

Overexpression of EphB4, EphrinB2, and epidermal growth factor receptor in papillary thyroid carcinoma: A pilot study

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ABSTRACT: *Background.* The purpose of this study was to examine the differential expression of *EphB4*, *EphrinB2*, and epidermal growth factor receptor (*EGFR*) genes in papillary thyroid carcinoma (PTC) and evaluate their association with lymph node metastasis.

Methods. *EphB4*, *EphrinB2*, and *EGFR* expression in 21 matched tumors and surrounding normal thyroid tissues were evaluated by complementary DNA (cDNA) microarray, Western blot, and immunohistochemistry (IHC).

Results. We noted a statistically significant overexpression of *EphB4*, *EphrinB2*, and *EGFR* in tumor versus normal tissue based on cDNA microarray, Western blot, and IHC analysis. *EphB4* and *EphrinB2* overex-

pression were significantly associated with the presence of lymph node disease.

Conclusion. Overexpression of *EphB4*, *EphrinB2*, and *EGFR* are associated with PTC, whereas *EphB4* and *EphrinB2* overexpression are associated with lymph node metastases. These genes may be potential biomarkers for identification of subclinical lymph node involvement in PTC and potential small-molecule targets for pharmacotherapy research. © 2014 Wiley Periodicals, Inc. *Head Neck* 37: 964–969, 2015

KEY WORDS: papillary, thyroid, gene, Ephb4, Ephrinb2, epidermal growth factor receptor (EGFR)

INTRODUCTION

Thyroid cancer has the fastest rising incidence among major human cancers in the United States; the American Cancer Society estimates over 56,000 new cases of thyroid cancer reported in 2012.¹ Papillary thyroid carcinoma (PTC) is the most common type of thyroid malignancy, accounting for 70% to 80% of cases. The overall 5-year survival rate for PTC is approximately 94.2% to 98.7% after surgery and, when indicated, radioiodine therapy.² Evidence-based guidelines published by the American Thyroid Association state that surgery is the primary mode of therapy for patients with PTC.¹ However, although total thyroidectomy for primary tumors at least 1.0-cm in diameter and confined to the thyroid gland is largely curative, there is no standardized treatment protocol for patients with locoregional or distant metastatic disease.

Studies have shown a correlation between nodal metastases and increased risk of locoregional tumor recurrence.^{3,4} In patients with clinically node-negative disease,

a prophylactic central neck (level VI) dissection remains controversial.⁵ A widely accepted approach to treatment of PTC with known nodal metastasis is compartmentalized modified neck dissection (levels II, III, IV, and Vb). However, the molecular mechanisms involved in nodal spread of PTC are incompletely defined. Aside from neck ultrasound, which is limited in its ability to visualize level VI lymph nodes, there is no method to predict or diagnose early lymph node involvement in order to formulate a treatment strategy.

It is well understood that rearrangements or mutations in genes encoding for proteins in the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase signaling cascade play a critical role in the development and progression of PTC.^{6,7} Chromosomal translocation of rearranged during transfection (RET)/PTC and NTRK1 tyrosine kinases, point mutations of B-RAF, RAS, and p53 oncogenes, as well as overexpression of fibroblast growth factor receptor and epidermal growth factor receptor (EGFR) have been implicated in PTC tumorigenesis.^{8–13} However, the precise molecular mechanisms leading to PTC invasion and metastasis remain undefined.

EGFRs are monomer cell-surface receptors, which are part of the ErbB family of receptor tyrosine kinases (RTK). After ligand binding and EGFR dimerization, intracellular signal transduction cascades (notably MAPK, Akt, and Jun-terminal kinase pathways) lead to a variety of physiologic processes, including cell proliferation, differentiation, migration, adhesion, and apoptosis.^{14,15} Mutations leading to EGFR overexpression have been associated with a variety of malignancies, including head

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and neck cancers, esophageal, ovarian, cervical, lung, and bladder cancers.¹⁶ The role of EGFR overexpression in PTC is also well documented in the literature.^{17–19} In 2008, Croyle et al²⁰ demonstrated that RET/PTC-induced cell growth is partly mediated by EGFR activation.

Eph receptors, the largest known subfamily of RTKs, are transmembrane proteins that are subdivided into class A and class B based on sequence homology and their binding affinity for 2 distinct types of membrane-bound ephrin ligands (EphrinA and EphrinB). The Eph/ephrin interaction results in bidirectional signaling from the Eph receptor (forward signaling) and from the Ephrin ligand (reverse signaling).^{21,22} In this manner, Eph and ephrins each exhibit both ligand and receptor functionality. As members of the RTK family, Eph receptors have been implicated in a diverse range of physiologic processes critical to embryologic development, including axon guidance, cell migration, and segmentation.^{21,23} Eph/ephrin signaling has also been linked to the regulation of various physiologic processes in adults, including stem cell differentiation, normal tissue homeostasis, cell migration, and angiogenesis.^{22–26} Early studies have shown that EphB4/EphrinB2 play a role in angiogenic remodeling and arterial vein specification in embryonic development.^{27,28} Endothelial-specific knockout of either EphB4 or EphrinB2 in mice has been shown to affect retinal angiogenesis and lymphangiogenesis in dermal skin.²⁹ In vivo studies noted EphB4 and EphrinB2 overexpression and specifically implicated EphrinB2 reverse signaling in tumor angiogenesis of a variety of pathologies.^{30–32} Recently, Xuqing et al³³ were the first to report EphB4 overexpression in PTC and its role in stimulating cell migration. Although upregulation of Ephs have been noted in a wide range of malignancies, including breast, prostate, glioma, pancreatic, esophageal, and melanoma,^{34–39} studies have also shown that EphB signaling arrests tumor invasion in colorectal cancer.⁴⁰ Thus, the complex and paradoxical functions of Eph receptor signaling depends on a variety of factors, including receptor and ligand homology, forward versus reverse signaling, and tissue type.

In 2006, our group described the relationship between EGFR and EphB4/EphrinB2 signaling in head and neck squamous cell carcinoma (HNSCC).⁴¹ We treated HNSCC cell lines with EGFR-specific kinase inhibitor, finding marked reduction in EphB4, but not EphrinB2 levels. HNSCC cell lines were also cultured in presence of chemical inhibitors of several pathways downstream of EGFR, including Akt, extracellular signal-regulated kinase, and p38 kinase. We noted inhibition of EGF-induced EphB4 expression in the presence of Akt pathway inhibitor only. Akt, a serine/threonine-specific protein kinase, is part of the PI3K/Akt/mTOR intracellular signaling pathway known to regulate apoptosis. Thus, the Akt pathway plays a role in EGFR-mediated regulation of EphB4 signaling in HNSCC and possibly PTC.

Dysregulation of molecular pathways involved in PTC offers new paradigms by which gene biomarkers can be identified for targeted therapy. To date, no study has investigated the expression of both EphB4 and its ligand EphrinB2 in PTC. Our goal was to identify key molecular constituents in the pathogenesis and spread of PTC so

that these biomarkers may be used as early predictors of lymph node involvement and potential molecular targets for future pharmacotherapy studies.

MATERIALS AND METHODS

Patient selection

Twenty-one adult patients with newly diagnosed PTC were prospectively entered into the study. The study was approved by the appropriate institutional review board at Keck Hospital of University of Southern California. All 21 patients had no history of previous thyroid surgery, radiation exposure, smoking, alcohol abuse, or family history of thyroid disease. Demographic data were collected from the patient's hospital records. The staff members who collected the demographic data had no knowledge of the status of EphB4, EphrinB2, or EGFR expression in the patient tumors.

Perioperative data included the TNM stage of the thyroid cancer (stages I–IV, according to the staging system of the American Joint Committee on Cancer), the size of the primary tumor estimated by neck ultrasound or CT, and the preoperative pathologic diagnosis by fine-needle aspiration. All patients underwent total thyroidectomy with central neck (level VI) dissection. Matched tumor and normal adjacent tissue were collected in all 21 cases. To control for the amount of histologically normal tissue within the tumor sample, we studied tumor tissue sections that showed greater than 70% tumor cells by hematoxylin-eosin staining.³ Each tissue was given a unique identification number and sent to the laboratory for analysis. The laboratory was blinded to the status of the tissue specimen.

Reagents

Antibodies to EphB4 (C-16), EphrinB2 (P-20), and EGFR (1005) were purchased from Santa Cruz Biotech (Santa Cruz, CA).

Microarray

Eight of 21 samples were classified as high stage (stage III–IV) and selected for complementary DNA (cDNA) microarray analysis of matched tumor and normal thyroid tissue. The mRNA was isolated from fresh tissue sections, as previously described.^{42,43} Each patient's matched normal tissue was used as a control for all experiments. The mRNA was processed and analyzed via GeneChip U133 Plus 2.0 array (Affymetrix, Santa Clara, CA) against 47,500 genes. Raw data was imported to microArray database (mAdb) and analyzed by software tools provided by the Center for Information Technology, National Institutes of Health.

Western blot

Western blot was performed on all 21 samples of matched tumor and normal tissue. Cell lysates were prepared, as previously described.⁴⁴ Typically, 10- μ g proteins from whole cell lysate were fractionated on a 4% to 20% Tris glycine polyacrylamide gel, electrotransferred to polyvinylidene difluoride membrane, and probed with a primary antibody overnight. The blot was stripped with

Western blot stripping buffer (Restore; Pierce Biotechnology, Rockford, IL) and reprobed with β -actin to confirm equivalent loading and transfer of protein. Signal was detected using a substrate (Super Signal West Femto Maximum Sensitivity Substrate; Pierce Biotechnology). β -actin was used as a loading control, and was measured for all genes. Western blots of tissue from primary tumor and uninvolved tissue were performed to determine the relative levels of EphB4, EphrinB2, and EGFR protein in these sites. Western blots were digitized, and the relevant protein bands of EphB4, EphrinB2, and EGFR were normalized with β -actin and quantified (Fluro-S Multi-Imager System; Bio-Rad Laboratories, Hercules, CA).

Immunohistochemistry

Immunohistochemical (IHC) analysis was conducted on all 21 samples. Fresh frozen tissue embedded in optimal cutting temperature (OCT) were sectioned at 5 mm and fixed in phosphate-buffered 4% paraformaldehyde and washed in phosphate-buffered saline. Endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide in phosphate-buffered saline for 10 minutes, followed by blocking of nonspecific sites with SuperBlock blocking buffer (Pierce Biotechnology) for 1 hour, both at room temperature. Sections were then incubated with primary antibody to EphB4 (1:100 dilution), EphrinB2 (1:200), and EGFR (1:200) overnight at 4°C. After washing in phosphate-buffered saline, antibody binding was localized with appropriate biotinylated secondary antibody and avidin/biotinylated horseradish-peroxidase complex (Vector Laboratories, Burlingame, CA). Sections were then stained with DAB reagent (Vector Laboratories) and counterstained with hematoxylin-eosin. Routine negative controls included deletion of primary and secondary antibody and substitution of normal immunoglobulin G isotype for primary antibody. The degree of antibody staining in tumor and normal tissue was quantitated using integrated optical density (IOD) measurements on Image-Pro Premier (Media Cybernetics, Rockville, MD). IOD is a quantitative measure within a range of intensity values based on the bit depth of the image (eg, 8-bit image range is 0–255, with 0 = black and 255 = white).^{45,46} In our IHC analysis, IOD is the density of antibody staining, and is calculated as the area multiplied by the average intensity. The antibodies used in IHC analysis do not react with other members of the EphB/EphA or EphrinB/EphrinA families.

Statistical analysis

Extracted microarray data was normalized using a 50th percentile (median) normalization method. The fold change of all genes was calculated by comparing tumor gene expression with negative control counterpart. A 2-tailed *t* test was used to compare tumor versus normal gene expression levels. A fold change ≥ 2.0 and α level of 0.05 was defined for statistically significant upregulation. IHC data (IOD measurements) was analyzed under a log₂ linear regression model; the base model was adjusted for race (age and sex were not found to be confounding variables). The Shapiro–Wilk test showed IOD data for all 3 genes followed a normal distribution. The paired *t*

TABLE 1. Characteristics of 21 study patients.*

Characteristic	No. of patients (%)
Tumor size, cm	
≤ 1	5 (24)
1.1–2	8 (38)
2.1–4	6 (29)
> 4	2 (10)
Multifocal disease	
Yes	10 (48)
No	11 (52)
Lymph node disease	
Yes	10 (48)
No	11 (52)
Extracapsular extension	
Yes	7 (33)
No	14 (67)
Neurovascular invasion	
Yes	4 (19)
No	17 (81)
Clinical stage*	
I	13 (62)
II	0 (0)
III	8 (38)

* Staged by the TNM staging system of the American Joint Committee on Cancer.

test was used to compare gene expression between tumor and normal tissue. Multivariate regression analysis (adjusted for race) of raw IOD data examined the relationship between independent variables (tumor size, focality, lymph node disease, extracapsular extension, neurovascular invasion, and clinical stage) and gene expression from tumor samples. For all purposes, a 2-sided *p* value $< .05$ was considered statistically significant. All statistical analysis was performed using SAS software (version 9.3; SAS Institute, Cary, NC).

RESULTS

Patient characteristics

Twenty-one adult patients (age 23–83 years; 81% women) with PTC were treated with total thyroidectomy and central neck dissection at Keck Hospital of University of Southern California, and were enrolled in this study. Clinicopathologic features of the tumor samples are listed in Table 1.

Complementary DNA microarray

The cDNA microarray analysis of 8 high-stage (III) samples showed differential overexpression of EphB4, EphrinB2, and EGFR in tumor compared to normal tissue. Table 2 is a compiled average fold change for these 8 samples. Other genes (not shown) known to play a role in tumorigenesis and metastasis that showed overexpression in our microarray included the matrix metalloproteinase family, interleukin cytokines, cell cycle regulating genes (cyclin-D1, etc.), and various ligands for RTKs, such as transforming growth factor- α and epidermal growth factor.

Western blot

EphB4, EphrinB2, and EGFR expression was observed in all 21 cases of tumor tissue, with varying band density.

TABLE 2. Microarray analysis of EphB4, EphrinB2, and epidermal growth factor receptor expression in tumor versus normal tissue samples.

Accession ID	Gene name	Symbol	Fold change*	<i>p</i> value
NM_004444	Eph receptor B4	EPHB4	2.49	.004
NM_004093	Ephrin-B2	EFNB2	2.42	.012
NM_005228	EGFR [†]	EGFR	2.86	.003

Abbreviation: EGFR, epidermal growth factor receptor.

* Fold change ≥ 2.0 with α -level of .05 was considered significant. The *p* values are from 2-tailed *t* test.

[†] Erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian.

Based on digitized quantification of Western blots by an imaging system (Fluro-S Multi-Imager System), patients were divided into high-expressing and low-expressing EphB4, EphrinB2, and EGFR groups. For all 3 genes, we noted a trend of higher band density in tumor compared to matched bands in normal tissue. Figure 1 shows 3 randomly selected samples depicting increased EphB4, EphrinB2, and EGFR protein in tumor compared to matched normal tissue.

Immunohistochemistry

Figure 2 shows a representative case of PTC with matched normal tissue, depicting minimal baseline expression of all 3 biomarkers in normal tissue and increased expression in tumor tissue. After a log₂ transformation of IOD data, a linear regression model was adjusted for race as a confounding variable (age and sex were not found to be significant covariates). We found statistically significant overexpression of EphB4 (fold change, -3.93; *p* < .0001), EphrinB2 (fold change, -3.66; *p* < .0001), and EGFR (fold change, -2.47; *p* < .0001) in tumor compared to normal tissue (Table 3). After log₂ transformation, matched samples with equivalent expression values would have a ratio of 1 and log₂ fold change of zero. A matched sample with 2-fold upregulation from normal to tumor tissue has a ratio of 0.5 (normal/tumor) and fold change of -1. Thus, a negative log₂ fold change indicates upregulation from normal to tumor. Multivariate analysis of raw IOD measurements showed a statistically significant association between EphB4 overexpression in

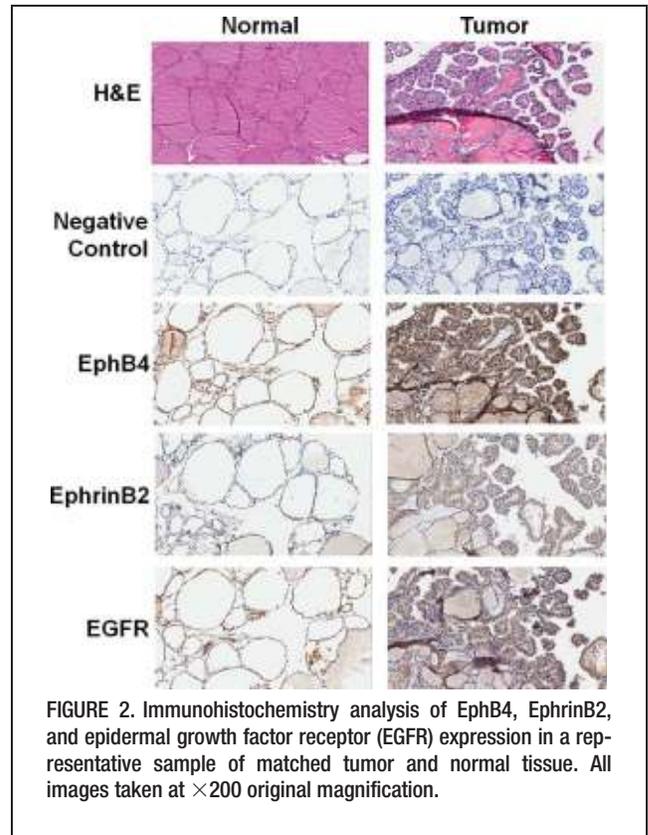


FIGURE 2. Immunohistochemistry analysis of EphB4, EphrinB2, and epidermal growth factor receptor (EGFR) expression in a representative sample of matched tumor and normal tissue. All images taken at $\times 200$ original magnification.

tumor tissue and lymph node disease (*p* < .0001) and extracapsular spread (*p* < .0001). EphrinB2 overexpression in tumor was found to be significantly associated with lymph node disease (*p* < .0001), and marginally associated with extracapsular extension (*p* = .071). We also noted a significant negative correlation with EphrinB2 and neurovascular invasion (*p* = .033). EGFR overexpression in tumor was significantly associated with clinical stage (*p* = .001), and marginally associated with lymph node disease (*p* = .07; Table 4).

DISCUSSION

In the last decade, our understanding of genetic alterations involved in the pathogenesis of differentiated thyroid cancer has grown. Exclusive point mutations and chromosome rearrangements in genes involved in the MAPK pathway have been shown to contribute to PTC tumorigenesis.^{6,7} However, the inciting events and precise molecular mechanisms leading to PTC invasion and spread remain unknown. EphB4, EphrinB2, and EGFR are well-studied genes in tumor angiogenesis, a basis for early invasion and metastasis in various cancers.²⁶⁻³² Previous studies by our group have demonstrated increased expression of EphB4 and its ligand EphrinB2 in HNSCC.^{3,41,47,48} The findings of this study are the first to demonstrate concurrent overexpression of EphB4, EphrinB2, and EGFR in PTC, and the association of these genes with lymph node metastases.

EGFR dysregulation is known to be an active mediator of tumor cell proliferation, invasion, and metastasis. Studies have implicated the role of EGFR-mediated

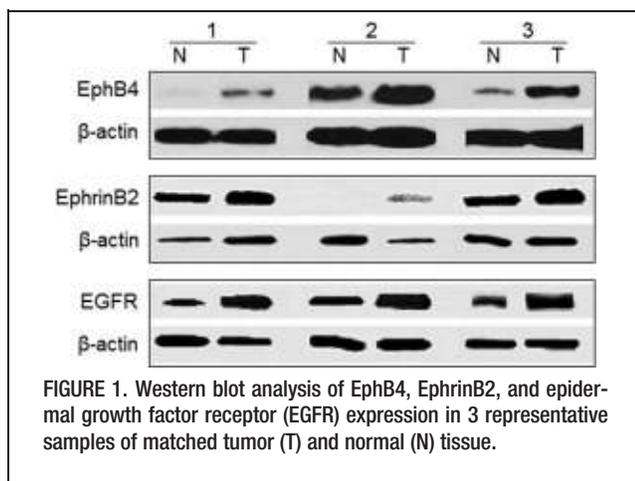


FIGURE 1. Western blot analysis of EphB4, EphrinB2, and epidermal growth factor receptor (EGFR) expression in 3 representative samples of matched tumor (T) and normal (N) tissue.

TABLE 3. Immunohistochemistry analysis of EphB4, EphrinB2, and epidermal growth factor receptor expression in tumor vs normal tissue samples.*

Gene	Fold change (SE)	<i>p</i> value [†]
EphB4	−3.93 (0.14)	< .0001
EphrinB2	−3.66 (0.15)	< .0001
EGFR	−2.47 (0.07)	< .0001

Abbreviation: EGFR, epidermal growth factor receptor.

*Fold change calculated as log₂ difference between normal and tumor integrated optical density (IOD). A 2-fold increase in expression from control to tumor yields a control:tumor ratio of 0.5 and log₂ fold change of −1. A negative log₂ fold change indicates upregulation in gene expression from control to tumor.

[†]From the paired *t* test (adjusted for race).

angiogenesis in the growth and invasion of epidermal tumors.⁴⁹ Furthermore, Hamada et al⁵⁰ describe the role of EphB4/EphrinB2 signaling in regulating tumor angiogenesis. All 3 genes belong to the RTK family of cell membrane receptors, which activate a multiplicity of downstream signaling pathways leading to nuclear gene transcription and, in the event of genetic mutation, uncontrolled cellular proliferation and invasion. Previous studies by our group have shown that activation of EGFR induces EphB4 via activation of Akt in HNSCC.⁴¹ Thus, the Akt pathway may play a role in epidermal growth factor-mediated EphB4 upregulation in PTC. Our results show the concurrent overexpression of EphB4 and EGFR in PTC, suggesting molecular crosstalk and synergy that allows PTC cells to differentiate, and eventually migrate to regional lymph nodes.

By cDNA microarray and IHC quantitative analysis, we found significant overexpression of EphB4, EphrinB2, and EGFR in PTC. This finding was verified by quantitative analysis of Western blots. Multivariate analysis revealed a significant association between gene expression in tumor and histopathologic markers of aggressive

disease: extracapsular extension (associated with EphB4 and EphrinB2 overexpression) and neurovascular invasion (EphrinB2 overexpression). We believe these findings implicate the role of Eph/ephrin signaling in locoregional invasion of PTC, thus denoting EphB4 and EphrinB2 as biomarkers for lymph node metastases.

We demonstrated an association between EphB4, EphrinB2, and EGFR gene expression in tumor tissue and lymph node metastases. The presence of pathologic lymph nodes has been reported in up to 80% of cases of PTC at the time of diagnosis, highlighting the propensity of PTC cells to migrate to lymph nodes early in the disease course.⁵¹ Although survival statistics for patients with PTC and nodal involvement are favorable, nodal involvement is an independent risk factor for tumor recurrence.⁵² Recurrent disease treated with repeat surgical resection is encumbering to patients and their quality of life. Incomplete knowledge of the molecular basis of PTC invasion and lymph node spread limits the long-term management and prevention of PTC recurrence.

Although our results show a considerably higher expression of EphB4, EphrinB2, and EGFR in PTC, we recognize a few potential limitations to our pilot study. Our sample size of 21 patients limits our ability to include multiple independent variables in our linear regression model. Thus, we chose to adjust for the potential confounding effect of race only and eliminated variables that were not found to be significant (tumor size, focality) from the regression model. Further studies could include analysis of gene expression from pathologic lymph nodes.

In summary, our findings offer strong preliminary evidence of the molecular activity involved in PTC invasion and lymphangiogenesis. Understanding the mechanism of PTC spread to lymph nodes may help clinicians predict the presence of lymph node involvement before radiographic or clinical evidence of nodal disease. Furthermore, identification of biomarkers involved in PTC

TABLE 4. Covariate association with EphB4, EphrinB2, and epidermal growth factor receptor gene expression in papillary thyroid carcinoma.*

Variables	EphB4		EphrinB2		EGFR	
	β (SE)	<i>p</i> value	β (SE)	<i>p</i> value	β (SE)	<i>p</i> value
Race						
White	1944.22 (1755.04)	.284	1860.78 (2687.51)	.499	−2814.24 (1138.45)	.024
Asian	5270.41 (2122.97)	.025	4459.14 (3063.97)	.166	−2256.45 (1576.12)	.170
Hispanic			Reference			
Lymph node disease						
Yes	8436.96 (1558.66)	< .001	10429.02 (2429.49)	.001	3551.21 (1778.50)	.069
No			Reference			
Extracapsular extension						
Yes	6591.78 (1232.23)	< .001	3784.57 (1943.52)	.071	1031.74 (1027.99)	.335
No			Reference			
Neurovascular invasion						
Yes	−2032.74 (1853.41)	.294	−6201.15 (2587.12)	.033	66.26 (1583.73)	.967
No			Reference			
Stage						
I–II	−2544.53 (1514.19)	.119	−868.21 (2383.10)	.722	−4113.91 (1054.0)	.001
III–IV			Reference			

Abbreviation: EGFR, epidermal growth factor receptor; β , regression coefficient; SE, standard error of β .

*Linear regression model of raw data, adjusted for race only.

metastases may guide future small-molecule targeting therapies.

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