



Association of epidermal growth factor receptor protein expression with histopathological and clinical parameters in carcinoma of the larynx

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Background: Laryngeal cancer is one of the most commonly occurring cancers in head and neck malignancies with the majority of tumors being squamous cell carcinomas. Despite sharing similarities with other head and neck tumors, laryngeal tumors require a special approach in treatment due to the need for organ preservation. Non-surgical procedures, including chemotherapy, radiotherapy and novel targeted therapies are alternative options. However, the selection of appropriate treatment modality is crucial for recurrence-free survival. With the availability of targeted therapies, the biomarker expression pattern has become a vital tool in the selection of treatment and prognosis. The present study aims to study the variation of protein [epidermal growth factor receptor (EGFR), phosphatase and tensin homolog (PTEN) and p16^{INK4}] expression and human papillomavirus (HPV) infection status in larynx carcinoma patients and correlate it with histopathological and clinical parameters.

Methods: This is a retrospective study comprising laryngeal carcinoma tumor samples from 18 patients. The samples were analyzed for EGFR, PTEN and p16^{INK4} expression by immunohistochemistry and HPV status by chromogenic in-situ hybridization (CISH).

Results: The EGFR over expression was observed in the 83.3% of patients. This high EGFR expression was associated with lymph node positivity (P=0.045) and advanced disease stage (P=0.035). Furthermore, the smoking status of the patients is correlated with a better prognosis (P=0.048). The PTEN was negatively expressed in 94.4% patients.

Conclusions: The association of EGFR over expression with lymph node status and advanced stage of the disease supports the role that EGFR plays in the tumor metastasis and invasion in laryngeal carcinoma patients. Therefore, the use of anti-EGFR targeted therapy in cases of laryngeal carcinoma may improve the prognosis of these patients. The other studied proteins p16^{INK4}, PTEN and HPV infection did not show any correlation with prognosis and other clinical parameters.

Keywords: Epidermal growth factor receptor (EGFR); p16^{INK4}; prognosis; phosphatase and tensin homolog (PTEN)

Submitted Feb 01, 2019. Accepted for publication Jul 11, 2019.

doi: 10.21037/tcr.2019.07.26

View this article at: <http://dx.doi.org/10.21037/tcr.2019.07.26>

Introduction

Laryngeal carcinoma is the third most common head and neck malignancy after oral cavity and pharynx cancer (1). Approximately 85–95% of laryngeal tumors are squamous cell carcinomas and 60% of patients present in the advanced stages (2) at the time of diagnosis. Laryngeal tumors are more prevalent in men than women (3) and ethnic variations have been observed in different populations with African Americans showing a higher incidence compared to Caucasians (4).

Alcohol consumption and tobacco usage is the major etiology accounting for 80% of head and neck squamous cell carcinomas (HNSCC) (5). Consequently, the global incidence of HNSCC is 4.6% and among HNSCC the oral cavity and larynx cancer incidence is 42.5% and 21.2%, respectively (6). Human papillomavirus (HPV) infection is also a risk factor in HNSCC, especially as it is associated with oropharyngeal cancer compared to other subsites of HNSCC (7).

The overall goal of therapeutic management of laryngeal carcinoma is to preserve the function of the larynx and in order to attain this it is important to assess the recurrence risk and prognosis of the disease in each case (8). Chemo-resistance is a major obstacle in the management of the disease (9). Even with the availability of various treatment modalities for laryngeal carcinoma, the five-year survival rate has declined in the past four decades (2). Tumor management by chemotherapy and radiotherapy has become feasible options and for localized tumors, surgery is the standard treatment plan. In the event of metastasis and for organ preservation, novel therapeutic agents will play a potential role in addressing laryngeal carcinoma (2).

The genetic alterations in the form of mutations and varied expression patterns in the oncogenes and tumor suppressor genes have gained importance in cancer management and for the designing of targeted therapy. These genetic alterations in laryngeal and oral cancer are complex and interrelated. Activation of proto-oncogenes, inactivation of tumor suppressor genes and altered expression of both genes play a major role in the prognosis and recurrence rate (10).

The epidermal growth factor receptor (EGFR), a cell surface receptor, was for the first time reported to be associated with HNSCC in 1986 (11). Its activation and over expression transform the downstream functions, such as cell proliferation, metastasis and angiogenesis. EGF and transforming growth factor β promote epithelial

to mesenchymal transition (EMT) which is a dynamic cellular process essential for the metastasis development by regulating a specific set of transcription factors (12). The abnormal expression of EGFR protein has been observed in several cancers and has gained importance in terms of drug design to treat various cancers by anti-EGFR molecules. Monoclonal antibodies (Cetuximab, Panitumumab) and small molecule tyrosine kinase inhibitors (Gefitinib, Erlotinib, Lapatinib, Canertinib) are used in the treatment of various cancers including colorectal cancer and HNSCC for the former and non-small cell lung cancer and pancreatic cancer for the latter (13).

Phosphatase and tensin homolog (PTEN) and p16^{INKA} are tumor suppressor genes. PTEN regulates phosphoinositide 3-kinase signaling pathway by dephosphorylating the lipid signaling intermediate phosphatidylinositol-3,4,5-triphosphate (PIP3). Due to the altered expression or loss of PTEN gene function, elevated PIP3 is observed in cells. The increase in PIP3 concentration leads to cell survival, growth and proliferation (14). PTEN loss of function is observed as a result of transcriptional silencing, somatic mutations and deletions (15). PTEN reduced expression also plays a role in cancer susceptibility (16). P16^{INKA} is a member of Ink4 family of cyclin dependent kinase inhibitors. P16^{INKA} acts as a negative regulator of the cell cycle by “S phase” inhibition (17). Genetic alterations and the over expression of p16^{INKA} play an essential role in several tumors, such as HNSCC, breast, colon, skin, bladder and cervix (18). The development of biomarker signatures may pave the way for novel treatment regimens to treat laryngeal carcinoma. Hence, this study is focused on analyzing the expression of EGFR, p16^{INKA}, PTEN and HPV infection to determine the association of these markers with histopathological parameters and prognosis in laryngeal carcinoma patients.

Methods

Study cohort

A total of 18 formalin fixed paraffin embedded (FFPE) tissue samples from Saudi patients with confirmed laryngeal carcinoma were obtained from King Fahd Hospital of the University. The FFPE blocks were reanalyzed by hematoxylin and eosin (HE) staining to reconfirm the diagnosis and to check for tumor content. This study was approved by the Institutional Review Board of Imam Abdulrahman Bin Faisal University, Dammam, Saudi Arabia

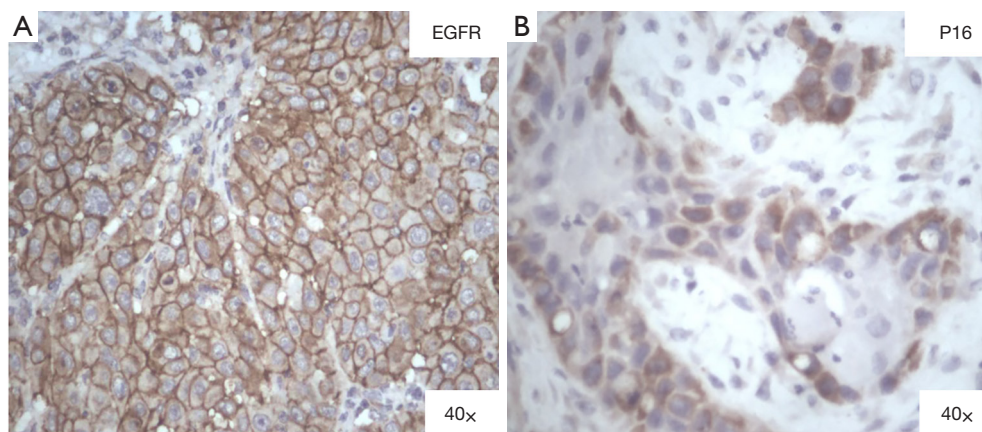


Figure 1 Typical EGFR and p16^{INK4} immunohistochemical staining profile. Images are taken at $\times 40$ magnification. (A) High expression of EGFR; (B) high expression of p16^{INK4}. EGFR, epidermal growth factor receptor.

(IRB number: IRB-2014-08-044).

Immunohistochemistry

FFPE sections of 5 μm thickness were cut using Leica microtome (Leica, USA) and these sections were mounted on poly-L-lysine coated slides (Sigma, USA). The slides were incubated at 60 $^{\circ}\text{C}$ for 12 hours, deparaffinization was carried out using xylene and rehydrated with various concentrations of ethanol. Primary blocking was performed by incubating in 3% hydrogen peroxide (Sigma, USA) solution for 30 minutes. Antigen retrieval was performed by a heat inducing method using a microwave. Secondary blocking reaction was carried out by incubating in 3% bovine serum albumin solution (Sigma, USA) for 30 minutes. Primary monoclonal antibodies, PTEN (Biocare medical, USA), p16^{INK4} (Biogenex, USA) and EGFR (Biogenex, USA) were used in different concentrations in the respective reactions as per the manufacturer's instructions. The slides were incubated with the secondary antibody conjugated with horse radish peroxidase polymer followed by 3,3'-diaminobenzidine (DAB) substrates (Biogenex, USA). Later, the slides were counter stained with hematoxylin, followed by dehydration and the cover slip was mounted on a slide.

The stained slides were observed under a microscope and each slide was scored based on the membrane staining. The EGFR scoring pattern that was followed is: 0= no staining or weak staining; 1+ = faint staining; 2+ = moderate and 3+ = strong staining of tumor cells. The cases that presented with >10% of staining were considered as having a positive

staining (Figure 1). For p16^{INK4}, the nuclear and cytoplasmic staining was considered, and the score was calculated as follows: 0= no staining, 1= weak, 2= intermediate and 3= strong staining of tumor cells. The results were dichotomized as negative (0 and 1) and positive (2 and 3) for p16^{INK4} expression. PTEN expression interpretation was dichotomous (negative and positive) and if a case showed strong positive staining in the majority or all of the tumor cells it was considered to be positive and if tumor cells showed no staining but the adjacent normal cells showed positive staining, it was considered to be negative for PTEN.

In-situ hybridization

HPV infection in the FFPE tissue sections were detected using *in-situ* hybridization. Tissue sections were treated with the nucleic acid retrieval system (Biogenex, USA) as per the manufacturer's instructions to expose the DNA sequence. The slides were then treated with fluorescein labelled probes for HPV16 and 18 (Biogenex, USA) followed by the detection of probe target hybrid by the immunohistochemistry procedure. Anti-fluorescence antibody was added to the slide followed by secondary antibody enzyme conjugate and substrate (DAB). Brown coloration was developed at the location of the probe target hybrid (Figure 2). Interpretation, which was performed by two independent pathologists, resulted in 100 nuclei being counted on each slide.

Data analysis

SPSS version 20 software (IBM, USA) was used to analyze

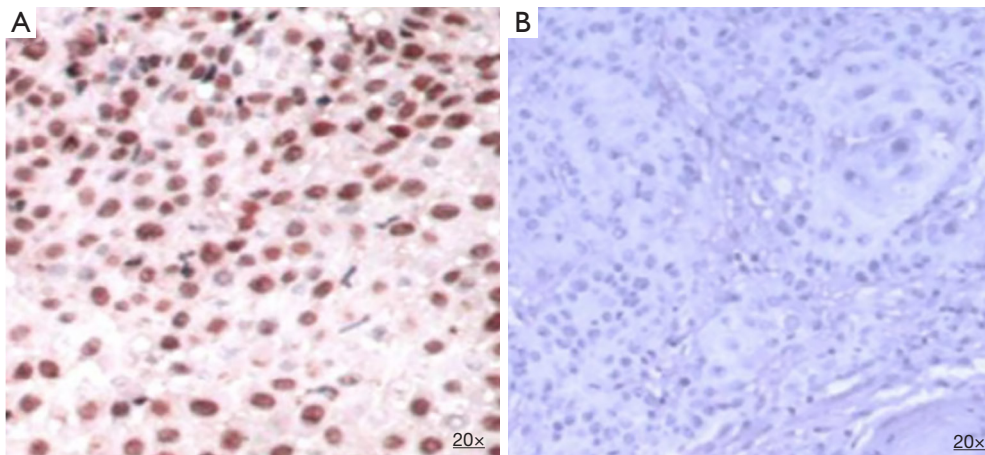


Figure 2 HPV detection by CISH. Representative HPV staining images by CISH. Images are taken at $\times 20$ magnification. (A) HPV CISH of an HPV16 positive laryngeal squamous cell carcinoma; (B) HPV CISH hybridization of an HPV16 negative laryngeal squamous cell carcinoma. HPV, human papillomavirus; CISH, chromogenic in-situ hybridization.

Table 1 Clinical and biomarker expression characteristics of the patients

Parameter	Classification	Samples, n (%)
Smoking status	Smokers	9 (50.0)
	Non-smokers	9 (50.0)
Stage	3	8 (44.4)
	4	10 (55.5)
Node status	Positive	14 (77.8)
	Negative	4 (22.2)
Prognosis	Good	6 (33.3)
	Bad	12 (66.7)
Treatment regimen	RT+CT+S	7 (38.9)
	RT+S+CT	1 (5.6)
	S+CT+RT	4 (22.2)
	S+RT+CT	6 (33.3)
HPV	Positive	5 (27.8)
	Negative	13 (72.2)
EGFR	Positive	15 (83.3)
	Negative	3 (16.7)
P16 ^{INK4}	Positive	10 (55.6)
	Negative	8 (44.4)
PTEN	Positive	1 (5.6)
	Negative	17 (94.4)

RT, radiotherapy; CT, chemotherapy; S, surgery; HPV, human papillomavirus; EGFR, epidermal growth factor receptor; PTEN, phosphatase and tensin homolog.

the data, which was represented in category variables and checked for distribution of proportions across category variables using Fisher exact test. The bivariate relationship between the variables, such as smoking status, stage, prognosis, node positivity, HPV status, EGFR, p16^{INK4} and PTEN protein expression, was determined by Spearman's rank correlation test. The results were considered significant if the P value was <0.05 .

Results

All the samples were obtained from patients who had been clinically diagnosed with carcinoma of the larynx. The mean age of the patient population was 54 ± 10 years. Prognosis was calculated based on the follow-up of the patient for 5 years. Recurrence or death due to disease was considered as a poor prognosis and based on this, 66.7% of the patients had a bad prognosis (Table 1).

According to the treatment modality, patients were classified into four groups; (I) 38.9% were treated by radiotherapy and chemotherapy prior to surgery; (II) 5.6% underwent surgery followed by radiotherapy and chemotherapy; (III) 22.2% underwent surgery followed by chemotherapy; and (IV) 33.3% had radiotherapy and surgery followed by chemotherapy and more radiotherapy. HPV and overexpression of EGFR was seen in 27.8% and 83.3% of patients, respectively. Tumor suppressors p16^{INK4} and PTEN was negatively expressed in 44.4% and 94.4% of patients, respectively. All the HPV positive cases showed over expression of EGFR and negative expression

Table 2 Correlation of molecular markers with the smoking status, node positivity, pathological stage, prognosis and treatment regimen

Clinicopathologic parameters	HPV status	EGFR expression	P16 ^{INK4} expression	PTEN expression
Smoking status	0.624	0.555	1.000	0.332
Node positivity	0.896	0.045	0.814	0.608
Prognosis	0.486	0.201	0.201	0.496
Stage of the disease	0.827	0.035	0.621	0.276
Treatment regimen	0.221	0.858	0.893	0.275

HPV, human papillomavirus; EGFR, epidermal growth factor receptor; PTEN, phosphatase and tensin homolog.

of PTEN. Of the HPV positive cases, 40% presented with p16^{INK4} negative expression. Lymph node positivity was observed in 80% of the HPV positive cases.

Bivariate analysis revealed that EGFR over expression was associated with lymph node positivity (P=0.045) and stage of the disease (P=0.035) (Table 2). However, the EGFR over expression was not associated with prognosis and smoking status. P16^{INK4}, PTEN protein expression and HPV infection did not yield any significant correlations with smoking status, node positivity, disease stage, treatment regimen and disease prognosis. Disease prognosis was observed to be associated with smoking status (P=0.048).

Discussion

Majority (95%) of HNSCC cases are squamous cell carcinomas, which advocate HNSCC to be a homogenous disease. However, the various subclasses of HNSCCs differed at histological level and genomic and transcriptome studies revealed molecular heterogeneity in HNSCC (19). TP53 mutations were seen in 39–53% of HNSCC tumors overall and 56.7% in laryngeal carcinoma, these mutations were associated with poor survival (20). *NOTCH1* gene inactivating mutations seen in 15% of HNSCC cases and this pathway regulates cell cycle and survival by acting as a tumor suppressor. *NOTCH3* gene was associated with poor disease and overall survival in laryngeal carcinoma (21).

DNA damage repair biomarkers including MAP17 and pH2AX overexpression found to be associated with better overall survival and laryngoesophageal dysfunction free survival (22). Nucleotide excision repair (NER) pathway involved in removing DNA cross links caused by chemo and radiotherapy, excision repair cross-complementation group 1 gene mutations of this pathway increase the laryngeal cancer risk (23). Signal transducer and activator of transcription 3 pathway activation regulates cell

proliferation and tumor survival and found to be associated in laryngeal cancer (24).

Several studies have emphasized the need for molecular biomarkers to aid in determining the prognosis for patients with HNSCC. However, HNSCC subsite specific data is scarce. Hence, this study focused on carcinoma of the larynx and its association with specific molecular markers, namely EGFR, PTEN expression and HPV infection and p16^{INK4}.

EGFR activation leads to cell proliferation, metastasis, invasion and angiogenesis via tyrosine kinase mediated activation of PI3K, PTEN/AKT, MAPK, ERK and JAK/STAT pathways. Hence, the activation of EGFR is crucial to the carcinogenesis (25,26). EGFR over expression was observed in >80% of HNSCC subjects (13); 68.6% (27) in laryngeal carcinoma and 91.5% in glottic laryngeal carcinoma patients (28). Similarly, our cohort exhibited EGFR over expression in 83.3% of cases. Other studies have attributed EGFR over expression to dephosphorylation and reduced degradation of EGFR protein and/or the high percentage of exon 19 and exon 20 insertions (26,29).

An advanced stage of the disease was associated with EGFR overexpression (P=0.035) in the present study which is consistent with studies conducted by Kontić *et al.* (27) and Ding (30). EGFR protein expression is elevated in lymph node positive group (P=0.045), which is similar to an earlier study conducted by Ding (30). Clinical correlation of EGFR over expression is the key to finding the association with histopathological parameters, prognosis and survival. Also, this correlation between EGFR over expression at the time of the diagnosis with advanced stage, lymph node involvement aids in the estimation of the tumor progression, metastasis and the potential for recurrence of the disease. Hence, EGFR protein status can be considered as an independent prognostic factor in laryngeal carcinoma.

Loss of PTEN expression is a commonly occurring event in HNSCC, overall, along with PIK3CA mutations (31).

The correlation between the PIK3CA mutation and PTEN gene expression was assessed in our previous study (25) in which we reported that 29% of cases presented with PIK3CA mutations and 60% of cases exhibited PTEN gene down regulation in the HNSCC cohort. The present study focused on laryngeal carcinoma and it was found that 94% of cases included in the study presented with low expression of PTEN. However, this percentage is higher than the study by Mastronikolis *et al.* (32) which reported that 56% of laryngeal tumors had abnormal (low) PTEN expression. In our study, the high percentage of low expression of PTEN may be due to the advanced grade of the tumors. However, we did not find any significant correlation with the prognosis of the disease and node positivity.

HPV is known to be involved in the development of cervical cancer (33). The mechanism of carcinogenesis begins with the viral oncogenes E6 and E7 which inactivate the retinoblastoma and tumor protein 53, which in turn causes cell cycle dysregulations and leads to carcinogenesis (34). Further research has revealed HPV involvement in HNSCC and the presence of HPV increases the risk of HNSCC (35). In the subtypes of HNSCC, HPV infection was seen less frequently in laryngeal cancers and oral cavity cancers compared to oropharyngeal cancers (7,36). In Laryngeal carcinoma, the average prevalence of HPV is 28% (ranging from 6.8% to 58.8%) (37-40). The wide range of HPV prevalence is due to the non-specificity of detection method used in these studies. The availability of novel sensitive technologies (*in-situ* hybridization) to detect HPV is also the reason for the reduced prevalence of HPV.

The combination of testing of HPV and p16^{INK4} is now promoted extensively as marker for disease progression (41). In the present study, this algorithm was employed by using highly sensitive technique *in-situ* hybridization to detect the HPV infection in tumor samples along with p16^{INK4} expression profiling. In the present study, we observed that 27.8% of laryngeal cancer cases presented with HPV infection. Among the HPV positive cases, 60% showed high p16^{INK4} protein expression. Based on our results, we postulated that there is a weak correlation between the HPV infection with p16^{INK4} with the prognosis unlike in oropharyngeal carcinoma.

Conclusions

High prevalence of HPV infection in studied population strengthens the idea of HPV role in etiology of laryngeal cancer. Similarly, the low expression of PTEN and higher expression of p16^{INK4} expression signature was observed

in the present study but did not yield any correlation with prognosis and other clinical parameters. The association of EGFR over expression with lymph node status and advanced stage of the disease supports the role that EGFR plays in tumor metastasis and invasion in laryngeal carcinoma patients. Therefore, the use of anti-EGFR targeted therapy in cases of laryngeal carcinoma may improve the prognosis of these patients.

Acknowledgments

The authors would like to thank Mr. Mohammed H. Al-Shamlan for his technical support.

Funding: This work was supported by the Deanship of Scientific Research, Imam Abdulrahman Bin Faisal University (#2014064, 2016-111-MED).

Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/tcr.2019.07.26>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the Institutional Review Board of Imam Abdulrahman Bin Faisal University, Dammam, Saudi Arabia (IRB number: IRB-2014-08-044) and written informed consent was obtained from all patients. The study outcomes don't affect the future management of the patients.

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Cite this article as: Vatte C, Al-Amri AM, Cyrus C, Chathoth S, Alsulaiman A, Al Sayyah A, Al-Ali A. Association of epidermal growth factor receptor protein expression with histopathological and clinical parameters in carcinoma of the larynx. *Transl Cancer Res* 2019;8(4):1395-1402. doi: 10.21037/tcr.2019.07.26