RESEARCH COMMUNICATION

High p16 Expression Predicts a Positive Response to Chemoradiotherapy in Stage IVa/b Head and Neck Squamous Cell Carcinoma

Yi-Ju Chen[1], Kun-Ming Rau[2,5], Chih-Yen Chien[3,5], Fu-Min Fang[4,5], Tai-Lin Huang[2,5], Tai-Jan Chiu[2,5]*

Abstract

Background: The objective of this study was to evaluate the effect of p16 expression on response to chemoradiation in stage IVa/b head and neck squamous cell carcinoma (HNSCC) patients. Methods: We retrospectively identified 64 patients with stage IVa/b HNSCC who received chemoradiation. Eligibility criteria included presence of biopsy-proven stage IVa/b HNSCC without a prior history of chemotherapy or radiotherapy. Immunohistochemistry was used to assess p16 protein expression in pretreatment biopsy specimens. Results: Of the 64 patients, 38 showed high p16 expression, and 50 patients responded to chemoradiotherapy, 32 exhibiting a complete and 18 a partial response. Response was significantly associated with p16 expression (P<0.001) and multivariate analysis indicated that that p16 expression (HR: 2.62, 95%C.I.: 1.14-6.06; P=0.024) was an independent prognostic factor for overall survival. Conclusions: High p16 expression predicts a better response to chemoradiation in patients with stage IVa/b HNSCC.

Keywords: Head and neck squamous cell carcinoma - p16 - chemoradiotherapy response

Asian Pacific J Cancer Prev, **12**, 649-655

Introduction

The etiology of head and neck squamous cell carcinoma (HNSCC) is multifactorial. Genetic factors, diet, occupational exposure, and life style have been implicated in the development of these cancers (Gillison, 2007). Although tobacco and alcohol consumption are traditionally considered the main risk factors in the majority of all HNSCC, infection with human papillomavirus (HPV) is thought to play a role in a subset of HNSCC (Gillison et al., 2008). HPV was detected more frequently in oropharyngeal cancers when compared with cancers in the oral cavity or other HNSCC subsites (Begum et al., 2005; Kreimer et al., 2005; Machado et al., 2010).

Unlike cervical cancer, HPV-16 is the most prevalent HPV type found in HNSCC (Gillison et al., 2000). HPV types 18, 31, 33, and 35 are rare (Kreimer et al., 2005; Licitra et al., 2006; Fakhry et al., 2008; Machado et al., 2010). Patients with HPV-positive HNSCCs are clinically distinct from their HPV-negative counterparts; they are more responsive to radiotherapy with or without chemotherapy despite often presenting with locoregionally advanced disease (Smith et al., 2004; Fakhry et al., 2008; Worden et al., 2008). The risk of death from HNSCC has been shown to be lower in these patients when compared with HPV-negative patients (Gillison et al., 2000). Since patients with HPV-positive HNSCCs differ in their response to therapy, knowledge of the HPV status of a patient may be a useful tool while choosing a particular treatment strategy. However, determining the presence of HPV in HNSCC remains challenging largely due to the expense and difficulty of the techniques employed.

p16, a tumor suppressor encoded by the CDKN2A gene is an important cell cycle regulatory protein and p16 mutations have been associated with increased risk of melanoma (Nobori et al., 1994; Stone et al., 1995). Immunohistochemical evaluation of p16 expression is often used as a surrogate for the presence of HPV (Shi et al., 2009), since p16 expression has been significantly associated with the presence of HPV-16 (Begum et al., 2005). In addition, since p16 was shown to be overexpressed in a high percentage of HPV-positive head and neck cancer patients, it has been proposed as a surrogate biomarker of HPV infection in these cases (Smith et al., 2010).

Treatment for HNSCC is highly complex, not only due the variety of tumor subsites, but also due to the anatomic constraints of the head and neck region and the importance of preserving organ function. A multidisciplinary approach that includes radiotherapy, chemotherapy, and surgery is commonly used to treat locoregionally advanced HNSCC (stages III or IVab). However, patients with unresectable, advanced HNSCC and those who are not surgical

*[1]Department of Pathology, [2]Department of Medical Oncology, [3]Department of Otolaryngology, [4]Department of Radiation Oncology, [5]Kaohsiung Chang Gung Head and Neck Oncology Group, Cancer Center, Chang Gung Memorial Hospital-Kaohsiung Medical Center, Kaohsiung, Taiwan *For correspondence: taijanchiu@163.com*

candidates have a poor prognosis (Adelstein et al., 2003; Agarwala et al., 2007). It has been reported that 87% of patients with oropharyngeal squamous cell carcinoma, who failed to respond to chemoradiation, died (Nichols et al., 2009).

Although p16 is a strong independent prognostic factor in patients with oropharyngeal squamous cell carcinoma (Kumar et al., 2008; Fischer et al., 2010a), the prognostic value of p16 in determining response to chemoradiation in HNSCC remains unclear. We undertook the present study to determine the correlation of p16 expression levels with response of locally advanced HNSCC tumors to chemoradiation. Specifically, p16 expression levels were evaluated immunohistochemically in tumors from patients with stage IVa/b HNSCC treated with chemotherapy and intensity-modulated radiation therapy.

Materials and Methods

Patients and Samples

In this study, we retrospectively analyzed 64 patients with locally advanced stage IVa or IVb HNSCC, who were treated with chemoradiotherapy at Chang Gung Memorial Hospital-Kaohsiung Medical Center in Kaohsiung, Taiwan between August 2008 and July 2009. All patients had histologically- and image-confirmed locally advanced HNSCC without evidence of distant metastases or a prior history of chemotherapy or radiotherapy. As part of the staging work-up, patients underwent the following examinations: complete physical examination, computed tomography (CT) or magnetic resonance imaging (MRI) of the head and neck, ultrasonography of liver, chest radiography, bone scan, and basic hematological and biochemical assessments.

Patient data collected included age at diagnosis, gender, betel nut use, smoking and drinking status, and American Joint Committee on Cancer (AJCC, the sixth edition) TNM stage as determined by computed tomography or magnetic resonance imaging analyses of the head and neck region. Smoking status was reported as never or ever smoked (including current and former smokers), and betel nut chewing was reported as never or ever chewed (including current and former chewers). Alcohol use was recorded as history of alcohol drinking or non-drinking. Informed consent was obtained from study participants and the study was approved by the Chang Gung Memorial Hospital Institutional Review Board, Kaohsiung, Taiwan.

HNSCC Treatment

All patients were treated with chemotherapy administered concurrently with radiation therapy. Chemotherapy included two cycles of induction chemotherapy with daily cisplatin (70 mg/m2) and 5-fluorouracil (700mg/m2) administered by continuous intravenous infusion over 4 days in a 28-day interval. Subsequent chemotherapy consisted of weekly doses of cisplatin (40 mg/m2). Radiotherapy was generally delivered as intensity-modulated radiation therapy administered five times weekly in daily fractions of 1.8 to 2 Gy for a total of 20-36 fractions using standard treatment protocols (Pignon et al., 2000; Pignon et al., 2009). Patients were evaluated weekly over the course of the treatment period. A head and neck CT or MRI scan was used to evaluate treatment response. Complete response (CR) was defined as the disappearance of all lesions; partial response (PR) was defined as an estimated 50% decrease in lesions (WHO criteria). Salvage surgery was advised when patients had a partial response.

Immunohistochemistry

Blocks containing pretreatment biopsies from the study participants were retrieved for p16 protein analysis. Sections of formalin-fixed paraffin-embedded tissues were cut to a thickness of 5 μm. Sections were deparaffinized, rehydrated and microwaved at 90°C for 15 minutes in 10 mM citrate buffer, pH 6.0 for antigen retrieval. Endogenous peroxidase activity was blocked with 3% H2O2. After rinsing, sections were incubated with a 1:500 dilution of a mouse monoclonal antibody specific for p16[INK4a] (BD Biosciences, San Jose, CA) for 60 min at room temperature followed by incubation with a secondary horseradish peroxidase-conjugated goat anti-mouse antibody (Dako Corp. Carpenteria, CA, USA) at room temperature for 1 h. Sections were immunoperoxidase labeled using the streptavidin-biotin peroxidase method (Super Sensitive Link-Label HRP Detection Systems, BioGenex Laboratories, San Ramon, CA) according to the manufacturer's instructions and then counterstained with hematoxylin.

All determinations of p16 expression were performed by the same pathologist. Strong nuclear and cytoplasmic staining of p16 in > 60% of tumor cells was regarded as high expression whereas low expression was defined as <60% of tumor cells staining positive for p16 (Figure 1) as previously described (Reimers et al., 2007).

Statistical Analysis

Correlation of clinical/histopathological characteristics and p16 expression levels were examined using Fisher's exact test for categorical variables, and data were represented by number (n) and percentage (%). Progression-free survival (PFS) was calculated from start of radiotherapy until the date of first distant relapse, local recurrence, regrowth, regional lymph node metastasis, death from any cause or the last follow-up examination. Overall survival (OS) was calculated from the start of radiotherapy until death of any cause or last follow-up

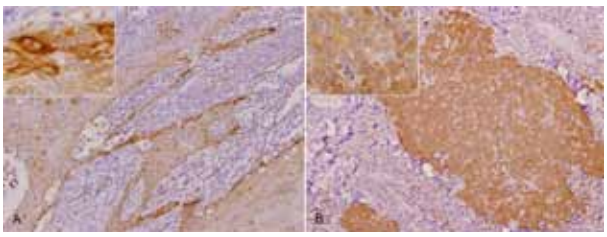

Figure 1. Analysis of p16 Expression in Head and Neck Squamous Cell Carcinoma. p16 expression was determined using immunohistochemistry. A) Low p16 expression (100× magnification). B) High p16 expression (100× magnification). The insets demonstrate cytoplasmic and nuclear staining of p16 (400× magnification)

Table 1. Analysis of Patient Characteristics by p16 Status (N=64)

	Total (n=64)	p16 Low (n=26)	p16 High (n=38)	p-value
Age (years)	53.0±9.2	51.1±8.5	54.3±9.5	0.166
Gender[2]				0.510
Male	62 (96.9)	26 (100)	36 (94.7)	
Female	2 (3.1)	0 (0.0)	2 (94.7)	
Site[3]				0.651
Oral cavity	27 (42.2)	12 (46.2)	15 (39.5)	
Oropharynx	24 (37.5)	8 (30.8)	16 (42.1)	
Hypopharynx	13 (20.3)	6 (23.1)	7 (18.4)	
Tumor Stage[2]				1.000
Iva	11 (17.2)	4 (15.4)	7 (18.4)	
IVb	53 (82.8)	22 (84.6)	31 (81.6)	
T stage, n (%)2				1.000
1 or 2	8 (12.5)	3 (11.5)	5 (13.2)	
3 or 4	56 (87.5)	23 (88.5)	33 (86.8)	
N stage[3]				0.847
0	14 (21.9)	6 (23.1)	8 (21.1)	
1, 2 or 3	50 (78.1)	20 (76.9)	30 (78.9)	
RTO dose (cGy)				
< 6000	24 (37.4)	8 (30.8)	16 (42.1)	0.358
≥ 6000	40 (62.5)	18 (69.2)	22 (57.9)	
Smoking, n (%)2				
Never	10 (15.6)	7 (26.9)	3 (7.9)	0.076
Ever	54 (84.4)	19 (73.1)	35 (92.1)	
Alcohol use[2]				
No	11 (17.2)	4 (15.4)	7 (18.4)	1.000
Drinking	53 (82.8)	22 (84.6)	31 (81.6)	
Betel nut, n (%)3				
Never	23 (35.9)	8 (30.8)	15 (39.5)	0.476
Ever	41 (64.1)	18 (69.2)	23 (60.5)	
Result, n (%)3				
CR+PR	50 (78.1)	15 (57.7)	35 (92.1)	0.001*
PD+SD	14 (21.9)	11 (42.3)	3 (7.9)	

P values are based on 1 independent two sample t test; 2 fisher's exact test and 3chi-square test; CR, complete response; PR, partial response; SD, stable disease; PD, disease progression; RTO, radiotherapy

Table 2. Univariate analysis for Progression-free and Overall Survival

	Progression-free			Overall		
	HR	(95% CI)	P-value	HR	(95% CI)	P-value
Age	0.98	(0.94, 1.02)	0.240	0.95	(0.91, 0.99)	0.024*
Site						
Oral	Ref			Ref		
Oroph	0.60	(0.27, 1.32)	0.204	0.61	(0.25, 1.45)	0.260
Hypoph	0.59	(0.23, 1.50)	0.266	0.24	(0.05, 1.05)	0.057
Tumor Stage						
Iva	Reference			Ref		
IVb	1.73	(0.61, 4.94)	0.305	5.98	(0.81, 44.3)	0.080
T stage						
1 or 2	Reference					
3 or 4	1.42	(0.43, 4.62)	0.565	1.75	(0.41, 7.46)	0.447
N stage						
0	Reference			Ref		
1, 2 or 3	1.05	(0.45, 2.43)	0.913	1.10	(0.41, 2.94)	0.854
RTO dose (cGy)						
< 6000	Reference			Ref		
≥ 6000	0.61	(0.30,1.23)	0.167	0.94	(0.41, 2.14)	0.874
Smoking						
Never	Reference					
Ever	1.46	(0.51, 4.16)	0.480	1.40	(0.42, 4.70)	0.584
Alcohol use						
No	Reference					
Yes	0.98	(0.40, 2.37)	0.956	1.14	(0.39, 3.34)	0.809
Betel nut						
Never	Reference					
Ever	1.23	(0.59, 2.56)	0.577	1.56	(0.65, 3.77)	0.319
P16 expression						
High	Reference					
Low	2.83	(1.38, 5.74)	0.004*	2.95	(1.29, 6.75)	0.011*

HR, Hazard ratio; CI, confidence interval; RTO, radiotherapy

examination. The survival rates were calculated by the Kaplan–Meier method and the differences between the survival curves were examined by the log-rank test.

Univariate Cox proportional hazards regressions were applied to estimate the individual hazard ratio (HR) for the PFS and OS. The significant variables in the univariate analyses (P<0.05) were taken for multivariate analysis. The HR with 95% confidence interval was measured to estimate the hazard risk of individual factors. A P-value of less than 0.05 was considered to be statistically significant. All statistical analyses were performed using the SPSS statistical software program (version 15.0; SPSS Inc., Chicago, IL).

Results

Association of patient characteristics with p16 expression

The average age at the time of diagnosis was 53.0±9.2 y (range 32-73 y). As shown in Table 1, we examined the correlation between patient characteristics and p16 expression levels. Of the 64 patients included in this study, 38 (59.4%) expressed high levels of p16. The primary tumor sites included the oral cavity (27 patients, 42.2%), oropharynx (24 patients, 37.5%), and hypopharynx (13 patients, 20.3%). However, the location of the primary tumor was not associated with p16 levels. Patient age at diagnosis, gender, T stage, N stage, smoking status, alcohol use, and betel nut chewing were not associated with p16 levels. Fifty patients (78.1%) responded to chemoradiotherapy; 32 patients (50.0%) had a complete response (CR), and 18 patients (28.1%) experienced a partial response (PR). We showed that response to chemoradiotherapy was significantly associated with p16 expression (P<0.001).

Survival analysis

The OS of the 64 HNSCC patients was 62.5% (40/64), with 24 deaths observed in the follow-up period. The univariate analysis for progression-free survival was shown in Table 2, in which the significant difference was only observed in between high vs. low p16 expression. We used univariate analysis to show that patients with localized HNSCC and low p16 expression levels had a significantly lower PFS than those with high p16 expression levels (P=0.002, Figure 2A). The OS was also significantly lower in patients with low p16 expression levels when compared with patients who had high p16 expression levels (P=0.006, Figure 2B). We additionally showed that only age (P=0.024) was associated with OS (Table 2).

Multivariate analysis was performed using the Cox

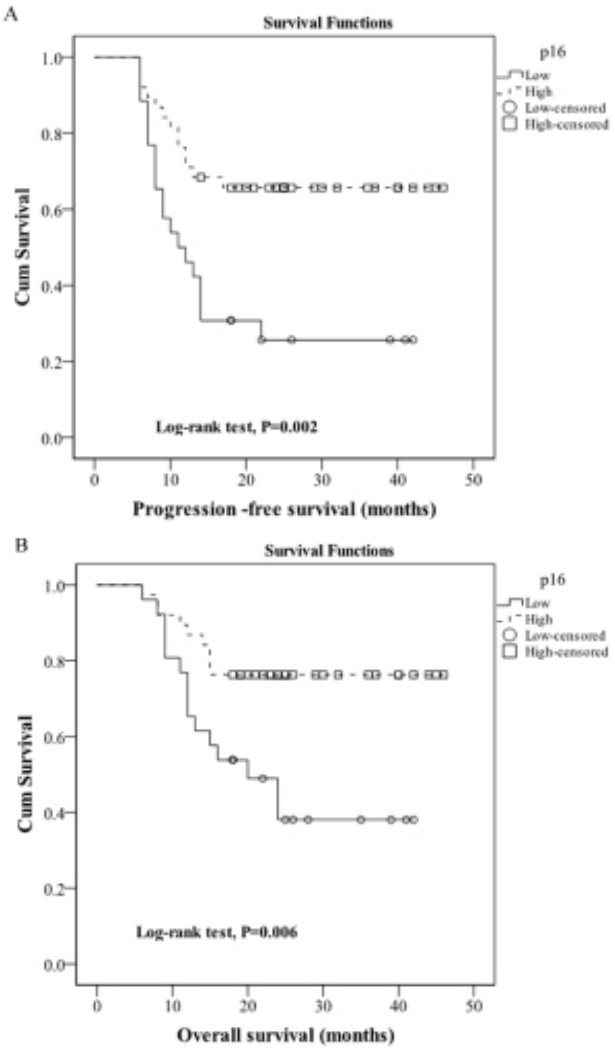

Figure 2. Correlation of p16 expression with survival curves of patients with locally advanced stage IVa or IVb HNSCC

proportional hazards model for all of the significant variables in the univariate analysis. The results from the multivariate analysis showed that p16 expression (HR: 2.62, 95%C.I.: 1.14-6.06; P=0.024) was an independent prognostic factor for OS.

Discussion

The present study was undertaken to determine the role of p16 expression in HNSCC stage and response to therapy. We showed that 55.8% of the patients enrolled in the study had high p16 levels and that high p16 levels were associated with 1) lower tumor stage (IVb versus IVa) and 2) a better response to chemoradiation therapy. In the United States, oropharyngeal squamous cell carcinoma is the most common of head and neck cancers (Ernster et al., 2007).Despite the decreasing rate of tobacco use, the prevalence of oropharyngeal squamous cell carcinoma has been increasing (Frisch et al., 2000; Shiboski et al., 2005). In Taiwan, oral, oropharyngeal, and hypopharyngeal cancers together represented the fourth leading cause of cancer deaths among males in 2006 (Bureau of Health Promotion DoH, the Executive Yuan, Taiwan, 2009). The incidence of HNSCC is closely correlated with the use of cigarettes, alcohol, and betel nuts and the poor prognosis among these patients is due to the fact that patients present to the clinic at an advanced stage. In most HNSCC patients, surgery is the first choice of treatment. However, since some stage IVa and most stage IVb patients are considered inoperable, a combination of chemotherapy and radiotherapy represents the only treatment option for these patients with advanced, inoperable head and neck cancers. We selected stage IVa or IVb patients rather than stage III (surgery) or IVc (metastasis) patients in our study because patients who respond well to chemotherapy and radiotherapy usually have a favorable long time survival, while patients who fail to respond to chemoradiation have poor survival rates. Identifying patients who respond to specific therapeutic regimens and designing individually tailored, optimized therapeutic strategies would clearly be an important way in which to greatly improve treatment response and patient survival.

A subset of HNSCC is caused by HPV infection and is distinct from non-HPV HNSCC in terms of its epidemiologic, clinical, and histologic characteristics (Gillison, 2004). HPV positive HNSCC patients are more responsive to radiotherapy with or without chemotherapy (Smith et al., 2004; Fakhry et al., 2008; Worden et al., 2008). Early determination of the patients HPV status is therefore essential, in order to develop patient-specific therapeutic strategies. Immunohistochemical detection of p16 expression is often used as a surrogate for HPV determination because 1) p16 expression was shown to be significantly associated with the presence of HPV-16 (Begum et al., 2005) and 2) it is an inexpensive, simple procedure when compared with HPV detection techniques (Shi et al., 2009). However, since it was not clear if p16 expression was a significant prognostic factor in HNSCC in terms of therapeutic response, we asked if p16 expression levels in patients with locally advanced HNSCC correlated with response to chemoradiation. We showed a significant relationship between p16 expression and response to chemoradiation. To the best of our knowledge, this is the first study showing the predictive significance of p16 expression in the response of patients with locally advanced, stage IVa and IVb head and neck cancers to chemoradiation therapy. Using a surrogate marker such as p16 to evaluate HPV status in HNSCC patients is especially significant in developing countries where HPV sequencing or ISH may not be affordable.

p16 expression was previously suggested to have a prognostic significance in HNSCCs (Ernoux-Neufcoeur et al., 2011). Lindel et al. (2001) reported an association between HPV-positivity and radiosensitivity of the oropharynx. In addition, Shah et al. (2009) reported that patients with oral cancer whose tumors expressed low levels of p16 were 2.08 times more likely to develop recurrence as compared to those with p16 overexpression. Furthermore, Fischer et al. (2010a) reported that p16 was a stronger prognostic marker than primary tumor extension, lymph node involvement, distant metastases, and clinical stages I-V, in oropharyngeal squamous cell carcinoma. These data are consistent with our study which included a more heterogeneous population of patients with respect

to primary tumors site. However, the scarcity of such patients and low patient enrollment make it a challenge to draw broad conclusions, and make it necessary to carry out additional, larger studies.

Although p16 and HPV have been shown to be associated with HNSCC, the roles of p16 and HPV in HNSCC continue to be dissected by a number of groups (Lassen et al., 2009; Ang et al., 2010; Fischer et al., 2010b; Lassen et al., 2010). We would like to emphasize some important differences between these studies and ours. The studies cited above demonstrated a correlation between p16 expression levels and response to radiotherapy. However, the tumor stages in these studies were heterogeneous (stage I to stage IV) and their primary tumor sites did not include oral squamous cell carcinoma (SCC). This may be because USA and Europe have a higher prevalence of oropharyngeal cancer compared to oral cancer and may explain why studies conducted in these countries do not conclusively show that p16 can predict treatment outcome in oral squamous cell carcinoma. We believe that an important distinguishing factor of our study is the inclusion of patients with oral SCC, which is the most common form of cancer in Taiwan and South Asia (India).

Additionally, some early stage patients in these previous studies underwent surgical intervention, resulting in good, long-term survival. In contrast, the patients in our study were inoperable (stage IVa or IVb) and concurrent chemoradiotherapy (CCRT) was the first choice of treatment. It has shown that patients who received CCRT had better survival rates when compared with patients who received only radiotherapy (Krstevska, 2009). Although p16 has been documented as a predictive marker in HNSCC patients who receive radiotherapy, it is not clear if p16 levels can predict treatment outcome in inoperable HNSCC patients receiving CCRT.

It is important to note that there are data that showed that p16 was not a predictive marker in HNSCC (Fountzilas et al., 2009). Such contradictions make it important to understand the role of p16 in HNSCC. The mechanisms underlying the association between p16 and therapy response remain unclear. It was recently shown that head and neck cancer patients with high levels of p16 responded well to radiotherapy and showed better long term survival (Lassen et al., 2009). HPV-positive tumors often express wild type p53 that is functionally inactivated by E6 oncoprotein (Fakhry and Gillison, 2006), whereas the p53 gene in HPV-negative tumors often shows specific mutations that are induced by smoking (Mellin et al., 2000). Since radiation and chemotherapeutic agents may decrease the capacity of E6/E7 to interfere with p53-pRb association, these cancers may be more susceptible to therapy (Moore, 1971).

Smoking, alcohol and betel nut use are well-established risk factors for poor outcomes in squamous cell cancers of the entire upper aerodigestive tract (Weber et al., 2003; Worden et al., 2008). However, we found no significant associations between these risk factors and prognosis in patients with high p16 expression, suggesting that tobacco, alcohol, or betel nut use may not significantly influence the molecular mechanisms underlying HPV-related carcinogenesis. It is possible that these risk factors may be related to the patients' overall and disease-free survival rates, but not to treatment response. Furthermore, high p16 expression may be an independent factor predictive of the therapeutic status of HNSCC patients. Further studies may help clarify this issue.

In the present study, the treatment effect in older patients (≥60 y) was not significantly different from that observed in patients less than 60 years of age, indicating that the treatment regimen should not be dependent on patient age. Primary tumors localized to sites outside the oropharynx which overexpress p16, responded well to chemoradiation. Furthermore, although salvage surgery after chemoradiation is associated with considerable morbidity, it can provide good locoregional control and improve overall survival in patients with residual disease after primary therapy (Richey et al., 2007; Nakamura et al., 2009). These data make it important to consider surgical options in patients who respond partially at the primary site after chemoradiation.

Given the remarkable predictive power of p16 expression with respect to tumor stage and response to chemoradiation, we suggest that locally advanced HNSCC patients should be evaluated for p16 expression prior to initiation of treatment. Tumors expressing low levels of p16 may require more aggressive management. In a study of patients with oropharyngeal squamous cell carcinoma, 87% of those who failed to respond to chemoradiation died, suggesting a low efficacy for salvage therapy. It is possible to improve outcomes in patients with inoperable HNSCC, by using simple tumor markers such as p16 to predict response to CCRT and plan novel therapeutic strategies. The most important clinical contributions of our study are 1) the identification of p16 as a marker to predict treatment response in inoperable HNSCC patients 2) the inclusion of oral SCC patients.

The present study has some limitations that warrant discussion. The correlation between p16 expression levels and the presence of HPV infection was not assessed. However, p16 expression has previously been shown to be highly correlated with HPV infection in multiple studies (Begum et al., 2005). Another limitation was that the presence of other HPV types was not analyzed in this study; Machado et al. (2010) observed other HPV types, including 6, 18, 33, 35, 45, 52/58, in oral cavity cancers. It is therefore possible that, although HPV-16 is often the most frequently observed type in HNSCCs (2000), our study participants in the p16 low expression group were actually infected with other HPV types.

In conclusion, high p16 expression was found in 56% of patients with HNSCC in this study. Stage IVa/b HNSCC patients with high p16 expression exhibited a better response to chemoradiation as compared with patients who had low p16 expression. These results suggest that p16 status is an important prognostic factor for therapeutic response determination in patients with advanced HNSCC.

Acknowledgements

The authors of the manuscript declare that they have no conflict of interest.

References

Adelstein DJ, Li Y, Adams GL, et al (2003). An intergroup phase III comparison of standard radiation therapy and two schedules of concurrent chemoradiotherapy in patients with unresectable squamous cell head and neck cancer. *J Clin Oncol*, **21**, 92-8.

Agarwala SS, Cano E, Heron DE, et al (2007). Long-term outcomes with concurrent carboplatin, paclitaxel and radiation therapy for locally advanced, inoperable head and neck cancer. *Ann Oncol*, **18**, 1224-9.

Ang KK, Harris J, Wheeler R, et al (2010). Human papillomavirus and survival of patients with oropharyngeal cancer. *N Engl J Med*, **363**, 24-35.

Begum S, Cao D, Gillison M, Zahurak M, Westra WH (2005). Tissue distribution of human papillomavirus 16 DNA integration in patients with tonsillar carcinoma. *Clin Cancer Res*, **11**, 5694-9.

Bureau of Health Promotion DoH, the Executive Yuan, Taiwan. http://www.bhp.doh.gov.tw/BHPnet/Portal/StatisticsShow.aspx?No=200911300001. Accessed December 2, 2009.

Ernoux-Neufcoeur P, Arafa M, Decaestecker C, et al (2011). Combined analysis of HPV DNA, p16, p21 and p53 to predict prognosis in patients with stage IV hypopharyngeal carcinoma. *J Cancer Res Clin Oncol*, **137**, 173-81.

Ernster JA, Sciotto CG, O'Brien MM, et al (2007). Rising incidence of oropharyngeal cancer and the role of oncogenic human papilloma virus. *Laryngoscope*, **117**, 2115-28.

Fakhry C, Gillison ML (2006). Clinical implications of human papillomavirus in head and neck cancers. *J Clin Oncol*, **24**, 2606-11.

Fakhry C, Westra WH, Li S, et al (2008). Improved survival of patients with human papillomavirus-positive head and neck squamous cell carcinoma in a prospective clinical trial. *J Natl Cancer Inst*, **100**, 261-9.

Fischer CA, Kampmann M, Zlobec I, et al (2010a). p16 expression in oropharyngeal cancer: its impact on staging and prognosis compared with the conventional clinical staging parameters. *Ann Oncol*, **21**, 1961-6.

Fischer CA, Zlobec I, Green E, et al (2010b). Is the improved prognosis of p16 positive oropharyngeal squamous cell carcinoma dependent of the treatment modality? *Int J Cancer*, **126**, 1256-62.

Fountzilas G, Kalogera-Fountzila A, Lambaki S, et al (2009). MMP9 but Not EGFR, MET, ERCC1, P16, and P-53 Is Associated with Response to Concomitant Radiotherapy, Cetuximab, and Weekly Cisplatin in Patients with Locally Advanced Head and Neck Cancer. *J Oncol*, 2009, 305908.

Frisch M, Hjalgrim H, Jaeger AB, Biggar RJ (2000). Changing patterns of tonsillar squamous cell carcinoma in the United States. *Cancer Causes Control*, **11**, 489-95.

Gillison ML (2004). Human papillomavirus-associated head and neck cancer is a distinct epidemiologic, clinical, and molecular entity. *Semin Oncol*, **31**, 744-54.

Gillison ML (2007). Current topics in the epidemiology of oral cavity and oropharyngeal cancers. *Head Neck Aug*, **29**, 779-92.

Gillison ML, D'Souza G, Westra W, et al (2008). Distinct risk factor profiles for human papillomavirus type 16-positive and human papillomavirus type 16-negative head and neck cancers. *J Natl Cancer Inst*, **100**, 407-20.

Gillison ML, Koch WM, Capone RB, et al (2000). Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. *J Natl Cancer Inst*, **92**, 709-20.

Kreimer AR, Clifford GM, Boyle P, Franceschi S (2005). Human papillomavirus types in head and neck squamous cell carcinomas worldwide: a systematic review. *Cancer Epidemiol Biomarkers Prev*, **14**, 467-75.

Krstevska V (2009). Radiotherapy and chemotherapy in locally advanced head and neck squamous cell carcinoma. *J BUON*, **14**, 361-73.

Kumar B, Cordell KG, Lee JS, et al (2008). 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*, **26**, 3128-37.

Lassen P, Eriksen JG, Hamilton-Dutoit S, et al (2009). Effect of HPV-associated p16INK4A expression on response to radiotherapy and survival in squamous cell carcinoma of the head and neck. *J Clin Oncol*, **27**, 1992-8.

Lassen P, Eriksen JG, Hamilton-Dutoit S, et al (2010). HPV-associated p16-expression and response to hypoxic modification of radiotherapy in head and neck cancer. *Radiother Oncol*, **94**, 30-5.

Licitra L, Perrone F, Bossi P, et al (2006). High-risk human papillomavirus affects prognosis in patients with surgically treated oropharyngeal squamous cell carcinoma. *J Clin Oncol*, **24**, 5630-6.

Lindel K, Beer KT, Laissue J, Greiner RH, Aebersold DM (2001). Human papillomavirus positive squamous cell carcinoma of the oropharynx: a radiosensitive subgroup of head and neck carcinoma. *Cancer*, **92**, 805-13.

Machado J, Reis PP, Zhang T, et al (2010). Low prevalence of human papillomavirus in oral cavity carcinomas. *Head Neck Oncol*, **2**, 6.

Mellin H, Friesland S, Lewensohn R, Dalianis T, Munck-Wikland E (2000). Human papillomavirus (HPV) DNA in tonsillar cancer: clinical correlates, risk of relapse, and survival. *Int J Cancer*, **89**, 300-4.

Moore C (1971). Cigarette smoking and cancer of the mouth, pharynx, and larynx. A continuing study. JAMA, 218, 553-8.

Nakamura K, Tahara M, Kiyota N, et al (2009). Phase II trial of concurrent chemoradiotherapy with S-1 plus cisplatin in patients with unresectable locally advanced squamous cell carcinoma of the head and neck: Japan Clinical Oncology Group Study (JCOG0706). *Jpn J Clin Oncol*, **39**, 460-3.

Nichols AC, Faquin WC, Westra WH, et al (2009). HPV-16 infection predicts treatment outcome in oropharyngeal squamous cell carcinoma. *Otolaryngol Head Neck Surg*, **140**, 228-34.

Nobori T, Miura K, Wu DJ, et al (1994). Deletions of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. *Nature*, **368**, 753-6.

Pignon JP, Bourhis J, Domenge C, Designé L (2000). Chemotherapy added to locoregional treatment for head and neck squamous-cell carcinoma: three meta-analyses of updated individual data. MACH-NC Collaborative Group. Meta-Analysis of Chemotherapy on Head and Neck Cancer. *Lancet*, **355**, 949-55.

Pignon JP, le Maître A, Maillard E, Bourhis J (2009). Meta-analysis of chemotherapy in head and neck cancer (MACH-NC): an update on 93 randomised trials and 17,346 patients. *Radiother Oncol*, **92**, 4-14.

Reimers N, Kasper HU, Weissenborn SJ, et al (2007). Combined analysis of HPV-DNA, p16 and EGFR expression to predict prognosis in oropharyngeal cancer. *Int J Cancer*, **120**, 1731-8.

Richey LM, Shores CG, George J, et al (2007). The effectiveness of salvage surgery after the failure of primary concomitant chemoradiation in head and neck cancer. *Otolaryngol Head Neck Surg*, **136**, 98-103.

Scheffner M, Werness BA, Huibregtse JM, Levine AJ, Howley PM (1990). The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation

of p53. *Cell*, **63**, 1129-1136.
Shah NG, Trivedi TI, Tankshali RA, et al (2009). Prognostic significance of molecular markers in oral squamous cell carcinoma: a multivariate analysis. *Head Neck*, **31**, 1544-56.
Shiboski CH, Schmidt BL, Jordan RC (2005). Tongue and tonsil carcinoma: increasing trends in the U.S. population ages 20-44 years. *Cancer*, **103**, 1843-9.
Shi W, Kato H, Perez-Ordonez B, et al (2009). Comparative prognostic value of HPV16 E6 mRNA compared with in situ hybridization for human oropharyngeal squamous carcinoma. *J Clin Oncol*, **27**, 6213-21.
Smith EM, Ritchie JM, Summersgill KF, et al (2004). Age, sexual behavior and human papillomavirus infection in oral cavity and oropharyngeal cancers. *Int J Cancer*, **108**, 766-72.
Smith EM, Rubenstein LM, Hoffman H, Haugen TH, Turek LP (2010). Human papillomavirus, p16 and p53 expression associated with survival of head and neck cancer. *Infect Agent Cancer*, **5**, 4.
Stone S, Jiang P, Dayananth P, et al (1995). Complex structure and regulation of the P16 (MTS1) locus. *Cancer Res*, **55**, 2988-94.
Weber RS, Berkey BA, Forastiere A, et al (2003). Outcome of salvage total laryngectomy following organ preservation therapy: the Radiation Therapy Oncology Group trial 91-11. *Arch Otolaryngol Head Neck Surg*, **129**, 44-9.
Worden FP, Kumar B, Lee JS, et al (2008). Chemoselection as a strategy for organ preservation in advanced oropharynx cancer: response and survival positively associated with HPV16 copy number. *J Clin Oncol*, **26**, 3138-46.