PERGAMON

European Journal of Cancer 38 (2002) 1717–1723

European Journal of Cancer

www.ejconline.com

Supraglottic and glottic carcinomas: clinically and biologically distinct entities?

P. Hirvikoski[a,*], J. Virtaniemi[b], E. Kumpulainen[c], R. Johansson[c], V.-M. Kosma[d]

[a]*Department of Pathology, Oulu University Hospital, PO Box 50, Oulu, FIN-90029 OYS, Finland*
[b]*Department of Otorhinolaryngology, University of Kuopio and Kuopio University Hospital, Kuopio, Finland*
[c]*Department of Oncology, University of Kuopio and Kuopio University Hospital, Kuopio, Finland*
[d]*Department of Pathology and Forensic Medicine, University of Kuopio and Kuopio University Hospital, Kuopio, Finland*

Received 2 January 2002; received in revised form 17 March 2002; accepted 2 April 2002

Abstract

This study aimed to examine the clinical and biological differences in 198 patients with either T1-T2 local glottic or supraglottic squamous cell carcinomas. The patients with supraglottic cancer had a poorer prognosis, as well as more advanced and histologically aggressive tumours than the patients with glottic tumours. They also had lower levels of haemoglobin and a poorer nutritional and performance status. Expression of α-catenin, hyaluronan, CD44, p53, p21/WAF1 and bcl-2 in the primary tumour were not associated with the site of the laryngeal carcinoma. In supraglottic tumours, the rate of spontaneous apoptosis and mitotic indices were significantly higher than in glottic tumours. The results suggest that clinical parameters including the haemoglobin level of the patient together with the tumour cell kinetics (mitotic and apoptotic rates) may contribute to the aggressive nature of supraglottic carcinoma. © 2002 Published by Elsevier Science Ltd.

Keywords: Laryngeal neoplasms; Mitotic index; Apoptosis; Haemoglobins; Cell adhesion; p53; bcl; Performance status; Body mass index; Prognosis

1. Introduction

In the treatment of localised laryngeal squamous cell carcinoma (LSCC), the site of the primary tumour has particular importance. The intricate laryngeal voice box provides functional uniformity in delicate forms of speech and swallowing, but it is anatomically divided into three parts (supraglottis, glottis and subglottis) on an embryological basis. The supraglottic site has profuse lymphatics and an easier entrance for invasion, while elastic layers and sparse lymphatics might hinder the spread of glottic LSCC. Patients with local T1-T2N0M0 supraglottic LSCC usually have a worse prognosis than their glottic counterparts due to the aforementioned anatomical reasons [1,2]. Individual LSCC patients with a similar histology and stage of the primary tumour may have completely different outcomes despite adequate treatment. In general, the presence of anaemia together with chronic diseases, gender, age, use of alcohol, tobacco smoking and performance status may affect patients' outcome. In addition, a legion of biological factors associated with cell adhesion, apoptosis and cell proliferation, for example, have been introduced to explain the aggressiveness of the LSCC tumour phenotype and to provide potential prognostic value in addition to traditional staging and histological grading.

An uncontrolled cell proliferation rate is, by definition, characteristic for malignancy. The rapid cell proliferation rate in LSCC can be determined by means of a volume corrected mitotic (M/V) index [3,4]. The progression and outcome of LSCC patients are also associated with a disturbed control of the cell cycle and apoptotic cell death. Many factors associated with these processes have been studied, for example the involvement of p53 has been examined [3,5–9]. The tumour suppressor gene *p21/WAF1* is a downstream factor of p53 (reviewed in Refs. [6,10,11]), and seems to be involved in the differentiation of LSCC [10,11]. The tumour suppressor effects of these genes may result in cell cycle arrest, DNA repair, and apoptotic cell death. The intricate pathways of apoptosis, by contrast, may

* Corresponding author. Fax: +358-8-315-2177.
E-mail address: pasi.hirvikoski@ppshp.fi (P. Hirvikoski).

0959-8049/02/$ - see front matter © 2002 Published by Elsevier Science Ltd.
PII: S0959-8049(02)00115-6

be prevented by overexpression of genes such as *bcl-2*. The overexpression of the anti-apoptotic protein bcl-2, commonly detected in follicular B-cell lymphomas, permits the acquisition of genetic alterations by prolonging cell survival and thus promoting malignant conversion (reviewed in Refs. [6,8,12,13]). The prognostic significance of the bcl-2 protein has been examined in LSCC [6–9,12].

The adhesive properties of tumour cells can not be overestimated in the processes of invasion and formation of metastases. One of the most important cell-cell adhesion molecules is the Calcium-dependent E-cadherin/catenin complex. The zipper-like E-cadherin/catenin complex establishes a firm connection between epithelial cells and α-catenin connects the complex to the cytoskeleton (reviewed in Refs. [14,15]). Of additional significance are the adhesive interactions between the tumour cells and the extracellular matrix. The ubiquitously expressed glycosaminoglycan hyaluronan is the major component of the tumour stroma. Hyaluronan, with its transmembranic receptor CD44, is involved in a variety of functions such as cell binding to the extracellullar matrix, cell migration, healing wounds and inflammation (reviewed in Refs. [16,17]). Similar to the aberrant expression of α-catenin [15], irregular expression of HA and CD44 has been associated with the aggressive and metastatic phenotype of LSCC [16].

The current study aimed to examine patients with local LSCC in order to reveal clinical and biological differences between supraglottic and glottic sites.

2. Patients and methods

2.1. Study population and clinical evaluation

Our study cohort consisted of 198 patients with T1-T2 local supraglottic or glottic LSCC without any evidence of nodal (N0) or distant metastases (M0). The patients were diagnosed and treated for primary LSCC in Eastern Finland between 1975 and 1995. The exact site and stage of disease were recorded according to the TNM classification [18]. The performance status of the patients at the time of diagnosis was evaluated according to the scale of Karnofsky and colleagues [19], and the presence of any chronic diseases was recorded. The duration of symptoms, use of alcohol and tobacco, and results of preoperative blood tests were recorded.

2.2. Tumours and (immuno)histochemical stainings

Histopathological differentiation was graded in three categories according to the World Health Organization (WHO) classification [20]. All of the staining results were available from databases that were designed for previous studies and included all stages of LSCC with a focus on the prognostic significance of these factors [3,10,12,15,16].

In brief, primary monoclonal antibodies had been used as follows: 1:170 for anti-α-catenin (Transduction Laboratories^{SM}, Kensington, KY, USA) [15], 1:100 for anti-CD44 (Hermes 3, kindly donated by Prof. S. Jalkanen, University of Turku, Finland) [16], 1:1000 for anti-p53 (D O7, Dako, Denmark) [3], 1:20 for anti-WAF1 (Novocastra Laboratories, UK) [10] and 1:200 for anti-bcl-2 (Clone 124, Dako) [12]. A histochemical staining using a specific probe for hyaluronan had been used as described in detail in Ref. [16]. Apoptotic cell death had been determinated using terminal deoxytransferase-mediated bio-deoxyuridine triphosphate (dUTP) nick labelling (TUNEL) [12].

The volume corrected mitotic index (M/V) was determined in sections stained with haematoxylin and eosin [21]. Similarly, the number of cells positive for TUNEL together with the apoptotic morphology were counted using the volume corrected apoptotic index (A/V) utilising the same [21] mathematical formula [12]. Both the M/V and A/V indices were used as continuous variables in the analyses. All other staining results available from the files had been semi-classified into two categories: For α-catenin, tumours $\geqslant 20\%$ of cells with cytoplasmic staining were categorised as positive [15]. Focal reductions in the staining signal for hyaluronan and CD44 were considered as irregular staining patterns [16]. Tumours with $\geqslant 10\%$ positive cells for bcl-2 [12] and $\geqslant 20\%$ nuclei for p53 [3] were considered to overexpress these proteins. For p21/WAF1 staining, a histoscore was counted as the percentage of positive nuclei multiplied by the intensity of the staining. Positive were the tumours with an index $\geqslant 25$ [10].

2.3. Statistics and ethics

The statistical tests used are reported in the footnotes of the corresponding tables. Chi square test for independence, Mann–Whitney U test and regression model were used. The Kaplan–Meier method was used to evaluate patient survival. Exact tests were used, where appropriate. The Ethical committee of Kuopio University and Kuopio University Hospital approved the research plan. Due to the retrospective nature of the study, the patients or their relatives were not contacted.

3. Results

The origin of glottic tumours ($n = 136$; 69% of the patients) was the vocal cords in 95% of the patients, while the anterior commissure was the origin in the other 5% of the cases. The primary origin of supraglottic tumours ($n = 62$; 31% of the patients) was epiglottis in 52%, arytenoids in 14% and false vocal cords in 34%

of the cases. The median and mean age of the patients was 63 years (range 31–85 years). 14 (7%) patients were female. The treatment of the patients according to subsite and stage was as follows: for glottic Tis-T1 patients, 89% received radical radiotherapy, 8% surgery and 3% of the cases surgery with adjuvant radiotherapy. Similar percentages were 51, 21 and 28% for the glottic T2 tumours, 55, 15 and 30% for the Tis-T1 supraglottic, and 38, 12 and 45% for the T2 supraglottic tumours, respectively. In addition, chemotherapy was given to 2 patients with supraglottic T2 tumours. All the patients with supraglottic tumours were smokers, while 9% of patients with glottic tumours were non-smokers ($P=0.04$). There was no difference in the consumption of alcohol ($P=0.7$), duration of symptoms ($P=0.3$), age ($P=0.6$) or the presence of chronic diseases ($P=0.1$) between the patient groups. Patients with supraglottic tumours had a poorer performance status and a lower body mass index (BMI) than patients with glottic tumours ($P=0.01$ and $P=0.005$, respectively). The results are summarised in Table 1.

The patients with supraglottic tumours had lower haemoglobin values ($P=0.01$) and lower erythrocyte mean cell volumes ($P=0.03$) than patients with glottic tumours. The difference in haemoglobin values was further demonstrated in a multivariate analysis after adjustment for tumour extent ($P=0.007$ for site; multiple regression model). This difference in the haemoglobin values stratified by tumour extent is illustrated in Fig. 1.

In general, supraglottic tumours were of a more advanced stage ($P<0.0005$) and were histologically less differentiated ($P=0.002$) than glottic tumours. Mitotic (M/V) indices were higher in supraglottic than in glottic tumours ($P=0.001$). This difference in the M/V indices was observed even after adjustment for tumour extent in a multivariate analysis ($P=0.03$ for site; multiple regression model), and is illustrated in Fig. 2. Similarly, apoptotic (A/V) indices were lower in glottic tumours than in supraglottic tumours ($P=0.02$). Due to the small number of cases available with an A/V index, no multivariate analysis was performed. The expression of

Table 1
Clinical parameters of patients and laryngeal tumours

Variable	Number of cases (%)		
	Glottic	Supraglottic	Significance
Extent of primary tumour (T)			$P<0.0005$[a]
T *in situ* and T1	97 (71)	20 (32)	
T2	39 (29)	42 (68)	
Recurrence of laryngeal cancer			$P<0.0005$[a,b]
Local	27 (87)	9 (43)	
Neck	1 (3)	11 (52)	
Distant	3 (10)	1 (5)	
Karnofsky performance status of patients[d]			$P=0.01$[a]
Poor (70 or less)	84 (64)	49 (82)	
Good (80 or more)	48 (36)	11 (18)	
Use of alcohol[d]			$P=0.7$[a,b]
Non-users/moderate users	9 (15)	2 (9)	
Heavy users	51 (85)	21 (91)	
Smoking habits			$P=0.04$[a,b]
Non-smokers	11 (9)	0	
Smokers	117 (91)	50 (100)	
Histological differentiation grade of the tumours[d]			$P=0.002$[a]
I well (*in situ*)	64 (62)	17 (35)	
II moderate	34 (33)	21 (44)	
III poor	6 (6)	10 (21)	
Median duration of symptoms (range) in months	4.0 (0–200) $n=132$	3.0 (0–120) $n=58$	$P=0.3$[c]
Median body mass index (range) kg/m^2	25.1 (17.6–37.0) $n=91$	23.4 (17.5–32.9) $n=40$	$P=0.005$[c]
Median haemoglobin level (range) g/l	145 (105–184) $n=92$	138 (83–166) $n=35$	$P=0.01$[c]
Median erythrocyte cellular volume (range) fl	92.0 (82–107) $n=92$	89.5 (73–99) $n=34$	$P=0.03$[c]

n, number of cases.
[a] Chi square test for independence.
[b] Exact test.
[c] Mann–Whitney U test.
[d] Data is missing for some patients in these categories.

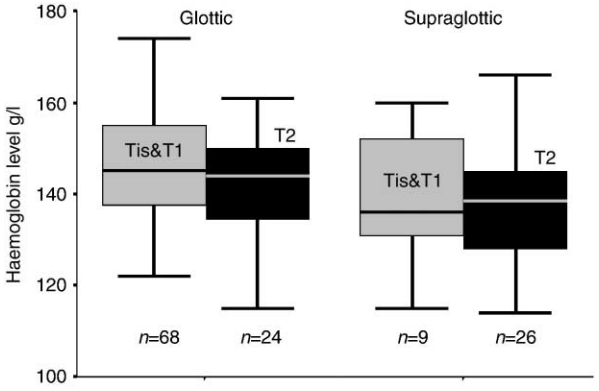

Fig. 1. Distribution of haemoglobin levels grouped by site and tumour (T) category of laryngeal cancer. *n*, number of cases.

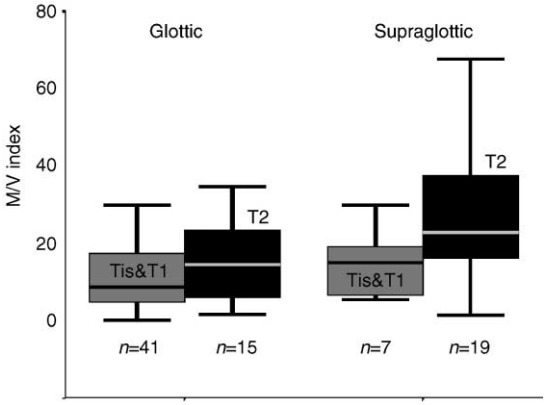

Fig. 2. Distributions of volume corrected mitotic (M/V) indices stratified by site and tumour (T) category of laryngeal cancer. *n*, number of cases.

α-catenin ($P=0.1$), hyaluronan ($P=0.6$), CD44 ($P=0.1$), p53 ($P=0.6$), bcl-2 ($P=0.9$) and p21/WAF ($P=0.2$) showed no statistically significant differences between glottic and supraglottic tumours. The results are summarised in Table 2.

During a median follow-up of 76.5 (range 1.7–258.6) months, 52 (26%) patients relapsed and 27 (14%) patients died due to LSCC. Glottic tumours were more prone to local recurrence, while the neck was the most common site of recurrence for supraglottic tumours ($P<0.0005$). The curves for overall cumulative corrected survival rates, stratified by site and extent of the tumour, are presented in Fig. 3. The patients with supraglottic tumours were more likely to relapse and die ($P=0.004$ and $P=0.001$, respectively; Kaplan–Meier) due to LSCC than patients with glottic carcinomas.

4. Discussion

This study was introduced to explore the clinical and biological differences between local supraglottic and glottic LSCC. In addition to a more advanced stage and histologically poorer differentiation grade of the tumours, the patients with supraglottic disease had clinical indicators commonly associated with a poor prognosis such as poorer performance status, lower haemoglobin levels and body mass indices. However, the expression of α-catenin, hyaluronan, CD44, p53, p21/WAF1 and bcl-2 were not statistically different between the two groups of LSCC patients. Nevertheless, supraglottic tumours had significantly higher apoptotic and mitotic rates than glottic tumours.

The prognosis of patients with glottic T2 tumours was nearly comparable to patients with supraglottic T1 tumours, while patients with glottic T1 tumours had a favourable survival and patients with supraglottic T2 tumours had a relatively poor survival (Fig. 3). This observation reflects the appropriate recording of clinical data. In this study, only 29% of glottic tumours were T2, in comparison to 68% of the supraglottic tumours. The more extended stage of the supraglottic LSCC might have affected the results. However, in the regression models, higher M/V indices and lower haemoglobin levels in the supraglottic LSCC were independent of the tumour extent.

In this study, the duration of symptoms was not different between the sites of LSCC. Glottic LSCC usually presents early with hoarseness, whereas dysphagia and sore throat may be symptoms for supraglottic LSCC [1,2]. This might provide a simple explanation for the clinical condition of patients with supraglottic tumours who were slimmer with a poorer performance status at presentation than patients with glottic LSCC. Although the small number of non-smokers and moderate users of alcohol limited definitive conclusions in our study, it is of importance to note that none of the patients with supraglottic disease were non-smokers. Indeed, glottic lesions have been more common in patients without a history of tobacco and alcohol [22]. In addition, the use of alcohol has been associated with supraglottic LSCC in particular [1], but the current study failed to show any correlation in the site of LSCC and the heavy consumption of alcohol.

Anaemia and disturbances in blood values are frequently seen in cancer patients. There seemed to be a trend towards microcytic anaemia in patients with supraglottic LSCC in our study. Haemoglobin levels may have a prognostic value for patients with irradiated LSCC [23–25], and patients with LSCC can have hypochromic microcytic anaemia during radiotherapy [23]. These aforementioned symptoms for supraglottic LSCC may explain the current observation, because the body mass index showed a correlation with the haemoglobin level ($P=0.02$). Patients with LSCC and low haemoglobin levels may have additional confounding medical problems [26], but in our study the presence of chronic diseases was not associated with haemoglobin levels ($P=0.9$). The biological associations between

Table 2
Results of immunohisto/cytochemical stainings by site of laryngeal tumours

Staining	Number of cases (%)		
	Glottic	Supraglottic	Significance
Staining patterns for α-catenin[d]			$P=0.1$[a]
Membranous	40 (77)	14 (58)	
Cytoplasmic	12 (23)	10 (42)	
Staining patterns for CD44[d]			$P=0.1$[a]
Normal	38 (76)	14 (58)	
Irregular	12 (24)	10 (42)	
Staining patterns for hyaluronan[d]			$P=0.6$[a]
Normal	39 (76)	17 (71)	
Irregular	12 (24)	7 (29)	
Expression of p53 protein[d]			$P=0.6$[a]
Normal	14 (26)	5 (20)	
Overexpression	40 (74)	20 (80)	
Expression of bcl-2 protein[d]			$P=0.9$[a]
Normal	41 (75)	19 (73)	
Overexpression	14 (25)	7 (27)	
p21/WAF1 index[d]			$P=0.2$[a,b]
Low	8 (17)	7 (33)	
High	38 (83)	14 (67)	
Volume corrected mitotic (M/V) index median count (range)	9.9 (0–61.2) $n=56$	19.5 (1.3–103.9) $n=26$	$P=0.001$[c]
Volume corrected apoptotic (A/V) index median count (range)	7.0 (0–58.8) $n=25$	18.4 (5.7–61.8) $n=11$	$P=0.02$[c]

n, number of cases.
[a] Chi square test for independence.
[b] Exact test.
[c] Mann–Whitney U test.
[d] Data is missing for some patients in these categories.

haemoglobin levels, tissue oxygenation and neoangiogenetic factors in LSCC form a complex integrity far beyond the context of these clinical observations.

The cell proliferative activity of LSCC, measured by counting mitotic indices [3,4,13,27] or by means of immunohistochemistry [3,5,7,14,27], may have some prognostic significance in LSCC [3–5,13], even though this is not supported by all reports in the literature [7,9,27]. In the current study, the M/V indices were higher in supraglottic than in glottic tumours after adjustment for T stage. In our clinical experience tumours with profuse proliferative activity may have a good primary response to radiotherapy, while the final outcome of the patient remains poor. In LSCC, a high cell proliferation rate has been associated with a high risk of occult neck metastases [14], which supports the current observation of high mitotic rates in supraglottic tumours with frequent recurrences into the cervical lymph nodes. High cell proliferative rates in supraglottic LSCC may also be associated with an aggressive phenotype and partly explain the poorer patient survival.

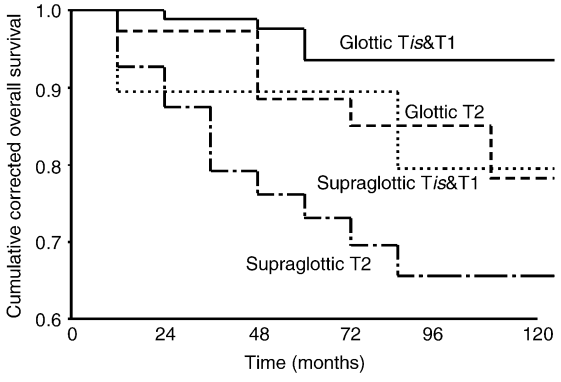

Fig. 3. Curves of disease-specific overall survival of the patients with laryngeal cancer. The patients are grouped by site and tumour (T) category (Kaplan–Meier analysis $P=0.001$).

In LSCC, increased rates of spontaneous apoptosis have been associated with a poor survival [5,12], although this has not been consistently demonstrated [9]. The higher A/V indices noted in the supraglottic tumours may be associated with the aggressive behaviour of LSCC. It is likely that cells in malignant

tumours with a stressful micro-environment have only few mechanisms for survival, which results in aggressive features, including increased rates of apoptosis and cellular proliferation. The overexpression of the anti-apoptotic protein bcl-2 has been more frequently observed in supraglottic than glottic tumours [8,13]. In the current study, however, the expression of bcl-2 and regulator proteins p53 and p21/WAF1 were not different in supraglottic and glottic LSCCs. The current observation of more frequent spontaneous apoptosis in supraglottic LSCC should be examined further, since it might be one of the fundamental biological factors associated with treatment outcome.

Disturbances in the expression of E-cadherin/catenin complex, as well as the tumour-associated hyaluronan and its receptor, CD44, have been associated with the behaviour of LSCC [7,14–16]. Despite this, supraglottic and glottic LSCC showed no difference in the expression pattern of these antigens in our study. Molecules associated with cell adhesion are numerous and the expression of syndecan-1 has been correlated with the site of LSCC [27]. In the present study, relapses in cervical lymph nodes were more commonly associated with a supraglottic site of the primary tumour. In fact, the aberrant expression of currently studied adhesion molecules may be associated with an increased capability to form cervical lymph node metastases [14–16].

The currently studied biological factors constitute only a few of the molecules associated with cancer, but are of importance in trying to phenotype LSCCs. Many of the observations in this study including the survival advantage of glottic tumours, are explained by anatomical structures and the more advanced stage of the supraglottic LSCCs. However, our patients with supraglottic tumours also had dismal clinical parameters such as higher mitotic and apoptotic rates and these parameters may have an additional value in determining the aggressive nature of supraglottic tumours.

Acknowledgements

This study has been supported by grants from the Research Foundation of the Orion Corporation.

References

1. Sessions RB, Harrison LB, Forastiere AAW. Tumors of the larynx and hypopharynx. In DeVita VTJ, Hellman S, Rosenberg S, eds. *Cancer: Principles and Practice of Oncology*. Philadelphia, J.B. Lippincott Company, 2001, 861–886.
2. Thawley S, Panje W, Batsakis J, Lindberg R. *Comprehensive Management of Head and Neck Tumors*. Philadelphia, W.B. Saunders Company, 1999.
3. Hirvikoski P, Kumpulainen E, Virtaniemi J, *et al*. p53 expression and cell proliferation as prognostic factors in laryngeal squamous cell carcinoma. *J Clin Oncol* 1997, **15**, 3111–3120.
4. Tomasino RM, Daniele E, Bazan V, *et al*. Prognostic significance of cell kinetics in laryngeal squamous cell carcinoma: clinicopathological associations. *Cancer Res* 1995, **55**, 6103–6108.
5. Lera J, Lara PC, Perez S, Cabrera JL, Santana C. Tumor proliferation, p53 expression, and apoptosis in laryngeal carcinoma: relation to the results of radiotherapy. *Cancer* 1998, **83**, 2493–2501.
6. Jin YT, Kayser S, Kemp BL, *et al*. The prognostic significance of the biomarkers p21WAF1/CIP1, p53, and bcl-2 in laryngeal squamous cell carcinoma. *Cancer* 1998, **82**, 2159–2165.
7. Spafford MF, Koeppe J, Pan Z, *et al*. Correlation of tumor markers p53, bcl-2, CD34, CD44H, CD44v6, and Ki-67 with survival and metastasis in laryngeal squamous cell carcinoma. *Arch Otolaryngol Head Neck Surg* 1996, **122**, 627–632.
8. Pruneri G, Pignataro L, Carboni N, *et al*. Clinical relevance of p53 and bcl-2 protein over-expression in laryngeal squamous-cell carcinoma. *Int J Cancer* 1998, **79**, 263–268.
9. Pulkkinen JO, Klemi P, Martikainen P, Grénman R. Apoptosis in situ, p53, bcl-2 and AgNOR counts as prognostic factors in laryngeal carcinoma. *Anticancer Res* 1999, **19**, 703–707.
10. Hirvikoski P, Kellokoski JK, Kumpulainen EJ, *et al*. Down-regulation of p21/WAF1 is related to advanced and dedifferentiated laryngeal squamous cell carcinoma. *J Clin Pathol* 1999, **52**, 440–444.
11. Pruneri G, Pignataro L, Carboni N, *et al*. Clinical relevance of expression of the CIP/KIP cell-cycle inhibitors p21 and p27 in laryngeal cancer. *J Clin Oncol* 1999, **17**, 3150–3159.
12. Hirvikoski P, Kumpulainen E, Virtaniemi J, *et al*. Enhanced apoptosis correlates with poor survival in patients with laryngeal cancer but not with cell proliferation, bcl-2 or p53 expression. *Eur J Cancer* 1999, **35**, 231–237.
13. Jäckel MC, Dorudian MA, Marx D, *et al*. Spontaneous apoptosis in laryngeal squamous cell carcinoma is independent of bcl-2 and bax protein expression. *Cancer* 1999, **85**, 591–599.
14. Franchi A, Gallo O, Boddi V, Santucci M. Prediction of occult neck metastases in laryngeal carcinoma: role of proliferating cell nuclear antigen, MIB-1, and E-cadherin immunohistochemical determination. *Clin Cancer Res* 1996, **2**, 1801–1808.
15. Hirvikoski P, Kumpulainen EJ, Virtaniemi JA, *et al*. Cytoplasmic accumulation of α-catenin is associated with aggressive features in laryngeal squamous cell carcinoma. *Int J Cancer* 1998, **79**, 546–550.
16. Hirvikoski P, Tammi R, Kumpulainen E, *et al*. Irregular expression of hyaluronan and its CD44 receptor is associated with metastatic phenotype in laryngeal squamous cell carcinoma. *Virchows Arch* 1999, **434**, 37–44.
17. Ioachim E, Assimakopoulos D, Goussia AC, *et al*. Glycoprotein CD44 expression in benign, premalignant and malignant epithelial lesions of the larynx: an immunohistochemical study including correlation with Rb, p53, Ki-67 and PCNA. *Histol Histopathol* 1999, **14**, 1113–1118.
18. Hermanek P, Sobin LH. UICC International Union Against Cancer. *TNM Classification of Malignant Tumors*, 4th edn, 2nd revision, Wiley-Liss, New York, 1992.
19. Karnofsky DA, Ableman WH, Craver LF, Burchenal JH. The use of nitrogen mustards in the palliative treatment of carcinoma. *Cancer* 1948, **1**, 634–656.
20. Shanmugaratnam K, Sobin LH. *Histological Typing of Tumours of the Upper Respiratory Tract and Ear*. Berlin, Springer-Verlag, 1991.
21. Haapasalo H, Collan Y, Pesonen Y. Volume corrected mitotic index (M/V index)—the standard of mitotic activity in neoplasms. *Pathol Res Pract* 1989, **185**, 551–554.
22. Agudelo D, Quer M, Leon X, Diez S, Burgues J. Laryngeal carcinoma in patients without a history of tobacco and alcohol use. *Head Neck* 1997, **19**, 200–204.
23. van Acht MJ, Hermans J, Boks DE, Leer JW. The prognostic

value of hemoglobin and a decrease in hemoglobin during radiotherapy in laryngeal carcinoma. *Radiother Oncol* 1992, **23**, 229–235.
24. Warde P, Sullivan B, Bristow RG, *et al*. T1/T2 glottic cancer managed by external beam radiotherapy: the influence of pretreatment hemoglobin on local control. *Int J Rad Oncol Biol Phys* 1998, **41**, 347–353.
25. Tarnawski R, Skladowski K, Maciejewski B. Prognostic value of hemoglobin concentration in radiotherapy for cancer of supraglottic larynx. *Int J Rad Oncol Biol Phys* 1997, **38**, 1007–1011.
26. Canaday DJ, Regine WF, Mohiuddin M, *et al*. Significance of pretreatment hemoglobin level in patients with T1 glottic cancer. *Radiat Oncol Invest* 1999, **7**, 42–48.
27. Pulkkinen JO, Penttinen M, Jalkanen M, Klemi P, Grénman R. Syndecan-1: a new prognostic marker in laryngeal cancer. *Acta Oto-Laryngol* 1997, **17**, 312–315.