Original Article

Systemic Levels of Neuropeptide Y and Dipeptidyl Peptidase Activity in Patients With Ewing Sarcoma—Associations With Tumor Phenotype and Survival

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BACKGROUND: Ewing sarcoma (ES) is driven by fusion of the Ewing sarcoma breakpoint region 1 gene (*EWSR1*) with an E26 transformation-specific (ETS) transcription factor (*EWS-ETS*), most often the Friend leukemia integration 1 transcription factor (FLI1). Neuropeptide Y (*NPY*) is an EWS-FLI1 transcriptional target; it is highly expressed in ES and exerts opposing effects, ranging from ES cell death to angiogenesis and cancer stem cell propagation. The functions of NPY are regulated by dipeptidyl peptidase IV (DPPIV), a hypoxia-inducible enzyme that cleaves the peptide and activates its growth-promoting actions. The objective of this study was to determine the clinically relevant functions of NPY by identifying the associations between patients' ES phenotype and their NPY concentrations and DPP activity. **METHODS:** NPY concentrations and DPP activity were measured in serum samples from 223 patients with localized ES and 9 patients with metastatic ES provided by the Children's Oncology Group. **RESULTS:** Serum NPY levels were elevated in ES patients compared with the levels in a healthy control group and an osteosarcoma patient population, and the elevated levels were independent of EWS-ETS translocation type. Significantly higher NPY concentrations were detected in patients with ES who had tumors of pelvic and bone origin. A similar trend was observed in patients with metastatic ES. There was no effect of NPY on survival in patients with localized ES. DPP activity in sera from patients with ES did not differ significantly from that in healthy controls and patients with osteosarcoma. However, high DPP levels were associated with improved survival. **CONCLUSIONS:** Systemic NPY levels are elevated in patients with ES, and these high levels are associated with unfavorable disease features. DPPIV in serum samples from patients with ES is derived from nontumor sources, and its high activity is correlated with improved survival. ***Cancer* 2015;121:697-707**. © *2014 American Cancer Society*.

KEYWORDS: Ewing sarcoma, neuropeptide Y, dipeptidyl peptidase IV, survival, disease phenotype.

INTRODUCTION

Ewing sarcoma (ES) is an aggressive malignancy of children and adolescents arising in bones or soft tissues. The presence of metastases is the most powerful adverse prognostic factor in ES, with a 5-year event-free survival (EFS) rate of 72% and a 3-year EFS rate of 27% for patients with localized and metastatic disease, respectively.[1,2] Although pulmonary metastases are the most common, the prognosis is worse for patients who have secondary bone tumors, particularly when both bone and lung metastases are present (EFS rate, 8%-14%).[1] Among patients who have localized disease, pelvic tumors carry a worse prognosis.[3]

Malignant transformation of ES is driven by chromosomal translocations resulting in fusion of the Ewing sarcoma breakpoint region 1 (*EWSR1*) gene with an E26 transformation-specific (ETS) transcription factor (*EWS-ETS*).[4] The most common fusion types include EWS–Friend leukemia integration 1 transcription factor (*EWS-FLI1*) transcripts, which vary in their fusion sites, and EWS–v-ets avian erythroblastosis virus E26 oncogene homolog (*EWS-ERG*).

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DOI: 10.1002/cncr.29090, **Received:** July 7, 2014; **Revised:** August 24, 2014; **Accepted:** August 26, 2014, **Published online** November 11, 2014 in Wiley Online Library (wileyonlinelibrary.com)

However, recent studies have identified other ETS proteins that fuse with EWS as well as novel translocations associated with ES and ES-related tumors that do not contain the *EWSR1* gene.[5]

Microarray analyses have identified multiple transcriptional targets of the EWS-FLI1 protein that are upregulated in ES, including neuropeptide Y (*NPY*) and its receptors: NPY receptor type 1 and type 5 (*Y1R* and *Y5R*).[6] NPY is a 36-amino-acid sympathetic neurotransmitter that regulates cell proliferation and differentiation and acts as an angiogenic factor.[7-9] Moreover, NPY controls bone homeostasis by blocking osteoblast differentiation.[10] There is also growing evidence of NPY's role in the regulation of tumor growth through both its angiogenic activity and its direct effects on tumor cells.[8,11-13] It is noteworthy that, in tumors of sympathetic origin, such as neuroblastoma and pheochromocytoma, NPY release manifested by its elevated systemic levels in patients has been associated with an aggressive phenotype of the disease.[14-17] No such correlations have bene observed for intratumoral NPY messenger RNA (mRNA) levels.

The role of NPY in ES remains unclear. Initial studies from our laboratory indicated that NPY could stimulate cell death through the activation of both Y1R and Y5R.[11,12] Consequently, exogenous NPY inhibited ES cell survival in vitro and the growth of primary tumors in an ES xenograft model.[11,12] However, we have also demonstrated that, in the hypoxic tumor microenvironment, the actions of endogenous NPY shift to Y2R/Y5R-driven effects that are known to promote tumor dissemination, such as ES cancer stem cell proliferation and migration, as well as angiogenesis.[13] This hypoxia-induced switch in NPY actions is mediated by increased Y2R and Y5R expression and also by the stimulation of dipeptidyl peptidase IV (DPPIV), a membrane protease that converts full-length NPY_{1-36} to the selective Y2R/Y5R agonist, NPY_{3-36}.[12,13] Thus, DPPIV is a key regulator of NPY actions in ES, shifting its activity from Y1R/Y5R-mediated growth inhibition to Y2R/Y5R-mediated, potentially prometastatic effects. However, the protease also modifies a variety of other peptides and augments the cellular immune response.[18,19]

High endogenous NPY expression in tumors often leads to its elevated systemic levels.[14-17] We have also demonstrated that high levels of DPPIV in ES xenografts result in its elevated activity in plasma.[12] Therefore, the objective of the current study was to assess NPY levels and DPPIV activity in sera from patients with ES and determine whether the pattern of their release correlates with a specific disease phenotype, providing insight into clinically relevant functions of the peptide. We have demonstrated for the first time that systemic NPY levels are highly elevated in patients who have ES with unfavorable disease features. Thus, our data corroborate results from previous experimental studies demonstrating a hypoxia-induced, prometastatic effect of NPY.[13] In contrast, DPPIV detectable in patients' sera is derived from nontumor sources, and its high activity is correlated with better EFS.

MATERIALS AND METHODS

Human Samples

In total, 232 serum samples from patients with ES and 21 serum samples from patients with osteosarcoma were received from the Children's Oncology Group (COG); 31 serum samples from healthy volunteer children, ages 6 to 18 years, were collected at the Georgetown University Clinical Research Unit; and human tissue sections from 17 archival, paraffin-embedded ES samples were collected from multiple institutions in Poland by 1 of the coauthors (E.I.-S.) in compliance with institutional ethical regulations. Use of these samples was approved by the Georgetown University Institutional Review Board.

Cell Culture

Human ES cell lines were obtained and cultured as previously reported.[12]

ES Xenografts

SK-ES1 or TC71 cells were injected orthotopically into gastrocnemius muscles of severe combined-immunodeficiency/beige (SCID/bg) mice.[13] Once tumors reached 1 cm^3 in size, the primary tumors were excised, and the tissues were collected for analyses.

Real-Time Reverse Transcriptase-Polymerase Chain Reaction

RNA was isolated using the High Pure RNA Isolation Kit (Roche Applied Science, Indianapolis, Ind). Complementary DNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, Calif) and was amplified using the ICycler iQ Detection System (Bio-Rad Laboratories), TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, Calif), and predesigned primers and fluorescein-labeled probes (Applied Biosystems). The results were calculated using the comparative Ct method with *β-actin* as a reference gene.

Tumor Translocations and Gene Expression Data

Translocation and gene expression profiling data for primary ES tumor samples were provided by the COG. Fusion type was known for 50 of the evaluated patients as determined from archived tumor specimens.[4] Gene

expression profiling of 56 archived COG tumor samples was performed using Affymetrix Human Exon arrays (Affymetrix, Santa Clara, Calif), and normalization and transcript summarization of data were achieved using Partek Genomics Suites software (Partek Inc., St. Louis, Mo).

NPY Enzyme-Linked Immunosorbent Assay

Conditioned media were collected from ES cells after 24 hours in culture, and the cells were trypsinized, counted, and lysed in enzyme-linked immunosorbent assay (ELISA) assay buffer. NPY concentrations in the cell extracts and culture media were determined using the Neuropeptide Y Enzyme Immunoassay Kit (Bachem Peninsula Laboratories, San Carlos, Calif) and were normalized per milligram of protein or cell number, respectively. Patient serum samples were extracted using C18 Sep-columns (Bachem Peninsula Laboratories), and NPY was measured using an ELISA as described above. For samples that reached the upper limit of detection (1.267 ng/mL) in the initial ELISA test and had sufficient volume, a second assay with a higher dilution was performed. For volume-limited samples, the upper limit of detection from the first ELISA test was used. Subsequent statistical analyses relied on categorized NPY data.

Dipeptidyl Peptidase Activity

Dipeptidyl peptidase (DPP) activity in human serum was measured colorimetrically at 405 nm, using 1 mM p-nitroanilide (pNA)-conjugated Gly-Pro dipeptide substrate (Sigma Chemical Company, St. Louis, Mo), as previously described.[12]

Immunohistochemistry

Immunostaining of ES xenografts and human tumors was performed on formalin-fixed, paraffin-embedded tissue samples using rabbit polyclonal anti-NPY antibody (Sigma Chemical Company).

Statistical Analyses

Statistical analyses were performed using SigmaStat (Systat Software, San Jose, Calif), GraphPad (GraphPad Software, La Jolla, Calif), and SPSS (IBM Corporation, Armonk, NY) software. For systemic NPY levels and DPP activity, measurements were divided into 4 rank groups or were analyzed as continuous variables. The comparisons were performed using the Kruskal-Wallis test, the Fisher exact test, the log-rank test, or a Cox proportional hazards model, as appropriate. For the Cox model, a stepwise variable-selection procedure was used, starting with sex, age at enrollment, primary site, randomized treatment assignment, rank group for NPY, and DPP as continuous variables. General NPY-immunoreactivity and DPP activity were compared using a 1-way analysis of variance and post hoc Bonferroni correction. The comparison of gene expression levels was performed using a *t* test.

TABLE 1. Demographic Characteristics of the Ewing Sarcoma Patient Population Included in the Study on Serum Neuropeptide Y Levels

Clinical Feature	No. of Patients (%)
Sex	
Female	94 (42.2)
Male	129 (57.8)
Age at enrollment	
<18	196 (87.9)
≥18	27 (12.1)
Race	
Black	2 (0.9)
White	198 (88.8)
Other	9 (4)
Unknown	14 (6.3)
Ethnicity	
Hispanic	18 (8.1)
Non-Hispanic	198 (88.8)
Unknown	7 (3.1)
Primary tumor site	
Nonpelvic	185 (83)
Pelvic	38 (17)
Randomized treatment assignment	
Standard	113 (50.7)
Intensive	110 (49.3)
First event in EFS analysis	
No event	156 (69.3)
Relapse	59 (26.5)
Second malignant neoplasm	5 (2.2)
Death	3 (1.4)
Follow-up for patients with "no event"	
Median [range]	4.9 [1.8-7.7]

Abbreviations: EFS, event-free survival.

RESULTS

Systemic NPY Levels are Elevated in Patients With ES

We previously demonstrated that ES cells expressed high levels of NPY. To determine whether this high NPY expression in tumors results in its secretion into the circulation, we measured NPY concentrations in sera from 232 patients with ES, including 223 patients with localized disease and 9 patients with metastatic disease. Table 1 summarizes the demographic characteristics of these patients. Patients with osteosarcoma (n = 21) and healthy children (ages 6-18 years; n = 31) served as reference populations.

NPY concentrations in sera from patients who had localized ES (mean, 0.940 ng/mL) and metastatic ES (mean, 1.212 ng/mL) were significantly higher compared with NPY concentrations in healthy controls (mean, 0.517 ng/mL), suggesting release of the peptide from ES tumors (Fig. 1A). Patients who had metastatic ES tended to have higher serum NPY levels compared with those

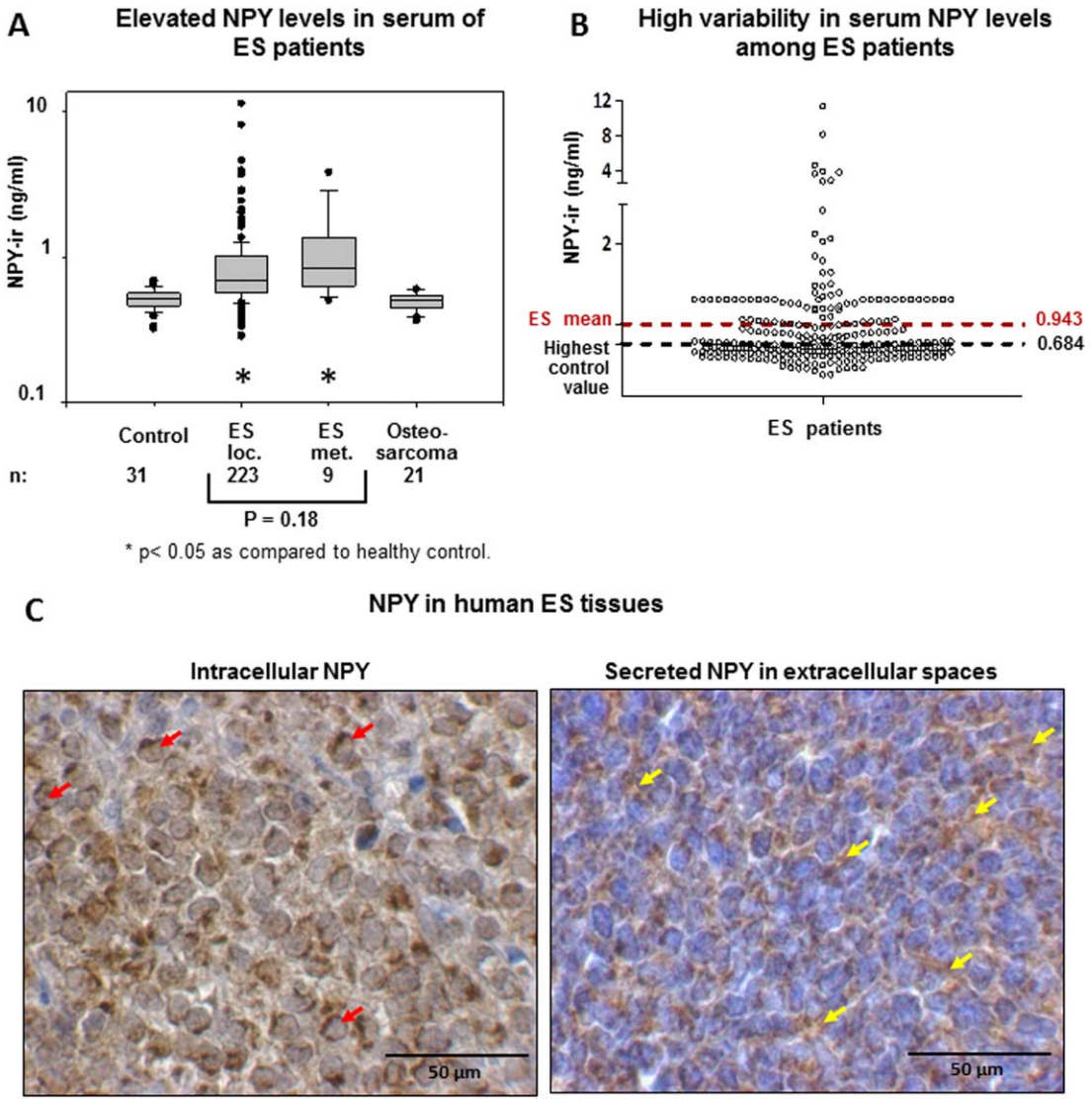

Figure 1. Ewing sarcoma (ES) tumors secrete various levels of neuropeptide Y (NPY) to the circulation. (A) NPY immunoreactivity (NPY-ir) was measured by using an enzyme-linked immunosorbent assay in sera from healthy children (ages 6-18 years), patients with localized ES and metastatic ES, and patients with osteosarcoma. (B) Variability in serum NPY concentrations among patients with ES (localized and metastatic patients combined) is illustrated. (C) Representative images of human ES tissues exhibiting different patterns of NPY immunostaining, including accumulation in tumor cell cytoplasm or in extracellular spaces. Red arrows indicate tumor cells with evident cytoplasmic NPY accumulation, and yellow arrows indicate NPY immunoreactivity in extracellular spaces.

who had localized ES ($P = .18$). However, because of the limited sample size, no definitive conclusion could be made. No increased serum NPY levels were observed in the patients with osteosarcoma (mean, 0.492 ng/mL).

Serum NPY Levels in ES Patients Are Highly Variable

Despite the overall elevated NPY levels in patients with ES, the NPY concentrations were highly variable within the ES population (Fig. 1B). Although 50% of patients with localized ES had NPY concentrations greater than the highest control value, the remaining patients were at the level of healthy controls. Variability in systemic NPY concentrations corresponded to a heterogeneous pattern of NPY immunostaining in human ES tissues (Fig. 1C). Eleven of 17 tested tissue samples (65%) exhibited prevalent intracellular staining; whereas, in 6 samples (35%), the staining was observed in both cytoplasm and extracellular spaces, suggesting increased release of NPY.

ES Cells Vary in NPY Release

The variability in NPY systemic levels and immunostaining patterns suggested differential NPY secretion from ES tumors. To test this, we compared NPY expression and release in a panel of ES cell lines. All tested cells had detectable levels of NPY mRNA and intracellular NPY protein (Fig. 2A,B); however, the cell lines varied in their release of the peptide: NPY was detectable in conditioned media from 5 of 9 cell lines (Fig. 2C), and its concentrations were positively correlated with mRNA levels (Fig. 2D). No significant correlation was observed between NPY mRNA and its intracellular levels.

To determine whether the differences in NPY secretion observed in ES cell lines are maintained in vivo, ES xenograft tissues derived from 2 cell lines that varied in NPY release (TC71 and SK-ES1 cells) were immunostained for NPY. In TC71 xenografts, NPY accumulated mainly in the cytoplasm of tumor cells; whereas, in SK-ES1 xenografts, NPY immunoreactivity was also detected in extracellular spaces, suggesting its secretion (Fig. 2E). This observation was in agreement with high concentrations of the peptide in SK-ES1-conditioned media but not in TC71-conditioned media (Fig. 2C). These results corroborated our data in human ES tissues, confirming variability in the release of NPY from ES tumors.

NPY Levels Are Increased in ES Patients With Various Translocation Types

Having determined the variability in systemic NPY levels among patients with ES, we sought to identify which disease phenotype was associated with elevated peptide levels. Because *NPY* is a transcriptional target of EWS-FLI1, we tested whether its release depends on the fusion type. Serum NPY levels were compared in a cohort of 50 patients who had known translocation status. All groups of patients with various EWS-FLI1 translocation types and EWS-ERG fusion had significantly elevated NPY levels compared with healthy controls (Fig. 3). NPY concentrations were particularly high in patients who were negative for EWS-FLI1 and EWS-ERG, although the limited number of samples precluded a reliable group comparison.

NPY Release Is Elevated in Pelvic ES

Because fusion type did not have an effect on NPY release, we correlated serum concentrations of the peptide with the patients' clinical characteristics (Table 1). No significant associations between sex, age, or randomized treatment assignment and NPY levels were observed. However, NPY was significantly elevated in patients who had pelvic tumors compared with those who had nonpelvic ES (Table 2). In total, 55.3% of patients with pelvic primary tumor sites had NPY levels above the 75th percentile, and only 18.4% had levels below the median ($P = 1.966 \times 10^{-5}$), indicating increased NPY release from these tumors.

NPY System Expression Is Increased in Bone ES

Aside from pelvic localization, bone origin of the primary tumor was also associated with increased NPY release (Fig. 4A). The comparison was performed in a subset of 66 patients who had known tumor localization. The difference between bone and extraosseous tumors achieved statistical significance within subsets of patients who had pelvic and axial tumors but not in those who had ES localized in the extremities. To determine whether these differences were correlated with the levels of *NPY* transcription, we interrogated gene expression microarray data from 56 human ES tumors (Fig. 4B). mRNA levels of NPY system elements were moderately but consistently up-regulated in bone tumors compared with extraosseous tumors. The differences in mRNA levels achieved statistical significance for DPPIV and Y2R ($P = .003$ and $P = .046$, respectively), whereas the difference was on the border of significance for NPY ($P = .052$).

Serum NPY Levels Had No Effect on Survival Among Patients with Localized ES

Patients with localized ES were grouped based on serum NPY levels into 4 approximately equally sized groups. On the basis of analyses performed using these quartiles, serum concentrations of NPY did not have an effect on patient survival (Fig. 5). Because of the small sample size, patients with metastatic ES were not included in this analysis.

Tumor DPPIV Does Not Affect Systemic DPP Activity in Sera From Patients With ES

Because DPPIV regulates NPY actions and its plasma levels are elevated in mice bearing DPPIV-rich ES xenografts, we tested DPP activity in sera from 179 patients with ES who were part of the cohort that we used to measure NPY. DPP activity in patients with ES did not differ significantly from the activity in our healthy control and osteosarcoma populations (Fig. 6A). These data indicate that the enzyme activity measured in these sera is derived from nontumor sources.

High Systemic DPP Activity Is a Strong Predictor of Better EFS in Patients With Localized ES

In contrast to NPY levels, DPP activity had a significant effect on EFS among patients with localized ES. The

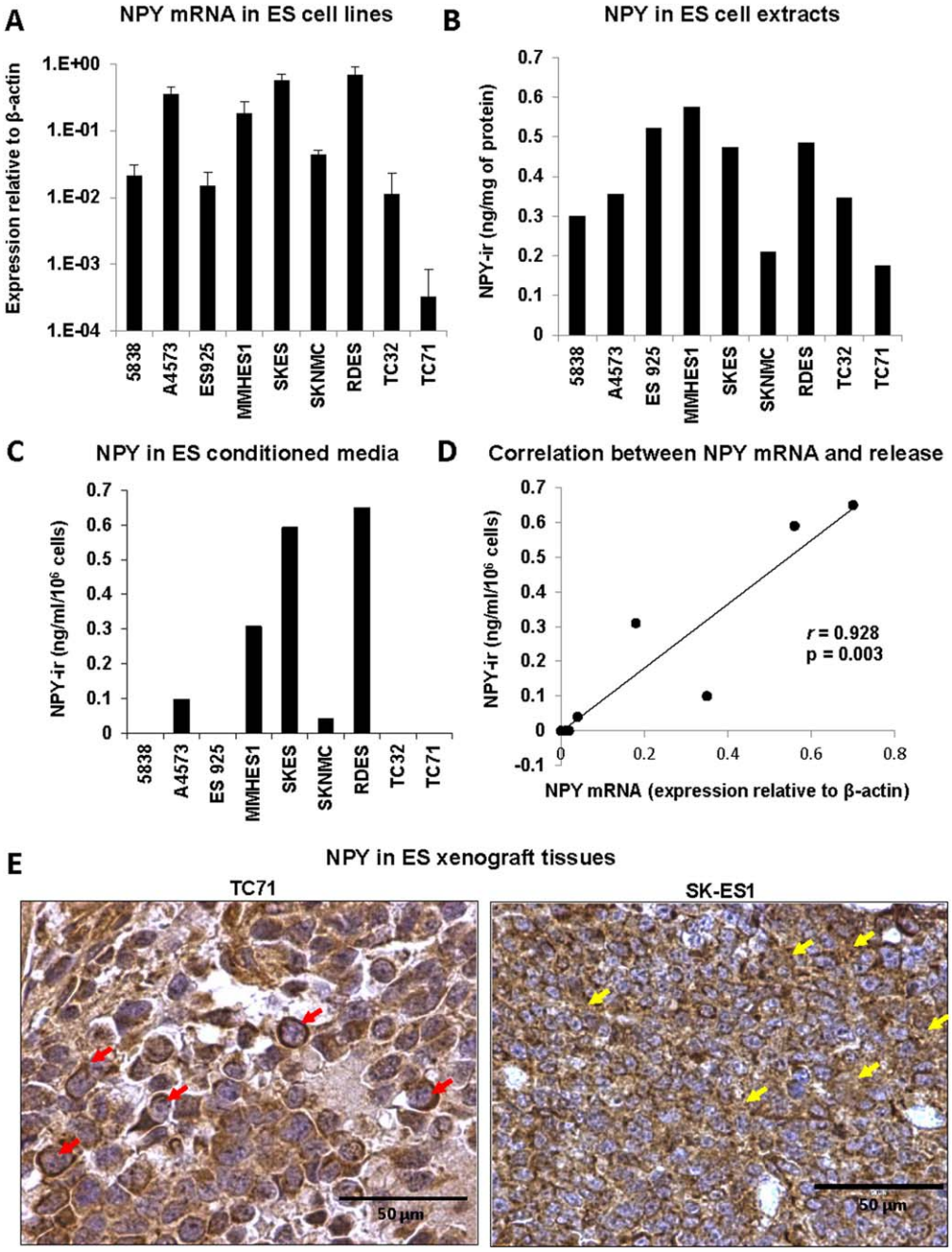

Figure 2. Ewing sarcoma (ES) cells constitutively express neuropeptide Y (NPY) but vary in its release. (A) NPY messenger RNA (mRNA) levels were measured in a panel of ES cell lines using real-time reverse transcriptase-polymerase chain reaction analysis. (B) NPY intracellular content was determined in cell extracts from ES cells using an enzyme-linked immunosorbent assay (ELISA). (C) Concentrations of NPY in conditioned media from ES cells were measured using an ELISA. (D) A positive correlation was observed between intracellular NPY mRNA and its concentrations in the corresponding conditioned media. (E) NPY immunostaining was performed in orthotopic xenograft tissues derived from 2 ES cell lines (TC71 and SK-ES1) that varied in NPY expression. Red arrows indicate tumor cells with cytoplasmic NPY accumulation, and yellow arrows indicate NPY immunoreactivity (NPY-ir) in extracellular spaces.

log-rank test for the effect on EFS of DPP activity levels categorized into quartiles had a *P* value of .0067, and the stepwise variable selection in the Cox proportional hazards model produced a model in which only the DPP level was statistically significant, with an estimated hazard ratio of 0.7546 per 0.1 change in DPP activity (Wald

95% confidence interval, 0.6626-0.8595; $P = 2.216 \times 10^{-5}$) (Fig. 6B). The effect on overall survival was less pronounced; the log-rank test for the effect on survival of DPP activity levels categorized into quartiles had a *P* value of .1154, and the stepwise selection process in the Cox model similarly identified only the DPP level as statistically significant, with an estimated hazard ratio of 0.8227 per 0.1 change in DPP activity (Wald 95% confidence interval, 0.6921-0.9779; $P = .0269$) (Fig. 6C).

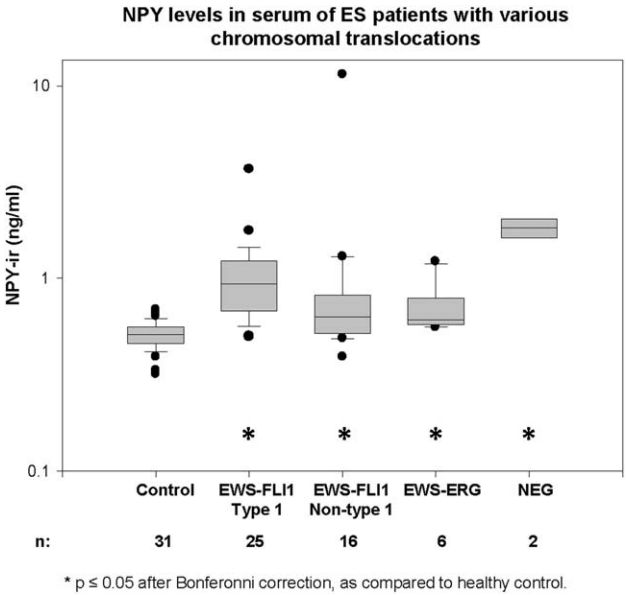

Figure 3. Serum neuropeptide Y (NPY) levels are elevated in patients with Ewing sarcoma (ES) independent of the Ewing sarcoma breakpoint region 1 (*EWSR1*) (EWS)-E26 transformation-specific (ETS) transcription factor (EWS-ETS) translocation type. NPY serum concentrations were compared between healthy controls (children ages 6-18 years) and patients who had ES with various types of EWS-ETS translocations. The study was performed in a cohort of 50 patients who were tested for the presence of the EWS–Friend leukemia integration 1 transcription factor (EWS-FLI1) fusion and the EWS–v-ets avian erythroblastosis virus E26 oncogene homolog (EWS-ERG) fusion. Two of the patients were negative (NEG) for both types of translocations.

DISCUSSION

NPY, as 1 of the EWS-FLI1 target genes, is highly expressed in ES. In these tumors, the peptide exerts several opposing effects, such as Y1R/Y5R-mediated cell death, Y2R/Y5R-dependent angiogenesis, and cancer stem cell proliferation and migration.[11-13] NPY functions are regulated by DPPIV and by a hypoxic tumor environment, both of which favor its Y2R/Y5R-driven actions.[13] Such complex activities of NPY raise a question regarding which functions of the peptide affect disease phenotype and the survival of patients with ES. To address this issue, we sought to identify the clinical features of ES associated with elevated NPY release and DPP activity.

The average serum NPY concentration was significantly elevated in ES patients compared with healthy controls. No such increase in NPY levels was observed in patients with osteosarcoma, a tumor that affects a patient population comparable to patients with ES, indicating that NPY release is a characteristic of ES. This observation is consistent with EWS-FLI1–driven expression of *NPY*.[6] The finding that NPY levels were elevated in patients who had ES with various types of *EWS-FLI1* rearrangements and *EWS-ERG* fusion implicates *NPY* as a universal EWS-ETS target.

Despite the overall elevated NPY levels in patients with ES, its serum concentrations varied greatly: 50% of patients who had localized disease had normal serum NPY levels. Our analysis of ES cell lines revealed significant differences in peptide secretion, suggesting that heterogeneous NPY release can underlie the variability in its systemic concentrations among patients with ES. This was further confirmed by extracellular NPY immunostaining observed both in ES xenografts derived from cell lines that release NPY and in some human ES tissues. Because NPY acts through cell membrane receptors, the ability of ES cells to secrete the peptide is an important mechanism regulating its actions. In ES cell lines, we observed a strong correlation between *NPY* mRNA levels and NPY concentrations in conditioned media. However, no such trend was observed

TABLE 2. Neuropeptide Y Concentrations in Sera From Ewing Sarcoma Patients With Pelvic and Nonpelvic Tumors

	No. of Patients (%)					
	NPY Levels by Percentile					
Primary Tumor Site	<25th	25th-50th	50th-75th	>75th	Total	*P*
Nonpelvic	51 (27.6)	53 (28.6)	46 (24.9)	35 (18.9)	185 (100)	
Pelvic	4 (10.5)	3 (7.9)	10 (26.3)	21 (55.3)	38 (100)	1.966×10^{-5}

Abbreviations: NPY, neuropeptide Y.

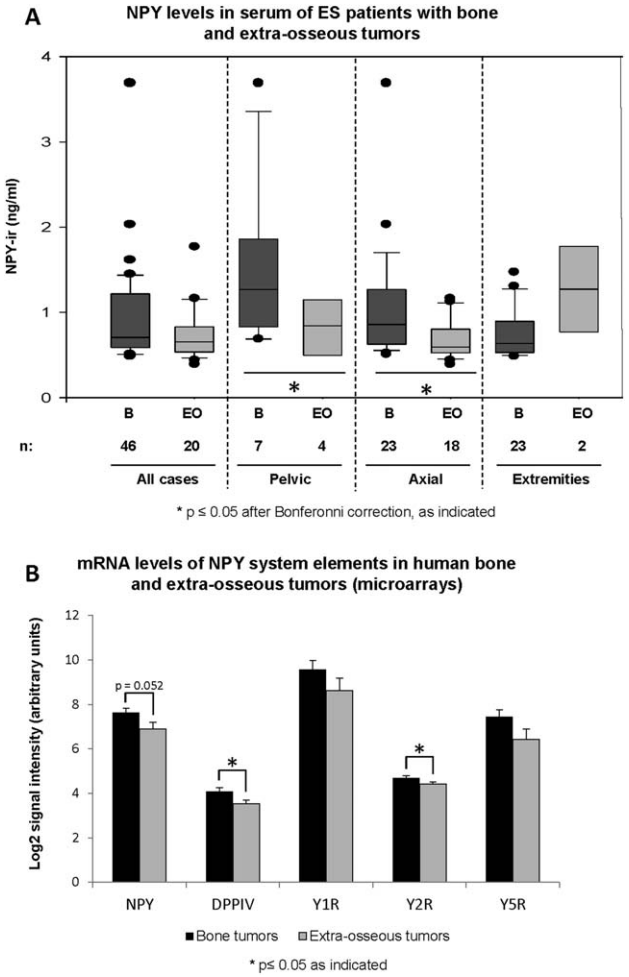

Figure 4. Systemic neuropeptide Y (NPY) expression is elevated in bone Ewing sarcoma (ES) compared with soft tissue ES. (A) Serum NPY concentrations were compared between patients with ES who had bone (B) or extraosseous (EO) primary tumors. These comparisons were made in the overall patient population and within subgroups that had particular tumor localization. (B) Messenger RNA (mRNA) levels of NPY and its receptors (NPY receptor type 1, 2 and 5 [Y1R, Y2R, Y5R]), and dipeptidyl peptidase IV (DPPIV) were measured by using gene expression microarrays in tissues from 56 human ES primary tumors originating from bone (n = 32) or soft tissues (n = 23) and were compared between the 2 groups.

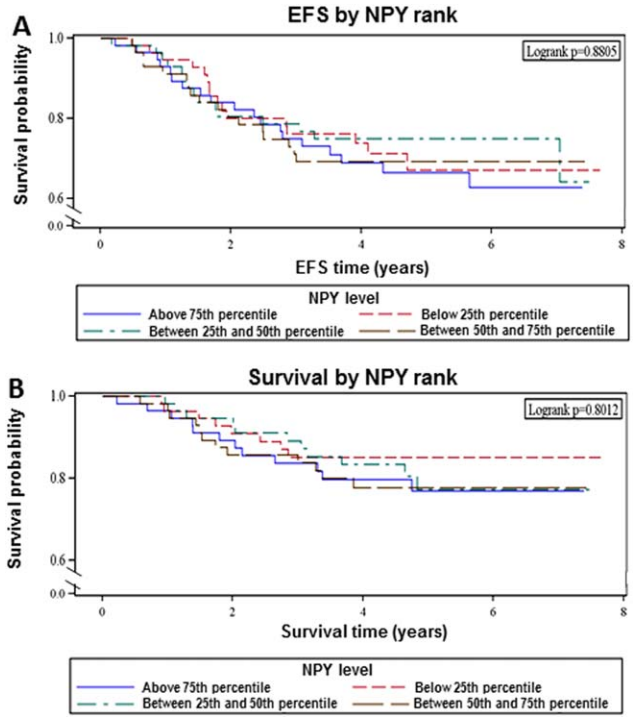

Figure 5. In patients with localized Ewing sarcoma (ES), serum neuropeptide Y (NPY) levels do not affect survival. (A) ES patients with localized disease were divided into 4 groups based on serum NPY levels (>75th percentile, 50th-75th percentile, 25th-50th percentile, and <25th percentile). Patients' event-free survival (EFS) was compared between these groups using the log-rank test, and no significant differences between groups were detected. (B) The same approach revealed no significant differences in overall survival between patients with ES who had various NPY levels.

in the small population (n = 6) of patients with ES who had matching data on *NPY* mRNA and serum levels (data not shown). Thus, NPY serum concentrations in patients may be further modified by tumor mass or by factors that promote its release in the tumor microenvironment.

One of the stromal factors known for regulating NPY synthesis and release is brain-derived neurotrophic factor (BDNF).[20] High levels of BDNF are present in the bone environment, which is in line with elevated expression and secretion of NPY from bone ES, at least in the pelvic and axial locations.[21] This observation suggests the potential involvement of NPY in ES bone invasion and is in agreement with the role of the peptide in regulating bone homeostasis. It has been demonstrated that NPY inhibits osteoblast differentiation, which impairs osteogenesis and decreases bone density.[10] Although bone lesions in ES are mainly osteolytic, blocking osteogenesis in pediatric and adolescent patients who have ongoing bone formation may shift bone homeostasis toward osteolysis and promote bone degradation. A similar phenomenon has been observed in neuroblastoma, another pediatric tumor with frequent bone metastases.[22]

Aside from bone ES, elevated NPY serum concentrations also were observed in patients with pelvic tumors, which carry a worse prognosis.[3] It is not clear, however, whether this unfavorable prognosis results from a greater tumor mass at diagnosis, the proximity of tumors to internal organs, frequent incomplete resection, or the different biology of these tumors. Similarly, increased NPY levels

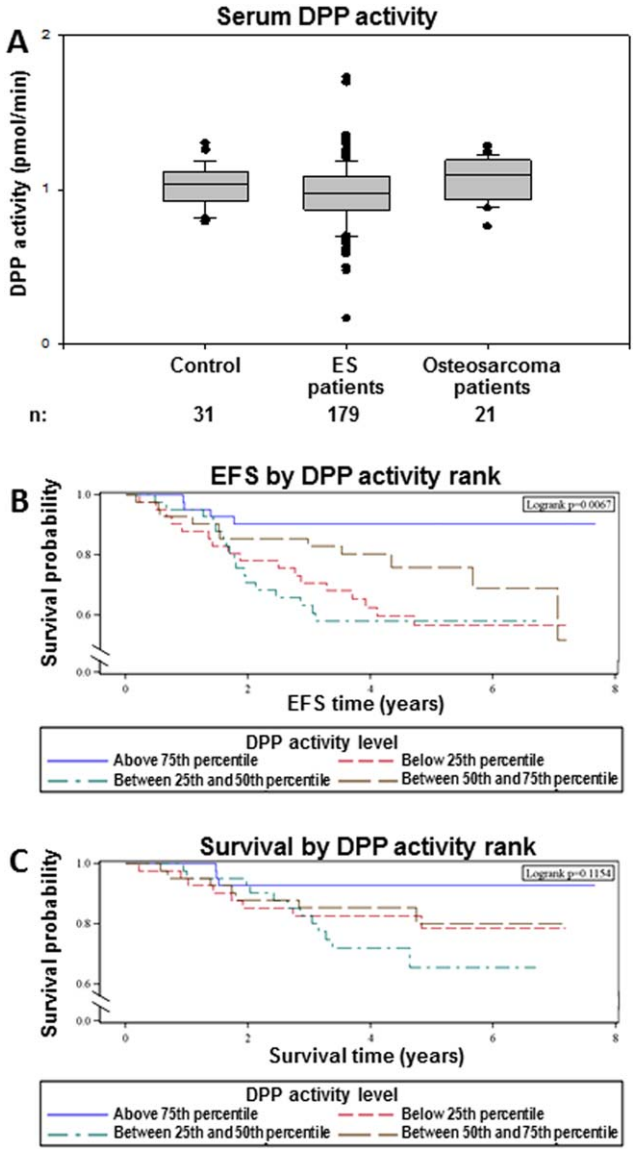

Figure 6. Dipeptidyl peptidase (DPP) activity is not elevated in sera from patients with Ewing sarcoma (ES), but its high levels are associated with better event-free survival (EFS). (A) DPP activity was measured in sera from healthy children (ages 6-18 years), patients with ES, and patients with osteosarcoma using a colorimetric method. No significant differences were observed between the experimental groups. (B) Patients with ES who had localized disease were categorized into quartiles based on their serum DPP activity. High DPP activity was associated with significantly better EFS among patients with ES, as determined by the log-rank test (P = .0067). (C) The effect of DPP levels on the overall survival of patients with ES who had localized disease, determined as described above, did not achieve statistical significance (P = .1154).

in these tumors may result from their greater size or more aggressive phenotype. The trend toward increased NPY serum concentrations in patients with metastatic ES suggest its elevated release associated with blood-borne disease dissemination. This notion is further supported by our previous experimental data indicating that a hypoxic microenvironment, which is known to increase ES malignancy, stimulates endogenous NPY and promotes its Y2R/Y5R-mediated growth-promoting and prometastatic actions.[13]

Analyses performed within a population of patients who had localized disease indicated no effect of serum NPY levels on the survival of patients with ES. However, given a trend toward increased peptide levels in patients with metastatic ES, the potential role of NPY as a prognostic factor in the overall ES population cannot be excluded. Nevertheless, even if further studies demonstrate no prognostic value for NPY, systemic levels of the peptide can be used to monitor disease progression and response to treatment in the subset of patients with elevated NPY release. This was proposed previously for patients with neuroblastoma and pheochromocytoma.[23,24] Moreover, high systemic levels of NPY may identify a subset of potential candidates for anti-NPY therapies among a population of patients with ES once the role of the endogenous peptide in these tumors is fully elucidated. Finally, the universal, fusion type-independent tissue expression of NPY may serve as a marker in differential diagnosis between ES and other small blue round cell tumors.

NPY actions are modified by DPPIV, which cleaves NPY and facilitates its Y2R/Y5R-mediated growth-promoting and prometastatic effects.[12,13] Because our previous studies indicated elevated DPP activity in plasma from mice bearing DPPIV-rich ES xenografts, we sought to determine whether the same measure would apply to patients with ES.[12] The assay used in our study measured the overall DPP activity in patients' sera and did not distinguish between different types of DPPs. However, DPPIV is the only known membrane DPP that can be converted to a soluble enzyme by its shedding from the cell surface, whereas other DPPs (DPP8 and DPP9) are intracellular proteases.[12] Therefore, DPP activity detectable in sera can be attributed mainly to DPPIV. Nevertheless, serum DPP activity in patients with ES did not differ significantly from that observed in healthy controls and patients with osteosarcoma. These data suggest that enzyme shedding from ES tumors is not sufficient to affect its activity in blood. Instead, the DPP activity detectable in serum depends on nontumor sources, such as endothelium and immune cells.[13,18,19] This discrepancy between animal studies and clinical data may be associated with the relatively greater tumor burden in mice than in patients.

Despite the nontumor origin of DPPIV detected in serum, its high activity was associated with significantly better EFS in patients with ES and trended toward such an effect on overall survival. This surprising discovery may be associated with the known role of DPPIV in regulating the immune response. DPPIV is a crucial factor in activation and propagation of T lymphocytes and natural killers, 2 key elements of the cellular immunity responsible for a host's antitumor response.[18,19] Thus, improved overall survival of patients with high DPPIV activity may reflect a more efficient immune response that inhibits disease progression. This observation strongly suggests that, in the event that prometastatic actions of NPY are confirmed in animal models, directly blocking this pathway will be a better therapeutic strategy than the previously proposed inhibition of multifaceted DPPIV activity.[12] It also raises a question regarding the safety of long-term administration of DPPIV inhibitors recently introduced as routine treatment for diabetes.[19]

In summary, here, we provide the first evidence for elevated systemic levels of NPY in patients with ES comparable to the levels described in patients with tumors of sympathetic origin.[14-17] Although we did not observe a direct effect of high NPY levels on the survival of patients with localized ES, the trend toward elevated NPY release in patients with unfavorable disease features suggests a potential role for the peptide in ES dissemination and warrants further survival analyses that include patients at the metastatic stage. This notion is also supported by our previous data indicating that a hypoxic tumor microenvironment activates the prometastatic actions of NPY in ES.[13] Moreover, increased systemic NPY levels and intratumor expression of the entire NPY system in bone tumors suggest a potential role of this pathway in ES bone invasion. Conversely, the association of high DPP activity with better survival implicates a potential role for DPPIV in anticancer immune response.

FUNDING SUPPORT

This work was supported by National Institutes of Health (NIH) grants UL1TR000101 (previously UL1RR031975) through the Clinical and Translational Science Awards Program, 1RO1CA123211, and 1R03CA178809 to J.B.K.; Children's Oncology Group (COG) Chair grant U10CA098543; Statistics and Data Center grant U10 CA98413-08; COG National Clinical Trials Network (NCTN) Group Operations Center grant 1U10CA180886; and COG NCTN Statistics and Data Center grant 1U10CA180899, as well as funding from the Children's Cancer Foundation (Baltimore, Md) to J.B.K. and J.A.T. Additional support came from the AFLAC Foundation; a Burroughs Wellcome Clinical Scientist Award in Translational Research; and NIH grants R01CA88004, R01CA133662, R01CA138212, and RC4CA156509 to J.A.T. The experiments were performed with use of Lombardi Comprehensive Cancer Center Shared Resources (Histopathology and Tissue, The Tissue Culture, and Biostatistics and Bioinformatics) supported by NIH/National Cancer Institute grant P30-CA051008, and support for statistical analyses was provided by a grant from the WWWW (QuadW) Foundation, Inc. to the COG.

CONFLICT OF INTEREST DISCLOSURES

The authors made no disclosures.

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