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Original Paper

Enhanced Apoptosis Correlates with Poor Survival in Patients with Laryngeal Cancer but not with Cell Proliferation, bcl-2 or p53 Expression

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The purpose of the current study was to analyse apoptosis and bcl-2 expression in laryngeal squamous cell carcinoma (SCC) with special reference to their prognostic significance, correlation with the clinical and pathological characteristics as well as cell proliferation and p53 accumulation. 172 patients with primary laryngeal SCC were followed-up for a median of 67 months. The volume corrected apoptotic (A/V) index was analysed using an *in situ* end labelling method (TUNEL) in 85 randomly selected patients. The expression of bcl-2 and p53 was analysed with monoclonal antibodies. The proliferative activity was measured both with Ki-67 (MIB-1) antibody and the volume corrected mitotic (M/V) index. The A/V index was not associated with p53 ($P = 0.6$) or bcl-2 ($P = 0.6$) expression or with proliferative parameters ($P = 0.9$ for M/V and $P = 0.3$ for MIB-1). The 10-year overall survival in patients with a high A/V index was poorer when compared with patients with a low index (47% versus 81%, $P = 0.005$), while accumulation of bcl-2 had no prognostic significance ($P = 0.5$). In Cox multivariate analysis of the whole cohort, stage ($P < 0.0005$) and histological grade ($P = 0.04$) were predictors of overall survival. In the subset of patients with an A/V index available, predictors of survival were stage ($P = 0.05$), A/V index ($P = 0.02$) and histological grade ($P = 0.04$). A high A/V index was an independent predictor of poor survival in laryngeal SCC. This effect was not associated with tumour cell proliferation. Accumulations of p53 and bcl-2 were not associated with apoptosis. Expression of bcl-2 lacks any prognostic significance in laryngeal SCC. We propose that assessment of the A/V index may help in selecting patients with poor prognosis. © **1999 Published by Elsevier Science Ltd. All rights reserved.**

Key words: laryngeal neoplasms, apoptosis, bcl, p53, prognosis, cell proliferation
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INTRODUCTION

PATIENTS WITH local laryngeal squamous cell carcinomas (SCC) have generally a good prognosis regardless of primary treatment (surgery or radical radiotherapy). However, within 10 years of diagnosis, approximately 30% of these tumours relapse. Despite an aggressive therapeutic approach, advanced tumours with nodal metastases usually have a poor prognosis. We have previously shown that accumulation of p53 protein is associated with a favourable outcome in laryngeal SCC [1]. The overexpression of p53 was not associated with tumour cell proliferation. The *p53* gene, however, may induce apoptosis. This prompted us to study the involvement of apoptosis in laryngeal SCC.

Apoptosis is an important biological consequence of exposure to ionising radiation and other DNA-damaging agents. Apoptosis can be distinguished biochemically and morphologically from necrosis by the following criteria: (1) chromatin condensation; (2) membrane blebbing; (3) appearance of apoptotic bodies; and (4) fragmentation of the genomic DNA [2]. The fragmentation of genomic DNA can be detected *in situ* by labelling 3′-OH ends with biotinylated deoxyuridine triphosphate (dUTP) through the action of terminal deoxynucleotidyl transferase (TdT) [3]. This method, later

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termed TUNEL (3′-OH nick end labelling, or more commonly TdT–mediated dUTP-biotin nick end labelling) has been used in a variety of neoplastic and physiological conditions.

bcl-2 and p53 are known to be involved in the complex mechanisms of radioresistance by modulating pathways leading to apoptosis. Mutated p53 lacks the normal function of p53, thus escaping G1 arrest and apoptosis. This escape of cells from normal activation of apoptosis allows a survival benefit for these cell clones that would otherwise be deleted [4]. By contrast, expression of bcl-2 may protect cells from p53-dependent apoptosis by suppressing signals that occur with transformation [5].

Most patients with laryngeal SCC are treated with either curative or adjuvant radiotherapy. In order to predict the response of SCC to radiotherapy, measurements of apoptotic cell death and markers of proliferative activity have been studied in cervical cancer, suggesting that increased apoptosis may be involved in the poor response to radiotherapy [6]. To date, there are no published reports correlating apoptosis with p53 and bcl-2 expression as well as with clinical outcome in a large series of laryngeal SCC patients with long-term follow-up. In the present study we analysed apoptosis by *in situ* 3′ end labelling of the DNA and compared its relationship with bcl-2 and p53 expression as well as with clinicopathological features and patient prognosis. Furthermore, extensive searches through databases were conducted in order to frame the significance of the results presented here.

PATIENTS AND METHODS

Study population

The retrospective study population was based on a cohort of 336 patients (316 men and 20 women) identified from the files of a nationwide, population-based Finnish Cancer Registry. The patients were diagnosed and treated for primary laryngeal SCC between 1975 and 1995 in eastern Finland. On the basis of availability of representative tumour tissue, 172 patients were included in the study. There were no differences in tumour (T1–T4) stage ($P = 0.23$), Karnofsky performance status ($P = 0.83$) or age ($P = 0.83$) between the patients selected in the current study ($n = 172$) and the patients in the geographical cohort ($n = 336$). Furthermore, 85 patients were selected by random order from the study cohort for determination of the apoptotic index.

Tumours

All the data from the patients' records were re-evaluated by the same oncologist and otorhinolaryngologist by co-operation. The exact site and stage of the disease were recorded according to the TNM classification [7] and based on the initial examination including indirect laryngoscopy, microlaryngoscopy, careful inspection and palpation of the neck and a chest X-ray. Only patients with histologically verified lymph nodes (pN+) were considered to have lymph node metastases. All patients were followed-up regularly by otorhinolaryngologists until death or until January 1997. The biopsies of primary tumours or laryngeal resections were fixed in 10% buffered formalin and embedded in paraffin. The histological diagnosis was confirmed by reviewing haematoxylin and eosin (H & E) stained original sections. The most representative block was selected to be cut into 3 μm thick sections. The tumours were graded as well ($n = 44$), moderately ($n = 85$) and poorly ($n = 43$) differentiated according to WHO classification [8].

Detection of apoptosis

For enzymatic *in situ* labelling of DNA strand breaks induced by apoptosis, the In Situ Cell Death Detection Kit, AP (Boehringer-Mannheim, Mannheim, Germany) based on the TUNEL method, was used. After a standard procedure of deparaffinisation and rehydration, the sections were incubated with proteinase K (20 μg/ml) for 45 min at 37°C and washed twice with Tris-buffer. The slides were incubated with TUNEL reagent for 1 h at 37°C, washed and incubated for 45 min with Converter-AP reagent at 37°C. After washing the colour reaction was introduced with Fast Red (Dako, Denmark) and counterstained with Mayer's haematoxylin and mounted. In each staining batch a SCC with morphologically demonstrable apoptotic figures was used as a positive control. In the cells with a positive signal the apoptotic figures were identified according to morphological features. For the apoptotic index (A/V), the method and formula originally introduced for the determination of the volume corrected mitotic index (M/V) [9] was used. Similarly to the M/V index, A/V expresses the number of apoptotic cells/mm^2 of neoplastic epithelium in the microscope. For the definition of the A/V index, 10 neighbouring fields were selected. Counting was performed using an objective magnification of 40× (field diameter 400 μm) by two observers simultaneously and a consensus was reached. Areas of inflammation, necrosis and dysplastic epithelia without distinct invasion were avoided.

Immunohistochemistry

Expression of p53 and bcl-2 proteins was analysed by monoclonal antibodies D 07 (Dako) and anti-bcl-2 (Clone 124, Dako). The sections were deparaffinised, rehydrated and washed in phosphate buffered saline (PBS). The sections were boiled in a microwave oven in 0.01 M citrate buffer (pH 6.0) twice for 5 min, cooled for 15 min in 0.05 M Tris buffered saline (TBS) (pH 7.49) and washed twice in PBS. Endogenous peroxidase was blocked by 5% hydrogen peroxide for 5 min and washed for 5 min with PBS. The tissue sections were incubated overnight at +4°C with the primary antibody (bcl-2 diluted 1:200 and D 07 1:1000) in PBS with 1% bovine serum albumin. The sections were washed twice with PBS and incubated for 30 min with biotinylated secondary antibody (ABC mouse Vectastain Elite kit; Vector Laboratories, California, U.S.A.) in PBS. The slides were washed twice in PBS for 5 min and incubated for 40 min in preformed avidin–biotinylated peroxidase complex. The sections were washed twice for 5 min with PBS and the colour developed with diaminobenzidine tetrahydrochloride substrate (Sigma, U.K.). The sections were lightly counterstained with Mayer's haematoxylin, dehydrated, cleared and mounted. In each staining batch positive controls (p53 positive colorectal adenocarcinoma and bcl-2 positive B-cell lymphoma) were used. In negative controls the primary antibody was omitted. The staining was evaluated by two observers simultaneously by a dual-head microscope.

Cell proliferation

For immunodemonstration of Ki-67, the monoclonal antibody MIB-1 (Immunotech, France) was used on deparaffinised and rehydrated sections. The sections were treated in a microwave oven in 0.01 M citrate buffer (pH 6.0) for

4×5 min and cooled for 30 min in the buffer. The primary antibody was used at a 1:40 dilution. The sections were incubated overnight at +4°C and the primary antibody was demonstrated with a streptavidin–biotin technique (Zymed Laboratories, U.S.A.). Counterstaining was performed with 0.4% ethyl green in acetate buffer for 15 min [1]. To determine the proliferation index (PI), an image analyser CAS 2000® (Becton Dickinson, U.S.A.) was used as previously described [1]. The M/V index was measured from H&E stainings by the method and formula originally introduced by Haapasalo and associates [9]. Briefly, the M/V index expresses the number of mitotic figures/mm^2 of neoplastic epithelium under the microscope. Mitotic figures were counted in the invasion front areas with the highest mitotic frequency, and 10 neighbouring fields were selected. Counting was carried out using an objective magnification of 40× (field diameter 400 μm) with an Olympus BH 2 microscope. Areas of inflammation, necrosis and dysplastic epithelia without distinct invasion were avoided.

Literature searches

An extensive literature search for pertinent publications was conducted via DataStar in Bern, Switzerland (Dialog Corporation) on the databases Medline, Cancerlit and Biological Abstracts from 1 January 1990 to 8 April 1998. In Medline and Cancerlit, the search profile was formed by combining MeSH (Medical subject headings) terms 'apoptosis' or 'cell death' and 'head and neck neoplasms' or 'otorhinolaryngologic neoplasms'. Another search was performed by combining 'bcl' with 'neoplasms'. All explodable terms were exploded. The search was first carried out on Medline and subsequently supplemented by Cancerlit. The search on Biological Abstracts was performed by the identical Boolean logic, but with free text words.

Statistics

To study the representativeness of the study population and the whole population and the association between bcl-2 and the variables studied, the chi-square goodness-of-fit test was used. For independence, the Kruskal–Wallis test and the Mann–Whitney *U* test were used to compare A/V and cell proliferation indices between the groups. Pearson correlation (*r*) was used to determine the linear association between cell proliferation and A/V indices.

The patterns of overall and disease-free survival were estimated by means of the product-limit method (Kaplan–Meier). Corrected survival rates were used, i.e. only deaths caused by laryngeal cancer were taken as outcome events and all other deaths as censored events. The date of recurrence was defined as the first documented date of symptoms or signs of the disease [10]. The statistical significance for the difference between Kaplan–Meier estimates was obtained using a log rank test. Multivariate analyses were performed using a Cox proportional hazards model by Wald's backward method. In the model, age and the A/V index were included as continuous variables.

Ethics

The research plan was approved by the Research Ethics Committee of Kuopio University and Kuopio University Hospital. To obtain data from the Finnish Cancer Registry, the study was approved by the Finnish Ministry of Social and Health (permission NR 117/07/95).

RESULTS

Patients, follow-up and treatment

The median and mean age of the patients was 64 years (range 31–85 years). The median time of follow-up was 67 months (range 1–241 months). For patients alive at the end of the follow-up ($n = 42$) the median time of follow-up was 128 months (range 72–241 months). There were 7 women (4%) in the study cohort. Relapses of laryngeal cancer were detected in 55 cases (32%). 58 patients (34%) died of laryngeal cancer. Other causes of death were as follows: second malignancies in 25 (15%), coronary heart disease in 26 (15%), pulmonary disease in 10 (6%), cerebrovascular disease in 3 (2%) and other causes (suicide etc.) in 8 (5%) patients. Surgical resection was the only treatment for 22 patients (13%): total laryngectomy in 19, supraglottic laryngectomy in 2, and frontolateral laryngectomy in 1 patient. 82 patients (48%) received radiotherapy as the only treatment with a median dose of 66 Gy (range 22–72 Gy). Pre- and postoperative radiotherapy was given in addition to surgical treatment to 11 (6%) and 48 patients (28%), respectively. Various combination treatments were given to 7 patients (4%). 1 patient did not receive any treatment because of poor general condition and another died of a sudden cardiac death.

A/V index

The median A/V index was 14.1 (range 0–89.1). There were no differences in the A/V index between stages (I–IV, $P = 0.2$), histological grades (I–III, $P = 0.3$), bcl-2/p53 immunostaining ($P = 0.6$ for both) or between the supraglottic versus glottic site of the primary tumour ($P = 0.1$) (Table 1). In addition, in the subgroup of patients with p53 negative tumours, the bcl-2 status showed no correlation with the A/V index ($P = 0.5$; Mann–Whitney *U* test). A similar result was obtained in a subgroup with p53 positive tumours ($P = 0.9$).

bcl-2

In two slides no invasive carcinoma tissue was left, leaving 170 cases for analysis. Positive immunostaining (≥10% positive cells) was seen in 58/170 cases (34%). A positive signal was always seen in tumour infiltrating activated lymphocytes, whilst there was no positivity in stroma. There was no relationship between bcl-2 immunopositivity and tumour stage ($P = 0.3$) or histological grade ($P = 0.1$). Supraglottic tumours were more frequently immunopositive (42%) than glottic tumours (23%) ($P = 0.01$). 5 of 6 non-smoking patients had bcl-2 negative tumours. Due to the small number of non-smokers, no statistical significance could be shown. Second malignancies were seen equally in both bcl-2 negative (15/112) and positive (9/58) groups ($P = 0.7$).

p53, M/V index and Ki-67

Overexpression of p53 protein (≥20% positive tumour cells) was seen in 116/167 (70%) cases. The median M/V index was 14.9 (range 0–103.9). The staining of Ki-67 (MIB-1) could be evaluated in 165 cases and the median PI was 39.4 (range 0–62.2). When the subgroup of patients with p53 positive tumours was analysed separately, there were no differences in MIB-1 PI ($P = 0.7$) or M/V indices ($P = 0.4$) between bcl-2 positive and negative groups. Similar results were detected in a subgroup of patients with p53 negative tumours ($P = 0.5$ and $P = 0.9$, respectively).

Table 1. *The relationship of bcl-2, p53, stage, histological grade and site of primary tumour with apoptosis and cell proliferation*

Variable	Median A/V index	Median M/V index	Median PI (Ki-67, MIB-1)
bcl-2 expression			
Positive	14.1 (27)*	16.6 (57)*	42.6 (56)*
Negative	15.3 (56)	15.1 (111)	38.5 (108)
Mann–Whitney *U* test	$P=0.6$	$P=0.6$	$P=0.4$
p53 expression			
Positive	14.5 (56)	14.9 (115)	39.4 (113)
Negative	14.6 (28)	16.2 (50)	41.8 (48)
Mann–Whitney *U* test	$P=0.6$	$P=0.8$	$P=0.8$
Stage			
I	7.3 (19)	8.6 (47)	38.4 (44)
II	12.9 (20)	18.7 (41)	36.4 (41)
III	18.2 (22)	13.7 (42)	41.5 (42)
IV	15.1 (24)	19.4 (40)	38.1 (38)
Kruskal–Wallis test	$P=0.2$	$P=0.001$	$P=0.9$
Site			
Glottic	11.9 (36)	10.8 (75)	37.1 (73)
Supraglottic	14.1 (45)	19.9 (83)	39.9 (79)
Mann–Whitney *U* test	$P=0.1$	$P<0.0005$	$P=0.3$
Histological grade			
I	12.4 (15)	7.0 (44)	33.9 (40)
II	11.7 (44)	15.9 (84)	39.8 (83)
III	17.0 (26)	30.2 (42)	45.6 (42)
Kruskal–Wallis test	$P=0.3$	$P<0.0005$	$P<0.0005$

*No. of patients shown in brackets. PI, proliferative index; A/V and M/V index, volume corrected apoptotic and mitotic index, respectively.

The relationship between parameters

Supraglottic tumours had significantly higher M/V indices than glottic tumours ($P<0.0005$) and the median M/V index was highest in stage IV tumours (Table 1). The PI measured by MIB-1 showed no association with any of the parameters, except histological grade (Table 1). In addition, the A/V index had no correlation with the M/V index ($r=0.01$; $P=0.9$) or MIB-1 ($r=0.1$; $P=0.3$). bcl-2 immunopositivity was not associated with p53 accumulation ($P=0.4$). The subgroups of patients with glottic versus supraglottic tumours were analysed separately to demonstrate differences in cell proliferative and apoptotic rates between p53 and bcl-2 positive and negative tumours, but no correlation was observed (data not shown).

Disease-free survival

During the follow-up 55 patients (32%) relapsed. Local recurrence was detected in 21 (38%), recurrence elsewhere in the neck in 21 (38%) and distant metastases in 13 cases (24%). For the univariate analysis, the A/V index and age were divided into two groups, with the median as the cut-off level, i.e. low (≤ 14.0) and high (>14.0), and 65 years as the cut-off level in age. In the univariate analysis, stage ($P<0.00005$), histological grade ($P=0.003$) and site of primary tumour ($P=0.0003$) were predictors of disease-free survival (Table 2).

Overall survival

There was no difference in overall survival between bcl-2 negative and positive groups ($P=0.5$; Figure 1). Significant predictors of overall survival were age ($P=0.02$), grade ($P<0.00005$), stage ($P<0.00005$), site of primary tumour ($P=0.0001$) and Karnofsky scale ($P=0.01$; Table 3). Patients with a low A/V index had a better prognosis

Table 2. *Cumulative* 10*-year disease-free survival rates in patients with laryngeal squamous cell carcinoma*

Variable	Surviving (%)	95% CI	*n*	Significance*
Stage				$P<0.00005$
I	83	(67–92)	46	
II	68	(50–80)	40	
III	60	(43–74)	42	
IV	24	(2–59)	32	
Histological grade				$P=0.003$
I	81	(64–91)	41	
II	67	(54–77)	79	
III	43	(26–59)	40	
Site of primary tumour				$P=0.0003$
Glottic	78	(65–87)	74	
Supraglottic	51	(38–63)	73	
Subglottic	50	(6–85)	4	
Transglottic	60	(20–85)	9	
Karnofsky performance scale				$P=0.3$
≤ 70	64	(53–73)	117	
>70	65	(47–78)	38	
A/V index				$P=0.06$
≤ 14.0	76	(57–87)	40	
>14.0	55	(38–70)	41	
bcl-2				$P=0.3$
Negative	67	(55–75)	105	
Positive	60	(45–72)	53	
Age				$P=0.4$
<65 years	64	(52–74)	88	
≥ 65 years	66	(53–76)	72	

*Log rank test. CI, confidence interval; A/V index, volume corrected apoptotic index.

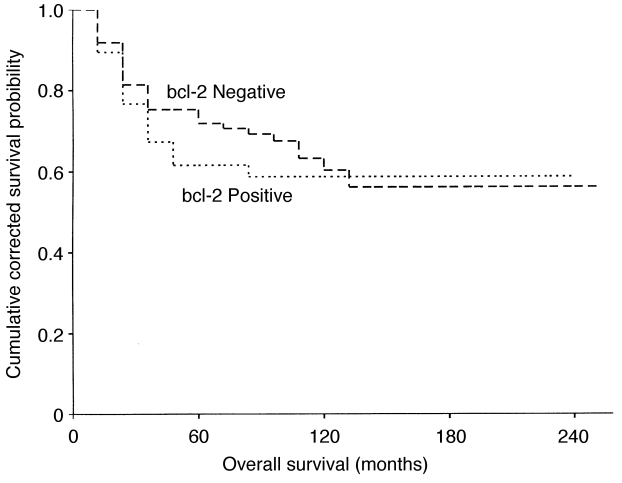

Figure 1. Overall survival of laryngeal squamous cell carcinoma patients with positive ($n = 58$) and negative ($n = 112$) staining for bcl-2 (log rank $P = 0.5$).

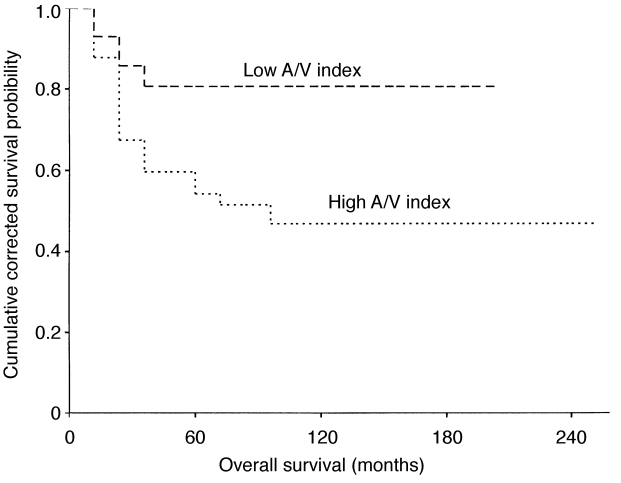

Figure 2. Patients with a high (n=43) A/V index had a poorer overall survival than patients with a low (n=42) A/V index (log rank P=0.005).

(Figure 2) than patients with a high A/V index ($P = 0.005$).

Two separate multivariate analyses (Table 4) were performed. First, an analysis with 165 patients and six variables including stage, site of primary tumour, histological grade, Karnofsky scale, age and bcl-2 immunostaining; then a second analysis with 79 patients and seven variables including the abovementioned variables and A/V index. In the first analysis, significant predictors of survival were stage ($P < 0.00005$) and histological grade ($P = 0.04$). In the second analysis, significant predictors of survival were the A/V index ($P = 0.02$), stage ($P = 0.05$) and histological grade ($P = 0.04$).

Table 3. Cumulative corrected 10-year overall survival rates in patients with laryngeal squamous cell carcinoma

Variable	Surviving (%)	95% CI	n	Significance*
Stage				$P < 0.00005$
I	89	(72–96)	48	
II	64	(39–81)	41	
III	52	(35–66)	43	
IV	28	(11–48)	40	
Histological grade				$P < 0.00005$
I	85	(68–93)	44	
II	60	(46–72)	85	
III	36	(20–53)	43	
Site of primary tumour				$P = 0.0001$
Glottic	78	(64–87)	76	
Supraglottic	50	(37–62)	83	
Subglottic	25	(1–67)	4	
Transglottic	44	(14–72)	9	
Karnofsky performance scale				$P = 0.01$
≤ 70	52	(39–62)	129	
> 80	79	(62–89)	38	
A/V index				$P = 0.005$
≤ 14.0	81	(65–90)	42	
> 14.0	47	(30–62)	43	
bcl-2				$P = 0.5$
Negative	60	(48–71)	112	
Positive	59	(44–71)	58	
Age				$P = 0.02$
< 65 years	69	(58–79)	90	
≥ 65 years	47	(31–62)	82	

*Log rank test. CI, confidence interval; A/V index, volume corrected apoptotic index.

DISCUSSION

Patients with laryngeal SCC suffer from notable social morbidity in the form of impaired quality of voice after radical

Table 4. Results of the Cox multivariate analyses for overall survival

Variable	HR	95% CI of HR	Significance
Model 1*			
Stage			$P < 0.0005$
I	1.0	–	–
II	2.8	(0.9–8.9)	$P = 0.09$
III	5.5	(1.9–16.3)	$P = 0.002$
IV	11.0	(3.7–32.4)	$P < 0.0005$
Histological grade			$P = 0.04$
I	1.0	–	–
II	1.9	(0.8–4.7)	$P = 0.1$
III	3.1	(1.2–7.8)	$P = 0.02$
Model 2†			
A/V index	1.02	(1.00–1.05)	$P = 0.02$
Stage			$P = 0.05$
I	1.0	–	–
II	1.3	(0.2–7.2)	$P = 0.8$
III	2.2	(0.5–11.0)	$P = 0.3$
IV	4.8	(1.0–22.6)	$P = 0.05$
Histological grade			$P = 0.04$
I	1.0	–	–
II	3.1	(0.4–24.1)	$P = 0.3$
III	7.1	(0.9–55.1)	$P = 0.06$

HR, hazard ratio; CI, confidence interval; A/V index, volume corrected apoptotic index (continuous variable). *The analysis included age, bcl-2, histological grade, Karnofsky scale, site of primary tumour and stage ($n = 165$). †The analysis included age, A/V index, bcl-2, histological grade, Karnofsky scale, site of primary tumour and stage ($n = 79$).

operation and oedema of the neck after radical radiation therapy. Patients with poor prognosis likely to relapse need aggressive therapeutic approaches, but patients with favourable prognosis should not be overtreated with such therapies affecting overall quality of life. In the current study, we demonstrated that enhanced apoptosis was highly associated with poor prognosis, and this effect was not associated with cell proliferation in laryngeal SCC patients. The major suppressor of apoptosis, bcl-2, was neither related to apoptosis nor survival. Furthermore, previously demonstrated favourable survival for patients overexpressing p53 protein [1] was not mediated by apoptosis in this large patient series with long-term follow-up.

The volume corrected apoptotic (A/V) index used in the current study is an adaptation of the M/V index [9]. The A/V index based purely on morphological identification of apoptotic cells has been used successfully in rat mammary carcinomas [11]. In the current study, the positive TUNEL staining was considered as a guide for the interpretation of the criteria required for morphological identification of apoptosis [2] for determining positivity. We believe that the characterisation of apoptosis by morphological criteria strictly on TUNEL positive cells determined by two observers simultaneously in a volume corrected way provides a reliable demonstration of apoptosis in laryngeal SCC. Using electron microscopy, the TUNEL method has been proven to provide a means for early detection of cells undergoing apoptosis even before the onset of gross apoptotic morphology [12]. The TUNEL positive cells, however, show two different patterns: one characteristic of apoptosis, the other pattern possibly representing the end stage of cell death [13]. It is also possible that cells during DNA replication could show TUNEL positivity. Any labelling of replicating DNA, however, would be relatively low in comparison with that of apoptotic cells and, thus, lost in the background [12].

Apoptosis is stimulated in stressful conditions. It could be speculated that activation of apoptotic cell death may contribute to tumour progression by facilitating the selection of cells with decreased or inhibited cellular differentiation and increased ability to survive in an unfavourable microenvironment. In breast carcinomas, enhanced apoptosis is associated with increased risk of lymph node metastases [14]. Furthermore, transformation of follicular lymphoma is linked to enhanced apoptosis [15], and high levels of apoptosis are more common in high grade head and neck neoplasms [16], suggesting that high apoptosis may indicate high turnover rates and, hence, the aggressiveness of the tumour. In the current study, the involvement of apoptosis (A/V) was independent of histological differentiation. The patients with low A/V indices had significantly improved survival. Furthermore, the A/V index was an independent predictor of survival in the multivariate analysis. Similar results of enhanced apoptosis related to poor survival have been detected in non-small cell lung cancer [17], cancers of the uterine cervix [6], breast [18] and bladder [19].

In some earlier studies using adaptations of the end labelling method [3], a significant correlation between apoptotic involvement and Ki-67 labelling index [20, 21] has been presented. Some reports [17, 22], however, have not shown this correlation. In studies that have used only morphological criteria without *in situ* techniques for the detection of apoptotic cells, apoptosis has been correlated with cell proliferation in several neoplasms [6, 19, 23, 24], but conversely data not indicating this correlation have also been presented [11, 25]. Both proliferative activity and apoptosis are involved in tumour growth and formation of metastases. In biopsies of oral and oropharyngeal mucosa and carcinomas, the apoptotic versus mitotic index is at its maximum in *in situ* carcinomas, suggesting that a change in apoptosis accompanies the onset of invasion in a premalignant lesion [26]. In the present study the absence of any association between the A/V index and cell proliferation suggests that apoptosis is a phenomenon distinct from cell proliferation in laryngeal SCC.

Apoptosis was neither associated with bcl-2 nor p53 expression in the current study. bcl-2 positivity, however, has been linked to decreased apoptosis in several neoplasms [14, 22, 23, 27], whereas the studies of ovarian [28], non-small cell lung [17, 29] and gallbladder carcinomas [25] parallel the current result. No association between apoptosis and p53 has been demonstrated in many neoplasms [17, 21, 25, 28, 29], including head and neck neoplasms [16], which is in accordance with the current observation.

The frequency of bcl-2 overexpression in head and neck cancers has varied from 16 to 42% [30–32], which is in line with our results. In early stage tumours it has been a predictor of poor survival [33, 34], but contradictory results have also been presented [32]. However, in previous series concentrating on laryngeal SCC with smaller patient groups than in the present study, the expression of bcl-2 was not associated with survival [35, 36]. The current study with a larger number of patients and long-term follow-up confirms these results.

Smoking as well as male gender are strongly associated with laryngeal SCC. The current study group included only 7 women and no correlation with gender in any of the parameters was detected. However, the small number of women is due to the whole geographical cohort, since only 20 (6%) of 336 patients were women. Although some doubt has been presented [37], smoking has been associated with bcl-2 positivity in head and neck cancers [31]. The current result of 5/6 non-smoker patients with negative bcl-2 staining parallels this observation, although the small number of patients limits the conclusions. The involvement of bcl-2 may be an early event in laryngeal SCC, since it can be, in some cases, detected in laryngeal keratoses of both dysplastic and benign histology [38]. Supraglottic tumours more often showed bcl-2 positivity in the present study. This could suggest that different biological properties are involved in the pathogenesis of supraglottic and glottic tumours.

Exhaustive searches using three online databases Medline, Cancerlit and Biological Abstracts were performed. The search for 'apoptosis' gave 271, 64 and 308 hits and 'bcl' gave 102, 24 and 73 hits, respectively. All the hits were carefully studied and only the journal articles concerning patients with laryngeal SCC or a mixture of SCCs of the head and neck were regarded as relevant. This approach resulted in a total of 10 articles [30–35, 39–42]. The search demonstrated that the current study introduces the largest cohort of head and neck cancer patients with survival data correlated with apoptosis and bcl-2.

In conclusion, we observed that increased apoptosis was a marker of poor prognosis in laryngeal SCC. In addition, the present notion that apoptosis was not associated with the expression of bcl-2 and p53 in laryngeal SCC, requires further studies. From the clinical point of view, the assessment of the A/V index in laryngeal SCC may help in selecting patients with poor prognosis.

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