EUROPEAN JOURNAL OF CANCER 46 (2010) 2088–2096

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.ejconline.com

Relationships between epidermal growth factor receptor expression and human papillomavirus status as markers of prognosis in oropharyngeal cancer

Angela Hong [a,b,*], *Timothy Dobbins* [c], *C. Soon Lee* [d,e,f], *Deanna Jones* [g,h], *Elise Jackson* [g,h], *Jonathan Clark* [g], *Bruce Armstrong* [i], *Gerald Harnett* [j], *Christopher Milross* [a,b], *Christopher O'Brien* [g], *Barbara Rose* [g,h]

[a] *Department of Radiation Oncology, Sydney Cancer Centre, Royal Prince Alfred Hospital, Sydney, NSW, Australia*
[b] *Central Clinical School, University of Sydney, Sydney, NSW, Australia*
[c] *Adult Cancer Program, Prince of Wales Clinical School, University of NSW, Sydney, NSW, Australia*
[d] *Discipline of Pathology, School of Medicine, University of Western Sydney, NSW, Australia*
[e] *Department of Anatomical Pathology, Liverpool Hospital, Sydney, NSW, Australia*
[f] *Cancer Pathology, Bosch Institute, University of Sydney, Department of Anatomical Pathology, Royal Prince Alfred Hospital, Sydney, NSW, Australia*
[g] *The Sydney Head and Neck Cancer Institute, Sydney Cancer Centre, Royal Prince Alfred Hospital, Sydney, NSW, Australia*
[h] *Department of Infectious Diseases and Immunology, University of Sydney, Sydney, NSW, Australia*
[i] *Sydney School of Public Health, University of Sydney, Sydney, NSW, Australia*
[j] *Pathwest Laboratory Medicine, QEII Medical Centre, Nedlands, Perth, Australia*

ARTICLE INFO

Article history:
Received 24 January 2010
Received in revised form 15 April 2010
Accepted 19 April 2010
Available online 25 May 2010

Keywords:
Oropharyngeal cancer
Epidermal growth factor receptor
Human papillomavirus
p16
Prognosis

ABSTRACT

Purpose: This study examines the prognostic significance of epidermal growth factor receptor (EGFR) expression in relation to human papillomavirus (HPV) status in oropharyngeal squamous cell carcinoma (SCC).

Materials and methods: Pathological diagnosis of 270 oropharyngeal SCCs was verified by the study pathologist; clinical details were extracted from institutional databases. Recurrence in any form or death from any cause was recorded for a median of 2.5 (range: 0–19.3) years after diagnosis. HPV status was determined by HPV E6-targeted multiplex real-time PCR/p16 immunohistochemistry; EGFR expression was evaluated by semiquantitative immunohistochemistry. Determinants of recurrence and mortality hazards were modelled using Cox regression with censoring at dates of last follow-up.

Results: Thirty-seven percent of cancers were HPV-positive (91% type 16). HPV was a predictor of loco-regional recurrence, event-free and overall survival after adjustment for clinicopathological variables and EGFR. Patients with EGFR-positive cancers were 5-fold more likely to have loco-regional failure relative to those with EGFR-negative cancers. Patients with HPV-negative/EGFR-positive cancers had an adjusted 13-fold increased risk of having a loco-regional failure, an almost 4-fold increased risk of having an event and more than a 4-fold increased risk of dying of any cause relative to those with HPV-positive/EGFR-negative cancers. There was weak evidence that the effects of EGFR on outcome were limited to patients with HPV-negative cancers.

* *Corresponding author at:* Department of Radiation Oncology, Royal Prince Alfred Hospital, Building 27 Missenden Road, Camperdown, NSW 2050, Australia. Tel.: +61 2 9515 8057; fax: +61 2 9515 8115.
E-mail address: ahon6809@mail.usyd.edu.au (A. Hong).
0959-8049/$ - see front matter
doi:10.1016/j.ejca.2010.04.016

Conclusions: HPV and EGFR are independent prognostic markers in oropharyngeal SCC. Combining testing for HPV and EGFR appears to provide additional prognostic information.

1. Introduction

For the past decade, clinical research in head and neck cancer has focused on targeting the molecular pathways. The addition of cetuximab, a monoclonal antibody inhibitor of epidermal growth factor receptor (EGFR) to radiation therapy, has improved outcomes in locally advanced head and neck squamous cell carcinoma (SCC) compared to radiation alone[1] although comparisons have not been made to chemoradiation, the standard of care.

EGFR is abnormally activated in approximately 80% of head and neck cancers.[2] EGFR expression has been associated with prognosis in head and neck cancer, particularly in patients treated with radiotherapy, although some studies have found no relationship between EGFR and treatment response or outcome.[2–6] These inconsistencies reflect factors such as the heterogeneity of disease within the head and neck cancer and differences in treatment. However, technical factors such as the sensitivity and specificity of the assays used for assessing EGFR, scoring methods and cut-off levels are also likely contributors.[2–6]

Human papillomavirus (HPV) is the cause of a subset of head and neck cancers with a favourable prognosis.[7–11] Its association is greatest in the oropharynx where HPV-positivity rates of up to 60% have been reported.[12,13] An inverse relationship between HPV status and EGFR expression (or amplification that leads to overexpression) has been demonstrated in several recent head and neck cancer studies,[5,14,15] although study found no association.[16] The results of studies evaluating the effect of EGFR on survival in relation to HPV status have been conflicting,[5,14,15] although in each of these studies a combination of HPV and EGFR was found to more accurately predict outcome than either alone.

In this study we have examined the prognostic significance of EGFR in relation to HPV in a large cohort of patients with oropharyngeal SCC. The combined effects of HPV and EGFR as predictors of outcome were also examined.

2. Materials and methods

2.1. Study population

The study group comprised 270 patients with biopsy proven SCC of the oropharynx who underwent curative treatment at hospitals in Sydney, Australia between 1987 and 2006. Two hundred and one patients were from Royal Prince Alfred Hospital. All consecutive patients with accessible tumour material and clinicopathological data in the study period were followed up for a median of 2.5 (range: 0–19.3) years. The study was approved by the ethics committees of Sydney South West Area Health Service (Protocols X05-0308, CH62/6/2006-041, 2006/055). Most of the data were retrieved from the database of the Sydney Head and Neck Cancer Institute at Royal Prince Alfred Hospital that covers the study period. Department of Radiation Oncology and Anatomical Pathology databases and hospital records were used to retrieve missing data. The study pathologist (CSL) reviewed the histology and tumour grade in all cases. Cancers were staged using the American Joint Committee on Cancer (AJCC) staging system.[17]

2.2. Laboratory studies

An HPV-positive cancer was defined as one testing positive for HPV DNA and overexpressing p16.[9,18] The presence and type of HPV DNA were determined by a E6-based multiplex tandem-PCR assay (MT-PCR) carried out on two to six 4–5 μm sections of tumour using modifications of the method described by Stanley and Szewezuk.[19] This assay simultaneously detects and identifies 21 HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, 70, 73, 82, 53, 6, 11 and 26). DNA was extracted using the QIAmp RNA viral mini kit (Qiagen, Hilden, Germany). The first step involved incorporation of all primer pairs into two PCR mixes and amplification for 20 cycles in a GeneAmp 2700 thermocycler (Applied Biosystems, Australia). Products from each of these mixes were then passed into triplex TaqMan real-time PCR assays with probes having FAM, VIC or Cy5 labels and amplification performed for 40 cycles in a RotorGene 6000 real-time thermocycler (Corbett Research, NSW, Australia). A Corbett CAS 1200 robot was used to inoculate samples and to make the transfers from the initial PCR into the TaqMan PCR mixes. Measured amounts of equine herpesvirus (EHV) were used to monitor the efficiency of DNA extraction and removal of PCR inhibitors. Paraffin sections were cut using stringent precautions to avoid cross contamination and water blanks were placed after every fifth tube to detect assay contamination.

Expression of p16 and EGFR was assessed by semiquantitative immunohistochemistry on fixed paraffin-embedded tissues using an autostainer (Dako Carpinteria, CA). For p16, antigen retrieval was carried out using Target Retrieval Solution, pH 9, (Dako) in a microwave oven for 10 min on high setting. After cooling, endogenous peroxidase was blocked using 0.3% v/v hydrogen peroxide in Tris-buffered saline (TBS); sections were incubated with primary antibody (1/200, clone JC2 Neomarkers, Fremont, CA) for 30 min, washed in TBS, treated with the EnVision™ Flex+ Dual Link horse radish peroxidase visualisation system (Dako) and then counterstained with haematoxylin. Expression of EGFR was determined using the PharmDx™ Kit for Autostainer (Dako) according to the manufacturer's instructions.

Immunostaining was evaluated by at least three independent observers including the study pathologist, all of whom were blinded to the clinical information. At least 20 high power fields (400×) were chosen at random and 2000 cancer cells assessed. The p16 antibody stained the nucleus and cytoplasm of cancer cells. Staining was strong and diffuse

and was essentially all or none. Weak focal staining was recorded as negative. EGFR-positivity was defined as membrane staining of at least moderate intensity of 10% of cancer cells.

2.3. Statistical analyses

Associations between HPV status and all recorded clinicopathological characteristics were assessed using a two-sample *t*-test for the continuous variable age and χ^2 tests for categorical variables. Time to loco-regional failure, an event and all-cause death were calculated from date of diagnosis. An event was defined as recurrence in any form or death from any cause, with only the first event taken into account. Patients without events were censored at the date of last follow-up. Univariate associations between patient characteristics and time to loco-regional failure, an event or death from any cause were summarised using Cox proportional hazards models. Multivariable survival models were constructed after inclusion of HPV status and other known or potential confounders of age, year of diagnosis, gender, tumour grade, primary site, T-stage, N-stage and treatment modality, defined as 'clinical variables'. Two multivariable analyses were conducted to examine the effect of EGFR individually: the first adjusted for the above variables while the second excluded T-stage and N-stage from the model because of the possibility that EGFR exerts an effect on outcome at least partly through T-stage or N-stage.[5,14,15] Kaplan–Meier survival curves were used to present the combined effects of HPV and EGFR on time to loco-regional failure, event and death. Interaction terms between HPV status and EGFR were used to assess whether HPV modified the effect of EGFR overexpression on survival. Results from these analyses are presented in the form of both joint-effects and stratified analyses. All analyses were conducted using the SAS System for Windows (SAS Institute, Cary, NC, USA).

3. Results

3.1. HPV status and type distribution

In all, 270 records were obtained with 268 records where both HPV DNA and p16 status were available. As there were only two patients with HPV-negative/p16-positive cancers, these records were excluded from the analyses leaving 266 patients. Thirty-seven percent (99/266) of the cancers were HPV-positive (HPV DNA-positive/p16-positive). HPV type 16 accounted for 91% (91/99) of the HPV-positive cases. Other HPV types were 18 (2), 35 (2) and 56 and 59 (one each). The HPV type in two HPV-positive cancers could not be determined. Six cancers positive for HPV 16 also contained a second HPV type (two had 35 and one each had 18, 33, 39, and 56). The 34

Table 1 – Demographic and clinical characteristics of the study population.

Variable		All[a] (*n* = 266)	HPV-positive (*n* = 99)	HPV-negative (*n* = 167)	*P*-value[b]
Mean age at diagnosis		59.8 (range 34–90)	55.3 (range 34–84)	62.5 (range 44–90)	<0.0001
Year of diagnosis	1996 or earlier	103 (39%)	24 (24%)	79 (47%)	0.0002
	1997 or later	163 (61%)	75 (76%)	88 (53%)	
Gender	Male	211 (79%)	79 (80%)	132 (79%)	0.88
	Female	55 (21%)	20 (20%)	35 (21%)	
Grade (1 missing datum)	1, 2	172 (65%)	47 (47%)	125 (75%)	<0.0001
	3	93 (35%)	52 (53%)	41 (25%)	
T-stage (4 missing data)	1	40 (15%)	16 (16%)	24 (15%)	0.67
	2	106 (40%)	40 (41%)	66 (40%)	
	3	73 (28%)	23 (24%)	50 (30%)	
	4	43 (16%)	18 (19%)	25 (15%)	
N-stage (3 missing data)	0	112 (43%)	25 (26%)	87 (53%)	<0.0001
	1	57 (22%)	22 (22%)	35 (21%)	
	2, 3	94 (36%)	51 (52%)	43 (26%)	
Stage (3 missing data)	1, 2	65 (25%)	12 (12%)	53 (32%)	0.0001
	3	79 (30%)	26 (27%)	53 (32%)	
	4	119 (45%)	59 (61%)	60 (36%)	
Tumour site	Tonsil	166 (62%)	74 (75%)	92 (55%)	0.0007
	Base of tongue	48 (18%)	17 (17%)	31 (19%)	
	Other	52 (20%)	8 (8%)	44 (26%)	
Treatment (3 missing data)	Surgery only	44 (17%)	12 (13%)	32 (19%)	0.37
	Radiotherapy only	90 (34%)	34 (35%)	56 (34%)	
	Surgery and radiotherapy	129 (49%)	50 (52%)	79 (47%)	
EGFR (17 missing data)	Negative	32 (13%)	21 (22%)	11 (7%)	0.0005
	Positive	217 (87%)	73 (78%)	144 (93%)	

[a] Two patients with HPV DNA negative/p16 positive cancers were not included in the analyses.
[b] Test for heterogeneity.

(13%) cancers testing HPV DNA-positive/p16-negative were regarded as HPV-negative.[18] Compared to patients with HPV-negative cancers, those with HPV-positive cancers were younger (mean age 55.3 versus 62.5 years, $P < 0.0001$), more likely to be diagnosed after 1996, more likely to have primary disease in the tonsil ($P = 0.0007$), higher grade cancers ($P < 0.0001$) and greater nodal disease ($P < 0.0001$) and hence more advanced AJCC stage disease ($P = 0.0001$).

3.2. EGFR versus clinicopathological variables and HPV status

The EGFR-positivity rate for the 249 cancers for which EGFR status could be determined was 87%. Patients with EGFR-positive cancers were more likely to have lower grade cancers (grade 1 or 2, 68%) compared to those with EGFR-negative cancers (38%, $P = 0.0008$). There were no statistically significant relationships between EGFR and age at diagnosis, gender, T-stage, N-stage or primary site. There was a strong inverse association between HPV status and EGFR ($P = 0.0005$) positivity (Table 1). The EGFR-positivity rate was 78% in HPV-positive patients and 93% in HPV-negative patients.

3.3. Survival analyses

Four patients were lost to follow-up. Loco-regional failure occurred in 74 (28%) of 262 patients. Failure occurred at the primary site in 45 patients and in the regional nodal area (with disease controlled at the primary site) in 29 patients. Eighteen patients developed distant metastasis, none of whom had loco-regional failure at the time of diagnosis of distant metastasis. There were 131 (50%) events and 108 (41%) deaths from any cause.

3.3.1. Effect of HPV on outcomes

Univariate analysis showed that patients with HPV-positive cancers were less likely to have loco-regional failure, an event or to die than those with HPV-negative cancers (Tables 2a–2c).

Table 2a – Multivariable associations of patient and disease characteristics with risk of loco-regional failure.

Characteristic	Category	Univariate		Multivariable[a]	
		HR (95% CI)	P-value[b]	HR (95% CI)	P-value[b]
Adjusting for clinical variables					
HPV status	Negative	1.0	<0.0001	1.0	0.0003
	Positive	0.34 (0.18, 0.58)		0.33 (0.17, 0.61)	
Age at diagnosis (years)	<60	1.0	0.55	1.0	0.70
	⩾60	1.15 (0.73, 1.82)		1.10 (0.67, 1.80)	
Year of diagnosis	1996 or earlier	1.0	0.0092	1.0	0.002
	1997 or later	0.54 (0.34, 0.86)		0.44 (0.26, 0.73)	
Gender	Male	1.0	0.061	1.0	0.005
	Female	0.57 (0.29, 1.02)		0.40 (0.19, 0.77)	
Grade	1, 2	1.0	0.079	1.0	0.79
	3	0.64 (0.37, 1.05)		0.93 (0.53, 1.58)	
T-stage	1	1.0	0.35	1.0	0.30
	2	0.98 (0.49, 2.14)		1.21 (0.58, 2.69)	
	3	1.46 (0.71, 3.22)		1.77 (0.82, 4.06)	
	4	1.59 (0.70, 3.74)		1.98 (0.80, 5.05)	
N-stage	0	1.0	0.69	1.0	0.22
	1	1.20 (0.67, 2.10)		1.50 (0.80, 2.77)	
	2, 3	0.91 (0.52, 1.58)		1.70 (0.89, 3.23)	
Primary tumour site	Tonsil	1.0	0.81	1.0	0.10
	Base of tongue	1.20 (0.66, 2.08)		1.28 (0.68, 2.31)	
	Other	0.97 (0.50, 1.75)		0.56 (0.27, 1.08)	
Treatment	Surgery only	1.0	0.043	1.0	0.010
	RT only	1.43 (0.75, 2.95)		0.95 (0.44, 2.13)	
	Surgery + RT	0.76 (0.39, 1.59)		0.44 (0.20, 1.02)	
Adjusting for clinical variables and EGFR					
HPV status	HPV+/–, P16–			1.0	0.0005
	HPV+, p16+			0.31 (0.15, 0.61)	
Adjusting for clinical variables and HPV					
EGFR	Negative	1.0	0.0003	1.0	0.006
	Positive	6.57 (2.06, 40.03)		5.16 (1.52, 32.29)	

[a] Clinical variables adjusted.
[b] Test for heterogeneity.

Table 2b – Multivariable associations of patient and disease characteristics with risk of failure or death.

Characteristic	Category	Univariate		Multivariable[a]	
		HR (95% CI)	P-value[b]	HR (95% CI)	P-value[b]
Adjusting for clinical variables					
HPV status	Negative	1.0	<0.0001	1.0	<0.0001
	Positive	0.36 (0.24, 0.54)		0.33 (0.20, 0.52)	
Age at diagnosis (years)	<60	1.0	0.027	1.0	0.061
	⩾60	1.47 (1.05, 2.08)		1.43 (0.98, 2.07)	
Year of diagnosis	1996 or earlier	1.0	0.0091	1.0	0.0005
	1997 or later	0.63 (0.45, 0.89)		0.51 (0.34, 0.74)	
Gender	Male	1.0	0.0044	1.0	0.0002
	Female	0.53 (0.32, 0.83)		0.40 (0.23, 0.66)	
Grade	1, 2	1.0	0.15	1.0	0.95
	3	0.77 (0.52, 1.10)		1.01 (0.67, 1.50)	
T-stage	1	1.0	0.0022	1.0	0.008
	2	0.74 (0.44, 1.30)		0.96 (0.55, 1.73)	
	3	1.30 (0.77, 2.27)		1.71 (0.96, 3.12)	
	4	1.84 (1.05, 3.31)		2.14 (1.12, 4.16)	
N-stage	0	1.0	0.84	1.0	0.006
	1	1.08 (0.68, 1.67)		1.38 (0.83, 2.25)	
	2, 3	1.12 (0.75, 1.67)		2.26 (1.37, 3.72)	
Primary tumour site	Tonsil	1.0	0.11	1.0	0.089
	Base of tongue	1.42 (0.91, 2.16)		1.61 (1.00, 2.54)	
	Other	1.47 (0.95, 2.23)		0.89 (0.55, 1.42)	
Treatment	Surgery only	1.0	0.074	1.0	0.008
	RT only	1.21 (0.74, 2.05)		0.80 (0.44, 1.47)	
	Surgery + RT	0.78 (0.48, 1.32)		0.46 (0.25, 0.87)	
Adjusting for clinical variables and EGFR					
HPV status	HPV+/−, P16−			1.0	<0.0001
	HPV+, p16+			0.32 (0.19, 0.52)	
Adjusting for clinical variables and HPV					
EGFR	Negative	1.0	0.0008	1.0	0.13
	Positive	2.75 (1.48, 5.86)		1.71 (0.86, 3.78)	

[a] Clinical variables adjusted.
[b] Test for heterogeneity.

After adjustment for age, year of diagnosis, gender, grade, T-stage, N-stage, primary site within the oropharynx and treatment modality, patients with HPV-positive cancers had one third of the risk of loco-regional failure (hazard ratio (HR) = 0.33, 95% confidence interval (CI): 0.17–0.61) and of having an event (HR = 0.33, 95% CI: 0.20–0.52) and a little more than one third the risk of dying of any cause (HR = 0.37, 95% CI: 0.21–0.62) relative to those with HPV-negative cancers. HPV status remained significant for all three outcomes after the additional adjustment for EGFR (HR = 0.31, 95% CI: 0.15–0.61, HR = 0.32, 95% CI: 0.19–0.52 and HR = 0.35, 95% CI: 0.19–0.62, respectively). Comparisons of outcomes over time incorporating year of diagnosis (<1997 versus 1997 and later) in multivariable models (Tables 2a–2c) showed that outcomes for patients with oropharyngeal cancer have improved over time.

3.3.2. *Effect of EGFR on outcomes*

On univariate analysis, patients with EGFR-positive cancers were more likely to have a loco-regional failure (HR = 6.57, 95% CI: 2.06–40.03) to have an event (HR = 2.75, 95% CI:1.48–5.86) and to die of any cause (HR = 4.24, 95% CI: 1.91–12.05) than those with EGFR-negative cancers (Tables 2a–2c). After adjustment for age, year of diagnosis, gender, grade, T-stage, N-stage, primary site within the oropharynx, treatment modality and HPV status, patients with EGFR-positive cancers were more than five times more likely to have loco-regional failure than those with EGFR-negative cancers (HR = 5.16, 95% CI: 1.52–32.29); there was a weaker association between EGFR and event-free survival (HR = 1.71, 95% CI: 0.86–3.78) and overall survival (HR = 2.48, 95% CI: 1.04–7.33) (Tables 2a–2c). When T-stage and N-stage were not included in the multivariable models, the HRs for EGFR were 5.49 (95% CI: 1.64–34.11; $P = 0.003$) for loco-regional failure, 2.20 (95% CI: 1.13–4.82; $P = 0.019$) for an event and 3.41 (95% CI: 1.47–9.93; $P = 0.003$) for death from any cause. Whether or not tumour grade was included in the models had little effect on the HRs for EGFR.

3.3.3. *Effect of combining HPV and EGFR on outcomes*

Effects of a combination of EGFR and HPV on outcomes are shown in Figs. 1a–c and were estimated in multivariable

Table 2c – Multivariable associations of patient and disease characteristics with risk of death from any cause.

Characteristic	Category	Univariate		Multivariable[a]	
		HR (95% CI)	*P*-value[b]	HR (95% CI)	*P*-value[b]
Adjusting for clinical variables					
HPV status	Negative	1.0	<0.0001	1.0	0.0001
	Positive	0.37 (0.23, 0.58)		0.37 (0.21, 0.62)	
Age at diagnosis (years)	<60	1.0	0.061	1.0	0.18
	⩾60	1.43 (0.98, 2.10)		1.32 (0.88, 2.00)	
Year of diagnosis	1996 or earlier	1.0	0.0079	1.0	0.001
	1997 or later	0.59 (0.40, 0.87)		0.49 (0.32, 0.75)	
Gender	Male	1.0	0.019	1.0	0.012
	Female	0.56 (0.33, 0.91)		0.51 (0.29, 0.87)	
Grade	1, 2	1.0	0.17	1.0	0.62
	3	0.75 (0.49, 1.12)		0.89 (0.56, 1.39)	
T-stage	1	1.0	0.0002	1.0	0.003
	2	0.70 (0.39, 1.29)		0.85 (0.46, 1.63)	
	3	1.24 (0.70, 2.28)		1.49 (0.80, 2.87)	
	4	2.31 (1.26, 4.35)		2.50 (1.24, 5.18)	
N-stage	0	1.0	0.71	1.0	0.021
	1	0.92 (0.55, 1.50)		0.98 (0.56, 1.67)	
	2, 3	1.14 (0.74, 1.75)		1.97 (1.16, 3.35)	
Tumour site	Tonsil	1.0	0.0081	1.0	0.036
	Base of tongue	1.73 (1.07, 2.73)		1.95 (1.16, 3.18)	
	Other	1.91 (1.19, 2.98)		1.40 (0.84, 2.32)	
Treatment	Surgery only	1.0	0.10	1.0	0.050
	RT only	1.22 (0.72, 2.16)		1.12 (0.60, 2.14)	
	Surgery + RT	0.77 (0.45, 1.38)		0.65 (0.34, 1.27)	
Adjusting for clinical variables and EGFR					
HPV status	HPV+/–, P16–			1.0	0.0002
	HPV+, p16+			0.35 (0.19, 0.62)	
Adjusting for clinical variables and HPV					
EGFR	Negative	1.0	<0.0001	1.0	0.039
	Positive	4.24 (1.91, 12.05)		2.48 (1.04, 7.33)	

[a] Clinical variables adjusted.
[b] Test for heterogeneity.

models (Table 3). After adjustment for age, year of diagnosis, gender, grade, T-stage, N-stage, primary site within the oropharynx and treatment modality, the best outcomes were seen in patients with HPV-positive/EGFR-negative cancers and the worst in those with HPV-negative/EGFR-positive cancers. Relative to patients with HPV-positive/EGFR-negative cancers, those with HPV-negative/EGFR-positive cancers had an adjusted 13-fold increased risk of having loco-regional failure (HR = 12.79, 95% CI: 1.70–96.26), an almost 4-fold increased risk of an event (HR = 3.89, 95% CI: 1.61–9.37) and more than a 4-fold increased risk of all-cause death (HR = 4.49, 95% CI: 1.34–15.03) (Table 3a). That the HRs for EGFR were substantially greater in HPV-negative than HPV-positive cancers and that the HRs for EGFR in HPV-positive cancers were close to 1.0, except for loco-regional failure, suggest that the impact of EGFR expression on outcome was limited to patients with HPV-negative cancers (Table 3b). The evidence for this proposition, however, is weak since the *P*-values for the interaction of HPV status and EGFR on outcome were high ($P = 0.73$ for loco-regional failure, $P = 0.20$ for an event and $P = 0.29$ for death from any cause). Furthermore, since only 7% of HPV-negative patients were EGFR-negative, this result is based on a small sample ($n = 11$) with limited events. The apparent inconsistency between the joint-effects and stratified analysis in Table 3 – HRs greater than unity for HPV-positive/EGFR-positive in joint-effects and less than unity in stratified analysis – appeared with addition of T- and N-stages to the model.

4. Discussion

This study has examined the prognostic significance of EGFR expression in relation to HPV status in the largest series of oropharyngeal cancers to date. Our data show that EGFR and HPV are independent prognostic markers in oropharyngeal cancer, although the effect of EGFR was more convincing for loco-regional control than for survival. We confirm previous reports of an inverse association between HPV and

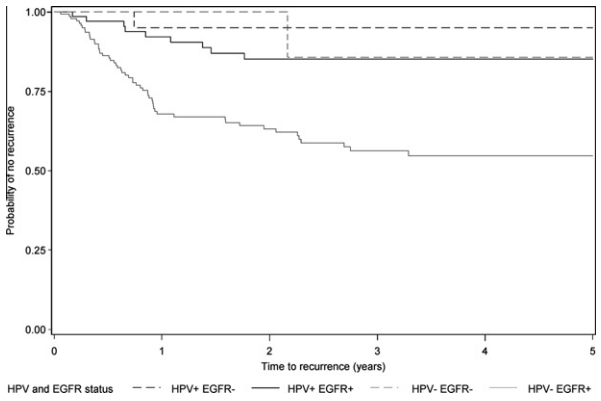

Fig. 1a – Probability of time free from loco-regional failure by HPV and EGFR status.

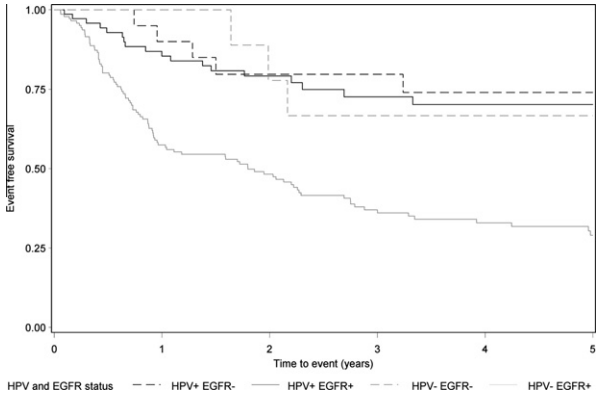

Fig. 1b – Probability of time free from event by HPV and EGFR status.

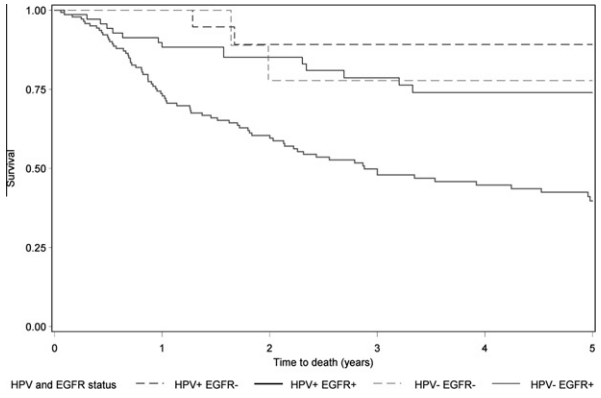

Fig. 1c – Probability of survival by HPV and EGFR status.

EGFR[5,14,15] and provide evidence that use of EGFR in combination with HPV status gives additional prognostic information particularly in terms of loco-regional control.

Loco-regional failure can profoundly impact the quality of life of patients with head and neck cancer. Ang and colleagues reported that the association between high EGFR expression and poor survival in a mixed series of head and neck cancers was due to poor loco-regional control and not distant metastasis.[20] Along similar lines, Bentzen and colleagues found that patients with head and neck cancers expressing high levels of EGFR benefited from accelerated radiation therapy in terms of loco-regional control.[21] However, in the meta-analysis of altered fractionation radiation therapy in 6516 patients with cancer in all subsites of the head and neck, an improvement in loco-regional control did translate to a small but significant overall survival benefit.[22]

In our series of 249 oropharyngeal cancer patients, EGFR was a marker of loco-regional failure after adjustment for clinicopathological variables. Our data also showed that the association was independent of HPV status. Several previous studies examined the role of EGFR as marker of survival in oropharyngeal cancers of known HPV status, but none assessed its effect on loco-regional failure.[5,14,15] Although all confirmed the effect prognostic effect of HPV, data on EGFR were conflicting possibly because of smaller sample size and variation in the number and nature of clinicopathological variables adjusted for in the multivariable analyses. In this study EGFR was a predictor of having an event or dying of any cause after adjustment for age, gender, grade, primary site within the oropharynx treatment modality and HPV, but the effect weakened after T- and N-stage were included in the multivariable model. Although stage is a well-recognised prognostic marker in head and neck cancer, EGFR is known to promote the growth of tumour cells, thus EGFR might exert an effect on outcome at least partly through T-stage or N-stage. However, published data on relationships between EGFR and stage are conflicting[5,14,15] and there was no statistically significant relationship between EGFR and stage in our study.

There is an increasing interest in the use of two or even three molecular markers to predict outcome in human cancer.[23,24] Despite the inverse relationship between HPV and EGFR, it seems likely that the molecular pathways are independent. Both Kumar and colleagues and Kong and colleagues found that a combination of EGFR and HPV was useful for stratifying disease-specific survival in head and neck cancer.[5,15] Reimers and colleagues[14] also concluded that testing for p16 (as surrogate for HPV status) in conjunction with EGFR had significant prognostic implications. We have extended these studies by assessing the combined effects of HPV and EGFR after adjusting for clinicopathological variables for all three outcomes. Patients with HPV-negative/EGFR-positive cancers were more likely to have loco-regional failure than those with HPV-negative/EGFR-negative cancers. The increased risk also applied to survival, but the effect was not as strong.

The retrospective nature of the study has allowed us to demonstrate improved outcomes from oropharyngeal cancer over time. This has occurred as the proportion of HPV-related cancers in our cohort has increased as in other western countries.[25,26] In common with some other retrospective studies, our study lacks data on alcohol and smoking history. However, our access to an institutional database that spanned the study period has addressed the potential shortcoming of inaccurate clinicopathological data.

The possibility that HPV and EGFR together define a subgroup of patients with HPV-negative tumours at particular risk of a poor outcome warrants further investigation in prospective studies with larger numbers. If our findings are

Table 3 – Association between EGFR and HPV status on time to loco-regional failure, event and death.

HPV and EGFR status	Time to loco-regional failure HR[a] (95% CI)	Time to event HR[a] (95% CI)	Time to death from any cause HR[a] (95% CI)
(a) Joint-effects presentation			
HPV-positive/EGFR-negative ($n = 21$)	1.00	1.00	1.00
HPV-positive/EGFR-positive ($n = 69$)	3.84 (0.48, 30.84)	1.08 (0.41, 2.85)	1.42 (0.39, 5.19)
HPV-negative/EGFR-negative ($n = 11$)	1.97 (0.12, 33.40)	1.35 (0.31, 5.79)	1.10 (0.17, 7.04)
HPV-negative/EGFR-positive ($n = 141$)	12.79 (1.70, 96.26)	3.89 (1.61, 9.37)	4.49 (1.34, 15.03)
(b) Stratified presentation			
HPV-positive/EGFR-negative ($n = 21$)	1.0	1.0	1.0
HPV-positive/EGFR-positive ($n = 69$)	4.10 (0.40, 42.46)	0.54 (0.16, 1.84)	0.58 (0.09, 3.76)
HPV-negative/EGFR-negative ($n = 11$)	1.0	1.0	1.0
HPV-negative/EGFR-positive ($n = 141$)	6.27 (0.80, 48.91)	3.22 (0.95, 10.93)	5.20 (1.18, 22.85)

[a] Clinical variables adjusted.

confirmed, pretreatment testing for EGFR in addition to HPV may help to better stratify patients with oropharyngeal cancer in the setting of tailored treatment. In particular, the group of patients with HPV-negative/EGFR-positive cancers might benefit from intensified treatment.

Conflict of interest statement

None declared.

Acknowledgements

This study was supported by grants from the Diagnostics and Technology Branch of the Australian Government Department of Health and Ageing with the support of Cancer Australia, The Cure Cancer Foundation Australia and The Sydney Head and Neck Cancer Institute, Sydney Cancer Centre, Royal Prince Alfred Hospital, Sydney. The HPV multiplex tandem-PCR assays were performed by Ms. Ethel Reyes.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2010.04.016.

REFERENCES

1. Bonner JA, Harari PM, Giralt J, et al. Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck. *New Engl J Med* 2006;**354**:567–78.
2. Thomas GR, Nadiminti H, Regalado J. Molecular predictors of clinical outcome in patients with head and neck squamous cell carcinoma. *Int J Exp Pathol* 2005;**86**:347–63.
3. Putti TC, To KF, Hsu HC, et al. Expression of epidermal growth factor receptor in head and neck cancers correlates with clinical progression: a multicentre immunohistochemical study in the Asia–Pacific region. *Histopathology* 2002;**41**:144–51.
4. Chang AR, Wu HG, Park CI, Jun YK, Kim CW. Expression of epidermal growth factor receptor and cyclin D1 in pretreatment biopsies as a predictive factor of radiotherapy efficacy in early glottic cancer. *Head Neck* 2008;**30**:852–7.
5. Kong CS, Narasimhan B, Cao H, et al. The relationship between human papillomavirus status and other molecular prognostic markers in head and neck squamous cell carcinomas. *Int J Radiat Oncol Biol Phys* 2009;**74**:553–61.
6. Fischer C, Zlobec I, Stockli E, et al. Is immunohistochemical epidermal growth factor receptor expression overestimated as a prognostic factor in head-neck squamous cell carcinoma? A retrospective analysis based on a tissue microarray of 365 carcinomas. *Human Pathol* 2008;**39**:1527–34.
7. Li W, Thompson CH, O'Brien CJ, et al. Human papillomavirus positivity predicts favourable outcome for squamous carcinoma of the tonsil. *Int J Cancer* 2003;**106**(4):553–8.
8. Gillison ML. Human papillomavirus and prognosis of oropharyngeal squamous cell carcinoma: implications for clinical research in head and neck cancers. *J Clin Oncol* 2006;**24**:5623–5.
9. Weinberger PM, Yu Z, Haffty BG, et al. Molecular classification identifies a subset of human papillomavirus – associated oropharyngeal cancers with favorable prognosis. *J Clin Oncol* 2006;**24**:736–47.
10. Fakhry C, Westra WH, Li S, et al. Improved survival of patients with human papillomavirus-positive head and neck squamous cell carcinoma in a prospective clinical trial. *J Natl Cancer Inst* 2008;**100**:261–9.
11. Lassen P, Eriksen JG, Hamilton-Dutoit S, et al. Effect of HPV-associated p16INK4A expression on response to radiotherapy and survival in squamous cell carcinoma of the head and neck. *J Clin Oncol* 2009;**27**:1992–8.
12. Gillison ML, Koch WM, Capone RB, et al. Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. *J Natl Cancer Inst* 2000;**92**:709–20.
13. Herrero R, Castellsague X, Pawlita M, et al. Human papillomavirus and oral cancer: the International Agency for Research on Cancer multicenter study. *J Natl Cancer Inst* 2003;**95**:1772–83.
14. Reimers N, Kasper HU, Weissenborn SJ, et al. Combined analysis of HPV-DNA, p16 and EGFR expression to predict prognosis in oropharyngeal cancer. *Int J Cancer* 2007;**120**:1731–8.
15. Kumar B, Cordell KG, Lee JS, et al. EGFR, p16, HPV titer, Bcl-xL and p53, sex, and smoking as indicators of response to therapy and survival in oropharyngeal cancer. *J Clin Oncol* 2008;**26**:3128–37.
16. Perrone F, Suardi S, Pastore E, et al. Molecular and cytogenetic subgroups of oropharyngeal squamous cell carcinoma. *Clin Cancer Res* 2006;**12**:6643–51.

17. Sobin LH, Hermanek P, Hutter RV. TNM classification of malignant tumors. A comparison between the new (1987) and the old editions. *Cancer* 1988;**61**:2310–4.
18. Smeets SJ, Hesselink AT, Speel EJ, et al. A novel algorithm for reliable detection of human papillomavirus in paraffin embedded head and neck cancer specimen. *Int J Cancer* 2007;**121**:2465–72.
19. Stanley KK, Szewczuk E. Multiplexed tandem PCR: gene profiling from small amounts of RNA using SYBR Green detection. *Nucleic Acids Res* 2005;**33**:e180.
20. Ang KK, Berkey BA, Tu X, et al. Impact of epidermal growth factor receptor expression on survival and pattern of relapse in patients with advanced head and neck carcinoma. *Cancer Res* 2002;**62**:7350–6.
21. Bentzen SM, Atasoy BM, Daley FM, et al. Epidermal growth factor receptor expression in pretreatment biopsies from head and neck squamous cell carcinoma as a predictive factor for a benefit from accelerated radiation therapy in a randomized controlled trial. *J Clin Oncol* 2005;**23**: 5560–7.
22. Bourhis J, Overgaard J, Audry H, et al. Hyperfractionated or accelerated radiotherapy in head and neck cancer: a meta-analysis. *Lancet* 2006;**368**:843–54.
23. Olopade OI, Grushko TA, Nanda R, Huo D. Advances in breast cancer: pathways to personalized medicine. *Clin Cancer Res* 2008;**14**:7988–99.
24. Johnston A, Salles G. Prognostic systems for lymphomas. *Hematol Oncol Clin North Am* 2008;**22**:839–61. viii.
25. Hong A, Grulich A, Jones D, et al. Squamous cell carcinoma of the oropharynx in Australian males induced by human papillomavirus vaccine targets. *Vaccine* 2010;**28**: 3269–72.
26. Nasman A, Attner P, Hammarstedt L, et al. Incidence of human papillomavirus (HPV) positive tonsillar carcinoma in Stockholm, Sweden: an epidemic of viral-induced carcinoma? *Int J Cancer* 2009;**125**:362–6.