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Correlation Between Clinical Outcome and Growth Factor Pathway Expression in Osteogenic Sarcoma

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Abstract

BACKGROUND: Multiple cell-signaling ligands and receptors—including vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF), endothelial growth factor (EGF), v-akt murine thymoma viral oncogene homo log (AKT), platelet-derived growth factor (PDGF), mitogen-activated protein kinase (MAPK), and 70-kilodalton (kD) protein S6 kinase (p70S6 kinase)—reportedly are variably expressed in osteogenic sarcoma. Expression of these proteins may have future implications for prognostication and targeted therapy. The objective of the current study was to determine the relation between clinical outcome and the expression of these proteins.

METHODS: A paraffin-embedded microarray of 48 human osteogenic sarcoma tissue specimens was stained with the antibodies against VEGF, IGF, EGF, AKT, PDGF, MAPK, and p70S6 kinase. Staining for each protein included the total protein and, when applicable, the phosphorylated version of the protein, immunohistochemical staining was then correlated with patient survival (overall survival [OS] and event-free survival [EFS]), histologic response to chemotherapy, and serum markers.

RESULTS: There was a negative correlation between VEGF receptor 3 (VEGF-R3) and both OS and EFS. VEGF-B was correlated with a poor histologic response to chemotherapy. Serum

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Conflict of Interest Disclosures

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markers were not correlated with any specific proteins. When using a *P* value of .05, multiple correlations were observed between proteins of various pathways.

CONCLUSIONS: The current results suggested that the VEGF pathway is a critical signaling pathway in osteogenic sarcoma. These data have identified specific proteins within these pathways toward which future investigations should be directed to further clarify their prognostic potential.*

Keywords

osteosarcoma; immunohistochemistry; survival; signal transduction

Osteogenic sarcoma is a primary malignancy of bone that affects children and young adults. When osteogenic sarcoma is treated with surgery and chemotherapy, 5-year survival rates range from 60% to 80%.^{1,2} Despite the availability of effective treatment, associated mortality in this disease remains relatively high. Clinically, the development of successful therapies is needed for those who respond poorly to standard chemotherapy regimens and for patients who present with disseminated disease, for whom current regimens result in an overall survival (OS) rate of only 11%.³

The etiology of osteosarcoma largely remains undefined because of profound genetic variability. Multiple cell-signaling transduction proteins (v-akt murine thymoma viral oncogene homolog 1 [Akt], mitogen-activated protein kinase [MAPK], and 70-kilodalton (kD) protein S6 kinase [p70S6 kinase]) as well as receptors (vascular endothelial growth factor [VEGF], insulin-like growth factor [IGF], and epidermal growth factor [EGF]) reportedly are expressed variably in osteogenic sarcoma. It remains to be determined whether these in vitro findings correlate with clinical parameters. Furthermore, to our knowledge, no study has adequately described a global picture of the cell-signaling proteins and downstream targets involved in the pathogenesis of osteosarcoma. The objectives of the current study were to understand the cell-signaling transduction pathways that potentially are involved in the pathogenesis of osteosarcoma by determining which are activated, how they relate to 1 another, and whether the expression of these proteins correlates with clinical outcome.

MATERIALS AND METHODS

Tissue samples were obtained from 48 patients (28 female, 20 male) who underwent resection of osteogenic sarcoma of the extremities, pelvis, and craniofacial bones. The mean age at the time of surgery was 22.1 years (range, 5-77 years). The samples included specimens that were taken at biopsy (15 specimens), during definitive resection of the tumor (25 specimens), and during resection of distant metastatic sites (8 specimens) for a total of 48 specimens. A variety of anatomic sites and histologic subtypes were included (Table 1). The majority of tumors (41 of 48 tumors) were high grade and extracompartmental and, thus, according to the Enneking classification, were classified as stage IIB.⁴ Seven patients had metastatic pulmonary disease at the time of diagnosis and, thus, were classified with stage III disease. Written informed consent in accordance with the Ethics Committee and Institutional Review Board of the hospital was obtained before tissue procurement. Immediately after procurement, samples of all specimens were decalcified, fixed in

formalin, and embedded in paraffin. A tissue microarray was constructed with the design based on previous osteosarcoma arrays.⁵ Sections from the paraffin-embedded microarray were cut and mounted on positively charged slides.

Positive control slides were selected for each immunohistochemical stain based on a review of the literature by using either established positive staining in each tissue for the given antibody or slides made commercially available by the distributor of a given antibody (Table 2). Slides that contained the paraffin-embedded microarray of osteogenic sarcoma served as negative controls. Negative control slides were treated with the same immunohistochemistry protocol as positive controls and test tissue; however, the incubation with primary antibody was omitted in negative controls.

Immunohistochemistry Technique

Slides that contained the paraffin-embedded microarray and control tissues first were baked at 60°C for 15 minutes and then deparaffinized using xylene followed by graded alcohols. Endogenous peroxidase activity was quenched using 3% hydrogen peroxide in phosphate-buffered saline (PBS). Next, antigenic proteins were unmasked by antigen retrieval using 10 mM sodium citrate in a water bath at 95 to 100°C for 25 minutes. Then, the tissue was blocked for 30 minutes at room temperature with isospecies serum. The specimens were stained with primary antibody diluted in 5% bovine serum albumin in PBS. For negative controls, the primary antibody was omitted, and tissues were treated with 5% bovine serum albumin in PBS only. All slides were placed in a humidified chamber during incubation with primary antibody. Dilutions and duration of staining were antibody-specific, as Table 2 demonstrates. Detection of the antibody-binding reaction was carried out with biotinylated secondary antibody coupled with streptavidin-horseradish peroxidase. Avidin-biotinylated enzyme complex (Vectastain ABC system; Vector Laboratories, Burlingame, Calif) was used according to the manufacturer's instructions. Then, the tissue was treated with 3,3'-diaminobenzidine (Biofx Laboratories, Owings Mills, Md) to identify sites of additional antibody binding. Counterstain with hematoxylin was performed. The tissue was then dehydrated with alcohol, permeated with xylene, and mounted with Permount organic mounting solution (Fisher Scientific Inc., Pittsburgh, Pa).

Outcome Measures

Immunohistochemical scoring—The intensity and location of tissue staining were assessed by a comparison between the positive and negative control slides. Staining was considered positive (2 points) only if the location of staining (membranous vs cytoplasmic) was consistent with that of the control tissue and if staining was of equal intensity to that of the positive control tissue. Tissue was considered negative (0 points) for a given antibody if there was a definitive absence of staining. Slides were considered equivocal (1 point) if staining was greater in intensity than that of negative controls but less than that of positive controls; equivocal results were treated as negative results for the purpose of this study. Tissue was assessed and graded by 3 blinded observers. The average of the values was assigned as the final grade. The degree of agreement among the observers was calculated with a kappa value of 0.96.

Clinical outcome measures—The primary clinical endpoint was survival. Staining characteristics were correlated to both OS and event-free survival (EFS).

Secondary endpoints included 3 parameters that some consider prognostic, including 1) Huvos grade of histologic response to chemotherapy (based on the percentage tumor necrosis determined at the time of definitive resection; a good response >90% necrosis, and a poor response is <90% necrosis),⁶ 2) lactate dehydrogenase (LDH), and 3) alkaline phosphatase (AP).^{6,7} Both serum markers are considered by some to be associated with decreased survival when elevated >90 U/L and with a 1.5-fold and 2-fold decrease in OS when elevated >400 U/L.⁷

Statistical Analysis

Kaplan-Meier survival curves were generated and log-rank tests were performed to compare EFS and OS among patients whose specimens stained positive with those whose specimens stained negative. Estimates of hazards ratios were obtained by fitting Cox proportional hazards models.

The Fisher exact test was used to evaluate the pairwise associations between the various antibodies as well as the associations between Huvos grade, LDH status (LDH >90 U/L), and AP status (AP >90 U/L) with each antibody. The target α error was set at .05. Because of the many statistical tests that were performed, we applied the approach of controlling the false-discovery rate to calculate the P values corrected for multiple tests.⁸

RESULTS

Of the 48 specimens, the percentage of samples that stained positive for a given protein ranged from 16.1% (p70S6 kinase) to 84.9% (MAPK) (Table 3).

OS and EFS probabilities for patients with localized disease at the time of presentation, as estimated by Kaplan-Meier analysis, were 71.2% and 67.1%, respectively, at a median follow-up of 7.9 years. Estimated OS and EFS probabilities for patients with metastatic disease at the time of presentation were 38.1% and 30.2%, respectively.

A positive stain for VEGF receptor 3 (VEGF-R3) was associated with reduced EFS (hazards ratio, 2.58; $P = .02$) (Fig. 1, top) and reduced OS (hazards ratio, 2.76; $P = .02$) (Fig. 1, bottom). However, after correcting for multiple tests using the false-discovery approach, these findings were no longer statistically significant (EFS, corrected $P = .45$; OS, corrected $P = .44$). None of the other antibodies tested were associated with EFS or OS.

Results of the Fisher exact tests revealed several antibodies for which expression depended on the expression of other antibodies (Table 4). Both positive and negative correlations were observed. These correlations existed between proteins and their downstream receptors as well as between proteins involved in distinct signaling pathways. However, after correcting for multiple tests using the false-discovery method, none of these correlations were statistically significant.

There was a correlation between a positive stain with VEGF-B and a poor response to chemotherapy (Huvos grade 1 or 2; $P = .02$) (Fig. 2). This correlation was not statistically significant after application of the false-discovery test (corrected $P = .58$). Elevated serum markers (LDH, >90 U/L; AP, >90 U/L) did not correlate statistically with any of the antibodies tested. Furthermore, when stratifying the group for patients with LDH and AP levels >400 U/L (values that reportedly are associated with a 1.5-fold and 2-fold increase in the risk of recurrence or death for these serum markers, respectively⁷), there still was no correlation with any of the proteins tested.

DISCUSSION

The results of the current study suggest that clinical outcomes in osteosarcoma may be associated with the expression of multiple cell-signaling ligands and receptors, as determined by immunohistochemistry. Our findings indicate that there may be a decrease in EFS and OS in patients who have tumors that stain positive for VEGF-R3.

The Huvos grade of histologic response to chemotherapy is a commonly used prognosticator for this disease. Our findings suggest that a poor response to chemotherapy corresponds with the expression of VEGF-B.

The current study was limited by its retrospective nature, small sample size, and subsequent reduced power. To acquire our cohort size, we included a heterogeneous group of patients with respect to location and stage, and we also included 2 patients with extraskeletal osteosarcoma. These limitations exemplify the need for well annotated tumor repositories of pediatric tumors. Lack of clinical outcome information is a significant hurdle to these efforts.

Another limitation of this study was the large numbers of statistical tests performed, which inherently increased the probability of obtaining false-positive results. To circumvent this possibility, the false-discovery approach for multiple tests was applied to protect against false-positive results. Because large numbers of statistical tests were applied to a relatively small sample size, the false-discovery method established that, ultimately, none of the findings were statistically significant.

The current study is the first to our knowledge that advances a global illustration of the myriad relations between the ligands and receptors involved in osteosarcoma. The technique of immunohistochemistry staining on tissue microarrays made it feasible to test for a large number of ligands, receptors, and their phosphorylated forms in a large number of specimens. However, we acknowledge that the assessment of staining is subjective, and defining criteria for positive staining is difficult. In an effort to reduce these biases, we used staining of control tissues for comparison of positive staining as well as multiple observers. The interobserver reproducibility was good ($\kappa = 0.96$).

Assessment of staining also may be less accurate in specimens that are taken after treatment, in which a “good” treatment response was obtained according to the Huvos classification (defined as $>90\%$ necrosis). It is unknown whether the 10% of viable tumor that remains is representative of the more chemosensitive cells that underwent necrosis. This limitation (the

absence of biopsy tissue in combination with >90% necrosis after treatment) was present in only 6 patients.

Our data are consistent with the current literature on the basis of involvement of the VEGF pathway in osteosarcoma. VEGF-positive tumors previously were correlated with an increased incidence of pulmonary metastasis, decreased OS, and decreased EFS compared with VEGF-negative tumors.⁹ VEGF expression in osteosarcoma has been correlated with vascular permeability, as determined by magnetic resonance imaging.¹⁰ Lee et al. reported that tumors expressing the VEGF-165 isoform of VEGF-A were associated with decreased OS and increased pulmonary metastasis. However, those authors did not report any association between VEGF-R1 or VEGF-R2 expression and survival.¹¹ Preclinical testing also has revealed in vivo antitumor activity in 80% of osteosarcomas when treated with AZD2171, a selective inhibitor of the VEGF receptor family. Our finding that VEGF-R3 is associated with reduced survival is consistent with the current literature suggesting that this angiogenic factor is prognostic in osteosarcoma.

Given the limitations of our study, the data imply that several ligands and receptors may be linked to survival. VEGF appears to be a critical signaling pathway. This study has provided us with a global snapshot that allows us to define which signaling proteins should be the focus of future investigations to further elucidate the factors involved in the pathogenesis of osteogenic sarcoma. Such factors, if confirmed as true prognostic indicators, may be used to guide treatment regimens in those who have had a poor initial response to chemotherapy or who have developed metastatic disease. Future directions of study will involve a prospective validation of the immunohistochemical findings. In addition, quantitative analysis of the gene expression of those proteins we have correlated with survival (VEGF-R3) and Huvos response (VEGF-B) will further validate these preliminary findings.

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FIGURE 1. Positive staining with vascular endothelial growth factor-receptor 3 (VEGF-R3) was correlated with (*Top*) reduced event-free survival and (*Bottom*) reduced overall survival.

FIGURE 2.

A correlation was noted between poor response to chemotherapy, as defined by the Huvos classification, and vascular endothelial growth factor B (VEGF-B).

Table 1.

Distribution of Tumors Based on Staging (Enneking Classification), Patient Demographics, Histologic Subtype, and Anatomic Location

Variable	No. of Patients
Mean age (range), y	22.1 (5-77)
Disease stage	
IIA	0
IB	0
IIB	41
III	7
Histologic subtype	
Conventional	36
Chondroblastic	7
Fibrohistiocytic	2
Giant cell rich	3
Anatomic location	
Distal femur	18
Proximal tibia	8
Pelvis	6
Humerus	7
Craniofacial	3
Proximal femur	1
Extraskeletal	1
Sacrum	1
Ulna	1
Metatarsals	1

Table 2.

Description of All Antibodies Tested, Including Testing Conditions (Concentration and Incubation Time), Control Tissue, Manufacturer Information, and Corresponding Secondary Antibody Used

Antibody	Manufacturer/Catalog No.	Control Tissue	Dilution/ Concentration	Incubation Time, min	Secondary Antibody/Catalog No.
VEGF-R1	Abcam (Cambridge, UK)/antibody 2350	Angiosarcoma	1/100	60	Donkey antirabbit IgG-B/sc-2089
VEGF-R2	Abcam/antibody 45010	Angiosarcoma	1/100	60	Donkey antirabbit IgG-B/sc-2089
P-VEGF-R2	Abcam/antibody 38473	Breast carcinoma	1/100	60	Donkey antirabbit IgG-B/sc-2089
VEGF-R3	Abcam/antibody 27278	Breast carcinoma	1/100	10	Donkey antirabbit IgG-B/sc-2089
VEGF-A	Lifespan Biosciences (Seattle Wash)/LS-C2929	Normal human placenta	1/100	60	Donkey antimouse IgG-B/sc-2098
VEGF-B	R&D Systems (Minneapolis, Minn)/MoAB751	Normal human heart	25 µg/mL	60	Donkey antimouse IgG-B/sc-2098
VEGF-D	R&D Systems/AF286	Normal human heart	15 µg/MI	60	Donkey antigoat IgG-B/sc-2042
PDGF-A	Santa Cruz Biotechnology Inc. (Santa Cruz, Calif)/(N-30) sc-128	Colon carcinoma	1/100	60	Donkey antirabbit IgG-B/sc-2089
PDGF-C	Santa Cruz Biotechnology Inc. (C-17) sc-18228	Normal human kidney	1/100	60	Donkey antigoat IgG-B/sc-2042
PDGF-BB	Abcam/antibody 15499	Angiosarcoma	1/100	10	Donkey antirabbit IgG-B/sc-2089
PDGF-Rα	Cell Signaling Technology (Danvers, Mass)/no. 3164	Ovarian carcinoma	1/100	60	Donkey antirabbit IgG-B/sc-2089
PDGF-Rβ	Cell Signaling Technology/no. 3169	Ovarian carcinoma	1/100	60	Donkey antirabbit IgG-B/sc-2089
P-PDGF-Rα	Santa Cruz Biotechnology Inc. (Tyr 754) sc-12911	Breast carcinoma	1/100	60	Donkey antirabbit IgG-B/sc-2089
p44/42 MAPK	Cell Signaling Technology/no. 9102	Breast carcinoma	1/100	60	Donkey antirabbit IgG-B/sc-2089
P-p44/42 MAPK	Cell Signaling Technology/no. 4376	NIH/3T3 cells	1/100	60	Donkey antirabbit IgG-B/sc-2089
p70 S6 kinase	Santa Cruz Biotechnology Inc. (H-9) sc-8418	Breast carcinoma	1/100	60	Donkey antimouse IgG-B/sc-2098
P-p70 S6 kinase	Santa Cruz Biotechnology Inc. (A-6) sc-8416	Colon carcinoma	1/100	60	Donkey antimouse IgG-B/sc-2098
P-p70 S6 kinase receptor	Santa Cruz Biotechnology Inc./sc-7984-R	Colon carcinoma	1/100	60	Donkey antirabbit IgG-B/sc-2089
IGF-1	R&D Systems/MoAB291	Normal human placenta	25 µg/MI	60	Donkey antimouse IgG-B/sc-2098
IGF-1-Rα	R&D Systems/MoAB1120	Normal human placenta	4 µg/MI	60	Donkey antimouse IgG-B/sc-2098
IGF-1-Rα	Cell Signaling Technology/no. 3027	Breast carcinoma	1/150	60	Donkey antirabbit IgG-B/sc-2089
IGF-2	Abcam/antibody 9574	Normal colon	1/100	60	Donkey antirabbit IgG-B/sc-2089
IGF-II-Rα	Santa Cruz Biotechnology Inc. (C015) sc-14410	Normal human placenta	1/100	60	Donkey antigoat IgG-B/sc-2042
P-IGF-1-Rβ	Abcam/antibody 39398	Breast Carcinoma	1/100	60	Donkey antirabbit IgG-B/sc-2089
EGF	Abcam/antibody 10409	Normal human kidney	1/500	60	Donkey antimouse IgG-B/sc-2098
EGF-R	Cell Signaling Technology/no. 4405	Breast carcinoma	1/50	60	Donkey antirabbit IgG-B/sc-2089
P-EGF-R	Cell Signaling Technology/no. 4407	MDA-MB468 cells	1/250	60	Donkey antirabbit IgG-B/sc-2089

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Antibody	Manufacturer/Catalog No.	Control Tissue	Dilution/ Concentration	Incubation Time, min	Secondary Antibody/Catalog No.
AKT-1	Cell Signaling Technology/no. 2967	Colon carcinoma	1/250	60	Donkey antimouse IgG-B/sc-2098
P-AKT-1	Cell Signaling Technology/no. 3787	Breast carcinoma	1/00	60	Donkey antirabbit IgG-B/sc-2089

VEGF-R1 indicates vascular endothelial growth factor receptor 1; IgG-B, immunoglobulin G-B; P-VEGF-R2, phosphorylated vascular endothelial growth factor receptor 2; PDGF-A, platelet-derived growth factor A; PDGF-R, platelet-derived growth factor receptor; MAPK, mitogen-activated protein kinase; P-PDGFR- α , platelet-derived growth factor receptor α ; p70 S6 kinase, 70-kilodalton (kD) protein S6 kinase; P-p70 S6 kinase, phosphorylated 70-kD protein S6 kinase; IGF, insulin-like growth factor; IGF-1-R α ; insulin-like growth factor 1 receptor α ; P-IGF, phosphorylated insulin-like growth factor; EGF, epidermal growth factor; EGF-R, epidermal growth factor receptor; P-EGF-R, phosphorylated epidermal growth factor receptor; AKT-1, v-akt murine thymoma viral oncogene homolog 1; P-AKT-1, phosphorylated v-akt murine thymoma viral oncogene homolog 1.

Table 3.

Positive Expression Rate for Each Antibody as a Percentage of the Total Number of Specimens

Antibody	Antibody Expression in Microarray, %
p70S6 kinase	16.1
VEGF-R3	21
VEGF-R1	24.2
VEGF-D	24.2
VEGF-A	27.4
P-EGF-R	27.4
VEGF-R2	30.7
PDGF-A	30.7
P-VEGF-R2	32.3
P-p44/42 MAPK	35.5
IGF-1-R- β	37.1
PDGF-BB	38.7
P-p70 S6 kinase	38.7
PDGF-R- β	40.3
PDGF-C	41.9
P-IGF-1-R	42
IGF-1-R- α	45.2
EGF	45.2
VEGF-B	50
PDGF-R- α	51.6
IGF-2	54.8
IGF-II-R	56.5
P-p70 S6 kinase receptor	58.1
EGF-R	59.7
IGF-1	62.9
P-AKT-1	62.9
P-PDGF-R- α	72.6
AKT-1	72.6
p44/42 MAPK	84.9

p70 S6 kinase indicates 70-kilodalton (kD) protein S6 kinase; VEGF-R3, vascular endothelial growth factor receptor 3; PDGF-A, platelet-derived growth factor A; P-EGF-R, phosphorylated epidermal growth factor receptor; P-VEGF-R2, phosphorylated vascular endothelial growth factor receptor 2; P-p44/42 MAPK, phosphorylated mitogen-activated protein kinase p44.42; IGF-1-R- β , insulin-like growth factor 1 receptor β ; P-AKT-1, phosphorylated v-akt murine thymoma viral oncogene homolog 1.

Table 4.

Relation Between Antibodies Tested *

Antibody	Correlating Antibody	Positive or Negative Correlation	Raw P	Corrected P
VEGF-A	None			
VEGF-B	VEGF-R-2	+	.01	.29
	P-MAPK	+	.03	.41
	PDGF-C	+	.002	.2
	IGF-IR- β	-	.003	.2
	P-IGF-IR	-	.007	.29
VEGF-D	P-p70S6	+	.006	.29
	VEGF-R3	+	.04	.46
VEGF-R1	VEGF-R2	+	.02	.39
	EGF	+	.02	.39
VEGF-R2	P-VEGF-R2	+	.04	.46
	P-p70S6	-	.03	.41
	P-EGF-R	+	.01	.29
VEGF-R3	P-IGF-IR	+	.03	.53
	IGF-IR- β	+	.03	.41
PDGF-A	None			
P-PDGF-R- α	P-AKT-1	+	.03	.41
PDGF-R- β	EGF-R	+	.004	.23
	IGF-IIR	+	.02	.39
PDGF-BB	P-MAPK	+	.05	.53
p70S6	P-p70S6	+	.003	.20
	AKT-1	-	.05	
	P-AKT-1	-	.03	.41
P-p70S6	IGF-1 R- α	+	.04	.46
P-p70S6-R	IGF-1 R- α	+	.02	.39
	P-IGF-1 R	+	.001	
IGF-1	None			
IGF-1-R- α	EGF-R	-	.05	.53

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Antibody	Correlating Antibody	Positive or Negative	Correlation	Raw P	Corrected P
P-IGF-1-R	IGF-2	+		.03	.41
	IGF-II-R	+		.03	.41
IGF-II-R	EGF-R	+		.02	.39
EGF	P-EGF-R	+		.01	.29
AKT-1	P-AKT-1	+		.02	.39

VEGF indicates vascular endothelial growth factor; VEGF-R-2, vascular endothelial growth factor receptor 2; +, positive; P-MAPK, phosphorylated mitogen-activated protein kinase; PDGF-C, platelet-derived growth factor C; IGF-IR-β, insulin-like growth factor 1 receptor β; P-p70S6, phosphorylated 70-kilodalton (kD) protein S6 kinase; EGF, epidermal growth factor; P-AKT-1, phosphorylated v-akt murine thymoma viral oncogene homolog 1.

* Contingency testing revealed several correlations between the proteins tested using a raw *P* value of .05. Increased expression of 1 protein was associated with either the increased or decreased expression of other proteins. A positive (+) correlation represented increased expression of 2 proteins, whereas a negative (−) correlation indicated an inverse correlation between 2 proteins. The false-discovery approach was used to correct for multiple tests.