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ORIGINAL PAPER

Expression of EGFR, VEGF, and NOTCH1 Suggest Differences in Tumor Angiogenesis in HPV-Positive and HPV-Negative Head and Neck Squamous Cell Carcinoma

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Abstract There is current interest in anti-angiogenesis therapies for head and neck squamous cell carcinomas (HNSCC), although the utility of these therapies in human papillomavirus (HPV) positive and HPV-negative HNSCC is unclear. Therefore, we explored heterogeneity in expression of a distal factor in angiogenesis (EGFR, the epidermal growth factor receptor), a proximal factor in angiogenesis (VEGF, the vascular endothelial growth factor) and a putative factor in angiogenesis (NOTCH1) in a HNSCC case series using immunohistochemistry in N = 67 cases (27 HPV-positive, 40 HPV-negative, by in situ hybridization). Box plots and the Wilcoxon rank sum or Kruskal–Wallis tests were used to compare staining scores (intensity × percent of cells staining) by HPV status and lifestyle factors. Associations between EGFR, VEGF, and NOTCH1 were assessed using box plots and Spearman correlation (ρ) in all cases, and stratified by HPV status. HPV-negative HNSCC over-expressed EGFR [median (range): 30 (0–300)] relative to HPV-positive HNSCC [7.5 (0–200)] ($P = 0.006$). VEGF and NOTCH1 were unrelated to HPV status ($P > 0.05$). EGFR was associated with VEGF in HPV-negative ($\rho = 0.40$, $P = 0.01$) but not HPV-positive HNSCC ($\rho = 0.25$, $P = 0.20$). NOTCH1 and VEGF were associated in HPV-negative ($\rho = 0.40$, $P = 0.01$) but not HPV-positive tumors ($\rho = -0.12$, $P = 0.57$). NOTCH1 was not associated with EGFR ($P > 0.05$). Our results are suggestive of heterogeneity in HNSCC angiogenesis. Future studies should explore angiogenesis mechanisms in HPV-positive and HPV-negative HNSCC.

Keywords Head and neck neoplasms · Receptor, epidermal growth factor · Receptor, NOTCH1 · Vascular endothelial growth factors · Angiogenic proteins

Introduction

Head and neck squamous cell carcinomas (HNSCC) are the sixth most common cancers and the eighth leading cause of cancer death worldwide [1]. Smoking is a primary cause of HNSCC and although HNSCC incidence decreased in

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developed nations concurrent with declines in smoking, an epidemic of oropharyngeal cancer emerged during the same time period [2–5]. It is now widely accepted that this epidemic is associated with the sexually transmitted human papilloma viruses (HPV) [6]. HPV-positive HNSCC have improved survival [1, 6], unique molecular characteristics, and are morphologically distinct from HPV-negative HNSCC [7]. However, further exploration of pathological heterogeneity in HNSCC is necessary to determine whether unique therapies may be appropriate for HPV-positive HNSCC [7].

One little-explored source of heterogeneity in HPV-positive and HPV-negative HNSCC that presents potential for clinical intervention is angiogenesis: the formation of new, tumor-infiltrating blood vessels from existing vasculature in response to release of growth factors from the tumor [8, 9]. Angiogenesis is required for tumor growth, and provides a path for cancer metastasis [8, 9]. The strongest biological evidence for differences in angiogenesis comparing HPV-positive and HPV-negative HNSCC comes from immunohistochemistry (IHC) studies of the epidermal growth factor receptor (EGFR), which is expressed at higher levels in HPV-negative compared with HPV-positive HNSCC [10–13]. EGFR is associated with angiogenesis in HNSCC through activation of the signal-transducer and activator of transcription 3 (STAT3) [14]. STAT3 induces transcription of the vascular endothelial growth factor (VEGF) [14], which is secreted by tumors [15]. VEGF stimulates angiogenesis by binding to receptors expressed on endothelial cells adjacent to the tumor [15]. IHC studies show VEGF is over-expressed in HNSCC and is associated with higher tumor stage, lymph node metastasis, and increased risk of death [16]. Only two studies reported on VEGF expression in HNSCC according to tumor HPV status and one study showed an association whereas the other did not [10, 17]. Few IHC studies have examined the EGFR–VEGF association in HNSCC, with one study reporting a positive association in a heterogeneous HNSCC case series [18], and two studies showing a null association in tonsil [10] and oral cavity cancer [19]. We are unaware of any studies examining the EGFR–VEGF association by tumor HPV status in HNSCC.

Recent studies suggest the NOTCH pathway, which controls cell fate and differentiation, may be associated with HNSCC angiogenesis [20, 21]. Of particular interest is NOTCH1, the second most commonly mutated gene in HNSCC after p53 [22, 23]. NOTCH1 is over-expressed in HNSCC relative to normal tissue [21, 24–27]. Evidence for the role of NOTCH1 in angiogenesis is preliminary, and to our knowledge no studies have made direct comparison between NOTCH1 and VEGF or EGFR. However, NOTCH1 has been associated with microvessel density (MVD) in oral tongue cancer, and MVD was associated with VEGF expression in the same study [21]. In another study, NOTCH1 expression was associated with well-differentiated oral tongue cancers, and these tumors were associated with low EGFR expression [28]. We are unaware of any studies comparing NOTCH1 expression in HNSCC by tumor HPV status.

To explore differences in angiogenesis comparing HPV-positive and HPV-negative HNSCC, we performed an IHC study of EGFR, VEGF, and NOTCH1 expression in a HNSCC case series derived from a case–control study of HNSCC etiology. Based on the existing literature described above, we hypothesized that EGFR is expressed at lower levels in HPV-positive tumors compared with HPV-negative tumors; that EGFR expression is positively associated with VEGF expression; and that VEGF expression is lower in HPV-positive compared with HPV-negative tumors. In addition, we engaged in a hypothesis-generating study of NOTCH1 expression in relation to EGFR and VEGF in HPV-positive and HPV-negative HNSCC.

Methods

Study Population

Between 2000 and 2010, N = 1,170 cases of HNSCC were recruited at University of Pittsburgh Medical Center otolaryngology clinics for participation in a case–control study of HNSCC etiology. Cases were age 18–79 at diagnosis with biopsy-verified primary squamous cell carcinoma (excluding in situ disease) of the lip, oral cavity (mouth or anterior tongue), pharynx (including base of tongue, soft palate, and uvula), larynx, nasal cavity, and paranasal sinuses within 1 year of interview. Cases completed an interviewer-administered questionnaire soliciting tobacco/alcohol use, anthropometry, and personal cancer history. This study provided the basis for the case series included in our report. Because our primary interest was the expression of NOTCH1 and markers of angiogenesis in HNSCC according to tumor HPV status, we began by searching for cases diagnosed during the time period when tumor HPV testing became common (starting in 2007), and later routine practice (2009), at our institution. Furthermore, we restricted our search to tumor sites most likely to be HPV-negative (oral cavity) or HPV-positive (oropharynx), and we selected only cases who self-reported no prior history of cancer to remove the effect of prior disease or treatment on our results. This search yielded 322 eligible cases. Among these cases, we identified 103 with tumor HPV status recorded in the pathology report, as determined by in situ hybridization (ISH) using a probe cocktail for HPV types 6, 11, 16, 18, 31, 33, 35, 45, 51, and 52 (Dako #Y1404). Formalin-fixed, paraffin-embedded tumors were requested

from storage for these 103 cases, and tumor blocks were retrieved for 71. These 71 cases formed the basis for our IHC experiments. Due to availability of HPV status or tumor blocks, oropharyngeal tumors, more recently diagnosed cases, and node-positive cases were more frequent among included than excluded cases (data not shown). All participants provided written informed consent and the study was approved by the University of Pittsburgh Institutional Review Board.

Immunohistochemistry

Paraffin tumor blocks were retrieved from storage (N = 71) and cut into 5-micron-thick sections. Slide preparation and immunostaining were performed by the Tissue and Research Pathology Services laboratory at the University of Pittsburgh Medical Center. Three slides were prepared per tumor block to be stained with commercially available antibodies to VEGF (Santa Cruz Biotechnology #SC-152), NOTCH1 (Cell Signaling #3608), and EGFR (Sigma Chemical #E3138). A single tumor block was available for 62 cases and two blocks for 9 cases. Slides were prepared as follows. Heat-induced antigen retrieval was performed in the Dako Biocare Decloaking Chamber using Biocare Medical Borg buffer (catalog #BD1000G1) (EGFR) or Dako PH6 citrate buffer (VEGF and NOTCH1). Engodenous peroxidase was blocked by quenching with 3 % hydrogen peroxide (Fisher Scientific) for 10 min, and the reagent was tapped off. Specimens were incubated with primary antibody as indicated in Table 1, followed by incubation with Biocare Mach 4 Universal HRP (EGFR), Dako Dual Envision+ (VEGF), and Dako Rabbit Envision+ (NOTCH1) secondary antibodies for 30 min. All specimens were washed for 5 min in tris-buffered saline. This was followed by incubation with Dako Substrate Chromagen (catalog #K3468) for 5 (NOTCH1) or 10 (VEGF and EGFR) min. All specimens where then washed with deionized water, counterstained with Harris hematoxylin for 10 s, washed in tap water, blued in ammonia and water, dehydrated, cleared and cover-slipped. Staining was performed on the Dako Autostainer Plus. All incubations were performed at room temperature. Paraffin-embedded tissues were used as positive controls (EGFR: HNSCC, VEGF: normal kidney, NOTCH1: lung cancer). Stains were interpreted as intensity (0 = no stain, 1 = weak stain, 2 = moderate stain, 3 = strong stain) and percentage of cells staining. Interpretation was done by a single pathologist (Lin Wang, MD, PhD) blinded to tumor HPV status.

Variable Definitions

Our IHC experiments revealed substantial variation in the percentage of cells staining and the intensity of stain for each marker. As suggested previously [29, 30], we constructed a measure that combined these two aspects of protein expression into a single semi-quantitative measure. We called this measure the staining score and defined it as the product of intensity and percentage of cells staining, with the average score used for cases with two tumor blocks. The primary independent variable in our analyses was tumor HPV status (positive or negative), as determined by ISH. For special analyses of VEGF, we defined an independent variable for combined EGFR/NOTCH1 expression as tertiles of the summed staining scores for these proteins, which we designated EGFR/NOTCH1-Low, EGFR/NOTCH1-Moderate, and EGFR/NOTCH1-High. We also defined the following variables to explore confounding and interaction: age at diagnosis (<50, 50–59, 60–69, and ≥70 years), sex (male or female), race (white or other/unknown), tumor site (oral cavity or oropharynx), clinical T-stage [1/2, 3/4, or X (not evaluable)], clinical N-stage [negative, positive, or X (not evaluable)], clinical M-stage [negative, positive, or X (not evaluable)], smoking status (ever/never, where ever-smoking was defined as smoking at least one cigarette per day for 6 months or longer), drinking status (ever/never, where ever-drinking was defined as drinking at least one drink per month for 1 year or longer), and body mass index (BMI) 1 year prior to diagnosis [<30 kg/m^2 (not obese) or ≥30 kg/m^2 (obese)].

Table 1 Antibodies used for immunohistochemistry

Protein	Antibody	Origin and binding site	Dilution	Positive control	Localization
VEGF	Santa Cruz Biotechnology SC-152	Rabbit polyclonal antibody to the N-terminus of VEGF-A Detects the 189, 165 (predominant), and 121 amino acid sequence isoforms of VEGF-A	1:400 for 60 min	Normal kidney	Cytoplasm and nuclei
NOTCH1	Cell signaling technologies 3608	Rabbit monoclonal antibody to proline 2439 Recognizes the whole (in-tact) NOTCH1 protein or the transmembrane/intracellular region	1:400 for 45 min	Lung cancer	Membrane and cytoplasm
EGFR	Sigma chemical E3138	Mouse monoclonal antibody to the intracellular domain of the receptor	1:7,500 for 60 min	Head and neck cancer	Membrane and cytoplasm

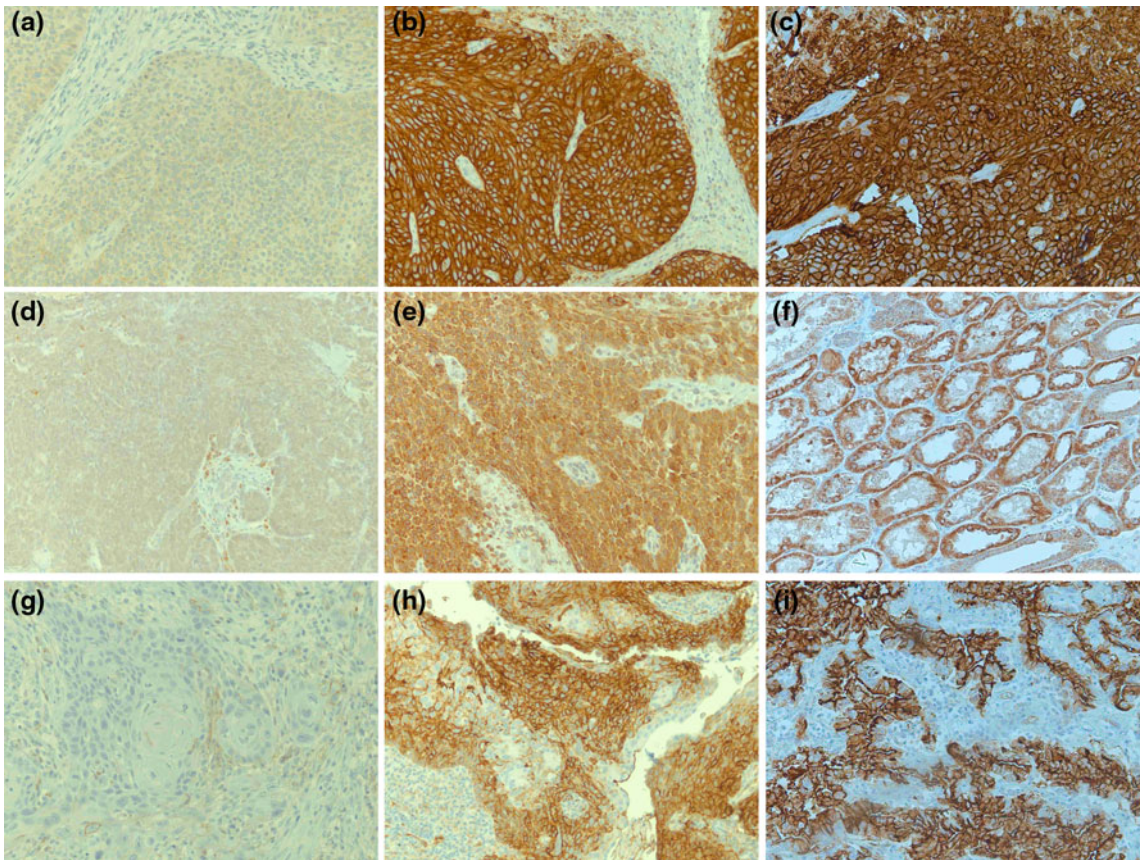

Fig. 1 **a** Low EGFR expression (×200). **b** High EGFR expression (×200). **c** Positive control for EGFR (head and neck cancer) (×200). **d** Low VEGF expression (×200). **e** High VEGF expression (×200). **f** Positive control for VEGF (normal kidney) (×200). **g** Low NOTCH1 expression (×200). **h** High NOTCH1 expression (×200). **i** Positive control for NOTCH1 (lung cancer)

Statistical Analysis

We followed a structured analytical approach that applied parsimonious yet appropriate methods to produce results that were easily comparable with existing literature. We began by characterizing the case series under study, as well as exploring differences in subgroups defined by demographic and pathological, and lifestyle factors using simple contingency table methods and Fisher's exact test. We then proceeded to investigate our hypotheses regarding protein expression. Given the exploratory nature of our research, we relied heavily on visual methods for detection of potentially important signals in the data. Specifically, we prepared box plots stratified by clinical T- and N-stage and tumor HPV status. The staining score for the protein of interest was plotted on the Y axis. Subgroups across which the score was to be compared were shown along the X axis. The box plots clearly demonstrate, for each subgroup, the range and the 25th and 75th percentiles of the staining score, and facilitate comparison of the median and mean score across subgroups. We used a conservative distribution-free approach to statistical inference, applying the Wilcoxon rank-sum test for 2-sample comparisons, and the Kruskal–Wallis test for 3 sample comparisons. In addition to box plots, we applied Spearman rank correlation (ρ) to examine two-way associations between proteins in the entire case series, and stratified by tumor HPV status. We then examined complex relationships between the markers under study using a linear regression technique within the context of the generalized linear models framework (i.e., normal errors and identity link). Specifically, we modeled VEGF expression based on combined EGFR/NOTCH1 expression, HPV status, and the HPV-EGFR/NOTCH1 interaction. Distributional assumptions of the model were verified graphically, and the likelihood ratio Chi square test was used to assess statistical significance. All statistical tests used a 2-sided $\alpha = 0.05$ and were performed in SAS 9.2 (SAS Institute, Cary, NC).

Results

A total of 67 of the 71 cases (94 %) for whom slides were prepared were included in this study. We excluded four cases

Table 2 Characteristics of cases evaluated by immunohistochemistry

	All (N = 67) n (%)	HPV negative (N = 40) n (%)	HPV positive (N = 27) n (%)	*P* value[a]
Age				0.31
<50	19 (28.4)	8 (20.0)	11 (40.7)	
50–59	27 (40.3)	17 (42.5)	10 (37.0)	
60–69	16 (23.9)	11 (27.5)	5 (18.5)	
≥70	5 (7.5)	4 (10.0)	1 (3.7)	
Sex				0.15
Male	50 (74.6)	27 (67.5)	23 (85.2)	
Female	17 (25.4)	13 (32.5)	4 (14.8)	
Race				0.64
Non-white/unknown	4 (6.0)	3 (7.5)	1 (3.7)	
White	63 (94.0)	37 (92.5)	26 (96.3)	
Tumor Site				<.001
Oral cavity	28 (41.8)	26 (65.0)	2 (7.4)	
Oropharynx	39 (58.2)	14 (35.0)	25 (92.6)	
T clinical				0.09
1/2	40 (59.7)	22 (55.0)	18 (66.7)	
3/4	25 (37.3)	18 (45.0)	7 (25.9)	
X	2 (3.0)	0 (0.0)	2 (7.4)	
N clinical				0.27
Negative	18 (26.9)	13 (32.5)	5 (18.5)	
Positive	49 (73.1)	27 (67.5)	22 (81.5)	
M clinical				>0.99
0	65 (97.0)	38 (95.0)	27 (100.0)	
1	1 (1.5)	1 (2.5)	0 (0.0)	
X	1 (1.5)	1 (2.5)	0 (0.0)	
Ever smoked				0.78
No	17 (25.4)	11 (27.5)	6 (22.2)	
Yes	50 (74.6)	29 (72.5)	21 (77.8)	
Ever drank alcohol				0.10
No	12 (17.9)	10 (25.0)	2 (7.4)	
Yes	55 (82.1)	30 (75.0)	25 (92.6)	
BMI 1 year pre-diagnosis				0.60
<30 kg/m^2	45 (67.2)	28 (70.0)	17 (63.0)	
≥30 kg/m^2	22 (32.8)	12 (30.0)	10 (37.0)	

[a] Fisher's exact test

because slides cut from the tissue blocks retrieved from pathology archives did not contain any tumor. Representative strong and weak stains for each marker, along with positive controls, are shown in Fig. 1a–i. As shown in Table 2, the majority of cases were aged 50–69 (64.2 %), male (74.6 %), and white race (94.0 %). Over half of tumors were oropharyngeal (58.2 %), and most were early clinical T-stage (59.7 % stage 1/2) and node-positive (73.1 %). Only one case had distant metastases. Most cases (74.6 %) reported ever-smoking or ever-drinking (82.1 %), and 32.8 % were obese 1 year prior to diagnosis. A total of 27 tumors (40.3 %) were HPV-positive (2 oral, 25 oropharyngeal) and 40 tumors (59.7 %) were HPV-negative (26 oral, 14 oropharyngeal). The HPV-positivity rate among oropharyngeal tumors was 64.1 %.

As expected, tumor site was associated with HPV-status (Table 2), with the majority of HPV-positive tumors (92.6 %) occurring in the oropharynx and only 35.0 % of HPV-negative tumors occurring in the oropharynx ($P < 0.001$). Although ever-smoking ($P = 0.78$) was not significantly associated with HPV status, smokers with HPV-negative tumors tended to have greater lifetime exposure [median pack-years = 41, inter-quartile range (IQR) = 24.5, N = 29 cases] compared to smokers with HPV-positive tumors (median pack years = 30, IQR = 26.3, N = 21 cases) ($P > 0.05$) (not tabulated). More HPV-positive cases (92.6 %) reported ever-drinking compared with HPV-negative cases (75.0 %), although this difference was not significant ($P = 0.10$). We noted no differences in drinks/day or years drinking comparing ever-

Table 3 Results of immunohistochemistry: analysis of protein expression and tumor stage

Marker	N[a]	Clinical T-stage	Score[b]
EGFR	65	All	20.0 (0–300)
	40	T-1/2	17.5 (0–300)
	25	T-3/4	30.0 (0–300)
		P value	0.28
VEGF	65	All	70.0 (0–200)
	40	T-1/2	90.0 (0–160)
	25	T-3/4	60.0 (0–200)
		P value	0.14
NOTCH1	64	All	40.0 (0–240)
	39	T-1/2	50.0 (0–240)
	25	T-3/4	20.0 (0–160)
		P value	0.01

EGFR epidermal growth factor receptor, *VEGF* vascular endothelial growth factor, *NOTCH1* notch receptor 1

[a] Two tumors were not evaluable for T-stage and are therefore not included in this table. In addition, one case was not evaluable for NOTCH1 due to insufficient tumor quantity

[b] *P* value is from a Wilcoxon Rank Sum test comparing T-1/2 and T-3/4 tumors

Table 4 Results of immunohistochemistry: analysis of protein expression and HPV status

Marker	HPV status	N[a]	Positive, n (%)[b]	Score[c,d]
EGFR	All	67	56 (83.6)	20.0 (0–300)
	Negative	40	37 (92.5)	30.0 (0–300)
	Positive	27	19 (70.4)	7.5 (0–200)
	P value[e]			0.006
VEGF	All	67	64 (95.5)	70.0 (0–200)
	Negative	40	38 (95.0)	60.0 (0–200)
	Positive	27	26 (96.3)	70.0 (0–180)
	P value[e]			0.82
NOTCH1	All	66	58 (87.9)	40.0 (0–240)
	Negative	40	35 (87.5)	42.5 (0–240)
	Positive	26	23 (88.5)	40.0 (0–160)
	P value[e]			0.68

EGFR epidermal growth factor receptor, *VEGF* vascular endothelial growth factor, *NOTCH1* notch receptor 1, *HPV* human papillomavirus

[a] One case was not stained for NOTCH1 due to insufficient tumor quantity

[b] Positive is defined as having greater than 0 % of cells staining

[c] Numbers are median (min–max)

[d] Score represents the product of percent staining and intensity

[e] *P* value is from a Wilcoxon Rank Sum test comparing HPV-positive and HPV-negative tumors

drinking HPV-positive and HPV-negative cases (not tabulated). Finally, T-stage and nodal status were not significantly associated with tumor HPV status.

Protein Expression According to Clinical Stage and Tumor Site

As shown in Table 3, NOTCH1 was over-expressed in T1/2 [median score (range): 50 (0–240)] compared with T3/4 tumors [median score (range): 20 (0–160)] ($P = 0.01$). This association was similar in analyses stratified by tumor site and HPV status (data not shown). EGFR and VEGF were unrelated to T-stage. Finally, wee observed no relationships between EGFR, VEGF, or NOTCH1 and N-stage or tumor site ($P > 0.05$ for all; data not shown).

Protein Expression According to Tumor HPV Status

As shown in Table 4, EGFR was expressed in 56/67 (83.6 %) of cases. We noted significant over-expression of EGFR in HPV-negative tumors [median score (range): 30 (0–300)] compared with HPV-positive tumors [median score (range): 7.5 (0–200)] ($P = 0.006$). Results were similar after restricting our analysis to oropharyngeal cases only (data not shown). VEGF was expressed in 64/67 (95.5 %) cases but was not associated with HPV status ($P = 0.82$ for all). NOTCH1 was expressed in 58/66 cases (87.9 %) and was also unrelated to HPV status ($P = 0.68$ for all).

Associations Between Expression of EGFR, VEGF, and NOTCH1 in All Cases and Stratified by Tumor HPV Status

Epidermal growth factor receptor was positively associated with VEGF in all cases combined [Kruskal–Wallis test (KW): $P < 0.01$], and in HPV-negative (KW: $P = 0.03$) but not HPV-positive (KW: $P = 0.16$) tumors (Fig. 2). Results of our correlation analysis (Table 5) were similar (HPV-negative: $\rho = 0.40$, $P = 0.01$; HPV-positive: $\rho = 0.25$, $P = 0.20$). NOTCH1 was not associated with VEGF in all cases combined (KW: $P = 0.11$; $\rho = 0.22$, $P = 0.08$) or in HPV-positive cases (KW: $P = 0.77$; $\rho = -0.12$, $P = 0.57$), but was associated with VEGF in HPV-negative cases (KW: $P = 0.02$; $\rho = 0.40$, $P = 0.01$) (Fig. 3; Table 5). NOTCH1 was not associated with EGFR in HPV-positive or HPV-negative cases (Table 5). Exploratory regression analyses revealed significant interaction between HPV and EGFR/NOTCH1 in predicting VEGF expression ($P = 0.02$; Supplementary Table 1). In HPV-negative tumors, VEGF expression was higher in EGFR/NOTCH1-moderate and EGFR/NOTCH1-high compared with EGFR/NOTCH1-low tumors ($P < 0.05$ for both) whereas expression of EGFR/NOTCH1 was generally unrelated to VEGF in HPV-positive tumors. Caution is warranted in interpretation of these results due to small sample sizes in categories defined by tumor HPV status and EGFR/NOTCH1 expression.

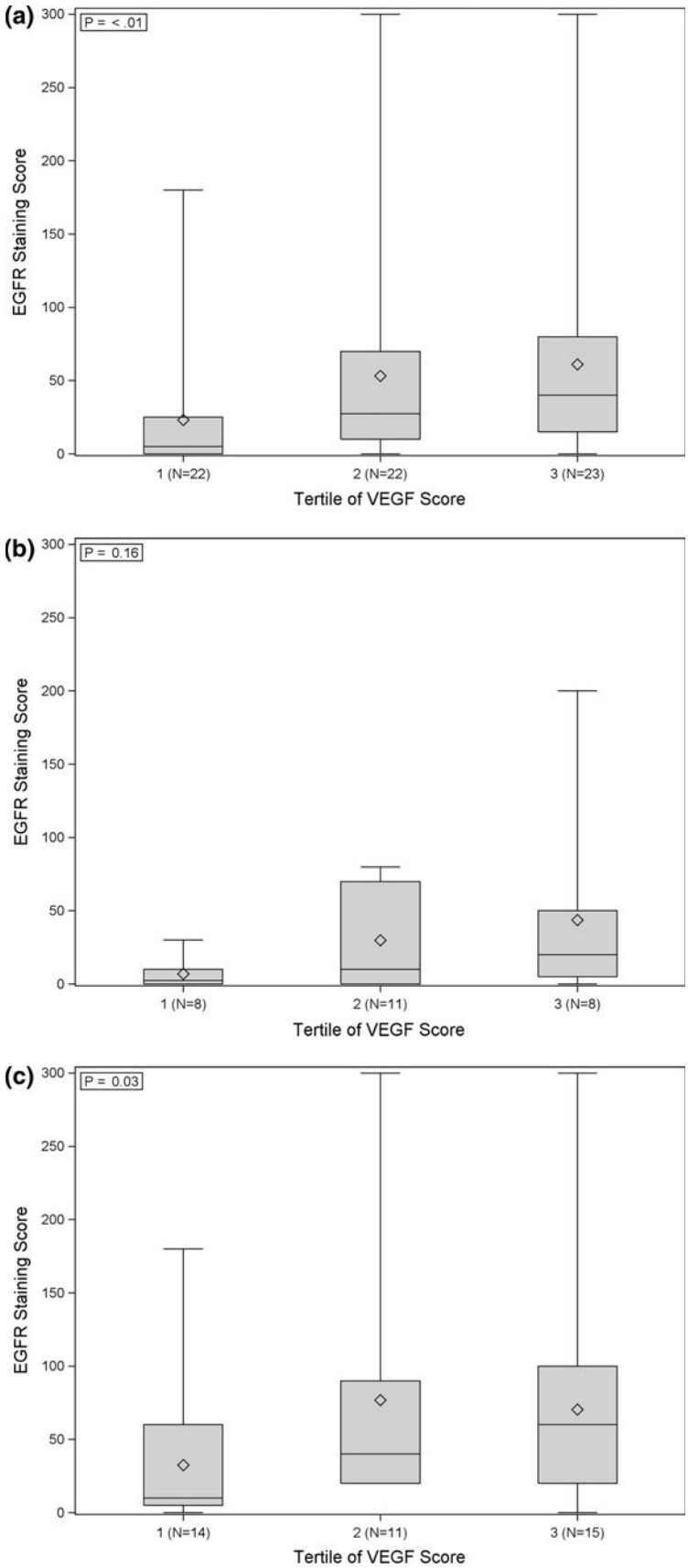

Fig. 2 Expression of EGFR by tertile of VEGF expression **a** all tumors, **b** HPV-positive tumors, **c** HPV-negative tumors. *P* value is from Kruskal–Wallis test

Table 5 Spearman correlations for markers detected by immunohistochemistry

	NOTCH1	EGFR	VEGF
All cases			
NOTCH1		0.11	0.22
		0.37	0.08
		66	66
EGFR	0.11		0.33
	0.37		0.007
	66		67
VEGF	0.22	0.33	
	0.08	0.007	
	66	67	
HPV-positive cases			
NOTCH1		0.32	−0.12
		0.11	0.57
		26	26
EGFR	0.32		0.25
	0.11		0.20
	26		27
VEGF	−0.18	0.25	
	0.57	0.20	
	26	27	
HPV-negative cases			
NOTCH1		−0.04	0.40
		0.79	0.01
		40	40
EGFR	−0.04		0.40
	0.79		0.01
	40		40
VEGF	0.40	0.40	
	0.01	0.01	
	40	40	

Numbers are: ρ (correlation coefficient), *P* value, N

Discussion

We used IHC to identify differences in expression of tumor angiogenesis markers comparing HPV-positive and HPV-negative HNSCC. We observed higher expression of EGFR—a cell surface receptor associated with angiogenesis through several downstream signal transduction pathways—in HPV-negative compared with HPV-positive HNSCC; and EGFR was associated with VEGF (a secreted angiogenesis mediator) in HPV-negative tumors. Our study also offers a preliminary report on NOTCH1—a cell surface receptor important in cell growth and differentiation—and angiogenesis markers in HNSCC. Our data show an association between NOTCH1 and VEGF in HPV-negative tumors, and no association between NOTCH1 and EGFR in HPV-negative or HPV-positive HNSCC.

Over-expression of EGFR in HPV-negative relative to HPV-positive HNSCC has been reported previously [10–13, 31, 32]. This may be attributable to higher EGFR copy number in HPV-negative compared with HPV-positive tumors [33–36]. EGFR signaling results in activation of several downstream pathways that may affect transcription or translation of VEGF, including the Ras/MAPK, PI3 K/Akt/mTOR, and STAT3 pathways [37, 38]. Of particular note is STAT3, which induces transcription of VEGF in HNSCC cell lines [14]. Therefore, assuming EGFR were truly under-expressed in HPV-positive HNSCC, one might expect a reduction in VEGF expression in HPV-positive tumors. However, our study and one other [10] showed no difference in VEGF expression comparing HPV-positive and HPV-negative HNSCC. While this result might be attributable to low statistical power, it is curious that we made this observation in the same sample that showed higher EGFR expression in HPV-negative HNSCC. Together, these findings are suggestive that HPV-positive HNSCC may be less dependent on EGFR for angiogenesis. To our knowledge, ours is the first study of HNSCC to investigate both the HPV-EGFR and HPV-VEGF association using IHC within the same sample of tumors. Our results suggest further study of these associations is warranted.

Our observation that EGFR is associated with VEGF is consistent with evidence from HNSCC cell lines [14] and a study of HNSCC tumors using a polymerase chain reaction (PCR) assay [18]. In our study, the EGFR–VEGF association was evident in all cases combined, but our data indicate this result was driven by an association in HPV-negative HNSCC. To our knowledge, ours is the first report of the EGFR–VEGF association by HPV status in HNSCC. However, a previous study of oral cancer, which is typically HPV-negative [6], reported no EGFR–VEGF association, but tumors in this study were slightly less likely to be positive for EGFR and VEGF compared with our sample [19]. A study of HPV-positive and HPV-negative tonsil cancer also reported no EGFR–VEGF association in all cases combined [12]. However, our sample included a higher proportion (60 %) of HPV-negative tumors (compared with 51 % in the aforementioned study [12]), possibly allowing us more power to detect an association [12].

In our study, NOTCH1 expression increased across tertiles of VEGF expression in HPV-negative tumors only. To our knowledge, ours is the first report of an association between NOTCH1 and VEGF according to HPV status in HNSCC. However, we are not the first to implicate NOTCH1 in angiogenesis in HNSCC [21]. One prior showed study NOTCH1 was associated with MVD in oral tongue cancer [21]. Although this study did not compare NOTCH1 and VEGF directly, it also showed an association between MVD and VEGF [21]. Tumor HPV status was not assessed in this study however [21], oral tongue cancer is frequently

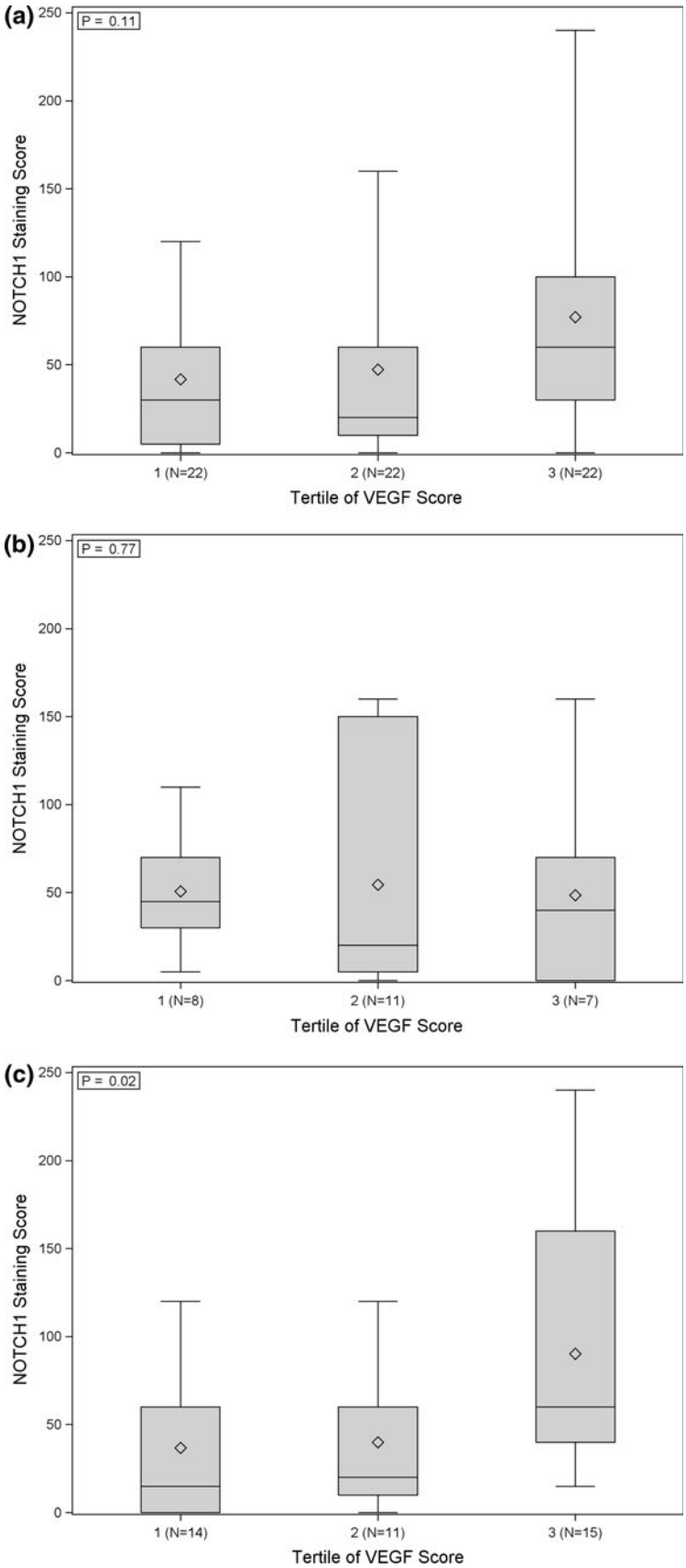

Fig. 3 Expression of NOTCH1 by tertile of VEGF expression **a** all tumors, **b** HPV-positive tumors, **c** HPV-negative tumors. *P* value is from Kruskal–Wallis test

Table 6 Working hypotheses regarding differences in tumor angiogenesis in HPV-positive and HPV-negative head and neck cancer—based on results of the present study

HPV-positive head and neck cancer	HPV-negative head and neck cancer
These tumors are less dependent on EGFR	These are EGFR-dependent tumors
Although angiogenesis is important in these tumors, angiogenesis is:	Angiogenesis is:
Unrelated to NOTCH1	Driven through independent effects of NOTCH1 and EGFR; and
Less dependent on EGFR	The effects of NOTCH1 and EGFR are possibly complimentary
Possibly driven by other mechanisms	
These tumors have more limited growth potential relative to HPV-negative head and neck cancers	These tumors have expanded growth potential relative to HPV-positive head and neck cancers

HPV-negative [6] and therefore we believe our results are in alignment with these findings. Our observation that NOTCH1 was associated with VEGF, but not EGFR, in HPV-negative tumors suggests NOTCH1 may be associated with angiogenesis independently of EGFR in HPV-negative tumors. This hypothesis is further supported by the results of our analysis of VEGF expression in relation to combined EGFR/NOTCH1 expression, which showed: (1) EGFR/NOTCH1 expression significantly predicted VEGF expression in HPV-negative tumors only; and (2) expression of VEGF in HPV-negative tumors was statistically significantly higher in tumors expressing higher levels of either or both EGFR and NOTCH1 compared with tumors expressing low levels of both receptors. We believe these results may have a biological basis as NOTCH1 expression has been positively associated with STAT3 expression in oral tongue cancer [39], and STAT3 activates transcription of VEGF [14]. We reviewed the literature on this topic and were unable to identify any IHC studies that examined EGFR, VEGF, and NOTCH1 simultaneously in HPV-positive and HPV-negative HNSCC. Therefore, our results should be considered preliminary.

Assessment of immunostains in our study was performed by a head and neck pathologist who was blinded to tumor HPV status. We used commercially available antibodies and positive controls. However, our study included a relatively small sample and we conducted many statistical tests without correction for Type I error. We must also point out that HPV-negative cases in our study included oral and oropharyngeal cancers. Due to small subgroup sizes, we were unable evaluate the role of tumor site in the EGFR–VEGF association among HPV-negative tumors. In addition, caution is warranted in interpretation of our results for NOTCH1. Specifically, our NOTCH1 antibody recognizes whole NOTCH1 protein, as well as the transmembrane or intracellular domain of NOTCH1 (Table 1). Because the intracellular domain of NOTCH1 is cleaved when the protein is activated [40], our observations of NOTCH1 expression likely included active and inactive protein. Furthermore, given the location of observed mutations in NOTCH1 in HNSCC [22, 23] and the target residue of our antibody (Table 1), it is possible that we have detected both mutated and wild type NOTCH1. The importance of these distinctions in comparing molecular phenotypes of HNSCC subgroups remains unclear because the relative frequency, as well as the true function, of NOTCH1 mutations in HPV-positive and HPV-negative HNSCC is still under investigation [22, 23].

We must also point out that our study did not delineate between different types of HPV infection. For example, others have used p16 IHC and HPV DNA detection to identify active HPV infection (tumors harboring HPV DNA and expressing p16) and inactive HPV infection (tumors harboring HPV DNA but not expressing p16) [41]. Although the in situ hybridization assay we used has several advantages, including the ability to locate HPV specifically within neoplastic tissue and the capability to visually distinguish between episomal and integrated virus [42], we could not distinguish between transcriptionally active or inactive HPV because p16 expression was not available in the pathology report for all of our cases. However, among the 21 of our 27 HPV-positive cases who did have data available, all expressed p16 (data not shown). This is in agreement with previous studies that show p16 expression in most tumors identified as HPV-positive by in situ hybridization [43]. Therefore, we believe the majority of HPV-positive cases in our study likely represent transcriptionally active HPV.

Finally, perhaps the greatest limitation of our study is that our results reflect associations between biological factors and cannot describe mechanisms through which these associations are produced. However, we view our study as providing the impetus to conduct future studies that evaluate such mechanisms.

In summary, we demonstrated differences in expression of proteins associated with angiogenesis comparing HPV-positive and HPV-negative HNSCC, and we showed an association between expression of NOTCH1 and angiogenesis-related proteins. We believe our results are suggestive of biological heterogeneity in HNSCC angiogenesis that has the potential to be exploited therapeutically. Based on the results of our investigation, we have developed a set of

working hypotheses (Table 6) that we hope will inform future study in this area. We expect a multifaceted approach will be required, including in vitro studies of tumor angiogenesis mechanisms in HPV-positive and HPV-negative HNSCC, along with histopathological studies of angiogenesis marker expression within clinical trials of anti-angiogenesis therapies in HPV-positive and HPV-negative HNSCC.

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