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Authors

Applebaum, Mark A
Thomas, Dafydd G
Hembrough, Todd
et al.

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Comparative evaluation of strategies for quantifying signaling pathway proteins in Ewing sarcoma

Mark A. Applebaum, MD[1], **Dafydd G. Thomas, MD, PhD**[4], **Todd Hembrough, PhD**[6], **Jon Burrows, PhD**[6], **Andrew E. Horvai, MD, PhD**[3], **Elizabeth R. Lawlor, MD, PhD**[5], and **Steven G. DuBois, MD**[2]

[1]Department of Pediatrics, University of Chicago, Chicago, Illinois

[2]Department of Pediatrics, University of California, San Francisco School of Medicine, San Francisco, California

[3]Department of Pathology, University of California, San Francisco School of Medicine, San Francisco, California

[4]Department of Pathology, University of Michigan Medical School, Ann Arbor, Michigan

[5]Department of Pediatrics, University of Michigan Medical School, Ann Arbor, Michigan

[6]Department of OncoPlex Diagnostics, Rockville, MD

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Introduction

Ewing sarcoma (EWS) is an aggressive malignancy of bone and soft tissues with a peak incidence in adolescence. Outcomes for patients with localized disease have improved over the past 20 years, with approximately 70% of patients disease-free 5 years from initial diagnosis 1 In contrast, outcomes for patients with initially metastatic disease remain poor. Only 20-25% of these patients survive disease-free 5 years from initial diagnosis.[1,2] Outcomes for patients with recurrent disease are also generally poor.[3-5] As a result, targeted therapies are being increasingly evaluated in these populations.

The insulin-like growth factor type 1 (IGF-1), epithelial growth factor (EGF) and mTOR pathways have been shown to play important roles in the growth of EWS.[6-9] Multiple preclinical models have shown EWS growth is inhibited by agents that interrupt these pathways by blocking IGF-1 receptor (IGF-1R) and mTOR.[10-12] Inhibition of epithelial

Corresponding Author: Steven DuBois, MD, Department of Pediatrics, UCSF School of Medicine, 505 Parnassus Avenue, M646, San Francisco, CA 94143-0106, Telephone: 415-476-3831, Facsimile: 415-502-4372, duboiss@peds.ucsf.edu.

growth factor receptor (EGFR) has been shown to be cytotoxic to EWS cells *in vitro* and increased EGFR expression may be one mechanism of resistance to IGF-1R inhibition.[13,14] In the case of IGF-1R inhibitors, only a subset of patients has a clinical response, though with significant improvements in disease burden.[15,16] Many groups have noted the need for improved use of biomarkers to identify patient subgroups who are most likely to respond to these targeted therapies.[17-19] Furthermore, there is evidence that tumor expression of some growth signaling proteins is correlated to overall survival and thus quantified measurements of these proteins may be useful as prognostic biomarkers.[20] However, the optimal modality for quantifying expression of these proteins in the type of tumor material most commonly available for evaluation, formalin-fixed paraffin embedded tissue, has not been determined.

Several methods are available for quantifying clinically-relevant signaling pathway proteins in archival paraffin-embedded tumor material: standard immunohistochemistry (IHC); automated quantitative analysis (AQUA) immunohistochemistry;[21] and mass spectrometry quantification.[22] While standard immunohistochemistry is widely available, it is only semi-quantitative and cannot be multiplexed. AQUA immunohistochemistry and mass spectrometry have the benefit of being both fully quantitative and can be tested as a multiplex assay. However, neither assay has been compared to IHC for use with signaling proteins in EWS.

The primary aim of the current study was to compare the performance characteristics of these techniques in tissue from EWS patients in quantifying IGF-1R, EGFR, and mTOR signaling pathway proteins. A secondary aim was to track expression of these antigens over the disease course. The results could then be used to inform the adoption of new technologies for use in clinical trials of targeted agents for EWS and potentially other childhood cancers.

Materials and Methods

Patients

Tissue samples were obtained by evaluating the records of 129 patients with confirmed EWS who had been previously treated at the University of California, San Francisco. From the potential pool of 129 patients, our study population was limited to those patients who had open diagnostic biopsies or surgical resection of their tumor and excluded patient samples obtained by needle biopsy. Patients who underwent open biopsy or resection at the time of relapse were also included. The presence of viable tumor cells readily visible microscopically was also required for inclusion. These criteria were designed to ensure sufficient tumor material for analysis. 63 patients were screened based on available material. 46 blocks of formalin-fixed tissue with viable tumor were available from 40 patients of the original 63. 34 blocks of skeletal origin were decalcified using EDTA and dilute HCL (Decal Stat, Decal Chemical Corporation, Tallman, NY). 28 of the 46 samples were obtained at time of initial diagnosis, 11 after initial neoadjuvant chemotherapy during primary surgical excision and seven from patients with relapsed disease. The remaining 23 patients were excluded because of insufficient viable tumor, typically as a result of chemotherapy effect (n=18). Five patients had tissue blocks that could not be located. Six of the 40 patients had samples at both time of diagnosis and after initial therapy. Five of these

patients had tissue obtained after initial neoadjuvant chemotherapy and one after treatment relapse. One pathologist (AEH) reviewed all cases to confirm the diagnosis of EWS based on histology, immunohistochemistry, and/or cytogenetic/molecular data.

Study Overview

The initial study design included evaluation of protein expression of IGF-1R, phospho-IGF-1R, phospho-AKT, PTEN, EGFR, and phospho-P70S6 kinase. These proteins were chosen as they are involved in signaling pathways of potential interest for targeted therapy for EWS, including IGF-1R, EGFR, and mTOR pathways.

All samples were evaluated by standard immunohistochemistry and AQUA immunohistochemistry including six patients with samples from both diagnosis and after treatment. 25 samples were also selected for mass spectrometry analysis using tissue from 20 patients. Each of the selected patients had their diagnostic sample tested by mass spectrometry. Five of these patients had paired samples from a second time point, four after chemotherapy and one at time of relapse that were evaluated to better understand the changes in these biomarkers after treatment.

Sample Preparation and Evaluation

Tissue microarrays (TMAs) were prepared for both standard and AQUA immunohistochemistry. Positive and negative controls included normal liver, kidney and tonsil tissue.

Immunohistochemical staining was performed on the DAKO Autostainer (DAKO, Carpinteria, CA) using DAKO LSAB+ or polymerized detection system (Envision+, DAKO) and diaminobenzadine (DAB) as the chromogen as noted in Supplementary Table 1. Serial sections of de-paraffinized TMA sections were labeled with antibodies listed in Supplementary Table 1. Microwave citric acid epitope retrieval was used for all antibodies with the exception of EGFR. Appropriate negative (no primary antibody) and positive controls were stained in parallel with each set of tumors studied and yielded expected positive and negative staining

Protein expression by standard immunohistochemistry was evaluated using a 0-8 scale, 8 being the strongest staining using a modified Allred scoring schema.[23] Standard IHC results were also categorized as low (0-2), medium (3-5) and high (6-8). Samples were evaluated by a single pathologist (DT). Scoring was done blinded to results from AQUA or mass spectrometry to prevent bias.

Double immunofluorescence staining for AQUA was performed as previously described.[24] Briefly, after deparaffinization and rehydration, TMA slides were subjected to microwave epitope retrieval in 1 mM EDTA buffer, pH8. After rinsing several times in 10 mM Tris HCL buffer, pH 8 containing 0.154 M NaCl (TBS), endogenous peroxidase activity was blocked with 2.5% (v/v) H2O2 in methanol for 30 mins. Non-specific binding of the antibodies was extinguished by a 30 min incubation with ‘Background Sniper” (BioCare Medical, Concord, CA). The TMA slide was then incubated with the tumor specific antibody, CD99 (species noted in Supplementary Table 2) overnight at 4C and each

antibody noted in Supplementary Table 2. Slides were then washed as described above and incubated with a combination of goat anti-mouse IgG conjugated to AF555 (Molecular probes, Carpinteria, CA, A21424, 1:200) in goat anti rabbit Envision+ (DAKO, Carpinteria, CA) or goat anti rabbit IgG conjugated to AF555 (Molecular Probes, Carpinteria, CA, A31630, 1:200) in goat anti mouse Envision+ (DAKO, Carpinteria, CA) for 60 minutes at room temperature in a dark humidity tray. The slides were then washed as described above and the target image is developed by a CSA reaction of Cy5 labeled tyramide (PerkinElmer, Waltham, MA, 1:50). The slides were washed with 3 changes of TBS and stained with the DNA staining dye 4′,6-diaminodo-2-phenylindole (DAPI) in a non-fading mounting media (ProLong Gold, Molecular Probes, Carpinteria, CA). The slides were allowed to dry overnight in a dark dry chamber and the edges were sealed.

The AQUA system (HistoRx, New Haven, Connecticut) was used for the automated image acquisition and analysis. Briefly, images of each TMA core were captured with an Olympus BX51 microscope at 3 different extinction/emission wavelengths. Within each TMA spot, the area of tumor was distinguished from stromal and necrotic areas by creating a tumor specific mask from the anti-CD99 protein, which was visualized from Alexafluor 555 signal. The DAPI image was then used to differentiate between the cytoplasmic and nuclear staining within the tumor mask. Finally, the fluorescence pixel intensity of the protein/ antibody complex was obtained from the Cy5 signal and reported as mean pixel intensity. Expression by AQUA was therefore measured as a continuous variable in arbitrary units.

For mass spectrometry analysis, 10 μm unstained sections of each sample were prepared on proprietary Director slides (OncoPlex Diagnostics, Rockville, MD – formerly Expression Pathology, Inc.). Liquid Tissue lysates were prepared from these slides according to the manufacturer's recommendations (OncoPlex Diagnostics).[22] Total protein content for each Liquid Tissue lysate was measured using a Micro BCA assay (Thermo Fisher Scientific Inc, Rockford, IL). Endogenous EGFR was quantitated using selected reaction monitoring (SRM)-MS by targeting the peptide IPLENLQIIR, which spans residues 98-108 of the EGFR extracellular domain. Likewise, IGR-1R was quantitated through the peptide GNLLINIR, which spans residues 358-365 of the protein's extracellular domain. A known amount of synthetic isotopically-labeled internal standard (5 fmol) for each peptide (IPLEN[13C15N]LQIIR and GNL[13C15N]LINIR) was added to the samples. The sample (1 mg total) was analyzed using a nanoAcquityLC system (Waters, Milford, MA) coupled directly on-line with a TSQ Vantage triple quadrupole mass spectrometer (Thermo Scientific, San Jose, CA). The SRM-MS assays were acquired using the following mass spectrometer conditions: Q1(FWHM);0.2, Q2(FWHM):0.7, dwell time;10 ms. The precursor and product ions monitored for SRM-MS analysis of EGFR and IGF-1R have been reported previously.[22] Each sample was analyzed in triplicate. PTEN was not included in the mass spectroscopy panel for technical reasons. The area under the curve (AUC) for the endogenous and isotopically-labeled standard peptide was used to calculate the absolute abundance of EGFR and IGF-1R in each sample. The concentration of endogenous SPARC peptide was calculated using the following formula:

$$\frac{\text{AUC endogenous peptide}}{\text{AUC isotopically}-\text{labeled peptide(internal standard)}} \times \frac{\text{amol isotopically}-\text{labeled peptide}*}{\mu\text{g total protein}**} = \text{amol endogenous peptide per}\mu\text{g}$$

*Quantity of spiked isotopically-labeled internal standard (amol) injected

**Quantity of total protein injected

Statistical Methods

Correlation coefficients were calculated by linear regression. One-sided ANOVA analysis was used to compare the mean AQUA expression for each analyte between categories of expression by IHC using single sample from each patient at the earliest time point in their disease. Two sided unpaired t-tests were used to compare means between groups at initial diagnosis and after initiation of chemotherapy. All statistical analyses were performed using STATA, version 11.

Results

Patient Characteristics

Samples were available from 40 patients whose characteristics can be seen in Table 1. The mean age of patients was 14 years (range 1-49 yrs) at time of diagnosis. 67.5% were male and 57.5% had localized disease. Overall survival for this cohort was 72.5% with a median follow up time of 51 months.

Protein Expression by Standard IHC, AQUA IHC and Mass Spectrometry

AQUA and standard immunohistochemistry were attempted for EGFR, IGF-1R, phospho-AKT, phospho-IGF-1R, phospho-70S6k and PTEN. Due to technical issues, results were only available for IGF-1R, EGFR and PTEN, an inhibitor of the Pi3K/AKT/mTOR pathway. One sample per patient was analyzed, with the sample from initial diagnosis included in the primary analysis if multiple samples were available in a given patient. Overall expression results can be seen in Table 2. Standard IHC yielded expression results in 35 of 46 samples for IGF-1R (76.1%), 36 samples for EGFR (78.3%) and 35 samples for PTEN (76.1%). AQUA yielded expression results in 34 of 46 samples for IGF-1R (73.9%), 37 samples for EGFR (80.4%) and 42 samples for PTEN (91.3%). A representative image for AQUA IGF-1R staining can be seen in Figure 1. Mass spectrometry yielded expression results in 7 of 25 samples (25%) for IGF-1R and 8 of 25 samples (32%) for EGFR. PTEN expression was not available using this methodology. To account for the possible loss of protein from decalcification, we compared samples from both skeletal and non-skeletal origin and saw similar ranges for all proteins and methodologies.

AQUA IHC Compared to Standard IHC

Mean IGF-1R expression by AQUA did not differ significantly between standard IHC expression categories (mean IGF-1R expression by AQUA for low IHC = 11,255, medium IHC = 11070, high IHC = 11023; p = 0.98) (Figure 2A). Mean PTEN expression by AQUA was higher in the medium and high IHC categories (mean PTEN expression by AQUA for

low IHC = 1229, medium IHC = 2715, high IHC = 2940; p = 0.064) (Figure 2B). Only two samples expressed EGFR by standard IHC (Figure 2C). These samples qualitatively had higher AQUA expression levels, but there were too few samples for a reliable determination of statistical significance.

Mass Spectrometry Compared to Standard IHC and AQUA IHC

Qualitatively, there appeared to be a trend towards higher mass spectrometry values correlating with higher standard IHC (Figure 3A) and AQUA (Figure 3B) expression, but there were too few samples with available mass spectrometry data to merit formal statistical analysis.

Protein Expression Levels over Time

In order to better understand the changes in these biomarkers after treatment, we compared six post-treatment samples, five after chemotherapy and one at time of relapse, to those obtained from the same patient at initial diagnosis by AQUA (Figure 4). Intra-patient variability in expression as detected by AQUA was minimal as levels after initiation of therapy were similar to those measured prior to treatment. There was also no difference in mean expression by AQUA for all diagnostic samples compared to those obtained after initiation of treatment for IGF-1R (10330 vs. 10947; p = 0.55), EGFR (2912 vs. 2238; p = 0.4) and PTEN (2317 vs. 2359; p = 0.93). These data suggest that individual tumors have similar expression levels of these three proteins over time when patients are treated with standard cytotoxic agents according to current therapeutic protocols and that chemotherapy does not change expression significantly in residual viable tumor.

Discussion

In this study, we demonstrated the feasibility of quantifying protein expression of IGF-1R, EGFR and PTEN in paraffin embedded tumor samples from patients with Ewing sarcoma. AQUA immunohistochemistry showed that tumors express these proteins across a wide range. Furthermore, although IGF-1R expression by AQUA was similar across a range of standard IHC expression categories, increased expression of PTEN and EGFR by AQUA showed a trend towards increased standard IHC expression.

In this initial descriptive study, it is not possible to determine if one approach should be favored over the other approaches as a tool for quantifying these pathways in Ewing sarcoma. Ultimately, a reliable quantitative method could be used for establishing relative expression values that are clinically significant. It is not known whether there are critical threshold levels of expression of these signaling proteins that correlate with clinical behavior of Ewing sarcoma. Our work lays the groundwork for future studies that seek to determine the clinical impact of expression of these proteins, particularly within the context of clinical trials of targeted therapies.

Importantly, there is not a gold standard to which the results of each assay could be compared. Therefore, other performance characteristics must drive selection of a particular assay for use in future testing. For example, the low success rate for mass spectrometry in this histology suggests that this platform may not be optimal for future study. AQUA may

be preferred over standard IHC as more objective and more quantitative, though it is important to note that correlation between these two approaches was poor for IGF-1R. These two methods are also capable of determining nuclear versus cytoplasmic protein expression levels and could be evaluated in future studies. [25]

Similar to AQUA, mass spectrometry also demonstrated a wide range of protein expression of IGF-1R and EGFR across samples. Higher mass spectrometric values appeared to correspond to high levels of expression on standard IHC. Unfortunately, this technique was not able to determine protein expression for a majority of the tumor samples tested. However, the mass spectrometry technique does define a lower limit of sensitivity and it may be that the low success rate also represents clinically insignificant levels of detection in these samples. Given previous success with this technique in other tumor types using frozen tissue [26,27], it is possible that the formalin fixation and, in a subset of cases, decalcification, may have interfered with this approach. While the protein expression levels in our study using tissue from skeletal and extraskeletal sources appeared similar, more samples would be needed to confirm this finding.

Interestingly, we observed stable expression levels of IGF-1R, EGFR, and PTEN when evaluated at multiple time points in therapy for an individual patient. This result is noteworthy as many centers are utilizing core needle biopsies or fine-needle aspiration to render an initial diagnosis of Ewing sarcoma. Therefore, paraffin-embedded tumor material may be scarce at diagnosis, but more plentiful at the time of definitive surgical local control. If our results are validated by other groups and in larger study cohorts, it may be feasible to evaluate tumor material from later in the disease course to infer expression at initial diagnosis.

The objective response rate of 10-15% for IGF-1R monoclonal antibodies in the treatment of patients with relapsed Ewing sarcoma motivates studies to understand possible resistance pathways to IGF-1R and other tyrosine kinase inhibitors. This experience also highlights the need to consider the potential for combining inhibitors of multiple different signaling pathways with each other and with conventional chemotherapy.[28-31] While IGF-1R, EGFR, and mTOR are all potential drug targets, it is yet to be fully determined if inhibition of any of these pathways can be augmented by simultaneously targeting other pathways.[32,33] The ability to quantify pathway components and establish clinically relevant levels will be important to determine how the expression of these proteins drive tumor growth, survival and drug sensitivity. Ultimately, finding the optimal targeted strategy for an individual patient may be informed by studies quantifying protein expression in that individual's tumor.

Prior studies have evaluated the clinical impact of some of these signaling proteins in Ewing sarcoma. The IGFBP-3:IGF-1 ratio has been correlated to younger age at time of diagnosis but this did not correlate to outcomes.[34] A separate study evaluating 45 paraffin-embedded samples showed higher standard IHC expression levels of p-mTOR and p27(KIP1) were significantly associated with improved outcome.[20] These levels were not compared to the upstream drug targets evaluated in our study. A different report showed that patients whose tumors only showed nuclear staining of IGF-1R had better overall survival in a group of patients treated with an IGF-1R inhibitor.[25] The extent to which more quantitative

approaches, such as AQUA, might improve upon the predictive ability of these markers remains to be established.

One limitation of our study was the pooling of data obtained from samples obtained before and after initiation of chemotherapy. The samples obtained after start of treatment are likely enriched for tumor cells that were not responsive to therapy and may have different biologic properties compared to those cells that were sensitive to chemotherapy. Data suggest that patients with poor chemotherapy induced necrosis, which are the specimens that likely had adequate post treatment tumor samples, have worse overall outcomes.[35] While our immunohistochemistry assays for phospho-AKT and phospho-P70S were not successful, other studies have been able to do so using formalin fixed paraffin embedded tissue from other tumor types.[36,37] This may be due either to biologic differences of the tumor type and/or differences in laboratory techniques. In addition, our sample size and retrospective study design make it difficult to determine the true correlation between quantitative methods of expression and standard IHC. Larger prospective studies are needed to clarify the optimal approach for each protein of interest.

Based on our findings, we conclude that growth signaling pathway proteins in Ewing sarcoma can be measured in a quantitative fashion. The three assays evaluated each have advantages and disadvantages that need to be considered in choosing an assay for a particular indication. Larger samples and a defined gold standard for comparison will be needed to determine the optimal approach for quantifying signaling proteins in Ewing sarcoma. These methods can then be used to aid the development of targeted therapies and evaluated as potential biomarkers predictive of response.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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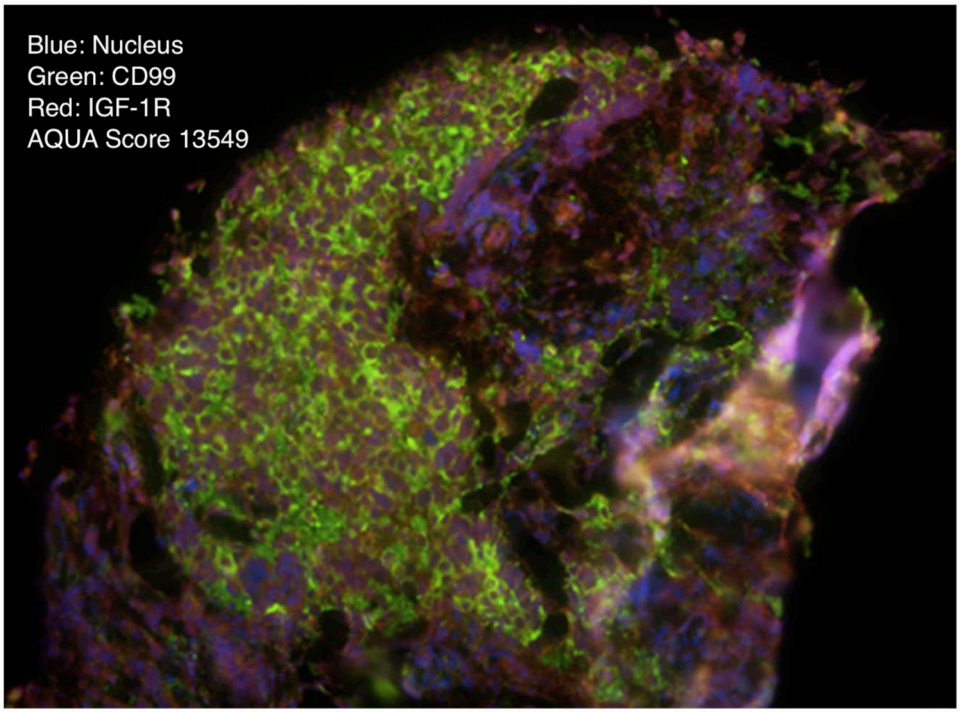

Figure 1.
Representative image of AQUA staining for IGF-1R.

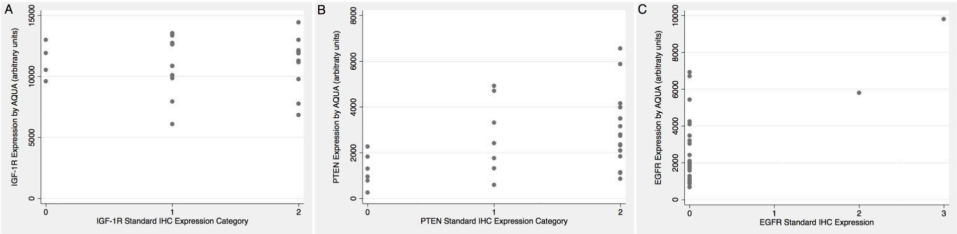

Figure 2.

Expression of signaling pathway proteins measured by AQUA immunohistochemistry compared to standard immunohistochemistry for IGF-1R (A), PTEN (B), EGFR (C).

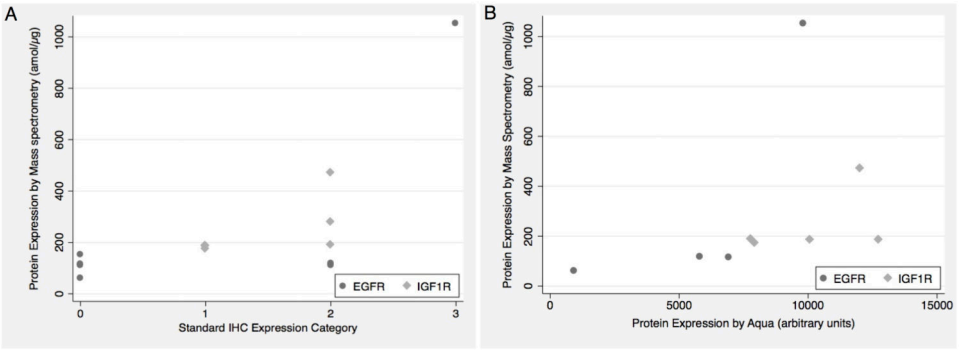

Figure 3.

Expression of signaling pathway proteins measured by mass spectrometry compared to standard immunohistochemistry (A) and AQUA immunohistochemistry (B) for IGF-1R and EGFR.

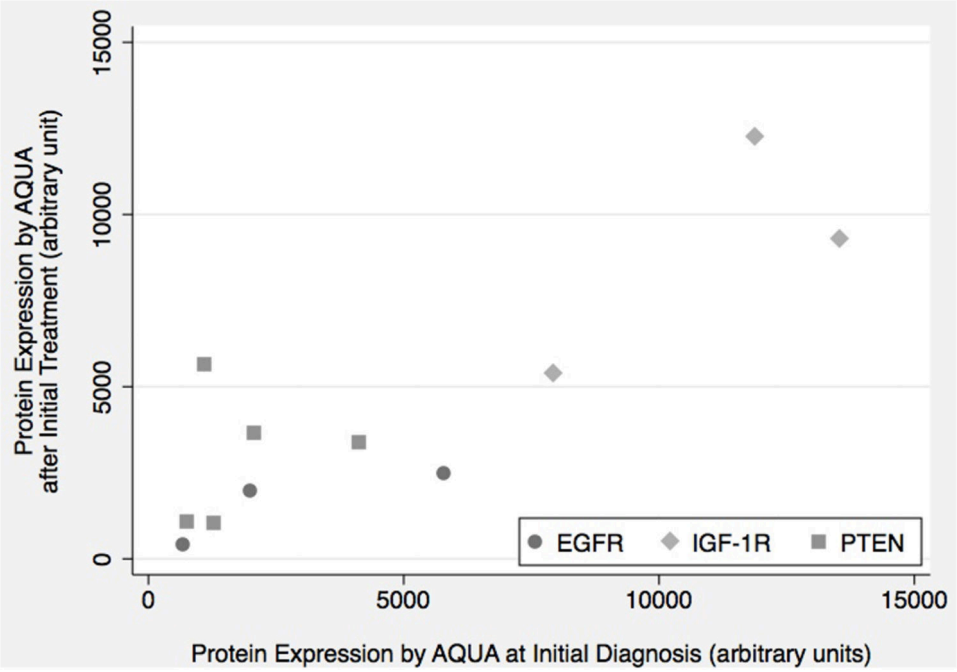

Figure 4.

Expression of signaling pathway proteins measured by AQUA immunohistochemistry in matched patient samples before and after initiation of chemotherapy.

Table 1

Characteristics of 40 patients with Ewing sarcoma and available tissue for quantification of signaling proteins.

Characteristic	All Patients n = 40	Patients with Tissue Evaluated by Mass Spectrometry n = 20
Median age at diagnosis (Range)	14 years (1-49 years)	13 years (1-45 years)
Median age at tissue collection (Range)	18 years (1-49 years)	15 years (1-46 years)
Male	67.5%	75.0%
Race		
White	67.5%	60.0%
Hispanic	17.5%	30.0%
Asian	10.0%	10.0%
African American	5.0%	0.0%
Stage		
Localized	57.5%	65.0%
Metastatic	30.0%	25.0%
Unknown	12.5%	10.0%
Tissue Origin		
Skeletal	72.5%	75.0%
Extraskeletal	27.5%	25.0%
Year of Diagnosis		
1990-1999	37.5%	40.0%
2000-2010	62.5%	60.0%

Table 2

Expression levels of signaling pathway proteins in paraffin-embedded Ewing sarcoma samples using three different methodologies.

Method	IGF-1R	PTEN	EGFR
Standard IHC Median Score (Range)	6 (0-8)	7 (0-8)	0 (0-3)
AQUA IHC Mean (Range)	10702 (393 – 14424)	2250 (251 – 6557)	2750 (672 – 9798)
Mass Spectrometry Mean (amol/μg) (Range)	246 (174 – 471)	N/A	234 (60 – 1052)