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Cyclin D1 protein expression is related to clinical progression in laryngeal squamous cell carcinomas

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Abstract

The expression of cyclin D1 gene was investigated in 74 laryngeal squamous cell carcinomas (LSCCs) in order to determine its clinical and prognostic value. Overexpression of cyclin D1 was detected immunohistochemically using DCS6 monoclonal antibody on formalin-fixed, paraffin-embedded tissue sections. Cyclin D1 expression was detected in 22 of the 74 cases investigated (30 per cent), thirteen of which presented nodal metastases (59 per cent); of the patients without any detectable cyclin D1 protein expression, six presented nodal metastases (12 per cent). Cyclin D1 protein expression was found in five per cent of the specimens of normal mucosa, eight per cent of those with low-grade dysplasia and 20 per cent of those with high-grade dysplasia. A statistically significant association was found between cyclin D1 expression and the supraglottic site ($p<0.05$), tumour extension ($p<0.001$), the presence of lymph node metastases ($p<0.001$), and advanced clinical stage ($p<0.001$). Cyclin D1 expression analysis is an important tool in the selection of LSCC patients with an aggressive clinical course.

Key words: Carcinoma, squamous cell; Larynx; Tumour markers, biological

Introduction

Laryngeal cancer represents the second most common malignant neoplasm of the respiratory tract after lung cancer (Cattaruzza *et al.*, 1996), and it is associated with risk factors such as smoking and alcohol consumption (Johnson, 1990). Traditional clinicopathological features such as tumour stage, histological grade and the presence of lymph node metastases do not seem to be sufficient to predict clinical outcome in patients with laryngeal squamous cell carcinomas (LSCCs) (Goldsmith and Pillsbury, 1991).

Recent studies have demonstrated that the activation of some proto-oncogenes and the inactivation of tumour suppressor genes are involved in the pathogenesis of these tumours (Field, 1992). The amplification of genes located in the chromosome 11 band q13 represents one of the most frequent genetic alterations in head and neck cancer (Bartkova *et al.*, 1995b); the genes mapped to this region include proto-oncogenes such as PRAD-1/cyclin D1 (Motokura *et al.*, 1991; Schuuring *et al.*, 1992), *int*-2/FGF3 (Casey *et al.*, 1986; Leonard *et al.*, 1991), *hst*-1/FGF4 (Adelaide *et al.*, 1988), and *ems*-1 (Patel *et al.*, 1996). The PRAD 1/cyclin D1 gene encodes for a protein that exerts its function in the late G1 phase of the cell cycle by complexing with *cdk* 4 and 6. The regulatory function of the cyclin D1/*cdk*s complex takes place through the phosphorylation of the proteins involved in cell cycle control, such as pRB (Bartkova *et al.*, 1995a; Gillett *et al.*, 1994). The cyclin D1-*cdk*s complex is negatively regulated by $p15^{INK4B}$ and $p16^{INK4A}$ genes (Cordon-Cardo, 1995), which are known to be mutated or deleted in many human malignancies, as well as by the p21 gene which, in its turn, is activated by wild type p53.

Deregulation of the PRAD-1/cyclin D1 gene is involved in the development of various types of tumour, such as parathyroid adenomas and non-Hodgkin lymphomas (Motokura *et al.*, 1991; Rosenberg *et al.*, 1991), as well as breast (Bartkova *et al.*, 1994; Gillett *et al.*, 1994), oesophageal (Shinozaki *et al.*, 1996), liver (Zhang *et al.*, 1993), ovarian (Courjal *et al.*, 1996) and head and neck carcinomas (Bartkova *et al.*, 1995b; Fracchiolla *et al.*, 1995; Bellacosa *et al.*, 1996).

Cyclin D1 amplification correlates well with mRNA overexpression (Jares *et al.*, 1994), and is associated with an increased expression of cyclin D1 protein that is detectable on archival samples by means of immunohistochemistry (Bartkova *et al.*, 1995a). Nevertheless, recent studies have underlined

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the fact that cyclin D1 is also overexpressed in the absence of any measurable increase in gene copy number, thus supporting the observation that mechanisms such as chromosomal translocations (Michalides *et al.*, 1995) can lead to deregulated expression; these data suggest that immunohistochemistry can be considered a useful and reliable methodological approach to the study of the abnormalities of this oncogene. Cyclin D1 expression has been immunohistochemically investigated in various tumours, including breast, colorectal, and uterine carcinomas, as well as melanomas and soft tissue sarcomas (Bartkova *et al.*, 1994; Bartkova *et al.*, 1995a; Michalides *et al.*, 1996). With regard to head and neck tumours, it has been shown that cyclin D1 gene amplification and/or protein expression are related to the presence of lymph node metastases (Jares *et al.*, 1994; Muller *et al.*, 1994), advanced clinical stage (Muller *et al.*, 1994), tumour recurrence and decreased survival (Schuuring *et al.*, 1992; Michalides *et al.*, 1995).

The role of cyclin D1 expression in laryngeal cancer has not yet been extensively investigated. The aim of the present study was to evaluate the incidence of cyclin D1 expression in a homogeneous group of laryngeal squamous cell carcinomas, and to correlate this expression with clinicopathological parameters, such as histological grade, clinical stage and lymph node metastases.

Materials and methods

Pathological samples

We studied a series of 74 patients with laryngeal squamous cell carcinomas admitted to Clinica ORL I of the University of Milan. Informed consent was obtained from all of the patients included in the study.

Pathological samples from each patient were collected during surgery and fixed in 10 per cent buffered formalin. The main clinical and pathological characteristics of the patients included in the study are shown in Table I. Seventy-three were male and one female, and their mean age was 63 years (range 46–80). Seventy of the cases presented with primary tumour; the remaining four cases had recurrent tumours. Sixty-nine patients had been exposed to risk factors such as tobacco smoke or alcohol; none of them had undergone previous radio- or chemotherapy. The diagnosis, clinical staging and identification of the anatomical site of the LSCCs were based on the UICC's TNM classification of malignant tumours (UICC, 1987): 28 tumours were supraglottic and 46 glottic; 32 were in stage I, 11 in stage II, 10 in stage III and 21 in stage IV. Nodal metastases were present in 19 cases. Macroscopically, 11 cases were characterized by an exophytic and/or ulcerative pattern of tumoral growth, and 63 by an infiltrative pattern. The histological grade was determined according to Shanmugaratnam and Sobin, (1991): 13 cases were well differentiated (G1), 39 moderately so (G2) and 22 poorly differentiated (G3). Normal laryngeal mucosa was histologically detectable in 44 specimens; low dysplasia in 36; high dysplasia in 20 (see Table I). The lymph node metastases corresponding to 12 primitive tumours were also immunohistochemically analysed.

TABLE I
CLINICO-PATHOLOGICAL PARAMETERS *vs* CYCLIN D1 EXPRESSION

	n.	Cyclin D1 expression	Comparison	*p* value
Sex				
Male	73	21		
Female	1	1		
Age (yr)				
<60	29	11 (38%)		>0.1
>60	45	11 (24%)		
Risk factors				
Yes	69	20 (29%)		>0.7
No	5	2 (40%)		
T				
X	0			
1	32	0		
2	14	5 (36%)	T_{1-2} *vs* T_{3-4}	<0.001
3	15	8 (53%)		
4	13	9 (69%)		
N				
0	55	9 (16%)		
1	5	4 (80%)	N_0 *vs* *N*+	<0.001
2	4	3 (75%)		
3	10	6 (60%)		
Stage				
I	32	0		
II	11	3 (27%)	I–II *vs* III–IV	<0.001
III	10	5 (50%)		
IV	21	14 (67%)		
Site				
Supraglottic	28	15 (54%)		<0.02
Glottic	46	7 (15%)		
Grading				
G1	13	3 (23%)		
G2	39	10 (26%)	G1 *vs* G2–3	>0.7
G3	22	9 (41%)		

Immunohistochemistry

The DCS6 (Novocastra Laboratories Ltd., Newcastle upon Tyne, UK) monoclonal antibody (MoAb) and a standard immunocytochemical ABC technique (Hsu *et al.*, 1981) was used to detect cyclin D1 expression. Briefly, the dewaxed sections were rehydrated, incubated with three per cent hydrogen peroxide in order to block endogenous peroxidase, and the slides were then treated for 15 minutes with 0.01 per cent pronase (Dako S2013). After extensive rinsings, the slides were incubated with: i) 20 per cent non-immune human serum for 20 minutes, at room temperature (r.t.); ii) MoAb (DSC6) (working concentration: 1mg/ml), overnight at 4°C; iii) biotin-labelled antiserum against mouse IgG (Vector, Burlingame, CA) at 1:20 dilution for 50 minutes at r.t.; iv) avidin-biotin-peroxidase complex (Vector, Burlingame, CA) at 1:100 dilution for 50 minutes at r.t. For the MoAb dilutions and slide rinsing 0.05 M TRIS buffered pH 7.6 saline (TBS) was used. Peroxidase activity was determined in a 0.03 per cent 3-3' diaminobenzidine tetrahydrochloride (Sigma

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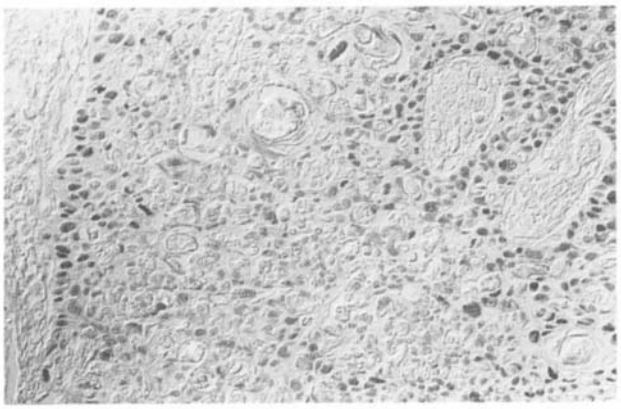

FIG. 1

An example of moderately differentiated squamous cell carcinoma showing numerous cells strongly immunoreactive to DCS6 antibody (original magnification 100 ×; haematoxylin counterstain).

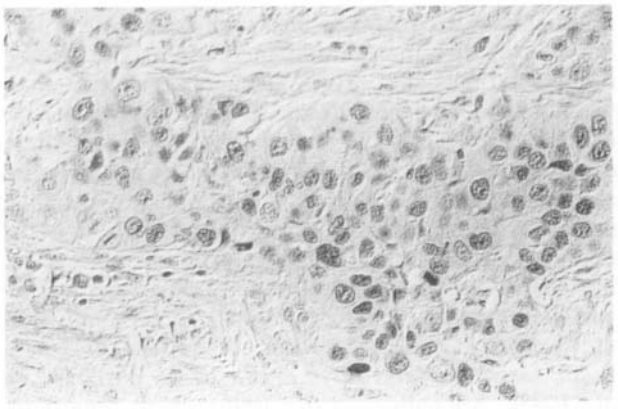

FIG. 2

In this case of poorly differentiated laryngeal squamous cell carcinoma, the cyclin D1 expression is limited to the nucleus, and its intensity ranges from weak to strong (original magnification 200 ×; haematoxylin counterstain).

Chemical Co., St Louis, MO) solution to which hydrogen peroxide was added. The slides were slightly counterstained using a 30 per cent Harris haematoxylin solution. The specific control tests included: a formalin-fixed, paraffin-embedded breast carcinoma as the positive control and the substitution of the MoAb with non-immune mouse serum as the negative control. The immunostained slides were independently evaluated by three of the authors (GP, NC, RB); in the few cases in which these assessments led to different results, a consensus was reached after re-examination. The number of the immunoreactive (IR) cells was counted using a gridded eye-piece at 250× magnification in at least 30 tumoral fields. Tumour staining was classified using the following scoring system: – = negative; 1+ = fewer than 10 per cent IR neoplastic cells; 2+ = 10–30 per cent IR neoplastic cells; 3+ = 30–50 per cent IR neoplastic cells; 4+ = more than 50 per cent IR neoplastic cells.

Statistical methods

Chi-squared tests with Yates' correction (Armitage and Berry, 1991) were used to evaluate the correlations between the overexpression of cyclin D1 and the evaluated clinico-pathological parameters.

Results

Cyclin D1 expression was detected by DCS6 MoAb in 22 of the 74 LSCCs investigated (30 per cent) (Figures 1 and 2, Table I). In most of the positive cases, the IR cells represented the minor component of the tumour: twelve cases (55 per cent) were scored 1+, six (27 per cent) 2+, and four (18 per cent) 3+; no tumour samples showed more than 50 per cent of IR neoplastic cells (Table I). The immunostaining pattern was predominantly nuclear, but some neoplastic cells also had a simultaneous cytoplasmic signal. The levels of cyclin D1 staining were generally heterogeneous (weak to strong), possibly due to variations in protein levels during cell-cycle progression.

Immunoreactivity was more pronounced amongst the most poorly differentiated tumours: of the positive cases, three G1 (14 per cent), 10 G2 (45 per cent) and nine G3 (41 per cent) LSCCs were immunoreactive to DCS6 MoAb. Furthermore, the majority of immunoreactive tumours (19/22; 86 per cent) were at an advanced clinical stage (III and IV).

Non-neoplastic laryngeal mucosa, with or without dysplastic changes, was available in most of the samples analysed (Table II). In particular, cyclin D1 expression was detectable in two out of 44 (five per cent) samples of normal mucosa, three out of 36 (eight per cent) samples with mild or moderate dysplasia, and four out of 20 (20 per cent) samples with high-grade dysplasia (Table I).

Interestingly, the corresponding neoplastic components of the two normal mucosa samples showing IR cells did not present cyclin D1 expression. On the other hand, the adjacent tumour component was immunoreactive in 90 per cent of the low and high-grade dysplastic cyclin D1-positive specimens (data not shown).

TABLE II

IMMUNOHISTOCHEMICALLY DETERMINED CYCLIN D1 PROTEIN LEVELS IN THE LSCC LESIONS INCLUDED IN THE STUDY

Tissue/tumour	No. of samples	Cyclin D1 immunoreactive cells +	++	+++
Normal mucosa	44	2	0	0
Low dysplasia	36	2	0	1
High dysplasia	20	1	3	0
Carcinoma	74	12 (55%)	6 (27%)	4 (18%)
Lymph node metastasis	12	3	0	2

Legend: + = fewer than 10% IR cells; ++ = 10–30% IR cells; +++ = 30–50% IR cells.

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Thirteen cases with cyclin D1 protein expression presented nodal metastases (13/22; 59 per cent); of the 52 LSCC patients without any detectable cyclin D1 expression, six had nodal metastases (12 per cent). A complete concordance in cyclin D1 expression was found between primitive tumours and their corresponding lymph node metastases.

Statistical analysis

Statistical analyses were performed to correlate the presence of cyclin D1 protein expression with a series of clinicopathological variables. No significant correlation was found with age, sex, exposure to risk factors (smoking habit, alcohol consumption), histological grade, or the pattern of tumoral growth (exophytic and/or ulcerated *vs* infiltrating). A statistically significant correlation was found between cyclin D1 expression and the anatomical site of the tumour (supraglottic *vs* glottic) ($p<0.05$). the extension of the tumour (T_1–T_2 *vs* T_3–T_4) ($p<0.001$), lymph node metastases (N_0 *vs* N+) ($p<0.001$) and clinical stage (stage I–II *vs* stage III–IV) ($p<0.001$).

Discussion

We found cyclin D1 overexpression in 30 per cent of the cases in a homogeneous cohort of 74 laryngeal squamous cell carcinomas; this rate is lower than that observed by others in different subsets of mainly advanced head and neck carcinomas (Bartkova *et al.*, 1995b; Michalides *et al.*, 1995). As we have recently reported that cyclin D1 expression never occurs in stage I glottic laryngeal carcinomas (Pignataro *et al.*, 1996), our finding could be ascribed to the fact that our series was characterized by a prevalence of early stage tumours (stage I and II).

A significant correlation was observed between the overexpression of cyclin D1 and tumour extension ($p<0.001$) and an advanced clinical stage ($p<0.001$). These results are in keeping with those of Michalides *et al.* (1995) who have recently reported a correlation between cyclin D1 protein expression, tumour recurrence and decreased survival in a panel of 47 head and neck squamous cell carcinomas. The reasons for these findings remain unexplained: however, cyclin D1 protein overexpression does lead 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.

Moreover, we found that cyclin D1 overexpression significantly paralleled metastatic lymph node involvement ($p<0.001$), a finding that has never been previously reported in immunohistochemically analysed tumour samples.

Jares *et al.* (1994) recently observed a correlation between mRNA overexpression and the presence of lymph node metastases in a series of LSCCs. If confirmed in a larger series, these data could have clinical implications because lymph node status is considered to be the most important prognostic factor in patients with head and neck cancer (Atula *et al.*, 1996), as this type of tumour is associated with a high incidence of cervical lymph node metastasis, possibly due to its rich lymphatic drainage. Since clinically negative lymph nodes have been reported to show a significant number of occult metastases, the elective treatment in N_0 LSCC patients remains controversial (Gallo *et al.*, 1996). This, together with other traditional diagnostic approaches such as cervical lymph node ultrasound (Atula *et al.*, 1996), the evaluation of cyclin D1 overexpression could represent an additional means of selecting N_0 patients to be submitted to selective functional neck dissection (Li *et al.*, 1996).

Davidson *et al.* (1996) have recently found a relationship between tobacco exposure and cyclin D1 mRNA expression in a heterogeneous group of head and neck carcinomas, thus further underlining the pivotal role of tobacco smoking in the molecular pathogenesis of this type of tumour. As the overwhelming majority of the patients in our retrospective series of consecutively-collected laryngeal cancers had been exposed to risk factors (Table I), we were not able to confirm this finding.

We detected cyclin D1 protein expression not only in tumour sections, but also in the normal (five per cent), low (eight per cent) and high grade (20 per cent) dysplastic mucosa surrounding the immunoreactive tumoral tissue. These data are in keeping with those of others, who have found cyclin D1 mRNA and/or protein expression in preneoplastic lesions of different anatomical sites, such as hyperplasia and *in situ* carcinoma of the breast (Motokura *et al.*, 1991; Bartkova *et al.*, 1994; Wang *et al.*, 1994; Bartkova *et al.*, 1995a). Taken together, these data support the hypothesis that a de-regulation of cyclin D1 expression may be involved in the early stages of tumorigenesis in various types of tumour, including laryngeal squamous cell carcinomas.

Conclusion

Overall, our results are consistent with the hypothesis that the cyclin D1 gene plays an important role in the development and progression of LSCCs. As recently described in a number of different tumours, cyclin D1 analysis could be used as an indicator of more aggressive laryngeal squamous cell carcinomas.

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