Published in final edited form as:

Cancer. 2011 November 1; 117(21): 4966–4976. doi:10.1002/cncr.26112.

Investigation of the Insulin-Like Growth Factor-1 Signaling Pathway in Localized Ewing Sarcoma:

A Report From the Children's Oncology Group

Scott C. Borinstein, MD, PhD¹, Donald A. Barkauskas, PhD², Mark Krailo, PhD³, Daniel Scher, MS⁴, Lauren Scher, BS⁴, Silke Schlottmann, PhD⁴, Bhaskar Kallakury, MD⁴, Paul S. Dickman, MD⁵, Bruce R. Pawel, MD⁶, Daniel C. West, MD⁷, Richard B. Womer, MD⁸, and Jeffrey A. Toretsky, MD⁴

¹Department of Pediatrics, Division of Pediatric Hematology/Oncology, Vanderbilt University, Nashville, Tennessee

²Children's Oncology Group, Arcadia, California

³Department of Preventive Medicine, Keck School of Medicine, University of Southern California, Los Angeles, California

⁴Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University, Washington, District of Columbia

⁵Department of Pathology, Phoenix Children's Hospital, Phoenix, Arizona

⁶Department of Pathology, The Children's Hospital Of Philadelphia, Philadelphia, Pennsylvania

⁷Department of Pediatrics, University of California, San Francisco School of Medicine, San Francisco, California

⁸Department of Pediatrics, Children's Hospital of Philadelphia, University of Pennsylvania, Philadelphia, Pennsylvania

Abstract

BACKGROUND—The insulin-like growth factor-1 (IGF-1) signaling pathway plays an important role in the pathology of Ewing sarcoma (ES). Retrospective studies have suggested that levels of IGF-1 and IGF binding protein 3 (IGFBP-3) are correlated with the outcome of patients with ES.

METHODS—The IGF-1 signaling pathway was investigated prospectively in 269 patients who had localized, previously untreated ES. Serum samples were obtained at diagnosis, and concentrations of IGF-1 and IGFBP-3 were determined by enzyme-linked immunosorbent assays. In addition, immunohistochemistry (IHC) was performed to assay for phosphorylated p70S6

© 2011 American Cancer Society

Corresponding author: Jeffrey A. Toretsky, MD, Departments of Oncology and Pediatrics, Lombardi Comprehensive Cancer Center, Georgetown University, 3970 Reservoir Road NW, Room 311 West, Washington, DC 20057-1469; Fax: (202) 687-1434; jat42@georgetown.edu.

CONFLICT OF INTEREST DISCLOSURES

The authors made no disclosures.

kinase, protein kinase B (Akt), and forkhead box protein O1 (FOXO1) and to determine the presence of protein tyrosine phosphatase-L1 (PTPL1). IHC findings along with IGF-1 and IGFBP-3 concentrations were correlated with age, tumor location, sex, event-free survival, and overall survival.

RESULTS—Patients aged >18 years tended to have higher levels of IGF-1 ($P = .10$), lower levels of IGFBP-3 ($P = .16$), and decreased IGFBP-3:IGF-1 ratios ($P = .01$). No correlations were observed between sex, tumor location, or outcomes and concentrations of IGF-1 or IGFBP-3. Phosphorylation of p70S6 kinase, Akt, and FOXO1 was detected in the majority of patient tissues but was not associated with age, sex, or tumor location. PTPL1 was present in >80% of tumors and also was not correlated with age, sex, or tumor location. There was no difference in survival with respect to the presence of phosphorylated p70S6 kinase, phosphorylated FOXO1, phosphorylated Akt, or PTPL1.

CONCLUSIONS—The baseline IGFBP-3:IGF-1 ratio was correlated with age but did not affect the outcomes of patients with ES. The authors concluded that additional investigation of the IGF-1 pathway is warranted in patients with ES, and especially in those who have received treatment with IGF-1 receptor antibody inhibitors.

Keywords

Ewing sarcoma; insulin-like growth factor 1; insulin-like growth factor binding protein-3; p70S6 kinase; Akt; forkhead box protein O1; protein tyrosine phosphatase-L1

Ewing sarcoma (ES) is a rare malignant neoplasm of bone and soft tissue that most often affects adolescents and young adults. Approximately 250 cases occur in the United States each year, requiring an intense, multimodal treatment approach.¹ Despite advances in surgery, radiation therapy, and chemotherapy, 25% of patients who have localized disease at diagnosis and >75% of patients who have metastatic disease will die of their malignancy.^{2, 3} Better treatments are needed to improve the outcome for these patients and will require the identification of new agents for the treatment of ES.

ES is characterized by the presence of a fusion protein between a member of the ETS and TET family of proteins, resulting in the generation of a novel transcription factor that is required for ES pathogenesis.⁴ The most common is the result of a t(11;22)(q24;q12) chromosomal translocation, which fuses the ES breakpoint region 1 (*EWSR1*) and Friend leukemia virus integration 1 (*Flil*) genes together. This abnormality is identified in 85% of patients.⁵ The product of this fusion gene, known as the EWS-FLI1 oncoprotein, is required for pathogenesis; however, it is not always sufficient independently to promote tumor formation, and additional mutations are required to evade EWS-FLI1-induced cellular senescence.^{6–8}

There is strong evidence that the insulin-like growth factor-1 (IGF-1) signaling pathway plays an important role in the malignancy of ES. The expression of the IGF-1 receptor (IGF-1R) is required for EWS-FLI1 transformation of mouse fibroblasts.⁹ IGF-1 and IGF-1R expression are increased in ES cell lines and primary tumors.^{10, 11} The down-regulation of IGF-1R inhibits transformation of mouse fibroblasts and in vivo growth of ES

xenographs.^{9, 12} Furthermore, mesenchymal stem cells (thought to be the cell of origin for ES) transfected with *EWS-Flil* express IGF-1 and depend on its expression for growth.^{13, 14}

IGF-1 activation of the IGF-IR initiates a complicated signal-transduction cascade that results in phosphorylation of many downstream targets, including forkhead box protein O1 (FOXO1), protein kinase B (Akt), and p70S6 kinase.^{15–17} IGF-1 signaling also is regulated by protein tyrosine phosphatase-L1 (PTPL1), which is a regulator of the IGF-1 signaling pathway.¹⁸ PTPL1 is highly expressed in ES cell lines and primary tumors, and it has been demonstrated that PTPL1 is up-regulated in cells transfected with the *EWS-Flil* oncogene.⁷

IGF-1 is bound in the serum by a family of proteins that limit the amount of free circulating IGF-1. The best characterized IGF-1 binding protein (IGFBP) is IGFBP-3, which plays an important regulatory role in IGF-1 signaling.¹⁹ An increased level of IGFBP-3 decreases the risk of developing adult cancers by limiting the amount of free circulating IGF-1.²⁰ In ES, the EWS-FLI1 oncoprotein binds to the IGFBP-3 promoter to decrease its expression.²¹ Treatment of ES cell lines with exogenous IGFBP-3 increases apoptosis.^{21, 22} To determine whether serum levels of IGF-1 and IGFBP-3 were correlated with survival in patients with ES, Toretsky et al analyzed serum IGF-1 and IGFBP-3 levels at diagnosis in 111 patients who were treated at the National Cancer Institute from 1972 to 1992.²³ Those authors reported higher IGFBP-3 to IGF-1 (IGFBP-3:IGF-1) ratios in patients who had metastatic ES compared with patients who had localized ES, and they also noted a trend toward better outcomes for patients with localized disease who had higher IGFBP-3:IGF-1 ratios.²³ Those results support IGF-1 signaling as important in the pathogenesis of ES, but they also suggest that the inhibition of IGF1 signaling may be an effective pathway for treatment.

Therefore, to better understand how IGF-1 and IGFBP-3 correlate with survival in patients with localized ES, we prospectively investigated the IGF-1 signaling pathway in 269 patients with localized (nonmetastatic), previously untreated ES who were enrolled on the Children's Oncology Group (COG) clinical trial AWES0031. Serum samples were obtained at diagnosis, and serum concentrations of IGF-1 and IGFBP-3 were determined. In addition, immunohistochemistry (IHC) was performed in 114 primary ES tumor samples to assay for phosphorylated p70S6 kinase (pS6), Akt (pAkt), FOXO1 (pFOXO1), and PTPL1. We correlated IHC findings along with IGF-1 and IGFBP-3 concentrations with age, location, sex, event-free survival (EFS), and overall survival (OS) to determine whether baseline IGF-1 signaling has any prognostic value in patients with localized ES.

MATERIALS AND METHODS

Patient Population and Acquisition of Tumor and Serum Samples

The COG clinical trial AEWS0031 and the associated biology study AEWS02B1 were approved by both local and central institutional review boards. One of the primary objectives of these studies was to acquire tumor and serum specimens from patients who were consented on the protocol to investigate the tumor biology of localized ES. Informed consent was obtained from the parent or legal guardian of all participants. Assents were approved from minors when appropriate. Patients with nonmetastatic ES were eligible for enrollment onto AEWS0031. All member institutions of the COG could participate in this

study. At the time of enrollment, patients were assigned randomly to receive standard-schedule chemotherapy with doxorubicin, vincristine, cyclophosphamide, ifosfamide, and etoposide (“standard timing”) or cycles of the same chemotherapeutic agents given on an every-14-day schedule (“intensive timing”). Submission of serum and tumor tissues obtained before the start of chemotherapy was encouraged but was not required for enrollment on AEWS0031. Furthermore, enrollment on AEWS02B1 was not required for participation on AEWS0031. The protocols specified collection and storage conditions for serum and tumor specimens. Details of AEWS0031 have been reported by Womer et al.³

Measurement of IGF-1 and IGFBP-3

Levels of IGF-1 and IGFBP-3 derived from ES patient serum samples were measured by comparison with standardized IGF-1 and IGFBP-3 levels using an enzyme-linked immunosorbent assay (ELISA) according to R&D Systems Quantikine protocol for human IGF-1 immunoassay (DG100; R&D Systems, Minneapolis, Minn). IGF-1 and IGFBP-3 concentrations were measured, and molar IGFBP-3:IGF-1 ratios were calculated. These parameters were compared across groups defined by age at diagnosis, sex, and tumor location (pelvic vs nonpelvic) in patients with localized ES. Patients were stratified into 4 quartiles with respect to IGF-1 and IGFBP-3 concentrations and IGFBP-3:IGF-1 ratios.

IHC Studies

IHC staining of confirmed ES patient tissue samples was performed for 4 different antibodies: PTPL1, pS6, pAkt, and pFOXO1. Five-micron sections from formalin-fixed, paraffin-embedded tissues were deparaffinized with xylene and rehydrated through a graded alcohol series. Heat-induced epitope retrieval was performed by immersing the tissue sections at 98°C for 20 minutes in 10 mM citrate buffer, pH 6.0, with 0.05% Tween. Immunohistochemical staining was performed using the Histostain Plus Kit (Invitrogen, Carlsbad, Calif) according to the manufacturer’s instructions. Slides were treated with 3% hydrogen peroxide and Zymed Solution A (Zymed Laboratories, South San Francisco, Calif) for 10 minutes each and exposed to primary antibodies for PTPL1 (Fap-1 [H-300]; 1:50 dilution; catalog no. sc-15356; Santa Cruz Biotechnology, Santa Cruz, Calif), phospho-S6 ribosomal protein (serine [Ser] 235/236) antibody (catalog no. 2211; 1:250 dilution; Cell Signaling Technology, Danvers, Mass), phospho-Akt (Ser 473 [D9E]) XP rabbit monoclonal antibody (catalog no. 4060; 1:25 dilution [special diluent needed]; Cell Signaling Technology), and pFOXO1 (Ser 256; 1:125 dilution; catalog no. sc-101681; Santa Cruz Biotechnology) for 1 hour at room temperature. The slides were exposed to Zymed Solutions B and C for 10 minutes each and to 3,3-diaminobenzidine as a chromogen (Dako, Carpinteria, Calif) for 5 minutes. Slides were counterstained with Harris modified hematoxylin (Fisher Scientific, Thermo, NJ) at 1:17 dilution for 2 minutes at room temperature, blued in 1% ammonium hydroxide for 1 minute at room temperature, dehydrated, and mounted with Acrymount (Statlab Medical Products, Lewisville, Tex). Consecutive sections with the primary antibody omitted were used as negative controls. Images were captured using an Olympus DP70 microscope (Olympus Imaging America, Melville, NY) at ×40x magnification. The results were scored by a pathologist (B.K., one of the coauthors of this article) based on both staining intensity and the approximate percentage of positive cells in each tissue sample. The procedure described above also was performed

on a microarray consisting of ES and non-ES tissue cores. The results were scored for both intensity and distribution of staining and were condensed into categories with either the absence (zero) or presence (nonzero) of staining.

Cells from the TC-32 and TC-71 ESFT cell lines were serum starved and treated with various amounts of IGF-1 or 10% fetal bovine serum for 3 minutes and then fixed with 4% paraformaldehyde in phosphate-buffered saline at 4°C overnight. Cellular organization was maintained for each condition when cells were scraped and resuspended in 4% agarose for paraffin block formation in preparation for IHC analyses. An array was constructed with the various conditions, and slices of paraffin-embedded cells were placed on slides and stained with various concentrations of PTPL1, pS6, pAkt, and pFOXO1 antibodies according to the protocol described above.

The same protocol was repeated on TC-32 and TC-71 ESFT cells with 10 nM IGF-1 for various time points between 0 minutes and 120 minutes. The cells were made into arrays on paraffin blocks and stained with various concentrations of PTPL1, pS6, pAkt, and pFOXO1 antibodies according to the protocol.

Statistical Analyses

Values for IGF-1, IGF-BP3, and the IGFBP-3:IGF-1 ratio were grouped into 4 groups (above the 75th percentile [$n = 56$], between the 50th and 75th percentiles [$n = 57$], between the 25th and 50th percentiles [$n = 57$], and below the 25th percentile [$n = 56$]). For each IHC measure, both intensity and distribution were either zero or nonzero, which we defined as the absence (“–”) or presence (“+”) of staining, respectively, for each of the 4 IHC characteristics. Each of the binary covariates paired with IGF-1 concentration measures (IGF-1 rank, IGFBP-3 rank, and IGFBP:IGF ratio rank) or IHC values were checked for associations by using the exact conditional test of proportions.²⁴ The outcomes in terms of EFS and survival were compared between patient groups. EFS was calculated from the date of enrollment to the date of disease progression, diagnosis of a second malignant neoplasm, death, or the last patient contact, whichever occurred first. Patients who experienced disease progression, a second malignant neoplasm, or death were considered to have experienced an EFS event; otherwise, the patient was considered as censored at last contact. Survival was calculated from the date of enrollment to the date of death or last patient contact, whichever occurred first. Patients who died were considered to have experienced a death event; otherwise, the patient was considered as censored at last contact. The risk for the outcomes as a function of time was estimated by using the Kaplan-Meier method.²⁵ Risks across groups defined by patient characteristics were compared using the log-rank test.²⁴ P values $< .05$ were considered statistically significant. The analyses were done in SAS software (version 9.2; SAS Institute, Inc., Cary, NC) using the PROC FREQ and PROC PHREG procedures.

RESULTS

Study Population

AEWS0031 was opened for enrollment in May 2001 and closed in August 2005. In total, 587 patients were enrolled, of which 568 patients were eligible (Fig. 1). Blood and/or tissue samples were available from 269 eligible patients. IGF-1 and IGFBP-3 levels were analyzed in sera from 226 patients. IHC analyses for pS6, pFOXO1, Akt, and PTPL1 were performed on 144 patient samples. Of these, 136 patients had all 4 IHC measurements, 3 patients had 3 of 4 IHC measurements, 4 patients had 2 of 4 IHC measurements, and 1 patient had only 1 IHC measurement. One hundred one patients had both IHC and IGF analysis, and 125 patients had IGF data only.

Serum Assay of IGF-1, IGFBP-3, and IGFBP-3:IGF-1 Ratios

Older patients tended to have higher levels of IGF-1 ($P = .10$) but lower levels of IGFBP-3 ($P = .16$) and significantly decreased IGFBP-3:IGF-1 ratios ($P = .01$), as indicated in Table 1. Males and females had similar levels of IGF-1 ($P = .93$) and IGFBP-3:IGF-1 ratios ($P = .77$), but females tended to have higher levels of IGFBP-3 ($P = .10$). Patients with pelvic tumors had higher levels of IGF-1 ($P = .25$) and lower IGFBP-3:IGF-1 ratios ($P = .24$), but these findings were not statistically significant. Tumor location was not associated with IGFBP-3 levels ($P = .69$).

There was no correlation between IGF-1 levels, IGFBP-3 levels, or the IGFBP-3:IGF-1 ratio and patient outcomes (Fig. 2; Table 2). EFS and OS were slightly worse for patients in the highest quartile of IGF-1 levels, but these findings were not statistically significant ($P = .43$ and $P = .49$ for EFS and OS, respectively). IGFBP-3 levels did not correlate with EFS or OS outcomes ($P = .63$ and $P = .67$, respectively). Although there appeared to be improved survival among patients with higher IGFBP-3:IGF-1 ratios, these findings failed to reach statistical significance ($P = .52$ and $P = .69$ for EFS and OS, respectively).

IHC Analysis of pS6, pAkt, pFOXO1, and PTPL1

IHC assays were developed for pS6, pAkt, PTPL1, and pFOXO1. To establish a relative quantification index of the antibodies, the ES cell line TC-71 was treated with IGF-1, resulting in the phosphorylation of p70S6 kinase (Fig. 3A). Akt phosphorylation was present maximally by 3 minutes (data not shown). TC-71 cells that were stimulated for 3 minutes with increasing concentrations of IGF-1 resulted in a dose responsive increase in pAkt (Fig. 3B). IHC assays also were developed for PTPL1 and pFOXO1. pFOXO1 demonstrated similar responsiveness to pS6; however, PTPL1 did not exhibit a time or dose responsive presence (data not shown). IHC was performed on 144 primary ES tumor samples. The IHC results were compared with the clinicopathologic attributes of the patients (Table 3), and neither age, sex, nor primary tumor location was associated with the presence of pS6 or pAkt ($P = .57$, $P = .23$, and $P = .44$, respectively, for pS6; and $P = .23$, $P = .58$, and $P = .78$, respectively, for pAkt). Most patients had evidence of pFOXO1 (>90%) and PTPL1 (>80%) expression regardless of age, sex, or tumor location. Next, EFS and OS were compared with the absence or presence of pS6, pAkt, pFOXO1, and PTPL1 (Fig. 4; Table 4). There was no difference in survival with respect to the presence of pS6, pFOXO1, pAkt, or PTPL1.

Correlation of IHC Status and IGF-1 and IGFBP-3 Analyses

To determine whether pS6, pFOXO1, pAkt, or the presence of PTPL1 correlated with IGF-1 concentrations, the results from IHC and IGF-1 measurements were compared in the 101 patients who had both analyses performed. No statistically significant correlations between IGFBP-3 levels or IGFBP-3:IGF-1 ratios and the presence of pAkt, pFOXO1, pS6, or PTPL1 were observed (data not shown).

DISCUSSION

In the current study, we investigated the IGF-1 signaling pathway in a prospective study of patients with newly diagnosed, localized ES. An ELISA was performed on serum samples that were isolated from 269 patients to determine whether IGF-1 levels, IGFBP-3 levels, or IGFBP-3:IGF-1 ratios were correlated with clinical data (age, sex, location of primary tumor) and patient outcomes (EFS and OS). Previous studies of IGF-1 analysis in ES have suggested that patients who have more extensive disease at diagnosis (eg, evidence of metastases) have lower levels of IGF-1 and higher IGFBP-3:IGF-1 ratios, prompting an investigation to determine whether these biomarkers could be used to identify patients with more widespread disease and, thus, a worse prognosis.²³ In our current analyses, we observed that IGF-1 levels, IGFBP-3 levels, and IGFBP-3:IGF-1 ratios were not correlated with EFS or OS. However, the prior study was confounded by significant changes in chemotherapy protocols during the 20-year timeframe when these patients were treated. In addition, patients with metastatic disease were excluded from participation on AEWS0031. Statistical analysis of the data did reveal that patient age was prognostic (data not shown), supporting previous studies establishing that older patients have poorer outcomes than younger patients.^{26, 27} Furthermore, we observed that patients aged <18 years tend to have higher IGF-1 levels ($P = .10$) but higher IGFBP-3:IGF-1 ratios (ie, lower levels of free IGF-1), suggesting that a possible contributor to the worse prognosis in older patients is IGF-1 signaling pathway activation. Very few older patients had IGFBP-3:IGF-1 ratios in the fourth quartile, suggesting that older patients have higher levels of free IGF-1.

We also investigated the presence of downstream IGF-1 signaling proteins (pS6, pFOXO1, pAkt, and PTPL1) to determine whether they had any association with prognosis. We prepared a set of calibration samples to test the dynamic range of the antibodies under various IGF-1 concentrations and exposure times. On the basis of these pilots, we decided to use 0, 1+, 2+, or 3+ as a scoring system. However, given the limited number of samples, these results were condensed to presence (nonzero) or absence (zero). Phosphorylation of Akt, p70S6 kinase, FOXO1, or presence of PTPL1 did not correlate with EFS or OS, which was surprising. However, there was a trend toward better EFS and OS in patients who had tumors that demonstrated PTPL1 staining compared with patients who had tumors that did not demonstrate PTPL1 staining ($P = .15$ and $P = .23$, respectively). It has been demonstrated that PTPL1 dephosphorylates insulin receptor substrate-1 (IRS-1), and its expression can decrease Akt phosphorylation and induce apoptosis in breast cancer cell lines.¹⁸ Chromatin immunoprecipitation experiments have demonstrated that EWS-FLI1 directly binds the PTPL1 promoter, and the reduction of PTPL1 in ES cell lines decreases their growth in soft agar and sensitizes them to the chemotherapy agent etoposide.²⁸ This finding suggests that

additional analysis of downstream IGF-1 signaling in ES tumor samples may provide a better understanding of how PTPL1 contributes to growth suppression.

There were several limitations to this study. First, <50% of our patients who had serum analysis had tumor samples available for IHC. An analysis of more tumor specimens would increase the statistical power of the study. Second, IGF-1 or IGFBP-3 levels in serum may not correlate with concentrations in the tumor microenvironment. Correlation with messenger RNA expression in ES tumor cells or assay of IGF-1R or IGFBP-3 by IHC or similar methodology in primary tumor cells may be able to characterize the status of IGF-1 signaling better in ES tumors. Third, a serial analysis of IGF-1 and IGFBP-3 levels throughout treatment and after the completion of therapy also would help elucidate whether and how IGF-1 signaling changes in treatment. Fourth, because this was a multicenter study, there may have been differences in the handling of tumor specimens at the time of biopsy. Protein phosphorylation is a dynamic process and may be affected by delays in tissue preservation. Finally, because the IGF-1 signaling pathway is complicated, an analysis of additional proteins (such as IRS-1, mammalian target of rapamycin [mTOR], phosphatase and tensin homolog [PTEN], 4E-binding protein-1, etc) could provide a more detailed analysis of IGF-1 pathway activation. An additional analysis of these proteins was not possible because of the absence of adequate antibodies and limitations on patient material.

Abrogation of the IGF-1 signaling pathway is an attractive target for cancer therapy and has prompted the development of humanized antibodies directed at the IGF-1R designed to inhibit IGF-1 signaling. Currently, these agents are being investigated intensively in preclinical, phase 1, and phase 2 clinical trials and have generated some promising results. One of 5 patients with relapsed ES who received treatment with MK-0646 (Merck, Whitehouse Station, NJ) demonstrated a response, and the antibody reportedly was safe and tolerable.²⁹ Robatumumab (SCH-717454; Schering-Plough, Kenilworth, NJ) reportedly has been effective in slowing the growth of pediatric tumor xenographs and resulted in a complete resolution in 1 ES model.³⁰ Cixutumumab (IMC-A12; ImClone Systems, New York, NY) slowed the growth of ES xenographs, which prompted the development of a multicenter clinical study in conjunction with the mTOR inhibitor temsirolimus.³¹ Three patients with ES were enrolled on that trial, and 2 demonstrated tumor reduction.³² Two patients with ES who were treated on a phase 1 study with the anti IGF-1R antibody R1507 (Roche, Basel, Switzerland) had a partial response that lasted at least 11.5 months.³³ A phase 2 investigation with R1507 has been completed in which 125 patients with relapsed or refractory ES were treated with R1507, and 14.4% of those patients had a complete or partial response with a median duration of 25 weeks.³⁴ Figitumumab (CP-751871; Pfizer Inc., New York, NY) was tested in a phase 1 study, and 9 patients with ES were enrolled. Two of those patients had stable disease for at least 3 months, and 1 patient had a partial response.³⁵ Finally, AMG-479 (Amgen, Thousand Oaks, Calif) was tested in a phase 1 trial in 12 patients with ES, and 2 patients demonstrated a decrease in tumor size.³⁶ A phase 2 clinical trial is currently underway using AMG-479 in patients with ES or desmoplastic small round cell tumors, and approximately 10% of patients have had a response.³⁷

Overall, all 6 IGF-1R antibody inhibitors have demonstrated some effect in patients with relapsed, heavily pretreated ES, and approximately 10% to 15% of patients have responded

to therapy. However, the mechanism responsible for IGF-1R antibody sensitivity is unclear, and what determines susceptibility also is unknown. To find these answers, it will be necessary to investigate the IGF-1 signaling pathway in tumor and serum specimens from patients who are treated with IGF-1R inhibitors to determine why some patients who receive these agents respond so dramatically. In addition, there is evidence that combining IGF-1R antibody inhibitors may result in a synergic antitumor effect.³⁸ Using IGF-1R antibody inhibitors with other drugs, such the small molecule YK-4-279, should be investigated. YK-4-279 is a novel agent that blocks binding of RNA helicase A with the EWS-FLI1 fusion protein, resulting in apoptosis in ES cell lines and mouse xenographs.³⁹ Finally, new technologies like mass spectrometry are available now to better quantify IGF-1R in tumor samples from patients who respond and do not respond to IGF-1R inhibitors. These investigations hopefully will shed more light and will result in the development of more effective treatments for patients with ES.

Acknowledgments

FUNDING SOURCES

This research is supported by the Chair's Grant U10 CA98543 and Human Specimen Banking Grant U24 CA114766 of the Children's Oncology Group from the National Cancer Institute, National Institutes of Health (NIH), Bethesda, Md. Additional support for research is provided by a grant from the WWW (QuadW) Foundation, Inc. (www.QuadW.org) to the Children's Oncology Group. In addition, support for our work came from the Children's Cancer Foundation (Baltimore Md); Go4theGoal; Dani's Foundation; Alex's Lemonade Stand Foundation; the Liddy Shriver Sarcoma Initiative, the Amschwand Sarcoma Foundation; the AFLAC Foundation; a Burroughs Wellcome Clinical Scientist Award in Translational Research (J.A.T.); and NIH Grants R01CA88004 (J.A.T.), R01CA133662 (J.A.T.), R01CA138212 (J.A.T.), and RC4CA156509 (J.A.T.).

REFERENCES

1. Horner, MJRL.; Krapcho, M.; Neyman, N., et al., editors. SEER Cancer Statistics Review, 1975–2006. Bethesda, MD: National Cancer Institute; 2009.
2. Grier HE, Krailo MD, Tarbell NJ, et al. Addition of ifosfamide and etoposide to standard chemotherapy for Ewing's sarcoma and primitive neuroectodermal tumor of bone. *N Engl J Med*. 2003; 348:694–701. [PubMed: 12594313]
3. Womer RB, West DC, Krailo MD, Dickman PS, Pawel B. for the Children's Oncology Group AEWS0031 Committee. Randomized comparison of every-2-week v. every-3-week chemotherapy in Ewing sarcoma family tumors (ESFT) [abstract]. *J Clin Oncol*. 2008; 26(15s) Abstract 10504.
4. Riggi N, Stamenkovic I. The Biology of Ewing sarcoma. *Cancer Lett*. 2007; 254:1–10. [PubMed: 17250957]
5. Delattre O, Zucman J, Plougastel B, et al. Gene fusion with an ETS DNA-binding domain caused by chromosome translocation in human tumours. *Nature*. 1992; 359:162–165. [PubMed: 1522903]
6. Deneen B, Denny CT. Loss of p16 pathways stabilizes EWS/FLI1 expression and complements EWS/FLI1 mediated transformation. *Oncogene*. 2001; 20:6731–6741. [PubMed: 11709708]
7. Lessnick SL, Dacwag CS, Golub TR. The Ewing's sarcoma oncoprotein EWS/FLI induces a p53-dependent growth arrest in primary human fibroblasts. *Cancer Cell*. 2002; 1:393–401. [PubMed: 12086853]
8. Riggi N, Suva ML, Suva D, et al. EWS-FLI-1 expression triggers a Ewing's sarcoma initiation program in primary human mesenchymal stem cells. *Cancer Res*. 2008; 68:2176–2185. [PubMed: 18381423]
9. Toretsky JA, Kalebic T, Blakesley V, LeRoith D, Helman LJ. The insulin-like growth factor-I receptor is required for EWS/FLI-1 transformation of fibroblasts. *J Biol Chem*. 1997; 272:30822–30827. [PubMed: 9388225]

10. Scotlandi K, Benini S, Sarti M, et al. Insulin-like growth factor I receptor-mediated circuit in Ewing's sarcoma/peripheral neuroectodermal tumor: a possible therapeutic target. *Cancer Res.* 1996; 56:4570–4574. [PubMed: 8840962]
11. Yee D, Favoni RE, Lebovic GS, et al. Insulin-like growth factor I expression by tumors of neuroectodermal origin with the t(11;22) chromosomal translocation. A potential autocrine growth factor. *J Clin Invest.* 1990; 86:1806–1814. [PubMed: 2174908]
12. Scotlandi K, Benini S, Nanni P, et al. Blockage of insulinlike growth factor-I receptor inhibits the growth of Ewing's sarcoma in athymic mice. *Cancer Res.* 1998; 58:4127–4131. [PubMed: 9751624]
13. Riggi N, Cironi L, Provero P, et al. Development of Ewing's sarcoma from primary bone marrow-derived mesenchymal progenitor cells. *Cancer Res.* 2005; 65:11459–11468. [PubMed: 16357154]
14. Cironi L, Riggi N, Provero P, et al. IGF1 is a common target gene of Ewing's sarcoma fusion proteins in mesenchymal progenitor cells [serial online]. *PLoS One.* 2008; 3:e2634. [PubMed: 18648544]
15. Pullen N, Thomas G. The modular phosphorylation and activation of p70s6k. *FEBS Lett.* 1997; 410:78–82. [PubMed: 9247127]
16. Martins AS, Ordonez JL, Garcia-Sanchez A, et al. A pivotal role for heat shock protein 90 in Ewing sarcoma resistance to anti-insulin-like growth factor 1 receptor treatment: in vitro and in vivo study. *Cancer Res.* 2008; 68:6260–6270. [PubMed: 18676850]
17. Biggs WH 3rd, Meisenhelder J, Hunter T, Cavennee WK, Arden KC. Protein kinase B/Akt-mediated phosphorylation promotes nuclear exclusion of the winged helix transcription factor FKHR1. *Proc Natl Acad Sci U S A.* 1999; 96:7421–7426. [PubMed: 10377430]
18. Dromard M, Bompard G, Glondou-Lassis M, Puech C, Chalbos D, Freiss G. The putative tumor suppressor gene PTPN13/PTPL1 induces apoptosis through insulin receptor substrate-1 dephosphorylation. *Cancer Res.* 2007; 67:6806–6813. [PubMed: 17638892]
19. Jones JI, Clemmons DR. Insulin-like growth factors and their binding proteins: biological actions. *Endocr Rev.* 1995; 16:3–34. [PubMed: 7758431]
20. Renehan AG, Zwahlen M, Minder C, O'Dwyer ST, Shalet SM, Egger M. Insulin-like growth factor (IGF)-I, IGF binding protein-3, and cancer risk: systematic review and metaregression analysis. *Lancet.* 2004; 363:1346–1353. [PubMed: 15110491]
21. Prieur A, Tirode F, Cohen P, Delattre O. EWS/FLI-1 silencing and gene profiling of Ewing cells reveal downstream oncogenic pathways and a crucial role for repression of insulin-like growth factor binding protein 3. *Mol Cell Biol.* 2004; 24:7275–7283. [PubMed: 15282325]
22. Kolb EA, Gorlick R. Development of IGF-IR inhibitors in pediatric sarcomas. *Curr Oncol Rep.* 2009; 11:307–313. [PubMed: 19508836]
23. Toretsky JA, Steinberg SM, Thakar M, et al. Insulin-like growth factor type 1 (IGF-1) and IGF binding protein-3 in patients with Ewing sarcoma family of tumors. *Cancer.* 2001; 92:2941–2947. [PubMed: 11753970]
24. Bishop, YMM.; Feinberg, SE.; Holland, PW. *Discrete Multivariate Analysis.* Cambridge, MA: MIT Press; 1975.
25. Kalbfleisch, JD.; Prentice, RL. *The Statistical Analysis of Failure Time Data.* New York: John Wiley and Sons; 2002.
26. Cotterill SJ, Ahrens S, Paulussen M, et al. Prognostic factors in Ewing's tumor of bone: analysis of 975 patients from the European Intergroup Cooperative Ewing's Sarcoma Study Group. *J Clin Oncol.* 2000; 18:3108–3114. [PubMed: 10963639]
27. Yock TI, Krailo M, Fryer CJ, et al. Local control in pelvic Ewing sarcoma: analysis from INT-0091—a report from the Children's Oncology Group. *J Clin Oncol.* 2006; 24:3838–3843. [PubMed: 16921035]
28. Abaan OD, Levenson A, Khan O, Furth PA, Uren A, Toretsky JA. PTPL1 is a direct transcriptional target of EWSFLI1 and modulates Ewing's Sarcoma tumorigenesis. *Oncogene.* 2005; 24:2715–2722. [PubMed: 15782144]
29. Atzori F, Tabernero J, Cervantes A, et al. A phase I, pharmacokinetic (PK) and pharmacodynamic (PD) study of weekly (qW) MK-0646, an insulin-like growth factor-1 receptor (IGF1R)

- monoclonal antibody (MAb) in patients (pts) with advanced solid tumors [abstract]. J Clin Oncol. 2008; 26(15s) Abstract 3519.
30. Kolb EA, Gorlick R, Houghton PJ, et al. Initial testing (stage 1) of a monoclonal antibody (SCH 717454) against the IGF-1 receptor by the Pediatric Preclinical Testing Program. *Pediatr Blood Cancer*. 2008; 50:1190–1197. [PubMed: 18260118]
 31. Houghton PJ, Morton CL, Gorlick R, et al. Initial testing of a monoclonal antibody (IMC-A12) against IGF-1R by the Pediatric Preclinical Testing Program. *Pediatr Blood Cancer*. 2010; 54:921–926. [PubMed: 20166202]
 32. Naing A, LoRusso P, Gupta S, et al. Dual inhibition of IGFR and mTOR pathways [abstract]. J Clin Oncol. 2010; 28(15s) Abstract 3007.
 33. Kurzrock R, Patnaik A, Aisner J, et al. A phase I study of weekly R1507, a human monoclonal antibody insulin-like growth factor-I receptor antagonist, in patients with advanced solid tumors. *Clin Cancer Res*. 2010; 16:2458–2465. [PubMed: 20371689]
 34. Pappo AS, Patel S, Crowley J, et al. Activity of R1507, a monoclonal antibody to the insulin-like growth factor-1 receptor (IGF1R), in patients (pts) with recurrent or refractory Ewing's sarcoma family of tumors (ESFT): results of a phase II SARC study [abstract]. J Clin Oncol. 2010; 28(15s) Abstract 10000.
 35. Olmos D, Postel-Vinay S, Molife LR, et al. Safety, pharmacokinetics, and preliminary activity of the anti-IGF-1R antibody figitumumab (CP-751,871) in patients with sarcoma and Ewing's sarcoma: a phase 1 expansion cohort study. *Lancet Oncol*. 2010; 11:129–135. [PubMed: 20036194]
 36. Tolcher AW, Sarantopoulos J, Patnaik A, et al. Phase I, pharmacokinetic, and pharmacodynamic study of AMG 479, a fully human monoclonal antibody to insulin-like growth factor receptor 1. J Clin Oncol. 2009; 27:5800–5807. [PubMed: 19786654]
 37. Tap WD, Demetri GD, Barnette P, et al. AMG 479 in relapsed or refractory Ewing's family tumors (EFT) or desmoplastic small round cell tumors (DSRCT): phase II results [abstract]. J Clin Oncol. 2010; 28(15s) Abstract 10001.
 38. Dong J, Demarest SJ, Sereno A, et al. Combination of 2 insulin-like growth factor-I receptor inhibitory antibodies targeting distinct epitopes leads to an enhanced antitumor response. *Mol Cancer Ther*. 2010; 9:2593–2604. [PubMed: 20716637]
 39. Erkizan HV, Kong Y, Merchant M, et al. A small molecule blocking oncogenic protein EWS-FLI1 interaction with RNA helicase A inhibits growth of Ewing's sarcoma. *Nat Med*. 2009; 15:750–756. [PubMed: 19584866]

Figure 1.

This chart illustrates the patient study tree for Children's Oncology Group clinical trial AWES0031. IDs indicates identifications; IGF, insulin-like growth factor; IHC, immunohistochemistry.

Figure 2.

Event-free survival (EFS) and overall survival are illustrated according to insulin-like growth factor-1 (IGF-1) levels, IGF binding protein-3 (IGFBP-3) levels, and the IGFBP-3:IGF-1 ratio.

Figure 3.

Immunohistochemistry of phosphorylated p70S6 kinase (pS6) and phosphorylated protein kinase B (pAkt) is illustrated. Immunohistochemical analysis was performed as described in the text (see Materials and Methods). A. Serum-starved TC-71 cells were exposed to 10 nM insulin-like growth factor 1 (IGF-1) for 0 minutes, 15 minutes, 60 minutes, and 120 minutes. (B) Serum-starved TC-71 cells were treated with increasing concentrations of IGF-1 (range, 0–100 nM) for 3 minutes and stained for pAkt.

Figure 4.

Event-free survival (EFS) and overall survival are illustrated according to the presence of phosphorylated p70S6 kinase (pS6), phosphorylated forkhead box protein O1 (pFOXO1), protein tyrosine phosphatase-L1 (PTPL1), and phosphorylated protein kinase B (pAkt). pFKHR indicates phosphorylated forkhead homolog 1 (rhabdomyosarcoma), also known as pFOXO1.

Table 1

Comparison of Insulin-Like Growth Factor-1 (IGF-1) Levels, IGF Binding Protein-3 (IGFBP-3) Levels, and the IGFBP-3:IGF-1 Ratio Ratio According to Age, Sex, Tumor Location, and Survival (N=226)

Characteristic	No. of Patients (%)				Total	<i>p</i> ^a
IGF-1 level						
Age, y						226
<18	54 (27.1)	47 (23.6)	49 (24.6)	49 (24.6)	199	
>18	2 (7.4)	10 (37)	8 (29.6)	7 (25.9)	27	.10
Sex						
Male	32 (24.6)	33 (25.4)	31 (23.8)	34 (26.2)	130	
Female	24 (25)	24 (25)	26 (27.1)	22 (22.9)	96	.93
Tumor location						
Nonpelvic	49 (26.2)	50 (26.7)	46 (24.6)	42 (22.5)	187	
Pelvic	7 (17.9)	7 (17.9)	11 (28.2)	14 (35.9)	39	.25
IGFBP-3 level						
Age, y						
<18	51 (25.6)	48 (24.1)	47 (23.6)	53 (26.6)	199	
>18	5 (18.5)	9 (33.3)	10 (37)	3 (11.1)	27	.16
Sex						
Male	39 (30)	34 (26.2)	27 (20.8)	30 (23.1)	130	
Female	17 (17.7)	23 (24)	30 (31.3)	26 (27.1)	96	.10
Tumor location						
Nonpelvic	48 (25.7)	45 (24.1)	49 (26.2)	45 (24.1)	187	
Pelvic	8 (20.5)	12 (30.8)	8 (20.5)	11 (28.2)	39	.69
IGFBP-3:IGF-1 ratio						
Age, y						
<18	50 (25.1)	45 (22.6)	49 (24.6)	55 (27.6)	199	
>18	6 (22.2)	12 (44.4)	8 (29.6)	1 (3.7)	27	.01
Sex						
Male	33 (25.4)	33 (25.4)	35 (26.9)	29 (22.39)	130	

Characteristic	No. of Patients (%)					Total	p ^a
	Q1	Q2	Q3	Q4			
Female	23 (24)	24 (25)	22 (22.9)	27 (28.1)		96	.77
Tumor location							
Nonpelvic	44 (23.5)	45 (24.1)	47 (25.1)	51 (27.3)		187	
Pelvic	12 (30.8)	12 (30.8)	10 (25.6)	5 (12.8)		39	.24

Abbreviations: Q, quartile.

^aFisher exact test.

Table 2

Analysis of Event-Free and Overall Survival According to Insulin-Like Growth Factor-1 (IGF-1) Levels, IGF Binding Protein-3 (IGFBP-3) Levels, and the IGFBP-3:IGF-1 Ratio

Variable	EFS		OS	
	HR	95% CI	HR	95% CI
IGF-1 level				
Q2	1.64	0.79–3.40	1.79	0.71–4.56
Q3	1.79	0.87–3.69	1.99	0.80–5.00
Q4	1.52	0.73–3.16	1.56	0.60–4.01
IGFBP-3 level				
Q2	1.11	0.55–2.22	0.95	0.41–2.19
Q3	0.95	0.46–1.94	0.69	0.28–1.70
Q4	1.42	0.72–2.77	1.19	0.53–2.66
IGFBP-3:IGF-1 ratio				
Q2	1.05	0.55–2.00	1.02	0.46–2.23
Q3	0.96	0.50–1.82	0.68	0.29–1.60
Q4	0.63	0.30–1.30	0.71	0.30–1.66

Abbreviations: CI, confidence interval; EFS, event-free survival; HR, hazard ratio; OS, overall survival; Q, quartile.

Table 3

Immunohistochemical Analyses of Ewing Sarcoma Tumor Samples at Diagnosis

Variable	No. of Patients (%)			<i>p</i> ^a
	IHC Positive	IHC Negative	Total	
p70S6 kinase phosphorylation, n=140				
Age, y				
<18	67 (53.2)	59 (46.8)	126	
>18	9 (64.3)	5 (35.7)	14	.57
Sex				
Male	40 (49.4)	41 (50.6)	81	
Female	36 (61)	23 (39)	59	.23
Tumor location				
Nonpelvic	65 (52.8)	58 (47.2)	123	
Pelvic	11 (64.7)	6 (35.3)	17	.44
FOXO1 phosphorylation, n=141				
Age, y				
<18	118 (92.9)	9 (7.1)	127	
>18	13 (92.7)	1 (7.1)	14	1.00
Sex				
Male	77 (92.8)	6 (7.2)	83	
Female	54 (93.1)	4 (6.9)	58	1.00
Tumor location				
Nonpelvic	116 (92.8)	9 (7.2)	125	
Pelvic	15 (93.8)	1 (6.3)	16	1.00
Akt phosphorylation, n=139				
Age, y				
<18	84 (66.7)	42 (33.3)	126	
>18	11 (84.6)	2 (15.4)	13	.23
Sex				
Male	58 (70.7)	24 (29.3)	82	
Female	37 (64.9)	20 (35.1)	57	.58
Tumor location				
Nonpelvic	83 (67.5)	40 (32.5)	123	
Pelvic	12 (75)	4 (25)	16	.78
PTPL1 presence, n=142				
Age, y				
<18	104 (81.9)	23 (18.1)	127	
>18	12 (80)	3 (20)	15	1.00
Sex				
Male	69 (83.1)	14 (16.9)	83	
Female	47 (80)	12 (20)	59	.66
Tumor location				

Variable	No. of Patients (%)		Total	<i>p</i> ^a
	IHC Positive	IHC Negative		
Nonpelvic	102 (81.6)	23 (18.4)	125	
Pelvic	14 (82.4)	3 (17.6)	17	1.00

Abbreviations: Akt, protein kinase B; FOXO1, forkhead box protein O1; IHC, immunohistochemistry; PTPL1, protein tyrosine phosphatase-L1.

^aFisher exact test.

Table 4

Analysis of Event-Free and Overall Survival According to Immunohistochemical Results in Patients With Ewing Sarcoma

Variable	EFS		OS	
	HR	95% CI	HR	95% CI
PTPL1 staining present	0.61	0.31–1.21	0.59	0.25–1.40
Akt phosphorylation present	1.04	0.54–2.01	0.90	0.40–2.01
FOXO1 phosphorylation present	0.70	0.25–1.95	0.59	0.18–1.96
p70S6 phosphorylation present	1.02	0.56–1.86	0.75	0.35–1.61

Abbreviations: Akt, protein kinase B; CI, confidence interval; EFS, event-free survival; FOXO1, forkhead box protein O1; HR, hazard ratio; OS, overall survival; PTPL1, protein tyrosine phosphatase-L1.