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Brief communication (Original)

Prognostic significance of p16, p53, Bcl-2, and Bax in oral and oropharyngeal squamous cell carcinoma

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Background: The proteins p16, p53, Bcl-2, and Bax are important cell cycle and apoptotic regulators involved in carcinogenesis and found to have prognostic significance in various cancers. However, the data for squamous cell carcinoma of oral cavity (OSCC) and of oropharynx (OPSCC) are conflicting.
Objective: We sought to determine if expression of p16, p53, Bcl-2, and Bax expression are associated with 5-year overall survival (OS) of patients with OSCC and OPSCC.
Methods: One-hundred thirty-seven cases of OSCC and 140 cases of OPSCC diagnosed from January 2002 to December 2004 at Songklanagrind Hospital, Songkhla, Thailand, were analyzed using a Cox proportional hazards model for 5-year OS in relation to immunohistochemical detection of Bcl-2, Bax, p53, and p16 proteins.
Results: The frequencies of p16, p53, Bcl-2, and Bax expression in OSCC were 13%, 45%, 4%, and 66%, and in OPSCC were 18%, 53%, 22%, and 75%, respectively. In univariate analysis, clinical variables including T stage, N stage and treatment were significantly associated with survival. In multivariate Cox regression, Bax overexpression was significantly associated with poor survival both in OSCC (HR 1.77, 95% CI 1.04–3.01) and in OPSCC (HR 2.21, 95% CI 1.00–4.85). We found no significant association of p16, Bcl-2, and p53 expression with survival.
Conclusion: The expression pattern of p16, p53, Bcl-2, and Bax are similar in OSCC and OPSCC. Only Bax expression has prognostic significance for both tumor sites.

Keywords: Bcl-2, Bax, immunohistochemistry, oral cancer, oropharynx, p53, p16, prognostic marker, squamous cell carcinoma

Oral cancer is an important health problem worldwide with an estimated 263,900 new cases and 128,000 deaths occurring in 2008 [1]. Oral cancer represents the twelfth and eighth most common type of cancer in developed and less-developed areas with an age-standardized incidence rate (ASR) of 6.9 and 4.6 per 100,000 males in 2008. It is also common in Thailand with an ASR of 8.3 per 100,000 males in Songkhla Province in southern Thailand [2]. Oropharynx cancer is less common, but it has a higher case-fatality rate than oral cancer. The survival outcome of oral and oropharynx cancers have only subtly increased during the past two decades, by contrast with the advances in their treatment [3]. Identification of biological factors to predict a patient's clinical outcome in planning effective therapeutic strategies is valuable for improvement of patient care.

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Apoptosis and cell cycle control are two intimately linked molecular pathways involved in carcinogenesis and progression of cancer. The anti-apoptotic Bcl-2 and pro-apoptotic Bax are important members of the Bcl-2 family of proteins that play a role in the regulation of apoptosis [4]. p53, a product of *p53* tumor suppressor gene, plays a role both in cell cycle control and apoptosis by inducing growth arrest and initiating apoptosis after exposure to DNA damage [5]. p53 regulates apoptosis via transcriptional activation of Bax and suppression of Bcl-2 [6]. p16 is a cell cycle blocker. It acts by inhibiting cyclin-D1-CDK4/6 complexes that drive G1-S transition of cell cycle [7].

Oral cavity squamous cell carcinoma (OSCC) and oropharyngeal squamous cell carcinoma (OPSCC) are known to share common etiologic factors including smoking and drinking alcohol. However, recent evidence denotes a substantial proportion of OPSCC being related to human papillomavirus (HPV) infection, which may result in different tumor characteristics and behavior [8, 9]. In this study, we assessed the

expression of the four proteins, Bcl-2, Bax, p53, and p16 by immunohistochemistry and separately evaluated their relationships to survival outcomes in OSCC and OPSCC.

Material and methods

The studied subjects included patients with histologically-proven primary OSCC and OPSCC who sought treatment at Songklanagarind Hospital from January 2002 to December 2004. Case findings and clinical data were collected from patient records in the Department of Otolaryngology Head and Neck Surgery, Faculty of Medicine, Prince of Songkla University. Follow-up information was obtained from this department and from the Cancer Registry Unit of the faculty. The study was reviewed and approved by our Institutional Ethics Committee.

Primary tumors, lymph node involvement, and stage determination were classified according to the International Union Against Cancer (UICC) classification, fifth Edition, 1997. Pretreatment evaluation included routine laboratory testing, chest X-ray and CT scan of head and neck region.

Mortality information was retrieved from the Department of Provincial Administration, Ministry of Interior. Census registration data is linked nationwide and can be assessed with authorized permission. The Cancer Registry Unit of the faculty updates the mortality information from the census registration data twice yearly. Patients not found dead in this database up to December 2008 were designated as being alive.

Immunohistochemistry

Immunohistochemistry was performed on paraffin sections. Antigen retrieval was accomplished by immersing slides in Tris-EDTA buffer pH 9 in a pressure cooker at 95°C for 4 minutes. Endogenous peroxidase was blocked using 3% hydrogen peroxide. The sections were incubated with primary antibodies against p53 (clone DO7, DakoCytomation; dilution 1:100), Bcl-2 (clone bcl-2/100/D5, Novocastra; dilution 1:80), Bax (polyclonal, DakoCytomation; dilution 1:150), and p16 (CINtec $p16^{INK4a}$ Histology Kit, DakoCytomation). The sections were then incubated with EnVision for 30 minutes. The slides were incubated with DAB for color development and counterstained with hematoxylin. Sections of esophageal squamous cell carcinoma that are known to be strongly positive for p53 expression and p16 expression were used as positive controls. Sections of endometrial hyperplasia were used as positive controls for Bcl-2 and Bax. In addition, infiltrating lymphocytes were used as internal positive controls for Bcl-2 and Bax expression.

Immunohistochemical evaluation was performed by one pathologist who was blinded to the clinical status and outcome of the patients. Immunoreactivity of Bax and Bcl-2 were observed in the cytoplasm, p53 expression in the nucleus and p16 expression in nucleus and cytoplasm. The percentage of positively stained tumor cells was estimated overall by assessing the whole slide. Staining equal to or less than 5% of Bax, Bcl-2, p16, and 10% of p53 was considered negative. Intensity of staining was assessed as negative, weak, modest, or intense.

Statistical analyses

Statistical analyses were conducted using the STATA statistical software package, version 6.0. Five-year overall survival (OS) of each category of variables was obtained using the Kaplan–Meier method and compared using a log–rank test. The starting date for the analysis was set at the date of definite clinical diagnosis, usually confirmed by pathological reports. The endpoint was the date of death up to December 2008. Cox proportional hazards regression was performed to obtain independent prognostic factors for survival. Protein expression categorized as ≤25%, 26%–50%, and >50% were used in Cox regression. The 5% level of significance was used for all statistical tests. When no significant difference between using percentage and intensity of expression was found, the percentage of expression was used in all analyses.

Results

Patients characteristics

One-hundred thirty-seven cases of OSCC and 140 cases of OPSCC were included for the analysis. The patients' characteristics are shown in **Table 1**. The proportion of cases in women was much greater for OSCC, whereas less-differentiated tumors were more frequently found in OPSCC. The majority of the patients were treated using radiation either with or without surgery. Approximately one-third of the patients were not treated because they refused treatment or did not show for their hospital visit after treatment planning.

Table 1. Clinicopathological features of the patients

Variables	Number of cases (%)	
	Oral cavity	Oropharynx
Age(y) mean (range)	65.3 (30–90)	64.9 (33–89)
Sex		
Male	85 (62)	136 (97)
Female	52 (38)	4 (3)
Stage		
I	17 (13)	10 (7)
II	24 (18)	27 (19)
III	28 (21)	37 (27)
IV	67 (49)	65 (47)
Treatment		
Radiation	37 (27)	66 (47)
Surgery	10 (7)	2 (1)
Radiation and surgery	53 (39)	27 (19)
Chemoradiation	1 (1)	7 (5)
Untreated	36 (26)	38 (27)
Differentiation		
Well	97 (71)	49 (35)
Moderate	30 (22)	65 (46)
Poor	10 (7)	26 (19)

Protein expressions in relation to clinicopathological parameters

The frequencies of p16, p53, Bcl-2, and Bax expression in OSCC were 13%, 45%, 4%, and 66% and in and OPSCC were 18%, 53%, 22%, and 75%, respectively. **Figure 1** shows immunochemical images of representative samples of the four proteins.

The correlations between protein expression and clinicopathological variables are presented in **Tables 2 and 3**. In OSCC, Bcl-2 expression was observed only in moderately-differentiated tumors. In OPSCC, p53 was inversely correlated with degree of differentiation.

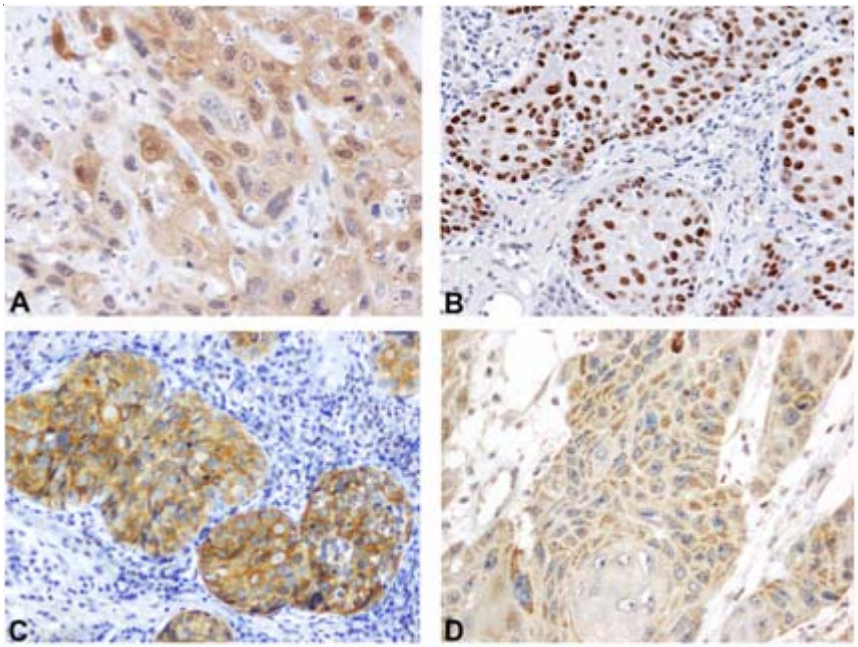

Figure 1. Representative examples of p16 expression (**A**), p53 (**B**), Bcl-2 (**C**), and Bax (**D**). Light microscope magnification 400x (A, C, D) and 200x (B).

Table 2. Clinicopathological variables in relation to protein expression in OSCC.

Variables	Percentage of cases with positive expression, [a]*P*							
	Bcl-2	***P***	**Bax**	***P***	**p53**	***P***	**p16**	***P***
Age, years								
<60	8	0.08	76	0.11	51	0.33	5	0.08
≥60	15		62		42		17	
Sex								
Male	5	0.39	65	0.89	65	0.89	13	0.70
Female	2		67		67		15	
T stage								
T1	4	0.94	48	0.09	57	0.45	18	0.69
T2	3		65		41		15	
T3–T4	4		72		43		11	
N stage								
N0	5	0.58	59	0.05	40	0.14	13	0.93
N1	4		85		62		16	
N2–N3	0		68		41		14	
Differentiation								
Good	0	0	65	0.87	38	0.03	14	0.95
Moderate	17		69		64		13	
Poor	0		70		60		10	

[a]Chi-squared test

Table 3. Clinicopathological variables in relation to protein expression in OPSCC

Variables	Percentage of cases with positive expression, [a]*P*							
	Bcl-2	***P***	**Bax**	***P***	**p53**	***P***	**p16**	***P***
Age, years								
<60	12	0.03	82	0.17	53	0.93	17	0.84
≥60	28		72		52		18	
Sex								
Male	22	0.88	75	0.25	75	0.25	18	0.71
Female	25		100		100		25	
T stage								
T1	37	0.11	93	0.07	58	0.86	19	0.45
T2	18		71		51		23	
T3–T4	18		70		52		14	
N stage								
N0	17	0.60	69	0.52	58	0.50	20	0.14
N1	25		78		52		6	
N2–N3	24		78		47		22	
Differentiation								
Good	23	0.07	69	0.41	57	0.13	7	0.01
Moderate	23		78		56		19	
Poor	36		81		34		35	

[a]Chi-squared test

Clinical variables and protein expression in relation to survival

Median survival time of the patients with OSCC was 13.2 months (range 0.13–84.7 months). The median survival time of OPSCC was 10.8 months (range 0.9–77.2 months). The 5-year OS rate of OSCC was 23% (95% CI, 16.7–30.8) and of OPSCC of 16% (95% CI, 10.3–23.0). The results of analyses in both OSCC and OPSCC are similar. In univariate analysis, only clinical parameters including T stage, N stage, and treatment, but none of the protein expression showed significant association with survival by log–rank tests (data not shown). In multivariate Cox regression (**Table 4**), however, Bax expression appeared to be significantly associated with poor survival. p16 expression tended to be related with favorable prognosis, but this was not significant. Bcl-2 and p53 expression also showed no significant association with survival.

Discussion

Evidence published during the last decade denotes a substantial proportion of HNSCC (about 20%), in particular in the oropharyngeal site (up to 45%) is HPV-induced, which is related to a more favorable prognosis compared with non-HPV-related tumor [10]. High-risk HPV oncoproteins disrupt the functional protein complex pRB-E2F, leading to the transcription of *p16* genes that promote cell proliferation. Immunohistochemically identified p16 overexpression is proposed to be a surrogate marker for HPV detection in HPV-related cancer, with a sensitivity and specificity of more than 90% and 80% respectively [11]. Our results showed that the frequency of p16 expression is slightly higher in OPSCC compared with OSCC, which is consistent with its probable link to HPV infection. However, the frequency is remarkably low compared with others [9]. HPV may probably not play an important role in Thai patients as reported

Table 4. Multivariate Cox regression analyses

Variables	Oral cavity			Oropharynx		
	HR	95% CI	*P*	HR	95% CI	*P*
Age ≤60 years	1			1		
Age >60 years	1.07	0.65–1.77	0.77	1.22	0.76–1.96	0.41
Male	1			1		
Female	1.61	0.99–2.61	0.054	1.52	0.35-6.65	0.58
T1	1			1		
T2	1.46	0.69–3.04	0.31	1.22	0.65–2.25	0.53
T3–T4	1.93	0.99–3.76	0.053	0.92	0.50–1.69	0.80
N0	1					
N1	1.23	0.69–2.17	0.48	1.83	1.04–3.24	0.04
N2–N3	1.89	1.04–3.42	0.04	4.15	2.48–6.93	<0.001
Untreated	1			1		
Radiation	0.78	0.45–1.36	0.39	0.28	0.17–0.47	<0.001
Surgery	0.24	0.08–0.75	0.014	N/A	-	-
Radiation and surgery	0.34	0.18–0.62	<0.001	0.18	0.09–0.37	<0.001
Chemoradiation	0.93	0.09–8.87	0.95	0.05	0.01–0.23	<0.001
Good differentiation	1			1		
Moderate differentiation	1.02	0.56–1.87	0.92	1.22	0.71–2.09	0.47
Poor differentiation	0.98	0.47–2.07	0.97	1.05	0.55–2.02	0.87
Bcl-2, ≤25%	1			1		
Bcl-2, 26%–50%	N/A	–	–	0.44	0.09–2.05	0.30
Bcl-2, >50%	1.77	0.34–9.28	0.50	0.64	0.32–1.25	0.19
Bax, ≤25%	1			1		
Bax, 26%–50%	1.58	0.69–3.63	0.28	2.21	1.00–4.85	0.049
Bax, >50%	1.77	1.04–3.01	0.04	1.09	0.66–1.81	0.72
p53, ≤25%	1					
p53, 26%–50%	1.37	0.62–3.01	0.44	1.09	0.54–2.22	0.81
p53, >50%	1.52	0.85–2.71	0.15	0.69	0.44–1.10	0.13
p16, ≤25%	1					
p16, 26%–50%	0.88	0.25–3.06	0.84	0.79	0.32–2.02	0.64
p16, >50%	0.89	0.31–2.58	0.83	0.77	0.39–1.53	0.45

after one study from Thailand where only one case positive for HPV-DNA was found in 32 cases of OSCC [12]. However, the true prevalence of HPV-related OPSCC in Thai patients needs to be confirmed in a larger sample with accurate techniques to clarify this point. Our results showed that p16 expression tended to be associated with a favorable prognosis, but this was not significant. The lack of significance of the low frequency of p16 expression is probably the result of a lack of sufficient power in the current sample size to reach significance.

The prognostic significance of p53 alterations in OSCC/OPSCC is inconsistent [13]. Consistent with some others [14-17], the current study revealed a nonsignificant association of p53 expression with survival. A recent meta-analysis has demonstrated a significant effect of p53 expression on overall survival in oral cancer [13]. However, most of the studies included in that systematic meta-analysis used a small number of subjects, therefore, the results may be subject to a sampling bias. The current study, which included more than one hundred patients, provides strength for our results. Together this evidence indicates a lack of promise for prognostic role for p53 expression in OSCC/OPSCC.

The low frequency of Bcl-2 and high frequency of Bax in the tumor studied is consistent with previous studies [18-20]. Bcl-2 expression is more likely to be seen in less-differentiated tumors, which was also reported after other studies [17, 18]. Consistent with most previous reports of OSCC/OPSCC [17, 20, 21], we found no prognostic significance of Bcl-2 expression in this cancer, although few studies reported significant association [19, 22].

Because Bax promotes apoptosis, Bax overexpression might be expected to be related to a better prognosis; however, only few studies support this [22], whereas others did not [19-21]. Surprisingly, we found high Bax expression (>25% of positive cells) was significantly associated with poorer survival compared with negative/low expression. To our knowledge, this direction of association has not yet been reported in oral cancer, but a similar finding has been reported in breast and rectal cancers [23, 24]. Aside from apoptotic function, some apoptotic proteins also involve in cell cycle control [25]. Bax has been found to play a role in cell proliferation by accelerating S-phase progression [26]. This may explain our finding of an adverse prognostic role for Bax. However, the actual mechanism contributing to this association needs to be explored further.

In conclusion, the present study demonstrates a similar expression profile for p16, p53, and Bax in OSCC and OPSCC. Bax expression was found to be associated with poor survival, which is rarely reported and needs to be explored further.

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