

Studies of the cell cycle regulatory proteins P16, cyclin D1 and retinoblastoma protein in laryngeal carcinoma tissue

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Abstract

Defects in the system controlling the cell cycle can lead to an increased proliferation of cancer cells. The aim of this study was to analyse the immunohistochemical expression of chosen cell cycle proteins (P16, cyclin D1 and retinoblastoma protein) and their connection with the clinical course of the disease in laryngeal squamous cell cancer (LSCC). Cancer tissue sections obtained from 58 patients after total laryngectomy served to determine the level of the proteins' expression using immunohistochemical staining and commercial antibodies. A decreased level of P16 expression in 47 per cent, of retinoblastoma protein in 12 per cent and strong cyclin D1 expression in 48 per cent of cases was revealed. Our results show significant correlation between decreased P16 expression and increased tumour dedifferentiation. Overexpression of cyclin D1 was statistically more common in locally advanced tumours (T₃–T₄). Low expression of retinoblastoma protein was significantly correlated with both positive P16 immunostaining and with strong cyclin D1 expression. Our study confirms that dysfunction of cell cycle regulation is a common event and may play a significant role in the development of squamous cell carcinoma of the larynx.

Key words: Larynx; Carcinoma, Squamous Cell; Protein P16; Cyclin D1; Retinoblastoma Protein

Introduction

Laryngeal squamous cell carcinomas (LSCCs) represent the second most common malignant neoplasm of the respiratory tract after lung cancer.¹ In Poland laryngeal cancer constitutes the third most common malignant neoplasm in men.² Clinico-pathological features such as TNM stage and histological grade seem to be insufficient to predict the clinical outcome in this group of patients.

Rapidly developing technology in the field of molecular biology enables estimation of control mechanisms of cancer cells at the molecular level. Much interest has focused on the proteins participating in the cell cycle control. Defects in the system controlling the cell cycle play an important role in the pathogenesis of cancer, resulting in an increased proliferation of cancer cells. Among these proteins, P16, retinoblastoma protein and cyclin D1 building the retinoblastoma protein-signalling pathway of cell cycle control, belong to the most important ones.^{3,4}

P16 was identified as an inhibitor of cyclin-dependent kinase 4, whose activity is critical in

regulation of normal cell cycle progression.⁵ Transient expression of P16 in several types of cultured cells leads to hypophosphorylation of retinoblastoma protein and inhibition of DNA synthesis.^{5–8} The gene *CDKN2A*-encoding P16 protein is localized on chromosome 9p21.⁵ Inactivation of the *CDKN2A* gene has been observed in many cancers.^{5,9}

The prototypic tumour suppressor gene is the retinoblastoma gene, which was identified because of its role in the development of childhood retinoblastoma. It is located on the long arm of chromosome 13 and exerts its suppressive effects during a defined window of time in the first two thirds of the G1 phase of the cell cycle. It is during this time that mammalian cells make most of their decisions about growth versus quiescence.¹⁰ It is widely accepted that the retinoblastoma gene is implicated in the pathogenesis and/or progression of a broad range of human malignancies.^{11–18}

Cyclin D1 is expressed during the G1 phase of the cell cycle and becomes associated with the catalytic partner CDK4 or CDK6.³ This results in a serine or threonine kinase activity of the cyclin D1. The CDK

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TABLE I
RELATION BETWEEN HISTOLOGICAL GRADING (G) AND EXPRESSION OF P16

Histological grading (G)	High expression of P16 (%)	Low expression of P16 (%)	Pearson's exact test
G1	6 (50)	6 (50)	<i>p</i> =0.06
G2	19 (63.4)	11 (36.6)	
G3	6 (37.5)	10 (62.5)	
Total	31 (55)	27 (45)	

complex participates in the transit through the G1 phase. The fundamental role of the cyclin D1 is to integrate extracellular signals with the cell-cycle regulating machinery. Increased expression of cyclin D1 has been associated with increased cell proliferation.^{19–21} Deregulation of cyclin D1 is likely to contribute to tumour development and it has been found in different types of tumours.^{22–26} It has been shown that cyclin D1 protein overexpression is correlated with poor prognosis in head and neck cancer patients.^{27–31}

The aim of this study was to investigate whether expression of P16, retinoblastoma protein and cyclin D1 is associated with clinical and pathological features in laryngeal cancer.

Material and methods

A total of 58 tissue samples of LSCC were obtained from patients (five women and 53 men) who had undergone surgical resection of a laryngeal tumour in the Department of Otolaryngology Wrocław Medical University during the years 1997–1999. The average age of the patients was 59 years (range 41–75 years). In 17 patients cervical lymph node metastases were detected. The histological grading of tumours on haematoxylin-eosin sections was as follows: G1–12, G2–30 and G3–16. Histologically all tumours were squamous cell carcinomas. TNM classification was evaluated according to the UICC criteria. Normal laryngeal epithelium cells obtained from 20 patients unaffected by cancer were used as the positive control group.

All specimens were fixed in 10 per cent formalin and routinely processed for paraffin embedding. Sections were cut at 3–4 µm and mounted on coated glass slides. Deparaffinized and rehydrated sections were incubated with 3 per cent H₂O₂. Primary monoclonal antibodies anti-cyclin D1 (DACO), anti-P16 (Santa Cruz) and anti-retinoblastoma protein (DACO) diluted 1:25, 1:25 and 1:100 respectively were applied. Tissue sections were incubated in humidified chambers for 60 minutes at room temperature. A three-step immunoperoxidase

method (ABC) was applied. As a chromogen 0.05 per cent 3,3'-diaminobenzidine tetrahydrochloride (DAB) in Tris buffer, pH 7.4 was used. Counter-staining was performed with haematoxylin. Intensity of cyclin D1 immunostaining was scored on a three-tiered scale as follows: (–) absent or very weak – 10–30 per cent cancer cells stained, (+) moderate – 30–50 per cent cancer cells stained, (++) strong – above 50 per cent cancer cells stained. Immunostaining of P16 and retinoblastoma protein was treated as positive when more than 20 per cent of cancer cells were stained.^{13,21,32,33} Statistical analysis using Chi-squared test and exact Fisher test was performed. Differences were considered statistically significant at *p* < 0.05.

Results

Decreased nuclear immunostaining for P16 was detected in 27 out of 58 (47 per cent) informative laryngeal carcinoma samples. In 11 cases no immunostaining was observed. There was normal immunostaining in epithelium adjacent to the tumour. In our study a significant association between the histopathological grading (G) of cancer and P16 expression was observed (Fisher's exact test, *p* < 0.05). Decreased P16 expression was noted more frequently among G3 patients (*p* = 0.06). In G1 samples 50 per cent of patients in the group showed lower expression, while 62.5 per cent of the G3 samples displayed such alteration as seen in Table I. No significant correlation between P16 immunostaining and TNM status was observed.

Strong nuclear immunostaining (over 50 per cent of cells) for cyclin D1 was detected in 28 out of 58 (48 per cent) informative laryngeal carcinoma specimens. Cyclin D1 protein was mostly immunolocalized in non-cancerous epithelium adjacent to the tumour. Only 7.1 per cent of the T₁–T₂ cases revealed overexpression of cyclin D1 whereas overexpressed cyclin D1 was detected in 51.9 per cent of T₃–T₄ cases (*p* = 0.001). No other correlation between cyclin D1 expression and clinical parameters was observed.

TABLE II
RELATION BETWEEN P16 AND RETINOBLASTOMA PROTEIN EXPRESSION

P16 expression	High expression of Rb (No. of cases)	Low expression of Rb (No. of cases)	Q Cochran test
High expression	25	6	<i>p</i> =0.0004
Low expression	26	1	

TABLE III
RELATION BETWEEN CYCLIN D1 AND RETINOBLASTOMA PROTEIN EXPRESSION

Cyclin D1 expression	High expression of Rb (No. of cases)	Low expression of Rb (No. of cases)	Q Cochran test
Absent or moderate expression	29	1	$P=0.00001$
High expression	22	6	

Positive nuclear immunostaining for retinoblastoma protein was detected in 51 out of 58 (88 per cent) informative laryngeal carcinoma samples. Low expression of retinoblastoma protein was significantly correlated with positive P16 immunostaining ($p = 0.0004$) (Table II). There was also a statistically significant correlation between cyclin D1 and retinoblastoma protein expression ($p = 0.00001$). Weak immunostaining of retinoblastoma protein was correlated with strong cyclin D1 expression (Table III). There was no relation between immunostaining of P16 and cyclin D1.

Among 58 informative cases, 15 patients had positive expression of all the proteins. Aberrant expression of at least one protein in the examined cell cycle protein (P16, cyclin D1, retinoblastoma protein) was observed in 25 patients (43.1 per cent) (in 14 cases for P16, in 10 cases for D1 and in one for retinoblastoma protein). Disturbance in simultaneous expression of two proteins was revealed in 17 patients (29.3 per cent) (in 12 cases for both P16 and D1; and in five cases for both cyclin D1 and retinoblastoma protein). We observed aberrant expression of all three analysed proteins in only one case. It can result from a low frequency of aberrant expression of retinoblastoma protein in patients with laryngeal cancer.

Laryngeal tissue serving as a negative control revealed correct expression for all P16, cyclin D1 and retinoblastoma protein in all cases ($p = 0.001$).

Discussion

Knowledge about the molecular basis of carcinogenesis has been developing over the last few years. Deregulation of cell cycle control seems to be one of the most important events in this process. This study presents the analysis of the cell cycle regulatory factors expression in patients with laryngeal cancer. The authors concentrated on the analysis of the proteins that are involved in the retinoblastoma protein pathway controlling the cell cycle as it moves into the DNA synthesis phase (G_1/S).^{4,34,35} The role of cell cycle regulatory factors in laryngeal cancer has not been elucidated. The papers dealing with this problem usually present results of estimation of one or two such factors.

P16 protein regulates the activity of CDK4/CDK6-cyclin D complex and indirectly phosphorylation of retinoblastoma protein. In normal cells the P16 protein is synthesized at a very low level. In cancerous cells inactivation of the retinoblastoma protein pathway results in an increase of P16 expression.⁵ Tekeuchi *et al.* revealed the correlation

between low expression of P16 protein and overexpression of cyclin D1.³⁶ Relation between the expression of these two proteins was observed in cell cultures.³⁷ In our study there was no correlation between P16 and cyclin D1 immunostaining. We found adverse correlation between P16 and retinoblastoma protein expression. The same adverse correlation was found between cyclin D1 and retinoblastoma protein expression by other authors.^{13-16,38,39}

Our results indicate that changes in immunostaining of P16, cyclin D1 and retinoblastoma protein are common. This confirms the important role of G1/S cell cycle phase regulation in laryngeal cancer. Low or absent immunostaining for P16 was detected both in cancer tissue and in surrounding dysplastic regions which indicate that dysfunction of this gene is an early event in carcinogenesis. These results are in accordance with the findings of Gallo *et al.*³² who additionally revealed that dysplastic lesions of the larynx with low P16 expression are prone to cancerous progression. The authors also found that immunostaining of P16 correlated with histological grading of the tumour. Low expression of P16 revealed 62.5 per cent of tumours in stage G3. It may indicate that in low differentiated tumours with high proliferation activity cell clones with dysfunction of the P16 gene dominate.

Low expression of retinoblastoma protein was detected in 12 per cent of our patients. This is very close to the results of other authors.¹³⁻¹⁶ This indicates that mutation of the retinoblastoma protein gene and loss of retinoblastoma protein expression are rare events in laryngeal cancer.

Overexpression of cyclin D1 results in the shortening of the G1 phase of cell cycle promoting cell proliferation. Abnormal expression of cyclins has been considered to be one of the most important factors in the tumorigenesis of various human malignancies. Strong expression of cyclin D1 was detected in head and neck tumours.²⁷ Cyclin D1 was found to be an independent prognostic factor in the study of Michalides *et al.*²² In other papers correlation between cyclin D1 overexpression and nodal metastases and advanced stage of disease was detected.^{21,27,40} We found overexpression of cyclin D1 in 48 per cent of the patients. These results are in accordance with those of other papers.^{21,26} There is strong evidence that overexpression of cyclin D1 rarely occurs in the early stage of laryngeal cancer.^{29,40} Cyclin D1 protein overexpression leads to increased cell proliferation, which would give neoplastic cells a growth advantage and may also

favour the occurrence of additional genetic lesions with potential oncogenic effects. In the present study overexpression of cyclin D1 in cases with early stage of the disease was found in 7.1 per cent of all cases with aberrant expression of cyclin D1 compared with 51.9 per cent of cases with advanced tumours that are characterized by abundant genetic lesions.

- **This is a study looking at the relative expression of three cell cycle proteins in tissues taken from patients suffering with laryngeal carcinoma**
- **It is widely understood that defects in the cell cycle play a role in the development of malignancy and the previous literature suggests that abnormalities of P16, cyclin D1 and retinoblastoma protein may either be implicated in the pathogenesis of malignant disease or may be correlated with a poor prognosis**
- **In this study 58 tissue samples were examined and compared to non-malignant laryngeal tissue. Diminished P16 expression was associated with a lack of differentiation and cyclin D1 expression associated with locally advanced disease. The authors also found that a low expression of retinoblastoma protein was correlated with positive P16 immunostaining and a strong expression of cyclin D1**
- **This study suggests that abnormalities in these proteins may have prognostic value in the assessment of laryngeal malignancy**

The authors did not find any correlation between examined factors and clinical features of the tumour. It may be explained to some extent by the low homogeneity of the examined group. Most of the patients were treated at an advanced stage of disease.

The results show significant correlations between decreased P16 expression and increased tumour dedifferentiation. Overexpression of cyclin D1 was statistically more common in locally advanced tumours (T₃–T₄). Low expression of retinoblastoma protein was significantly correlated with both positive P16 immunostaining and with strong cyclin D1 expression. This study confirms that dysfunction of cell cycle regulation is a very important event in head and neck tumours.⁴¹ The prognostic value of these factors should be confirmed in prospective studies.

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