane antigen immunoexpression in Ewing sarcoma, 20×. (C) Desmin negativity in Ewing sarcoma, 20×. (D) Strong nuclear Ki-67 expression in Ewing sarcoma, 10×.
however, molecular studies are needed to confirm the final diagnosis.  

**Cell cycle biomarkers**

The cell cycle pathway and its multiple protein components are frequently altered in cancer. In ESFT, genetic alterations affecting the regulators of the G1-phase cell cycle transition have been described, including deletion of both CDKN2A (INK4/ARF locus) and RB1. Kovar et al were the first to describe CDKN2A, finding it in 30% of primary tumors and 52% of ES cell lines. After this work, several retrospective studies have demonstrated an association between CDKN2A alterations and clinical outcome in ESFT patients. Patients in these studies were found to have a worse disease-specific survival in univariate and multivariate analysis. A meta-analysis examining the prognostic significance of CDKN2A alterations in ESFT based on six separated studies (N=188 patients) concluded that the pooled risk ratio for worse outcome with CDKN2A alterations was 2.17 (95% confidence interval, 1.55–3.03; P<0.0001). Hence, the evidence to support CDKN2A loss as a negative prognostic marker is strong and worthy of prospective validation.

The prognostic significance of TP53 point mutations in ESFTs has also been evaluated in retrospective series by immunohistochemistry and/or sequencing. TP53 overexpression is detected in approximately 10% of ESFT and has been associated with advanced disease at diagnosis, poorer treatment response, and a worse overall survival. Huang et al, in a series of 60 patients, reported TP53 mutations in 13% of cases, as well as CDKN2A homozygous deletions in another 13% of cases. These authors demonstrated that TP53 mutations and/or CDKN2A alterations were significantly associated with poor response to chemotherapy, and in a multivariate analysis, TP53 and/or CDKN2A alterations as a single, combined variable were identified as the most significant prognostic factors. Finally, our group, using immunohistochemistry and fluorescent in situ hybridization analyzed a series of cell cycle regulation markers (p53, p21, p27, and Ki-67) in a series of 324 genetically well-characterized ESs. In this study, we found that expression of p53 (P=0.025), p21 (P=0.015), and p27 (P=0.013) was higher in disseminated than in localized disease. In addition, Ki67 was expressed in 34% of cases (Figure 3D) and constituted an independent prognostic factor for progression-free and overall survival, independent of the type of treatment. In fact, the study demonstrated that Ki67 expression represents a valuable indicator of poor prognosis in localized ESFT.

As a whole, compelling data from several retrospective studies implicate alterations of TP53, CDKN2A, and Ki67 as negative prognostic biomarkers in ESFT. We are still waiting for the analysis of prospectively collected samples from clinical studies such as the AEWS0031 from the Children’s Oncology Group. If studies such as this confirm prior observations, then analysis of these cell cycle proteins will become a strong candidate for inclusion as a prognostic biomarker that can inform treatment decisions in future clinical trials.

**Growth signaling pathway expression in ESFT**

Mora et al described the relationship between clinical outcome and the immunohistochemical expression of proteins involved in active growth signaling pathways such as c-KIT (tyrosine–protein kinase KIT), AKT (protein kinase B), p-mTOR (phosphorylated mTOR), IGF-1R (insulin-like growth factor receptor 1), IGFBP-3 (IGF-binding protein 3), MAPK (MAP-Kinase), p-AKT, p27KIP1, and p70S6 kinase. These authors analyzed 45 ESFTs, finding that of all the variables analyzed, only presence of metastasis, p-mTOR, and p27KIP1 immunostaining were independent prognostic factors of outcome, with p-mTOR and p27KIP1 protein overexpression being associated with better survival.

Ahmed et al also studied the expression of therapeutic targets in 72 ESFTs. They performed immunohistochemical (IHC) staining of intracellular molecules mTOR, vascular endothelial growth factor (VEGF), Akt, nuclear factor κB, and BRAF and correlated these results with survival data. The tumors revealed heterogeneous protein expression with poor BRAF immunoreactivity. No prognostic significance was observed, except for VEGF, where decreased VEGF expression was significantly associated with better prognosis.

Expression of KIT protein is an uncommon event in ESFT. An immunohistochemical and mutational study of 71 ESFTs revealed positive expression by IHC in 38% of cases; however, activating mutations were found in only 2.6% of the tumors. This study confirms that c-kit mutations are not necessarily concordant with CD117 IHC expression in ESFT, as in other neoplasms. As far as we know, c-kit status has neither diagnostic nor prognostic significance in ESFT.

NOTCH pathway alterations have been described in ESFTs and are associated with abnormalities in tumor cell growth. Kovar et al studied the Notch pathway by gene expression profiling and immunohistochemistry in ESFT and found that although Notch receptors were highly expressed, Notch did not appear to be active. High expression of the Notch effector HES1 transcription factor, usually used as a
surrogate marker for active Notch, was also restricted to the outside of the nucleus in the majority of ESFTs. This indicates that HES1 is uncoupled from Notch in ESFT and supports a role for Notch in ESFT tumor suppression.

ERBB4 immunohistochemical expression has been studied by Mendoza-Naranjo et al\(^4\) in a cohort of 19 paired biopsies of primary and metastatic ESFTs from the same patients, as well as 94 (78 primary and 16 metastatic) nonpaired ESFTs. The immunohistochemical study revealed significantly higher ERBB4 expression in metastatic lesions (78.9%) compared with primary tumors (42.1%), and the Kaplan–Meier survival analysis demonstrated a direct correlation between high ERBB4 expression and poor outcome. These findings confirm a link between high ERBB4 expression and metastatic disease in ES patient samples. In addition, the same group had previously reported that activation of the ERBB4 tyrosine kinase suppresses anoikis, or detachment-induced cell death, and induces chemoresistance in ESFT cell lines in vitro.\(^5\)

Considering the probability of aberrant expression of ganglioside antigen GD2 in undifferentiated/primitive neuroectodermal tumors related with stem cells, Kailayangiri et al\(^6\) explored the GD2 expression in ESFT cell lines and primary tumors, using immunofluorescence. Surface GD2 was detected in ten of ten ESFT cell lines and three of three primary cell cultures. Furthermore, diagnostic biopsies from 12 of 14 patients also revealed GD2 expression. In the same study, they found that GD2-specific T cells had further activity against ES xenografts. In conclusion, GD2 surface expression is common in ESFT and provides a suitable target antigen for immunotherapeutic strategies to prevent metastasis and relapse.

**Biomarkers related with angiogenesis**

Angiogenesis is essential for sustained tumor growth and provides the systemic network that stimulates metastasis. Without the formation of supporting vasculature, tumor cells would be unable to obtain the nutrients and oxygen necessary for proliferation and would not be able to mediate metastatic spread. A delicately controlled balance between pro- and antiangiogenic factors typically regulates this process; environmental stressors or genetic changes such as hypoxia, acidosis, oncogene activation, and loss of tumor suppressor genes lead to dysfunction of this balance and result in angiogenesis.\(^5\)

To consider the potential utility of antiangiogenic and vascular targeting therapies, it would be helpful to understand the clinical effect of angiogenesis biomarkers in patients with EFST. Unfortunately, these data are relatively scarce and difficult to interpret. At least three groups have investigated tumor microvascular density as a prognostic factor in EFST. One group demonstrated a significant association between elevated microvascular density and poor event-free survival and overall survival (OS) in 29 patients with ES. In contrast, two other groups reported that tumor microvascular density does not correlate with metastatic status, disease-free survival, or OS.\(^6\)

VEGF is the best characterized proangiogenic factor and is considered the most important factor involved in the development of the vasculature. There are a number of different VEGF molecules (VEGFA–VEGFE) that bind to VEGF receptors (VEGFR1–VEGFR3).

Patients with ESFTs have increased circulating VEGF levels compared with controls.\(^4\) Interestingly, EWS-ETS fusion oncoproteins drive the expression of VEGF and may contribute to the increased VEGF levels observed in patients. In addition, a study of angiogenesis using immunohistochemistry\(^6\) demonstrated that VEGF expression is correlated with poorer overall survival; however, confirmation with a larger series is needed.

Other biomarkers of angiogenesis have not been studied systematically in patients with EFST. These markers include soluble VEGFR, thrombospondin, placental growth factor, and circulating endothelial cells. Larger studies that specifically include patients with ESFT will be required to further assess the utility of angiogenic biomarkers for prognostic and therapeutic guidance.\(^6\)

**Biomarkers related to epithelial mesenchymal transition**

An immunohistochemical study performed in a large series of ESFTs revealed that desmoplakin expression was correlated with improved PFS. In addition, expression of ZO-1 (zona occludens 1) or Snail was correlated with improved OS, and cytokeratin 8/18 immunoreactivity was associated with a poorer prognosis.\(^1\)

ZEB2 is a transcription factor that represses epithelial gene expression in ES cells, and this, in turn, represses the epithelial phenotype and facilitates metastasis. ZEB2 reduction in ES cells results in a decreased metastatic potential, using a mouse metastasis model.\(^5\)

Ezrin, a member of the ezrin/radixin/moesin protein family, plays a pivotal role in tumor invasion and metastasis. The prognostic significance of this protein has been studied in a large series of ESFTs (415 cases),\(^4\) in which IHC ezrin expression was found to represent a favorable prognostic factor in these tumors.
Tumor protein D52 (TPD52) is overexpressed in cancers of diverse cellular origins, and increased TPD52 expression is associated with increased proliferation and invasive capacity in different cell types. Although ESFT can reveal TPD52 immunoeexpression, no prognostic significance has been described.

**Biomarkers related to the tumor microenvironment**

The Leiden (Holland) working group of ESFT has demonstrated that the CXCR4/CXCL12 axis is frequently altered in ESFTs and affects tumor progression and patient survival by promoting cell growth. They demonstrated an expression level-dependent negative effect of CXCR4 protein expression on ESFT overall survival and provide evidence for a crucial role of the CXCR4–CXCL12 axis in promoting ESFT cell growth. The same group indicated that increased tumor infiltration of CD8+ T-cells is correlated with improved OS. In addition, Fujiwara et al., using IHC analysis of macrophage infiltration reported that high macrophage infiltration was correlated with poorer OS.

Immunohistochemical analysis of papillomavirus binding factor (PBF), previously defined as an osteosarcoma-associated antigen, was performed by Yabe et al in a series of 20 ESFTs. Ninety percent of the tumors were positive for PBF, and in the prognostic analysis, PBF overexpression was significantly associated with decreased disease-free and OS. Their finding demonstrates that PBF overexpression is a poor prognostic factor in ESFT. This result should be validated in large and prospective series.

CCN3 is a family of proteins that stimulate mitosis, adhesion, apoptosis, extracellular matrix production, growth arrest, and migration of multiple cell types. Perbal et al. examined the expression of CCN3 protein by IHC in a large series of ESFTs, and their results showed that expression of the full-length CCN3 in ESFTs is associated with a worse prognosis.

Lectin galactoside-binding soluble binding protein (LGALS3BP) is a matrixcellular protein with a role in tumor progression and metastasis. Zambelli et al. explored the possible correlation between LGALS3BP and event-free survival and overall survival in ESFT. In their study, IHC expression of this protein was correlated with improved EFS and OS. The association between LGALS3BP and prognosis was confirmed at the protein level, when expression of the molecule was determined in tumor tissues, but not in serum, indicating a role for the protein in the local tumor microenvironment.

Xg is a member of the CD99 family, expressed in ESFT cell lines and ESF primary tumors. Meynet et al. concluded that Xg immunoeexpression in ESFT defines a subgroup of patients with worse prognosis compared with those with Xg-negative localized tumors, indicating a clinical relevance of Xg expression in ESFT.

Connexins are building unit proteins of gap junctions that are prognostic markers in carcinomas. Bui et al. studied Connexin 43 immunoeexpression in a series of 36 ESFTs and found cytoplasmic reactivity in 78% of tumors. In addition, high expression was correlated with poorer OS.

The expression in ESFT of proteins, such as lysine demethylase specific, has recently been demonstrated, although studies are ongoing with the goal of defining whether or not there is a prognostic implication of these proteins in the ESFT.

The six-transmembrane epithelial antigen of the prostate 1 (STEAP1) is a membrane-bound mesenchymal stem cell marker highly expressed in ESFT. Grunewal et al. investigated the possible association between STEAP1 immunoeexpression and outcome in a series of 114 primary ESFTs. STEAP1 was positive in 62.3% of tumors, and high membranous STEAP1 immunoreactivity was correlated with better overall survival; thus, high membranous STEAP1 expression predicts improved outcome.

**Copy number alterations**

Genomic instability with subsequent copy number alterations (CNAs) has been well-characterized in ESFTs. The most commonly reported CNAs in ESFTs are trisomy of chromosome 8, trisomy of chromosome 12, and gain of chromosome 1q. Using a variety of platforms, several recurring regions of gains and losses with clinical relevance have been described. However, most of these results are derived from retrospective studies that use different approaches to identify CNAs. A prospective analysis of CNAs and clinical outcome has not yet been undertaken.

**Minimal residual disease**

Assessment of minimal residual disease (MRD) has been considered a critical part of therapeutic decision-making in many leukemic processes. Attempts to validate methodologies and prognostic correlations for MRD detection in ESFTs have primarily used reverse-transcriptase polymerase chain reaction and flow cytometry.

Reverse-transcriptase polymerase chain reaction assays for MRD are designed to identify specific ESFT-related fusion transcripts both in blood and/or bone marrow as evidence of
occult micrometastatic disease or persistent disease after systemic therapy. The largest published study examined EWSR1-FLI1 and EWSR1-ERG transcript levels in a total of 172 patients from a study of the French Society of Pediatric Oncology. Circulating transcripts were identified in 20% of patients at diagnosis and were more frequently observed in patients with large tumor burdens. In localized disease, the presence of fusion gene transcripts in blood or bone marrow correlated with significantly poorer outcomes.

To rigorously address whether the detection of circulating tumor transcript is of prognostic significance, the multicenter European Ewing Tumor Working Initiative of National Groups Ewing Tumour Studies 1999 trial prospectively collected bone marrow samples for more than 10 years. As the first large prospective trial examining MRD by reverse-transcriptase polymerase chain reaction in ESFT patients, the findings of this study will be critical for evaluating the feasibility and usefulness of this modality as a biomarker for ES.

Flow cytometry has also been used for some studies to define the presence of MRD in ESFT. These studies use the cell-surface glycoprotein CD99 to identify tumor cells. These studies confirm the feasibility of the approach and provide preliminary support for the potential prognostic significance of MRD.

### Predictive biomarkers in ES

CD133 has been described as a putative marker of mesenchymal stem cell in ESFTs, and its immunoexpression has been associated with chemoresistance in at least some cases of primary ESFT. Jiang et al have explored whether high levels of CD133 are associated with drug resistance in ESFT; their study revealed heterogeneity in PROM1/CD133 expression in tumors and cell lines and confirmed that high levels of PROM1 expression were associated in some cases with chemoresistant disease. Nevertheless, they suggested that further studies are required to elucidate the contribution of PROM1/CD133 expressing cells to therapeutic resistance in a large, prospective cohort of primary ESFT.

A number of biological targets and potentially promising novel agents have been identified for ESFT, with insulin growth factor receptor 1 (IGF1R) and poly(adenosine diphosphate-ribose) polymerase 1 (PARP1) being the better-identified targets in the context of ESFT. IGF1R is highly expressed by ES cells, and many studies have demonstrated the importance of the IGF1R pathway in ES tumor models.

### Table 2 Potential biomarkers in Ewing sarcoma

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(Continued)
antibodies resulted in dramatic responses in a few patients with refractory disease. However, in subsequent trials in unselected ES populations, response rates have been only about 10%, albeit in heavily pretreated patients. Thus, whether IGF1R targeted therapy has failed to provide significant response rates because of a lack of intended biologic activity against the tumor remains unknown.

The ability to predict whether a patient is likely to respond to a novel agent greatly increases the chance of success of a targeted therapy and encourages personalized medicine more generally. Such a biomarker does not exist for IGF1R-directed therapy in ESFT, although some studies have suggested that differential expression and activation of the insulin receptor and nuclear localization of phosphorylated IGF1R may be useful predictors of treatment response.

Another potential treatment to emerge from recent preclinical research is PARP1 inhibition. PARP1 is a key enzyme involved in single-strand repair DNA. In late 1990, it was demonstrated that PARP1 is overexpressed in ESFT, that its expression is regulated by ETS transcription factors, and that in vitro and in vivo models of ESFT are highly sensitive to PARP1 inhibitors alone and in combination with temozolomide. Because PARP1 inhibition has already been evaluated in numerous different adult-onset tumor types, a variety of potential biomarkers of DNA repair currently exist (PARP1 expression or γ-H2AX) and could be incorporated for evaluation in future pediatric trials.

Conclusion

Several diagnostic and prognostic biomarkers for ESFT have been published in recent years; Table 2 summarizes the biomarkers with references. Of particular relevance are studies of cell cycle proteins, epithelial components, and MRD; nevertheless, they all need to be validated in a series of prospective studies.

The findings that only a small proportion of patients with relapsed ESFT respond to targeted therapies serve as an example of the critical need for predictive biomarkers in this disease.

Table 2 (Continued)

<table>
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<th>Biological target</th>
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<td>Insulin growth factor receptor 1</td>
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<td>Poly(ADP-ribose)polymerase 1</td>
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Notes: Green, diagnostic support; red, prognostic significance; gray, prognostic significance requires validation.

Abbreviations: AKT, protein kinase B; MAPK, MAP-Kinase; p-mTOR, phosphorylated mTOR; VEGF, vascular endothelial growth factor; ADP, adenosine diphosphate; ZO-1, zona occludens 1.


