CANCER BIOLOGY & THERAPY
2016, VOL. 17, NO. 2, 181–187
http://dx.doi.org/10.1080/15384047.2015.1108489

Taylor & Francis
Taylor & Francis Group

RESEARCH PAPER

E6 viral protein ratio correlates with outcomes in human papillomavirus related oropharyngeal cancer

Angela Hong[a,b], Xiaoying Zhang[c], Deanna Jones[c], Mei Zhang[a,b], C. Soon Lee[d], J. Guy Lyons[a], Anne-Sophie Veillard[e], and Barbara Rose[c]

[a]Central Clinical School, Sydney Medical School, The University of Sydney, NSW, Australia; [b]Department of Radiation Oncology, Lifehouse, NSW, Australia; [c]Department of Infectious Diseases and Immunology, Sydney Medical School, The University of Sydney, NSW, Australia; [d]Department of Anatomical Pathology, Royal Prince Alfred Hospital, Camperdown, NSW, Australia; [e]NHMRC Clinical Trials Center, The University of Sydney, NSW, Australia

ABSTRACT

Background: The study aimed to identify prognostic markers to improve the management of patients with HPV positive OSCC Methods: We determined the ratio of HPV E6*I and E6*II splice variants by quantitative RT-PCR in 177 HPV positive OSCC and correlated the findings with other clinicopathological data Results: There was no significant difference in locoregional recurrence (HR 1.72 p = 0.24) and death (HR 1.65, p = 0.13) among patients whose tumors had an E6*I/*II ratio ≥1 compared with an E6*I/*II ratio of <1. Univariate analysis showed that patients with E6*I/*II ≥1 OSCC were more likely to have an event. In the multivariable analysis, there was a trend for more events in patients with E6*I/*II ratio ≥1 (HR 1.70, 95% CI 0.95-3.03, p = 0.07) Conclusion: Our data suggest that the use of HPV 16 spliced transcripts may help to predict for poorer outcomes in patients with HPV positive OSCC.

ARTICLE HISTORY
Received 20 May 2015
Revised 25 September 2015
Accepted 11 October 2015

KEYWORDS
E6; head and neck cancer; human papillomavirus; oropharyngeal cancer; p16

Introduction

Human papillomavirus (HPV) infection is now the main etiology of oropharyngeal squamous cell cancer (OSCC) in developed countries.[1,2] More than 90% of HPV positive OSCC is caused by the HPV type 16 genotype.[3] The prognostic significance of HPV in OSCC is well established, with HPV positive OSCC having significantly better outcomes after adjusting for other known prognostic variables.[4,5] The better outcome seen in HPV positive OSCC has been attributed to better response to chemotherapy and radiation therapy.[6] Despite the better prognosis, the 3-year locoregional recurrence rate for HPV positive OSCC is still 13.6% and the overall survival is 82%.[4] A more refined prognostic marker for HPV positive OSCC is needed to guide treatment.

HPV type 16 has 2 splice acceptor sites. The HPV 16 E6 gene encodes E6*I and E6*II transcripts as well as the full length E6 mRNA. The unspliced E6 transcript gives rise to a 19 kDa protein containing 2 putative zinc fingers, whereas E6*I and E6*II give rise to nearly identical 6 kDa proteins truncated within the first zinc finger domain.[7] Our previous study transfecting E6 isoforms individually or in combination into HPV negative OSCC cells showed that E6*I had the greatest radiosensitising effect and E6*II had no effect at all.[8] This suggested that E6*I may have a role in the radioresponsiveness of HPV positive OSCC and raised the possibility that E6*I level may predict clinical outcome. In support of the importance of E6 isoforms, cervix cancer studies have shown that higher grade cancers are more likely to have a higher E6*I/E6*II ratio.[9]

The role of established OSCC prognostic markers such as epidermal growth factor receptor (EGFR) and cyclin D1 as modifiers of the effect of HPV on outcome in OSCC also warrants further investigation. In HPV positive OSCC, the p53 and pRb pathways are inactivated by the viral oncoproteins. Retinoblastoma (pRb) pathway proteins (p16, cyclin D1 and pRb) have also shown potential as prognostic markers in head and neck cancer. In our previous study, patients with HPV positive and cyclin D1-positive cancers had up to an eightfold increased risk of poor outcome relative to those with HPV positive and cyclin D1-negative tumors. Others have reported similar findings.[10] EGFR is abnormally activated in approximately 80% of head and neck cancers.[11] EGFR expression has been associated with poorer prognosis in head and neck cancer, although previous study indicated that the effect of EGFR on outcome in OSCC may be limited to HPV negative OSCC.[12]

This study aimed to identify additional prognostic markers that could be used in conjunction with the existing clinicopathological markers to improve the management of patients with HPV positive OSCC. Our particular interest was in the role of E6*I/E6*II ratio to predict outcome and its relationships with cyclin D1 and EGFR expression.

Results

Patient characteristics

The baseline characteristics of the 177 patients are shown in Table 1. The median age at diagnosis was 55.3 years and 31.3 % were never smokers at the time of diagnosis. The primary

CONTACT Angela Hong angela.hong@sydney.edu.au

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

	All Patients (N = 177)	E6*I/*II ratio <1 (N = 73)	E6*I/*II ratio ≥ 1 (N = 104)	P-value for heterogeneity
Mean age at diagnosis (range)	55.3 (31-89)	55.2 (37-86)	55.4 (31-89)	0.91
Gender				0.88
- F	33 (18.6%)	14 (19.2%)	19 (18.3%)	
- M	144 (81.4%)	59 (80.8%)	85 (81.7%)	
Smoking status (Missing = 33)				0.63
- Non-Smoker	45 (31.3%)	22 (35.5%)	23 (28.0%)	
- Ex-Smoker	52 (36.1%)	21 (33.9%)	31 (37.8%)	
- Current Smoker	47 (32.6%)	19 (30.6%)	28 (34.1%)	
Alcohol status (Missing = 65)				*
- Non-Drinker	20 (17.9%)	12 (23.1%)	8 (13.3%)	
- Ex-Drinker	5 (4.5%)	1 (1.9%)	4 (6.7%)	
- Drinker	87 (77.7%)	39 (75.0%)	48 (80.0%)	
T stage (Missing = 2)				0.44
- 1	50 (28.6%)	24 (32.9%)	26 (25.5%)	
- 2	57 (32.6%)	22 (30.1%)	35 (34.3%)	
- 3	47 (26.9%)	21 (28.8%)	26 (25.5%)	
- 4	21 (12.0%)	6 (8.2%)	15 (14.7%)	
N stage				0.25
- 0	34 (19.2%)	18 (24.7%)	16 (15.4%)	
- 1	30 (16.9%)	13 (17.8%)	17 (16.3%)	
- 2-3	113 (63.8%)	42 (57.5%)	71 (68.3%)	
TNM Stage				0.23
- 1-2	17 (9.6%)	9 (12.3%)	8 (7.7%)	
- 3	36 (20.3%)	18 (24.7%)	18 (17.3%)	
- 4	124 (70.1%)	46 (63.0%)	78 (75.0%)	
Grade				0.87
- 1-2	74 (41.8%)	30 (41.1%)	44 (42.3%)	
- 3	103 (58.2%)	43 (58.9%)	60 (57.7%)	
Primary tumour tumor site				*
- Tonsil	155 (87.6%)	68 (93.2%)	87 (83.7%)	
- Base of Tongue	19 (10.7%)	3 (4.1%)	16 (15.4%)	
- Other Oropharyngeal site	3 (1.7%)	2 (2.7%)	1 (1.0%)	
Treatment				
- Radiotherapy +/− Chemo	85 (48.0%)	31 (42.5%)	54 (51.9%)	
- Surgery +/− Adjuvant Radiotherapy +/− chemo	92 (52.0%)	42 (57.5%)	40 (48.1%)	
EGFR (Missing = 1)				0.60
- Positive	126 (71.6%)	50 (69.4%)	76 (73.1%)	
- Negative	50 (28.4%)	22 (30.6%)	28 (26.9%)	
Cyclin D1				0.41
- Positive	62 (35.0%)	23 (31.5%)	39 (37.5%)	
- Negative	115 (65.0%)	50 (68.5%)	65 (62.5%)	

* no test was performed as the numbers were too small.

tumor was located in the tonsil in 87.6% of the cases and the majority presented with stage 3 or 4 disease. Definitive radiation therapy with or without concurrent chemotherapy was used in 48% of the patients. For those who were treated with chemotherapy, the main agents were cisplatin and 5-Fu. No patient was treated with cetuximab. Immunohistochemistry showed that 71.6% of the tumors were positive for EGFR and 35% were positive for cyclin D1. There was no significant difference in the clinico-pathological characteristics by EGFR and cyclin D1 status (Table 1 and 2). Patients were divided into those with E6*I/*II <1 (n = 73, 41.2%) or ≥1 (n = 104, 58.8%). The baseline characteristics of the 2 groups were similar with no statistically significant difference between the 2 groups by patient characteristics such as gender, age, smoking and alcohol intake status.(Table 1)

*Effect of E6*I/*II ratio on outcomes*

Loco-regional outcome data was incomplete in 5 patients. Overall loco-regional failure occurred in 12.8% of patients with failure at the primary site in 14 patients and in the regional nodal region (with disease controlled at the primary site) in 8 patients (Table 2). There were 57 (32.2%) events and 42 (23.7%) deaths from any cause.

There were more events among patients whose tumors had an E6*I/*II ratio ≥1 compared with an E6*I/*II ratio of <1 with the Kaplan-Meier estimate of 5-year event rates of 40% vs 26% (Fig. 1). Similarly there were non-significant differences in locoregional recurrence and death between the 2 groups. There were only 22 locoregional recurrences in total and the Kaplan-Meier estimate 5-year event rates of 17% vs 11%. Only 42

Table 2. Event rates at 5 year by E6*I/*II ratio and the Kaplan-Meier estimate of 5-year event rates are shown in square brackets.

	All Patients (n=177)	E6*I/*II ratio <1	E6*I/*II ratio ≥ 1 (n=104)
Locoregional recurrence	22 (12.8%) [14%]	7 (9.6%) [11%]	15 (15.2%) [17%]
Event (recurrence or death)	57 (32.2%) [34%]	17 (23.3%) [26%]	40 (38.5%) [40%]
Death	42 (23.7%) [23%]	13 (17.8%) [19%]	29 (27.9%) [26%]

deaths (31 died of this cancer and 11 were non cancer related deaths) were observed among the 177 patients and those with tumors having E6*I/*II ratio >1 were more likely to die (the Kaplan-Meier estimate 5-year death rate 26% vs 19%).

Univariate analysis showed that patients with E6*I/*II ≥1 OSCC were more likely to have an event than those with E6*I/*II <1 OSCC (Table 3B). After adjusting for confounders, there was a non-statistically significant trend in higher risk of event for patients with E6*I/*II ratio ≥1 (HR 1.70, 95% CI 0.95-3.03, p=0.07). E6*I/*II ratio did not predict locoregional recurrence or death on multivariable analysis (Tables 3A and 3C). N stage and EGFR status were strong predictors of locoregional recurrence. Gender and T stage were predictors of event-free survival. In term of overall survival, T stage and EGFR status were strong independent predictors.

Table 3A. Multivariable associations of patient and disease characteristics with risk of locoregional recurrence.

		Univariate		Multivariable*	
Characteristic	Category	HR (95% CI)	P-value	HR (95% CI)	P-value
Age at diagnosis	<60	1.00	0.19		
	≥60	0.48 (0.16, 1.42)			
Gender	Male	1.00	0.08		
	Female	0.17 (0.02, 1.27)			
Grade	1,2	1.00	0.35		
	3	0.67 (0.29, 1.54)			
T Stage	1	1.00	0.07		
	2	1.26 (0.30, 5.28)			
	3	3.20 (0.87, 11.85)			
	4	4.43 (1.06, 18.55)			
N Stage	0	1.00	0.06	1	0.033
	1	1.73 (0.56, 5.30)		1.71 (0.55, 5.31)	
	2,3	0.54 (0.18, 1.61)		0.47 (0.15, 1.43)	
EGFR	Negative	1.00	0.034	1	0.035
	Positive	8.77 (1.18, 65.33)		8.72 (1.17, 65.09)	
Cyclin D1	Negative	1.00	0.66		
	Positive	0.81 (0.32, 2.07)			
E6*I/*II ratio	<1	1.00	0.24	1	0.17
	≥1	1.72 (0.70, 4.23)		1.94 (0.75, 5.02)	

* Multivariable model is adjusted for age, gender, T stage, N stage, EGFR, treatment and E6*I/*II ratio.

Table 3B. Multivariable associations of patient and disease characteristics with risk of an event (recurrence or death).

		Univariate		Multivariable*	
Characteristic	Category	HR (95% CI)	P-value	HR (95% CI)	P-value
Age at diagnosis	<60	1.00	0.29		
	≥60	1.34 (0.78, 2.31)			
Gender	Male	1.00	0.034	1.00	0.020
	Female	0.37 (0.15, 0.93)		0.33 (0.13, 0.84)	
Grade	1,2	1.00	0.57		
	3	0.86 (0.51, 1.46)			
T Stage	1	1.00	0.005	1.00	0.004
	2	1.33 (0.56, 3.17)		1.41 (0.59, 3.38)	
	3	2.95 (1.31, 6.64)		2.98 (1.33, 6.70)	
	4	3.56 (1.43, 8.86)		3.90 (1.55, 9.86)	
N Stage	0	1.00	0.28		
	1	0.66 (0.29, 1.50)			
	2,3	0.61 (0.33, 1.13)			
EGFR	Negative	1.00	0.06		
	Positive	1.93 (0.97, 3.83)			
Cyclin D1	Negative	1.00	0.20		
	Positive	1.42 (0.83, 2.42)			
E6 ratio	<1	1.00	0.053	1.00	0.07
	≥1	1.76 (0.99, 3.11)		1.70 (0.95, 3.03)	

* Multivariable model is adjusted for age, gender, T stage, N stage, treatment and E6*I/*II ratio.

Table 3C. Multivariable associations of patient and disease characteristics with risk of death from any cause.

		Univariate		Multivariable*	
Characteristic	Category	HR (95% CI)	P-value	HR (95% CI)	P-value
Age at diagnosis	<60	1.00	0.09		
	≥60	1.73 (0.92, 3.26)			
Gender	Male	1.00	0.045		
	Female	0.30 (0.09, 0.97)			
Grade	1,2	1.00	0.24		
	3	0.69 (0.38, 1.28)			
T Stage	1	1.00	0.007	1.00	0.022
	2	1.84 (0.58, 5.90)		1.97 (0.61, 6.32)	
	3	4.55 (1.54, 13.47)		3.98 (1.34, 11.85)	
	4	4.83 (1.45, 16.07)		4.75 (1.41, 15.98)	
N Stage	0	1.00	0.11		
	1	0.49 (0.19, 1.30)			
	2,3	0.50 (0.25, 0.99)			
EGFR	Negative	1.00	0.045	1.00	0.037
	Positive	2.44 (1.02, 5.82)		2.56 (1.06, 6.19)	
Cyclin D1	Negative	1.00	0.10		
	Positive	1.68 (0.91, 3.13)			
E6 ratio	<1	1.00	0.13	1.00	0.26
	≥1	1.65 (0.86, 3.19)		1.50 (0.75, 3.00)	

* Multivariable model is adjusted for age, gender, T stage, N stage, EGFR, treatment and E6*I/*II ratio.

Discussion

This is the first study to examine the prognostic value of the E6 spliced transcripts in HPV type 16 positive OSCC. Type 16 is the main causative agent of HPV positive OSCC. Our previous data showed that up to 96% of HPV positive OSCC were related to HPV Type 16, therefore the result of this study is applicable to the vast majority of HPV positive OSCC.[3] The better outcome seen in HPV positive OSCC compared with HPV negative OSCC is well established.[13,14] However, further refinement of the prognostic value of HPV positivity is needed. Patients with HPV positive OSCC are known to have a better prognosis than patients with HPV negative OSCC, therefore there were fewer events and death in our study cohort. Nevertheless our data suggest that determination of E6*I/*II ratio might identify patients with HPV positive OSCC who are at higher risk of an event. Higher rates of locoregional recurrence and events were observed in patients E6*I/*II ≥1 OSCC as well as worse overall survival, however these results were not statistically significant.

The incidence of OSCC is increasing in Western countries despite declining tobacco use.[2,15-18] This is attributable to a rising incidence of HPV positive OSCC with up to 70% of OSCC caused by HPV and type 16 accounting for more than 90% of the cases.[14] The oncogenicity of HPV is directly related to the expression of E6 and E7. The E6 oncoprotein initiates degradation of the tumor suppressor protein p53.[19] and the E7 oncoprotein can cause inactivation of the retinoblastoma gene.[20] Together they cause a loss in cell cycle control. Except for type 16, all other high risk HPVs have only a single splice acceptor. In addition to the full length E6 mRNA, the type 16 HPV E6 gene encodes E6*I and E6*II transcripts by alternative splicing

within the E6 open reading frame. These transcripts utilize the same splice donor site at nucleotide 226 but different splice acceptor sites at nucleotide 409 and nucleotide 526. The primary role of splicing events is unclear. A recent publication by Ajiro et al. suggested that the novel splice form of E6 isoform can enhance cell proliferation and therefore make type 16 a particularly carcinogenic type of HPV.[21] In cervical cancer cell lines, splicing may help with the translation of the E7 oncoprotein.[22] Our previous in vitro study of OSCC cell lines showed that the different isoforms of E6 affect the radiosensitivity by different degrees, with E6*I providing greatest radiosensitisation and E6*II having no effect.[8] Based on these in vitro data, we expected that a high ratio of E6*I would correlate with radiosensitivity clinically and hence give better clinical outcomes. In contrast, this current study suggested that a higher E6*I/*II ratio may be a predictor of poorer outcomes. It is possible that differences between our in vitro data and the current study reflect limitations of in vitro studies. Apart from the radiosensitising effect, E6 isoforms could have other adverse effects on tumor progression and treatment response. It has been suggested that E6 might contribute to immunosuppression as it inhibits monocyte differentiation to Langerhans cells, which is responsible for induction of T cell-dependent immunity.[23]

The mechanism underpinning the poorer outcomes associated with a higher E6*I/*II ratio in this study is unknown. In one recent study, high grade cervix cancers had a higher E6*I/*II ratio than low grade cancers.[9] The authors concluded that the detection of E6*I/E6*II mRNAs may help to predict disease progression. However, the effect of E6*I/*II ratio on outcomes of cervical cancer was not reported. Nonetheless, the adverse effect of a high E6*I/E6*II ratio is consistent with results of the current study. There has been no other published study on the prognostic value of E6*I/E6*II ratio in OSCC. Our testing focused on the primary tumor, it is not known whether there is a concordance between primary tumors and metastatic sites.

Several previous studies have tried to identify additional prognostic factors in HPV positive OSCC. Our previous study on EGFR provided weak evidence that the effects of EGFR on outcome were limited to people with HPV negative OSCC.[12] Our current study showed that EGFR expression was a prognostic marker for locoregional recurrence and overall survival. Despite the inverse relationship between HPV and EGFR, it seems likely that the molecular pathways are independent. Both Kumar et al.[24,25] and Kong et al.[24,25] found that a combination of EGFR and HPV was useful for stratifying disease specific survival in head and neck cancer. Reimers et al.[26] also concluded that testing for p16 in conjunction with EGFR had significant prognostic implications. The increased risk also applied to survival, but the effect was not as strong. In cervical cancer, splicing of HPV16 E6 is regulated by the EGFR pathway. The inverse relationship between HPV status and cyclin D1 has been well documented.[10] It was therefore noteworthy that in the current study, cyclin D1 was not a significant prognostic factor after adjusting for E6*I/E6*II ratio in addition to known prognostic variables.

In conclusion, the detection of HPV 16 spliced transcripts may identify prognostic markers of poorer outcomes in patients with HPV type 16 positive OSCC and help future clinical trials design to improve the outcome of HPV positive OSCC. Due to few events in HPV positive OSCC, the results of the current study should be examined in a larger cohort.

Materials and methods

Study population

A total of 177 patients with stage 1-4 HPV type 16 OSCC treated with curative intent between 1985-2011 were included in the study. Ethics approval was obtained from the Sydney Local Health Service ethics committees (Protocols X12-0141 and HREC/08/RPAH/162). Demographic and clinicopathological data were retrieved from our prospective databases and medical records. Selection criteria included availability of primary tumor, baseline clinicopathological data and follow-up data. A HPV-positive cancer was defined as one testing positive for HPV DNA and with p16ink4 (p16) overexpression on immunohistochemistry.[27,28] Patients were followed up for the occurrence of an event (recurrence in any form or death from any cause) for a median of 62 months after diagnosis. The study pathologist confirmed the histology, and tumor grade, and scored the immunohistochemistry blinded to the outcomes. Cancers were staged using the American Joint Committee in Cancer Staging System 7th edition.

EGFR and cyclin D1 expression were determined by semi-quantitative immunohistochemistry using PharmDx^{TM} Kit for autostainer (Dako) and clone SP4 (Neomarkers) respectively.[10,12] EGFR positivity was defined as membrane staining of at least moderate intensity in 10% of cancer cells.[12] Cyclin D1 positivity was defined as nuclear staining of at least moderate intensity in 10% of cancer cells.[10]

Detection of HPV E6*I and E6*II splice variants by quantitative RT-PCR

Four to 8 sections of 10μm formalin fixed paraffin embedded samples were used for RNA extraction with RNeasy FFPE kit (Qiagen) according to manufacturer's instructions. RNA extracts were quantified and assessed using NanoDrop ND 1000 (Thermo Fisher Scientific, USA). To determine the relative expression levels of E6*I and E6*II, a 2-step Cyber Green qRT-PCR was performed using Rotor-Gene 6000 machine (Corbett Life Science). HPV 16 E6*I and E6*II specific primers were designed according to the literature with some modifications.[9] The forward primer for both E6*I and E6*II mRNA was 5′CAGTTATGCACAGAGCTGCA (nucleotide 121 to 139, GeneBank Accesion Number K02718). The reverse primers for E6*I and E6*II were 5′–GACAGTTAATACACCTCACGT and 5′–CGTGTTCTTATGATCTCACGT, which generated a 120bp and a 123 base pair product respectively. A B2M internal control gene specific primer set was purchased from Qiagen (PN: 249900).

A SuperScript^{TM}III Reverse Transcriptase kit (ABI, USA) was used for all cDNA syntheses. Each 20μl reaction mix contained 4μl of 5x FS buffer, 1μl of 10mM dNTP mix, 0.2μl of RNase inhibitor, 1 μl of 0.1M DTT, 650ng of total RNA, 300ng of the primer mix, and 1.0μl of Superscript^{TM} III Reverse Transcriptase. The cycle conditions were as suggested were suggested by

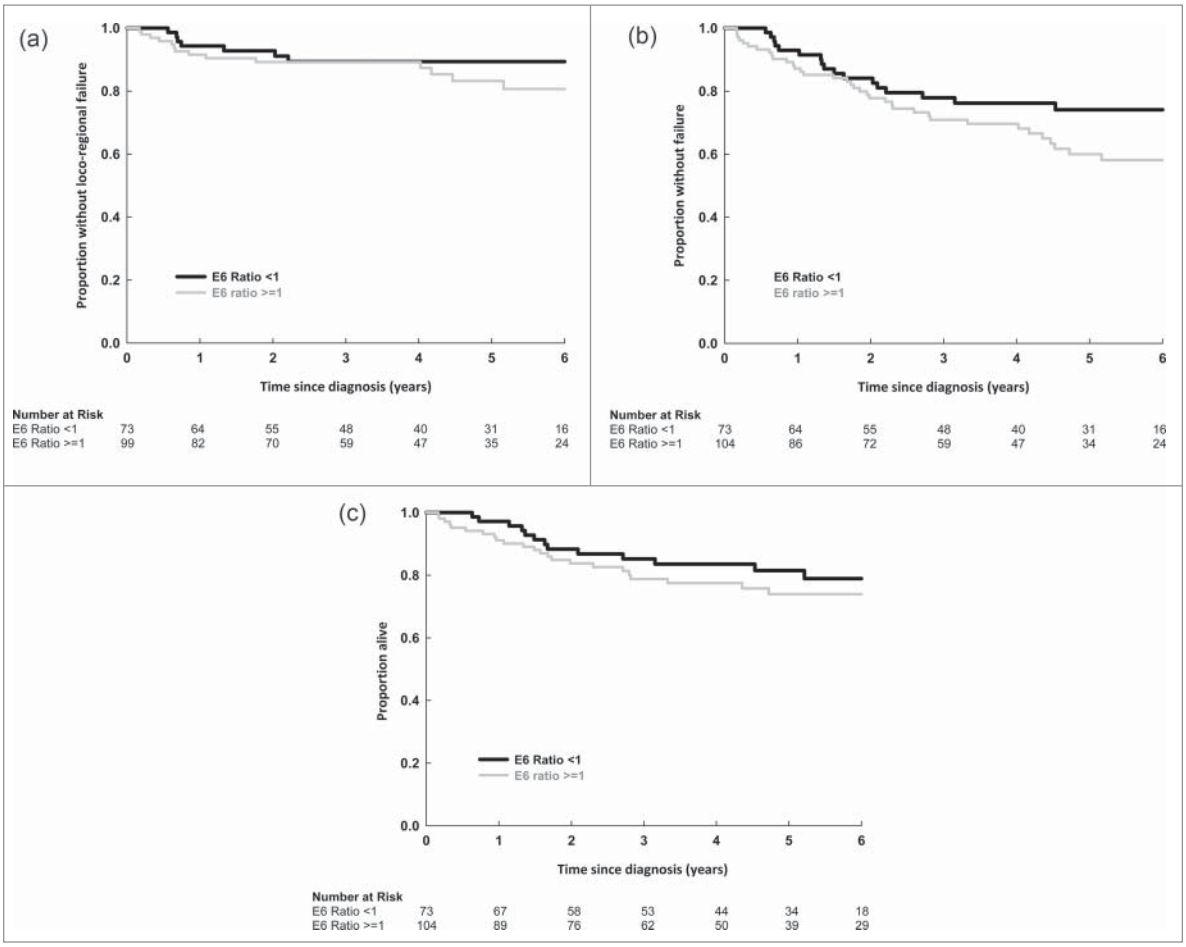

Figure 1A. Probability of remaining free of loco-regional recurrence by E6*I/*II ratio.

the manufacturer: 1) 25°C for 5 minutes, 2) 55°C for 60 minutes, 3) 70°C for 15 minutes, and 4) hold at 4°C. The resulting product was diluted at 1: 4 ratio with nuclease-free water stored at −20°C and used as a template in downstream qPCR.

E6*I and E6*II RNA transcripts were measured using the Cyber-Green assay according to the manufacturer's instruction. Each 10μl qPCR reaction mix contained 5μl of 2x Cyber-Green Master Mix (Invitrogen, USA), 4μl of 1 in 4 diluted cDNA products and each gene specific primer at a final concentration of 0.4μM. All reactions were conducted in duplicate, and a non-template control was included in each run. The cycle condition were optimised particularly for the E6*I and E6*II amplicons: 95°C for 10 minutes, followed by 40 cycles of 95°C for 10 seconds, 41°C for 15 seconds and 68°C for 20 seconds. The data were acquired at 68°C.

Relative expression level of a gene was calculated as described.[29] PCRs with similar efficiencies were judged by having similar amplification plots and then selected for the down-stream analysis. Mean Ct values were normalized using B2M and indicated as the Δ mean Ct. The relative expression level of a given gene was calculated using $2^{-\Delta}$ and presented as fold change. The E*I/E6*II fold change ratio was calculated.

Statistical methods

Categorical variables were compared with chi-square tests. Times to loco-regional failure, any event (recurrence or death) and death from any cause were calculated from date of diagnosis. For the analysis of time to loco-regional failure (defined as clinical, radiological and/or pathological evidence of recurrence at the primary site or in the regional nodal area), patients were censored at last follow-up, death or distal recurrence where applicable. Five patients were excluded as they had incomplete information on recurrence. For the analysis of time to death from any cause, patients were censored at last follow-up if they

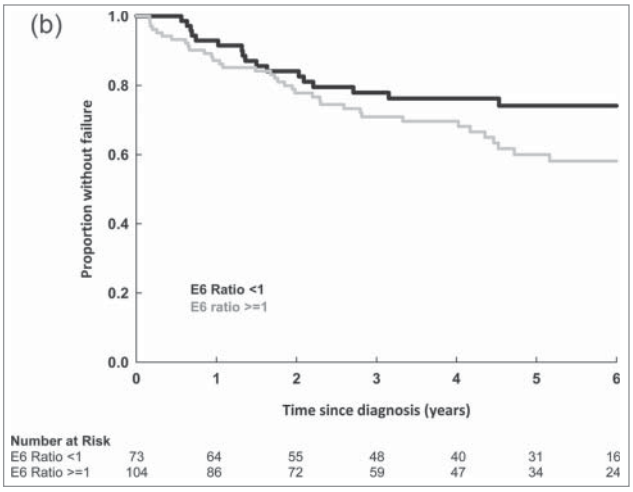

Figure 1B. Event free survival by E6*I/*II ratio.

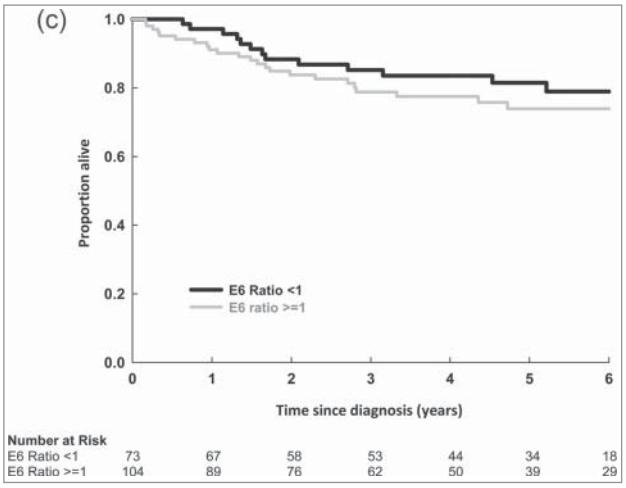

Figure 1C. Overall survival by E6*I/*II ratio.

were not known to have died. For the analysis of event-free survival, patients without events were censored at the date of last follow-up. An event was defined as recurrence of the OSCC in any form or death from any cause, with only the first event taken into account. Time-to-event analyses were undertaken using Cox proportional hazards regression modeling. Selection of predictor variables in the multivariable model was performed using the backward elimination method with E6*I/*II ratio forced into the final model.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Funding

The authors would like to thank the following funding bodies for their supports: Royal Australian and New Zealand College of Radiologists, Cancer Council NSW and Priority-driven Collaborative Cancer Research Scheme co-funded by the Cure Cancer Australia Foundation and the Australian Government through Cancer Australia.

References

1 Chaturvedi AK, Anderson WF, Lortet-Tieulent J, Curado MP, Ferlay J, Franceschi S, Rosenberg PS, Bray F, Gillison ML. Worldwide trends in incidence rates for oral cavity and oropharyngeal cancers. J Clin Oncol 2013; 31:4550-9; PMID:24248688; http://dx.doi.org/10.1200/JCO.2013.50.3870

2 Hong AM, Grulich AE, Jones D, Lee CS, Garland SM, Dobbins TA, Clark JR, Harnett GB, Milross CG, O'Brien CJ, et al. Squamous cell carcinoma of the oropharynx in australian males induced by human papillomavirus vaccine targets. Vaccine 2010; 28:3269-72; PMID:20226244; http://dx.doi.org/10.1016/j.vaccine.2010.02.098

3 Hong AM, Martin A, Armstrong BK, Lee CS, Jones D, Chatfield MD, Zhang M, Harnett G, Clark J, Elliott M, et al: Human papillomavirus modifies the prognostic significance of t stage and possibly n stage in tonsillar cancer. Ann Oncol 2013; 1:215-9; PMID:22887467; http://dx.doi.org/10.1093/annonc/mds205

4 Ang KK, Harris J, Wheeler R, Weber R, Rosenthal DI, Nguyen-Tan PF, Westra WH, Chung CH, Jordan RC, Lu C, et al. Human papillomavirus and survival of patients with oropharyngeal cancer. N Engl J Med 2010; 363:24-35; PMID:20530316; http://dx.doi.org/10.1056/NEJMoa0912217

5 Hong AM, Dobbins TA, Lee CS, Jones D, Harnett GB, Armstrong BK, Clark JR, Milross CG, Kim J, O'Brien CJ, et al. Human papillomavirus predicts outcome in oropharyngeal cancer in patients treated primarily with surgery or radiation therapy. Br J Cancer 2010; 103:1510-7; PMID:20959828; http://dx.doi.org/10.1038/sj.bjc.6605944

6 Fakhry C, Westra WH, Li S, Cmelak A, Ridge JA, Pinto H, Forastiere A, Gillison ML: 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; PMID:18270337; http://dx.doi.org/10.1093/jnci/djn011

7 Vaeteewoottacharn K, Chamutpong S, Ponglikitmongkol M, Angeletti PC: Differential localization of hpv16 e6 splice products with e6-associated protein. Virol J 2005; 2:50; PMID:15960845; http://dx.doi.org/10.1186/1743-422X-2-50

8 Pang E, Delic NC, Hong A, Zhang M, Rose BR, Lyons JG: Radiosensitization of oropharyngeal squamous cell carcinoma by human papillomavirus 16 oncoprotein e6 *i. Int J Radiat Oncol Biol Phys 2011; 79:860-5; PMID:21106305; http://dx.doi.org/10.1016/j.ijrobp.2010.06.028

9 Cricca M, Venturoli S, Leo E, Costa S, Musiani M, Zerbini M: Molecular analysis of hpv 16 e6i/e6ii spliced mrnas and correlation with the viral physical state and the grade of the cervical lesion. J Med Virol 2009; 81:1276-82; PMID:19475620; http://dx.doi.org/10.1002/jmv.21496

10 Hong AM, Dobbins TA, Lee CS, Jones D, Fei J, Clark JR, Armstrong BK, Harnett GB, Milross CG, Tran N, et al. Use of cyclin d1 in conjunction with human papillomavirus status to predict outcome in oropharyngeal cancer. Int J Cancer 2010; 128:1532-45; PMID:20503270; http://dx.doi.org/10.1002/ijc.25479

11 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; PMID:16309541; http://dx.doi.org/10.1111/j.0959-9673.2005.00447.x

12 Hong A, Dobbins T, Lee CS, Jones D, Jackson E, Clark J, Armstrong B, Harnett G, Milross C, O'Brien C, et al. Relationships between epidermal growth factor receptor expression and human papillomavirus status as markers of prognosis in oropharyngeal cancer. Eur J Cancer 2010; 46:2088-96; PMID:20537890; http://dx.doi.org/10.1016/j.ejca.2010.04.016

13 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; PMID:17179099; http://dx.doi.org/10.1200/JCO.2006.07.1829

14 Hong AM, Martin A, Chatfield M, Jones D, Zhang M, Armstrong B, Lee CS, Harnett G, Milross C, Clark J, et al. Human papillomavirus, smoking status and outcomes in tonsillar squamous cell carcinoma. Int J Cancer 2012; 132:2748-54; PMID:23180456; http://dx.doi.org/10.1002/ijc.27956

15 Chaturvedi AK, Engels EA, Anderson WF, Gillison ML. Incidence trends for human papillomavirus-related and -unrelated oral squamous cell carcinomas in the united states. J Clin Oncol 2008; 26:612-9; PMID:18235120; http://dx.doi.org/10.1200/JCO.2007.14.1713

16 Marur S, Forastiere AA: Head and neck cancer: Changing epidemiology, diagnosis, and treatment. Mayo Clin Proc 2008; 83:489-501; PMID:18380996

17 Hocking JS, Stein A, Conway EL, Regan D, Grulich A, Law M, Brotherton JM: Head and neck cancer in australia between 1982 and 2005 show increasing incidence of potentially hpv-associated oropharyngeal cancers. Br J Cancer 2011; 104:886-91; PMID:21285981; http://dx.doi.org/10.1038/sj.bjc.6606091

18 Chaturvedi AK, Engels EA, Pfeiffer RM, Hernandez BY, Xiao W, Kim E, Jiang B, Goodman MT, Sibug-Saber M, Cozen W, et al. Human papillomavirus and rising oropharyngeal cancer incidence in the united states. J Clin Oncol 2011; 29:4294-301; PMID:21969503; http://dx.doi.org/10.1200/JCO.2011.36.4596

19 Scheffner M, Werness BA, Huibregtse JM, Levine AJ, Howley PM. The e6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell 1990; 63:1129-36; PMID:2175676; http://dx.doi.org/10.1016/0092-8674(90)90409-8

20 Munger K, Phelps WC, Bubb V, Howley PM, Schlegel R. The e6 and e7 genes of the human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. J Virol 1989; 63:4417-21; PMID:2476573
21 Ajiro M, Zheng ZM: E6^e7, a novel splice isoform protein of human papillomavirus 16, stabilizes viral e6 and e7 oncoproteins via hsp90 and grp78. MBio 2015; 6:e02068-02014; PMID:25691589; http://dx.doi.org/10.1128/mBio.02068-14
22 Tang S, Tao M, McCoy JP, Jr., Zheng ZM: The e7 oncoprotein is translated from spliced e6*i transcripts in high-risk human papillomavirus type 16- or type 18-positive cervical cancer cell lines via translation reinitiation. J Virol 2006; 80:4249-63; PMID:16611884; http://dx.doi.org/10.1128/JVI.80.9.4249-4263.2006
23 Iijima N, Goodwin EC, Dimaio D, Iwasaki A: High-risk human papillomavirus e6 inhibits monocyte differentiation to langerhans cells. Virology 2013; 444:257-62; PMID:23871219; http://dx.doi.org/10.1016/j.virol.2013.06.020
24 Kumar B, Cordell KG, Lee JS, Worden FP, Prince ME, Tran HH, Wolf GT, Urba SG, Chepeha DB, Teknos TN, 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; PMID:18474878; http://dx.doi.org/10.1200/JCO.2007.12.7662
25 Kong CS, Narasimhan B, Cao H, Kwok S, Erickson JP, Koong A, Pourmand N, Le QT: 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; PMID:19427557; http://dx.doi.org/10.1016/j.ijrobp.2009.02.015
26 Reimers N, Kasper HU, Weissenborn SJ, Stutzer H, Preuss SF, Hoffmann TK, Speel EJ, Dienes HP, Pfister HJ, Guntinas-Lichius O, et al. Combined analysis of hpv-DNA, p16 and egfr expression to predict prognosis in oropharyngeal cancer. Int J Cancer 2007; 120(8):1731-8; PMID:17236202
27 Weinberger PM, Yu Z, Haffty BG, Kowalski D, Harigopal M, Brandsma J, Sasaki C, Joe J, Camp RL, Rimm DL, et al. Molecular classification identifies a subset of human papillomavirus–associated oropharyngeal cancers with favorable prognosis. J Clin Oncol 2006; 24:736-47; PMID:16401683; http://dx.doi.org/10.1200/JCO.2004.00.3335
28 Smeets SJ, Hesselink AT, Speel EJ, Haesevoets A, Snijders PJ, Pawlita M, Meijer CJ, Braakhuis BJ, Leemans CR, Brakenhoff RH: A novel algorithm for reliable detection of human papillomavirus in paraffin embedded head and neck cancer specimen. Int J Cancer 2007; 121:2465-72; PMID:17680565; http://dx.doi.org/10.1002/ijc.22980
29 Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative pcr and the 2(-delta delta c(t)) method. Methods 2001; 25:402-8; PMID:11846609; http://dx.doi.org/10.1006/meth.2001.1262