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Distribution and prognostic significance of cell cycle proteins in squamous carcinoma of the larynx, hypopharynx and adjacent epithelial hyperplastic lesions

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Abstract

Alterations of cell cycle proteins contribute to the development and biological behaviour of malignant tumours. We evaluated the distribution and prognostic significance of immunohistochemically detected proteins p53, p21, Rb, and cyclin D1 in 101 laryngeal and hypopharyngeal squamous cell carcinomas (SCC) and adjacent epithelial hyperplastic lesions (EHL). Protein expression was correlated with tumour grade and stage.

Varying patterns of protein expression were found in SCC. A significant correlation ($p<0.05$) was found between Rb expression and tumour grade. Different grades of EHL exhibited randomly distributed p53 and cyclin D1 positive cell clusters with no association to the pattern of their expression in SCC.

Our study demonstrated derailment of cell cycle regulation in almost all cases of SCC of the larynx and hypopharynx. However, only cyclin D1 expression had an independent prognostic value for cancer-specific survival. The results also suggest that Rb gene inactivation, although rare, might be more important in the development of SCC than previously thought.

Key words: Laryngeal neoplasms; Hypopharyngeal neoplasms; Cell cycle proteins

Introduction

Squamous cell carcinoma of the head and neck region (HNSCC) is a group of cancers with similar histology but, to some extent, different aetiology and biological behaviour, relating mainly to the site of origin.[1] Despite improvements in diagnosis and new modalities of treatment, the survival rate has remained relatively low over the last two decades.[2]

One of the most important properties of cancer cells is aberrant regulation of the cell cycle, which is regulated in normal cells by the coordinated activity of different growth promoting and suppressing factors. In the normal cell cycle, most of the regulatory actions occur at the so-called restriction point (R) in the late G1 phase. From this point, the cell cycle continues without interruption until it is completed.[3]

The most important cell cycle-promoting factors, which accelerate the transition through the R point, are the proto-oncogenes cyclins, of which cyclin D1 is the most significant. The braking activity of the cell cycle depends on the activity of the tumour suppressor genes (TSG), and Rb, p53 and p21 are among the most important of the agents suppressing transition through the R point.

Cyclin D1 in high concentration and combination with cyclin-dependent kinases (CDK4 and 6) accelerates the transition through the R point. The Rb gene protein is the primary substrate for cyclin D1. When phosphorylated, it binds to cyclin D1 and stops the cell cycle. Rb gene inactivity, which is a consequence of Rb gene mutation, deprives the cell of duplication control. This event can lead to uncontrolled proliferation, characteristic of malignant cells.[4] The phosphorylation of the Rb protein can therefore be interpreted as a key event in cell cycle control.

Another TSG involved in cell cycle arrest is the p21 gene, which is stimulated by the product of an additional TSG, the p53 protein.[5] As a response to DNA damage, the p53 protein is stabilized and bound to DNA. Their complex acts as a translator factor affecting the p21 gene. The p21 protein in turn inhibits the action of cyclin D1-CDK complexes and the cell's mechanisms are enabled to repair the DNA damage. The p21 protein is therefore the decisive mediator of a p53 response to DNA damage.[3]

The nuclear phosphoprotein p53, as already mentioned, participates in the R point arrest, allowing the cell to repair DNA damage before

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progression into the cell cycle. It acts in two directions; firstly, it influences the expression of the p21 protein, and secondly, when the damage of the cell DNA is irreparable, it activates the apoptotic pathway of the cell cycle, causing the cell's programmed death. The activity of p53 is dependent on a functional, wild type protein. When this is not the case, the mutant p53 protein cannot exert its normal activity.[6]

Different TSG and oncogenes involved in regulation of the cell cycle have recently been extensively studied.[4,6–12] Variable expressions of TSG proteins have been described in cancer cells, although in a normal laryngeal squamous epithelium, only p21 and Rb nuclear positivity are expected to be found in the suprabasal epithelial layer.[6,13] Since cyclin D1, Rb, p21, and p53 are the most important of the cell cycle regulatory genes, the different patterns of their protein expression in cancer cells could be used to indicate important alterations occurring in the development of SCC.

The aim of our study was, therefore, to analyse the immunohistochemical expression, distribution and possible prognostic significance of various cell cycle regulatory proteins (p53, p21, Rb, cyclin D1) in SCC of the larynx and hypopharynx and adjacent different grades of epithelial hyperplastic lesions (EHL).

Materials and methods

During the period from 1996 to 30 June 1999, surgical specimens of 123 patients with SCC of the larynx and hypopharynx, obtained from the Department of Otorhinolaryngology and Cervicofacial Surgery, Clinical Centre, Ljubljana, were included in the study. Of the 123 patients, 22 had been treated previously by irradiation, and were thus excluded from the study.

The entire pathological diagnostic procedure on the remaining 101 specimens was performed at the Institute of Pathology, Medical Faculty, Ljubljana, Slovenia. Formalin-fixed tissue specimens were stained with haematoxylin and eosin (H and E). SCC were graded according to the criteria of the WHO classification into three grades.[14] The disease stage was determined according to the UICC/TNM classification.[15]

Epithelial hyperplastic lesions (EHL) in adjacent mucosa

In 72 surgical specimens different grades of EHL were found adjacent to the cancer. EHL were graded according to the Ljubljana classification into different risk groups, as follows: simple, abnormal and atypical hyperplasia, and carcinoma *in situ*.[16]

Immunohistochemistry

Immunohistochemical staining for p53, p21, Rb, and cyclin D1 was performed on formalin-fixed, paraffin-embedded tissue sections of the cancer specimens and adjacent EHL. After pre-treatment, described separately for each antibody, the sections were stained by the protocol of streptavidin biotin labelling with diaminobenzidine chromogen using a Tech Mate™ 500/1000 (DAKO, Glostrup, Denmark), reagent and buffers Chem Mate (DAKO, Glostrup, Denmark), and counterstained with Mayer haematoxylin. All cases were stained simultaneously for each protein, with appropriate specimens as positive and negative control. p53-pretreatment with microwave POLAR PATENT PP-780 at 96°C for 10 minutes in EDTA buffer at pH 8.0 was performed before incubation with monoclonal antibody Pab 1801 (Oncogene Science, Uniondale, NY, USA) diluted at 1:80. p21-pre-treatment with autoclaving at 121°C for five minutes in citrate buffer at pH 7.3 was done before incubation with anti-p21 WAF1/Cip1 monoclonal antibody SX118 (Dakopatts, Glostrup, Denmark) diluted at 1:40. Cyclin D1-pre-treatment with microwave at 96°C for 10 minutes in EDTA buffer at pH 8.0 was used before incubation with anti-cyclin D1 P2D11F1 (Novocastra, England), diluted at 1:25. Rb- pre-treatment with autoclaving at 121°C for five minutes in citrate buffer at pH 7.3 was used before incubation with anti-retinoblastoma gene protein NCL-RB (Novocastra, England), diluted at 1:50. The specificity of reactions was determined using non-immune serum instead of primary antibody, as negative control, and SCC which previously exhibited over-expression of p53, and cyclin D1 as positive staining control. For p21 and Rb immunohistochemistry, we used normal laryngeal mucosa as a positive staining control.

Immunohistochemical scoring

Semi-quantitative scoring of immune reactivity in SCC was performed according to the percentage of nuclear positivity in the tumour cells, as follows: Score 0: zero per cent; Score 1:1–10 per cent; Score 2: 10–50 per cent; Score 3: more than 50 per cent of tumour cells with a positive nuclear reaction.

In EHL the principle of evaluation was different from that in SCC. In EHL, usually there was no immune reactivity for p53 or cyclin D1 (0). When present it was found in single basal cells and/or cell clusters (+) occupying no more than two thirds of the epithelial thickness. The analysis was carried out by a pathologist (MV) in a blind test without knowledge of other parameters.

Statistical analysis

Correlations between immunohistochemical scoring results and various clinicopathological features were assessed using the Kendall's tau test. Survival curves for disease-free (DFS) and cancer-specific survival (CSS) were constructed using the method of Kaplan and Meier and the differences between curves were assessed with the log-rank test. Cox's regression model was used to evaluate the predictive power of various factors in multivariate analysis. Due to the presumption that progression would most frequently occur within two years after surgical treatment,[17] the last patient who was included in the study, was operated in June 1999. The time between surgery and the first recurrence (distant or local) of the

disease was used for calculating DFS. For calculating CSS, the interval between surgery and SCC-related death was used; patients dying from causes unrelated to SCC were censored at the time of their death. The median follow up for censored cases was 38 months (three to 60 months). Data on patients' survival and cause of death were obtained from the Registry of Cancer for Slovenia, at the Institute of Oncology, Ljubljana, Slovenia, and from patients' records. All of the statistical calculations were done using the SPSS software package (SPSS, Chicago, IL).

Results

Of 101 patients, there were 94 men and seven women with 73 laryngeal and 28 hypopharyngeal SCC, with ages ranging from 36 to 80 years (mean ± SD; 57.7 ± 10.5 years). Tumour grading and staging characteristics are presented in Table I.

Tumour grade ($p = 0.004$), T ($p<0.0001$), and N stage ($p<0.0001$) showed a significant correlation with disease location. Patients with hypopharyngeal SCC had less differentiated tumours and were diagnosed at a more advanced stage than patients with laryngeal SCC. A higher tumour grade was associated with a higher T stage ($p = 0.023$).

Immunohistochemistry of SCC

The positivity of immunohistochemical reactions in tumour cell nuclei are shown in Table II. Co-expression of all four markers in SCC was present in 46 cases. Cyclin D1, p21 and Rb co-expression with negative p53 reaction was found in 33, and p53, p21 and Rb co-expression without cyclin D1 in six cases. Co-expression of only p21 and Rb was detected in 10, and p53 and p21 in two cases. Other patterns of reactions were determined in single cases each: Rb only; cyclin D1 and p21; cyclin D1 and Rb; and p21 only. Examples of p53, Rb, and p21 immune reactions in SCC and adjacent EHL are illustrated in Figures 1, 2 and 3.

TABLE I
DISEASE CHARACTERISTICS OF 101 PATIENTS WITH SQUAMOUS CELL CARCINOMA OF THE LARYNX AND HYPOPHARYNX

	No. of patients Larynx	No. of patients Hypopharynx	Total
Tumour grade			
Grade 1	21	1	22
Grade 2	41	20	61
Grade 3	11	7	18
Total	73	28	101
T stage			
T_1	5	0	5
T_2	25	1	26
T_3	27	16	43
T_4	16	11	27
Total	73	28	101
N stage			
N_0	41	2	43
N_1	9	2	11
N_2	23	21	44
N_3	0	3	3
Total	73	28	101

TABLE II
SEMI-QUANTITATIVE SCORING RESULTS OF p53, CYCLIN D1, p21 AND Rb IMMUNE REACTIONS IN TUMOUR CELLS OF 101 SQUAMOUS CELL CARCINOMAS OF THE LARYNX AND HYPOPHARYNX

	Score 0 0%	Score 1 1–10%	Score 2 10–50%	Score 3 >50%	Total positivity
p53	47	10	19	25	54/101
cyclin D1	20	41	29	11	81/101
p21	2	17	46	36	99/101
Rb	4	6	13	78	97/101

Significant correlations were present between cyclin D1 and p21 ($p = 0.007$), cyclin D1 and Rb ($p = 0.028$) expression, and Rb expression and tumour grade ($p = 0.046$). p53 expression did not correlate with any other protein expression nor with disease characteristics.

EHL in adjacent mucosa

EHL were present adjacent to cancer in 72 of 101 surgical specimens. Simple hyperplasia was detected in 54/72 cases (75 per cent), abnormal in nine, atypical hyperplasia in six, and carcinoma *in situ* in three cases.

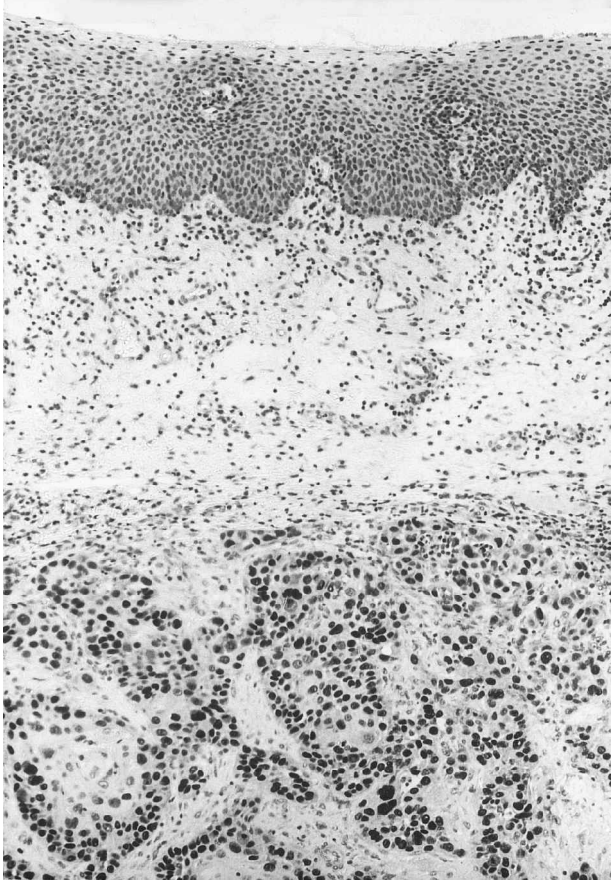

FIG. 1
SCC growing under p53 negative squamous epithelium with abnormal hyperplasia. More than 50 per cent of cancer cell nuclei stained positively for p53 protein (×100).

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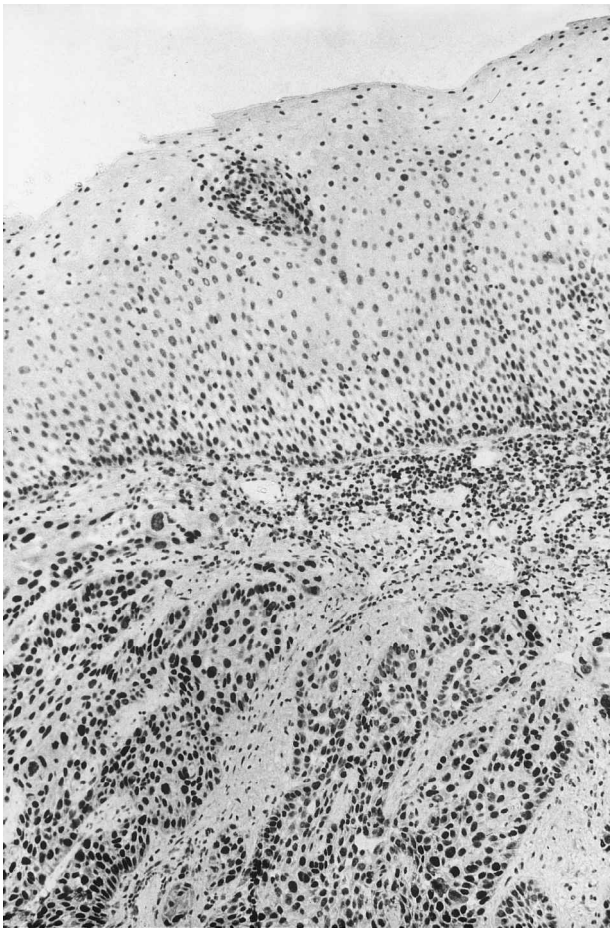

FIG. 2

More than 50 per cent of cells in SCC stained positively for Rb protein. The overlying squamous epithelium contains Rb positive cell nuclei in the suprabasal layers of simple hyperplasia (×100).

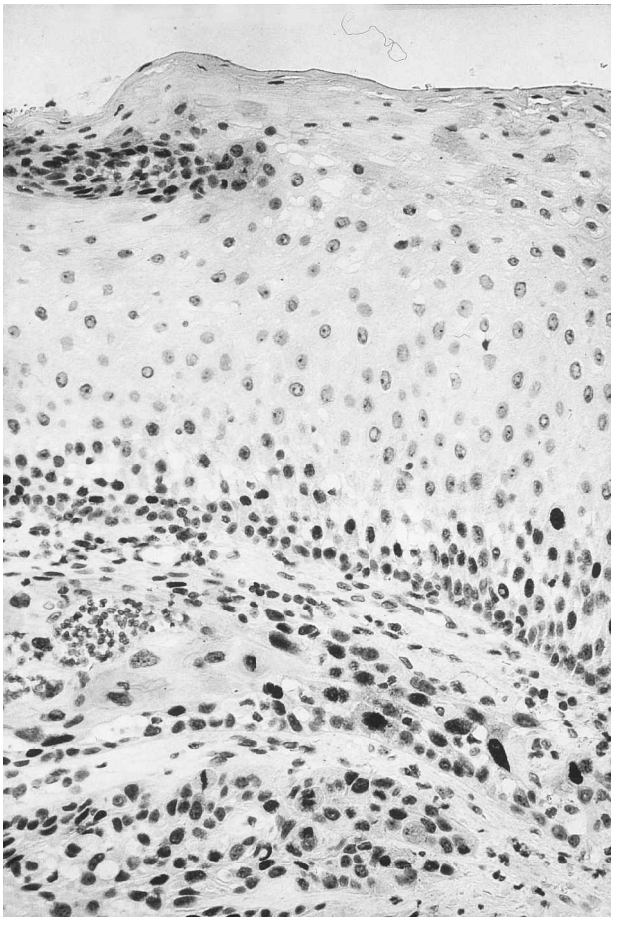

FIG. 3

More than 50 per cent of cells in SCC stained positively for p21 protein. The overlying squamous epithelium contains p21 positive cell nuclei in the suprabasal layers of simple hyperplasia (×200).

In all cases p21 and Rb nuclear positivity was found in the suprabasal epithelial layers. In addition individual or combined reactivity was observed for p53 and cyclin D1. The separate results of p53 and cyclin D1 immunohistochemistry are presented in Table III. The frequency of both cyclin D1 and p53 positivity revealed random distribution among different grades of EHL. Figure 4 illustrates the presence of a small cluster of cyclin D1 positive cells that was found in simple hyperplasia. Interestingly, in two cases with positive Rb staining in the suprabasal cells of simple hyperplasia, the tumour cells were clearly negative.

Disease-free and cancer-specific survival

During the study period, 33 patients had disease recurrence and 26 patients died of SCC. The three-year DFS was 65.1 per cent and CSS 73.7 per cent. The distribution of various clinical, pathological and

TABLE III

THE RESULTS OF p53 AND CYCLIN D1 IMMUNOHISTOCHEMISTRY IN EPITHELIAL CELL NUCLEI IN DIFFERENT GRADES OF EPITHELIAL HYPERPLASTIC LESIONS ADJACENT TO SCC

		Simple hyperplasia No. of cases	Abnormal hyperplasia No. of cases	Atypical hyperplasia No. of cases	Carcinoma in situ No. of cases	Total No. of cases
	0	41	5	5	2	53
p53	+	13	4	1	1	19
	Total	54	9	6	3	72
	0	41	9	4	0	54
cy. D1	+	13	0	2	3	18
	Total	54	9	6	3	72

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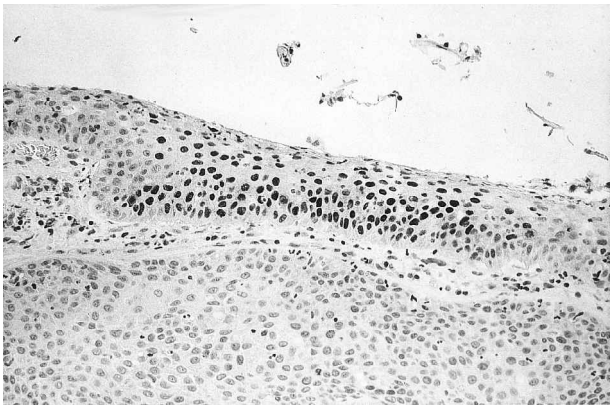

FIG. 4

A cluster of cyclin D1 positive epithelial cells in simple hyperplasia of the mucosa in the vicinity of SCC (×200).

immunohistochemical variables and their relation to survival in univariate analysis is shown in Tables IV and V. In multivariate analysis, N stage (N_0 *vs.* $N_{1–3}$) was the only significant independent predictive factor for DFS (relative risk 4.50; 95 per cent confidence interval 1.85–10.94; $p = 0.001$). On the other hand, in addition to N, cyclin D1 expression and tumour grade showed independent prognostic significance for CSS (Table VI).

Discussion

The results of the present study indicate that the cell cycle control is damaged in almost all cases of SCC of the larynx and hypopharynx. These data also suggest that derailment of both the growth-promoting (through cyclin D1) and/or growth suppressing pathway (through p53) of the cell cycle might be involved in the development of SCC.

The overall relationship between cyclin D1 and Rb expression in our investigation is in contrast with their expected appearance in the normal epithelium. Usually, in a normal epithelium we find Rb expression in the suprabasal layers and no cyclin D1 positivity.[18] Since there is a significant concordance between cyclin D1 and Rb immunohistochemical findings and molecular methods, immunohistochemistry can be used as a reliable marker of the gene status.[19] In addition to p21, there was high expression of Rb and cyclin D1 in eight cancers. This finding could be explained by cyclin D1 overexpression which has been described as being able to supersede Rb-mediated growth inhibition in SCC.[20] Accordingly, it has been found that overexpression of cyclin D1 in oesophageal cancer is associated with persistent expression of the Rb protein.[18]

TABLE IV

RESULTS OF DISEASE-FREE SURVIVAL (DFS) RATE CALCULATION FOR 101 PATIENTS WITH SQUAMOUS CELL CARCINOMA OF THE LARYNX AND HYPOPHARYNX. STATISTICALLY SIGNIFICANT VALUES (LOG RANK TEST) ARE MARKED WITH*

Prognostic factor		No. of patients	3 year DFS	*p* value
Sex	Male	94	63.4%	0.27
	Female	7	85.7%	
Age	⊆ 50 ys.	30	74.1%	0.26
	⊃ 50 ys.	71	61.4%	
Tumour grade	Grade 1	22	81.8%	0.0321*
	Grade 2	61	63.5%	
	Grade 3	18	46.7%	
T stage	T_1	5	80.0%	0.0044*
	T_2	26	88.1%	
	T_3	43	59.8%	
	T_4	27	48.2%	
N stage	N_0	43	84.9%	0.0001*
	N_1	11	54.6%	
	N_2	44	52.5%	
	N_3	3	0%	
Location	Larynx	73	75.0%	0.0005*
	Hypopharynx	28	34.4%	
p53	Score 0–2	76	67.5%	0.38
	Score 3	25	56.9%	
p21	Score 0–2	65	62.5%	0.41
	Score 3	36	70.2%	
cyclin D1	Score 0–1	61	69.1%	0.20
	Score 2–3	40	59.6%	
Rb	Score 0–2	23	76.3%	0.22
	Score 3	78	61.8%	

TABLE V

RESULTS OF CANCER-SPECIFIC SURVIVAL (CSS) RATE CALCULATION FOR 101 PATIENTS WITH SQUAMOUS CELL CARCINOMA OF THE LARYNX AND HYPOPHARYNX. STATISTICALLY SIGNIFICANT VALUES (LOG RANK TEST) ARE MARKED WITH*

Prognostic factor		No. of patients	3 year DFS	*p* value
Sex	Male	94	71.6%	0.12
	Female	7	100%	
Age	⊆ 50 ys.	30	86.4%	0.25
	⊃ 50 ys.	71	68.8%	
Tumour grade	Grade 1	22	86.4%	0.0193*
	Grade 2	61	75.1%	
	Grade 3	18	51.7%	
T stage	T_1	5	100%	0.0005*
	T_2	26	95.0%	
	T_3	43	67.1%	
	T_4	27	56.1%	
N stage	N_0	43	93.0%	0.0000*
	N_1	11	71.6%	
	N_2	44	59.1%	
	N_3	3	0%	
Location	Larynx	73	83.1%	0.0003*
	Hypopharynx	28	46.0%	
p53	Score 0–2	76	72.0%	0.789
	Score 3	25	78.9%	
p21	Score 0–2	65	70.9%	0.494
	Score 3	36	78.3%	
cyclin D1	Score 0–1	61	79.5%	0.026*
	Score 2–3	40	64.7%	
Rb	Score 0–2	23	82.4%	0.32
	Score 3	78	71.1%	

TABLE VI

INDEPENDENT PROGNOSTIC FACTORS FOR OVERALL SURVIVAL OF 101 PATIENTS WITH SQUAMOUS CELL CARCINOMA OF THE LARYNX AND HYPOPHARYNX AFTER MULTIVARIATE COX PROPORTIONAL HAZARD ANALYSIS

Independent prognostic factor	Relative risk	95% confidence interval	*p* value
N stage			0.01
N_0	1	–	
$N_{1–3}$	7.38	2.20–24.8	
cyclin D1			0.019
0–1	1	–	
2–3	2.57	1.17–5.64	
Tumour grade			0.037
1	1	–	
2	1.35	0.39–4.70	
3	3.85	1.01–14.70	

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The derailment of the apoptototic and/or growth suppressing pathway of the cell cycle, usually mediated by p53, is demonstrated by the overall correlation between cyclin D1 and p21 expression in tumour cells. Furthermore, there were no correlations between p53 and p21, nor between p53 and any other protein examined. In non-cancerous cells, p21 expression is dependent on the wild type p53 protein expression[6] and actually serves as an indirect indicator of the p53 gene status. Accordingly, in normal epithelium there is no p53 or cyclin D1 expression, and p21 is found in the basal and suprabasal epithelial layers. The results of our investigation of SCC support the suggestions of previous studies that the apoptototic pathway of the cell cycle in laryngeal and hypopharyngeal SCC is derailed, and the p21 expression independent of p53.[21] A possible mechanism of this derailment and the lack of correlation between p21 and p53 immunohistochemistry is mutation of the p53 gene which is not reflected by the expression of mutant p53 protein. As is already known, immune staining shows a positive reaction with both the wild type and mutant p53 protein. Due to the short half-time of the wild type p53 protein, it was postulated that most of the positivity is the result of p53 gene mutation. However, only missense mutations encode a mutant protein that can be detected immunohistochemically.[22] Other, nonsense and frameshift mutations cannot be detected using immunohistochemical methods. Additionally, approximately 30 per cent of the mutations occur in the noncoding, including the promotor, regions of the p53 gene, and are also not detectable by the use of immunohistochemical methods.[12] A p53 independent pathway of p21 induction has also been supported by the suggestion that p53 immunoreactivity may be associated with protein suppression rather than mutation of the p53 gene.[21] And finally, since cyclin D1 and p21 expressions correlate, it is possible that p21 expression is stimulated directly by cyclin D1. The upregulation of p21 in various carcinomas, provoked by cyclin D1, was reported by de Jong *et al.*[8] This mechanism might be a likely explanation for three of our cases with simultaneous high scores of p53, cyclin D1 and p21.

Our results highlight the central role of cyclin D1 in regulation of the cell cycle in SCC of the larynx and hypopharynx. It is likely that, when over-expressed, cyclin D1 is able to override most of the inhibitory action exercised by its antagonists. The higher expression of cyclin D1 had also predictive value for patients' CSS. This finding is consistent with previous publications that verified the prognostic significance of cyclin D1 gene amplification[7] and a correlation of its overexpression with tumour recurrence rate.[23] The predictive value of cyclin D1 immunohistochemistry for patients' CSS could actually be a consequence of cyclin D1 amplification, as already described. However, this finding could be interpreted in another way. It is possible that due to multiple, interacting suppressing agents, operating at the R point and suppressing the progression through the cell cycle, mutations and/or altered expressions among them combine. So different shares of their influence on the cell cycle are represented in different cases. Consequently, when compared to the single promoting agent, cyclin D1, the possible prognostic significance of the antagonists p21, p53 and Rb could be lost during statistical analysis.

As far as prognostic significance of only p53 gene expression is concerned, the results of different studies are contradictory. Some authors have claimed p53 to be associated with prognosis,[24] while other authors have been unable to demonstrate this.[25,26] Gene mutations but not overexpression were demonstrated to be associated with survival in HNSCC.[10] In the present study, p53 expression had no influence on patient survival. Similar results were obtained by Nadal *et al.*,[25] who found no correlation between p53 expression in SCC and their clinical and pathological characteristics. The reason for contradictory results among different studies could be the partial inaccuracy of p53 immunohistochemistry, already described in detail.

Changes in Rb expression which, in contrast to p53 expression, correlate with the presence of Rb mutations,[27] occur relatively rarely in the carcinogenesis of SCC. It is believed that they are an early event but play a less important role in the carcinogenesis of SCC.[13]

It has recently been published that Rb inactivation occurred only in SCC developing from premalignant lesions with the same Rb inactivation.[13] We found two cases with Rb inactivation in SCC but unchanged Rb expression in simple hyperplasia adjacent to the cancer. However, simple hyperplasia, as defined in the Ljubljana classification[16] represents a reactive and not a precancerous lesion. Nevertheless, loss of Rb expression in our SCC cases showed a significant association with tumour grade. The presented data therefore support the rarity of Rb gene inactivation, but also suggest that, although rare, it might be more important in the development of SCC than previously thought. Further studies are required on these cases to elucidate its role in the carcinogenesis of HNSCC.

In different grades of EHL adjacent to SCC, cell cycle proteins were randomly distributed. Our current findings are thus not completely in agreement with the previous studies of our group[28] and the group from Barcelona,[25] which showed an increased immune reactivity for p53, that correlated with the severity of laryngeal EHL. Furthermore, it was demonstrated that mucosal p53 expression can precede histopathological signs of dysplasia.[29] The authors showed a high complementarity between p53 expression in different grades of dysplasia and subsequent cancerous growth. It appeared that p53, over-expressed in a high number of laryngeal SCC, influences early stages of malignant transformation, but is probably not associated with further progression of the tumours.[29] Different from EHL in the present study, they evaluated epithelial changes, that developed over time into cancer. In contrast to that of p53, cyclin D1 over-expression was described as

appearing in late stage tumours and was not thought to participate in malignant transformation.[30] It was usually attributed to the progression of a fully developed tumour. The presented results are not in accordance with that suggestion, since cyclin D1 positivity in EHL was similar to that of p53. Since EHL in our cases were present in the vicinity of the cancerous lesion it is possible that they represent *de novo* genetic or epigenetic changes arising in an area of *field cancerization*.

The 101 laryngeal and hypopharyngeal SCC showed a clear correlation between the tumour grade and disease stage. According to the progression model of carcinogenesis, this correlation is a consequence of accumulating mutations in tumour cells, that become less differentiated over time. It has already been shown that prostate cancers become similarly less differentiated and of a higher Gleason score when growing for long time.[31]

The results of survival analysis performed on 101 SCC attest the prevalent prognostic value of tumour-based prognostic markers. In different studies, prognostic significance has been exhibited by the presence and extent of nodal metastases, T stage, tumour site, total tumour volume and tumour size.[32] The only independent prognosticator of DFS of our patients was the N stage. Additional independent prognostic value for CSS was demonstrated by the tumour grade and cyclin D1 expression, which was the only cell cycle protein with prognostic value. Smith *et al.*[26] found no prognostic value of cyclin D1 expression in oropharyngeal SCC. However, cyclin D1 expression has been reported to correlate with recurrence in surgically treatable SCC of the head and neck.[23] It has been suggested to be a useful prognostic factor in advanced cases of hypopharyngeal carcinoma[33] and considered to be a predictive marker of response to induction chemotherapy.[26] In the present study, it was one of the independent prognosticators for CSS but not DFS. Conversely, Pignataro *et al.*[34] demonstrated independent prognostic significance of cyclin D1 immunohistochemistry for DFS and borderline significance for CSS of patients with laryngeal SCC. The difference between the two studies is probably due to a different number of patients and follow-up duration.

In conclusion, the present study demonstrated derailment of the cell cycle control in almost all cases of SCC of the larynx and hypopharynx. It appears that cyclin D1 has a central role in the regulation of cell cycle in SCC. Additionally, it has an independent prognostic value for CSS. Our results also suggest that Rb gene inactivation, although rare, might be more important in the development of SCC than previously thought.

In EHL of the surrounding mucosa, p53 and cyclin D1 positive cells/cell clusters were randomly distributed and do not seem to have any association with their expression in SCC, indicating that they might result from new mutations in areas of field cancerization.

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