

Expression of Cyclin D1 and Ki-67 in Squamous Cell Carcinoma of the Penis

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Abstract. *Background:* Cyclin D1 plays an important role in regulating the progression of cells through the G1-phase of the cell cycle. The aim of the study was to investigate the expression of cyclin D1 and Ki-67 in squamous cell carcinomas (SCC) and in some premalignant lesions of the penis and to correlate it with clinicopathological parameters and patient survival. *Materials and Methods:* Formalin-fixed paraffin-embedded tissues from 21 SCC, 7 lichen sclerosus, 5 condyloma acuminatum and 2 erythroplasia of Queyrat were stained by immunohistochemistry for cyclin D1 and Ki-67. *Results:* Cyclin D1-positive nuclear staining was overexpressed in 13/21 SCC (61.9%) and in one case of erythroplasia of Queyrat. Strong reactivity for Ki-67 was found in 16 (76.2%) SCC, in 3 condyloma acuminatum and in one case of erythroplasia of Queyrat. A tendency for an association between cyclin D1 expression and tumour differentiation ($p=0.07$) but not the level of tumour invasion ($p=0.50$) was found. The Ki-67 expression was notably increased with the advance of tumour grade, but the difference did not reach a statistically significant level ($p=0.46$). A slight tendency towards a relationship between Ki-67 and cyclin D1 protein expression was observed ($p=0.32$). Two patients relapsed and one died from the disease over a median follow-up period of 4.6 years (range 0.1-10.3 years). *Conclusion:* Ki-67 antibody and cyclin D1 overexpression seem to parallel each other, supporting the concept that cyclin D1 serves as a cell cycle activator. Cyclin D1 overexpression may be used as a prognostic factor of poor outcome in penile carcinoma.

Squamous cell carcinoma (SCC) of the penis is a relatively rare malignancy, accounting for only approximately 0.4-

0.6% of all malignancies in men, and the carcinogenic mechanism is largely unknown (1). Lichen sclerosus and condyloma acuminatum are premalignant lesions associated with penile SCC, while erythroplasia of Queyrat represents squamous carcinoma *in situ*.

Alterations of genes involved in cell cycle control and subsequent deregulation of the G1/S transition may cause uncontrolled cell cycle progression and may be implicated in the development and progression of cancer (2).

Cell cycle progression is regulated by a group of cyclins and cyclin-dependent kinases (CDKs) acting at different phases of the cell cycle. Cyclins are divided into two main families. The G1 family includes cyclins C, D1, 2, 3 and E and the other family the cyclins A and B. Cyclin D1 (CCND1), through interaction with cyclin-dependent kinase, induces phosphorylation of the retinoblastoma gene protein (pRB) (3). Unphosphorylated pRB binds to and inactivates transcription factors such as E2F, and prevents the G1-S transition, whereas phosphorylated pRB does not interact with E2F and promotes gene expression (4).

Increased expression of cyclin D1, as a result of amplifications and rearrangements, has been reported in parathyroid adenomas (5), some B-cell lymphomas (6), and in oesophageal (7, 8), head and neck (9, 10), hepatic (11), colorectal (12), and some breast carcinomas (13, 14). These studies have suggested that overexpression of cyclin D1 shortens the G1-phase and accelerates cell growth, thus positively contributing to oncogenesis (15, 16).

The nuclear Ki-67 protein, which can be visualized using the MIB1 antibody, is expressed in all proliferating cells (G1-, S-, G2-, M0-phase) but not in quiescent cells (G0-phase) or in the early G1-phase (17).

Overexpression of cyclin D1 has been associated with poor prognosis in SCC of the larynx (18), head and neck tumours (10), and non-small cell lung cancer (NSCLC) (19). Other studies have not found any prognostic impact of cyclin D1 in NSCLC (20), oral cancer (21) and mammary carcinomas (22), while cyclin D1 was associated with a good

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prognosis in a subset of breast (23), transitional cell carcinoma (24) and NSCLC (25, 26).

Recent studies have raised the possibility that cyclin D1 actually functions as a negative regulator of cellular proliferation (15, 16, 27) and indicated that the potential oncogenic properties of cyclin D1 *in vivo* are unclear.

The above data prompted us to investigate the expression of cyclin D1 in relation to the proliferation activity determined by the Ki-67 antibody in SCC and in some pre-cancerous lesions of the penis and to correlate it with clinicopathological parameters and clinical outcome. To the authors' knowledge, no previous report has addressed the significance of cyclin D1 overexpression in penile SCC.

Materials and Methods

Patients and tissues. From April 1995 to October 2005, 35 men were admitted to the Department of Plastic Surgery of "Andreas Sygros" Hospital, due to a suspicious lesion of the penis and underwent excisional biopsy of the lesion. The patients' ages ranged from 26-96 years old (mean 63.5). At clinical examination enlargement of the inguinal lymph nodes was not found. The diagnosis and the staging according to the TNM system were assessed from formalin-fixed paraffin-embedded sections, stained with haematoxylin and eosin. The median follow-up of the SCC patients was 4.6 years (range, 0.1-10.3 years). Two out of 21 patients relapsed at 0.3 and 5.2 years, respectively and one died at 10.3 years after the surgery.

Immunohistochemistry. Immunostaining was performed on formalin-fixed, paraffin-embedded tissue sections, using the streptavidin-biotin peroxidase labelling procedure for the presence of Ki-67 and cyclin D1 protein. The cyclin D1 (clone SP4, Neomarkers, USA) and the Ki-67 (clone MIB1, Dako, Copenhagen, Denmark) antibodies were used at a dilution of 1:30 and 1:50, respectively, for 45 minutes. Microwave heating in a solution of sodium citrate, pH6 was performed prior to incubation with both the antibodies, as described in our previous study (28). Negative control slides were prepared by omitting the primary antibody. A cyclin D1 overexpressing SCC of the head and neck served as a positive control for cyclin D1.

Interpretation of the staining. Cyclin D1 was considered as overexpressed when >10% of cell nuclei were stained, according to published data (21).

The median value for the Ki-67 (which was 8% in this study) was used as the respective cut-off point and tumours were classified as either less than or greater than the median value. The proportion of positive cells was counted in ten high-power fields (corresponding to a total of at least 1000 tumour cells).

Statistical analysis. Two-way analysis was performed with the Fisher's Exact test in the case of nominal variables and with the Jonckheere-Terpstra test in the case of ordinal variables. Student's *t*-test was used for comparing means. Survival analysis was not performed, since the number of events (two relapses and one death) was not sufficient to produce meaningful results with the Kaplan-Meier method. A *p*-value of less than 5% was considered statistically significant. All analyses were performed using SAS Release 9.0 (SAS Institute Inc, Cary, NC, USA).

Table I. Cyclin D1 and Ki-67 expression in the patients of our study.

	Cyclin D1 (<i>p</i> =0.002)		Ki-67 (<i>p</i> =0.0001)	
	Total	>10% <10%	>Median <Median	
SCC	21	13 8	16 5	
Condyloma acuminatum	5	0 5	3 2	
Erythroplasia of Queyrat	2	1 1	1 1	
Lichen sclerosis	7	0 7	0 7	

Results

Histology. The histology revealed 21 cases of SCC of the usual type (according to the WHO classification), 7 lichen sclerosis, 5 condyloma acuminatum and 2 erythroplasia of Queyrat. Thirteen of the carcinomas were well-differentiated, 5 moderately-, and 3 were poorly-differentiated. One case of poorly-differentiated and another one of moderately-differentiated SCC invaded the corpus spongiosum and cavernosum (stage pT2). The remaining 19 carcinomas invaded the subepithelial connective tissue (stage pT1).

Immunohistochemistry. Focal and weak staining of cyclin D1 was observed in squamous epithelium adjacent to tumours, but was always restricted to the basal and parabasal cell layer of the epithelium. Stromal cells and accompanying inflammatory cells were negative for cyclin D1.

Cyclin D1 was overexpressed in 13/21 penile SCC (61.9%) (Table I and Figures 1, 2) and in one case of erythroplasia of Queyrat (Figure 3). The remaining 8 cases of SCC (Figure 4), condyloma acuminatum (Figure 5) and lichen sclerosis (Figure 6) showed <10% cyclin D1-positive nuclei.

Strong Ki-67 reactivity (greater than the median value of 8%) was found in 16 (76.2%) squamous cell carcinomas, in 3 condyloma acuminatum and in one case of erythroplasia of Queyrat (Table I).

There was strong evidence that Ki-67 and cyclin D1 were more often observed in SCC than in the premalignant lesions (*p*<0.0001 and *p*<0.002, respectively).

SCC patient descriptive statistics. Patients with cyclin D1 overexpression had a lower mean age (62.1 years) while the patients with strong Ki-67 reactivity had a higher mean age (66.1 years) than the others, but the differences were not statistically significant (Table II). A trend for an association between cyclin D1 expression and decreasing differentiation (*p*=0.07) was observed.

The greater Ki-67 expression with the advance of tumour grade was observed, but the difference did not reach a statistically significant level (*p*=0.46). There was no

Table II. Cyclin D1 and Ki-67 expression in SCC in relation to clinicopathological findings.

	Total	Cyclin D1			Ki-67		
		>10%	<10%	<i>p</i>	>Median	<Median	<i>p</i>
Age							
Mean	64.3	62.1	67.8	0.27 (NS)	66.1	58.6	0.26 (NS)
Std	12.9	15.4	8.9		13.9	7.5	
N	21	13	8		16	5	
Differentiation grade							
Well	13	6	7		9	4	
Moderate	5	4	1	0.07 (NS)	4	1	0.46 (NS)
Poor	3	3	0		3	0	
Level of invasion							
pT1	19	11	8	0.50 (NS)	14	5	1.0 (NS)
pT2	2	2	0		2	0	

NS: Not statistically significant at the 5% level; std: standard deviation.

statistically significant association between cyclin D1 or Ki-67 expression and the level of tumour invasion ($p=0.50$ and $p=1.0$, respectively).

The cyclin D1 expression was not statistically significantly associated with the Ki-67 ($p=0.32$) (Table III), however the odds of a patient with strong Ki-67 reactivity having cyclin D1 overexpression were 3.3 times greater than for a patient without strong Ki-67 reactivity.

Cyclin D1 expression seemed to be associated with a poor clinical outcome since the patients who relapsed or died showed greater than 10% positive cells.

Discussion

CCND1 gene amplification usually correlates with increased cyclin D1 mRNA and protein expression. However, a significant number of carcinomas exhibit cyclin D1 overexpression without amplification of the gene (29). Therefore, other factors are likely to be involved, such as a mutation in the promoter region, a post-transcriptional event, or alterations of a regulatory transcription factor (29). It has been assumed that such overexpression may lead to the propagation of unrepaired DNA damage, the accumulation of genetic errors, and a selective growth advantage for altered cells (30). Recent studies have indicated that cyclin D1 affects the activity of various other non-CDK-dependent cellular transcription factors, such as oestrogen, DMP1, STAT3, and BETA2/Neuro D (31).

In the present study cyclin D1 was overexpressed in 61.9% of SCC, which was higher than the percentage in oesophageal, NSCLC, cervical carcinomas (8, 32, 33) and similar to that in oral cavity, head and neck and breast carcinomas (30, 34-36).

Table III. Association of Ki-67 with Cyclin D1 expression.

Cyclin D1	Total	Ki-67 ($p=0.32$)	
		>Median	<Median
>10%	13	11	2
<10%	8	5	3

The tendency towards a relationship between cyclin D1 expression and differentiation grade was in agreement with another study (37), but not with others in breast (22, 23) and head and neck cancer (38).

In our patients, Ki-67 expression was more pronounced with the advance of tumour grade, a finding that is in accordance with that of Berdjis *et al.* (39).

In our study the finding that cyclin D1-positive cells were located in the proliferation active basal cell compartment of the squamous epithelium indicated that expression of cyclin D1 functions mainly in promoting cell proliferation. The most straightforward explanation may be that cyclin D1 overexpression, *via* hyperphosphorylation of the retinoblastoma protein, induces increased tumour cell proliferation and thereby corresponds to an aggressive phenotype.

In our study, a slight tendency for a statistically significant relationship between proliferative activity and cyclin D1 protein expression was observed. This is in accordance with the findings in thyroid carcinomas (40) and cervical squamous cell carcinomas (33), but not in agreement with results in mantle cell lymphomas and in breast carcinomas (35, 41). A cyclin D1-independent proliferation pathway is

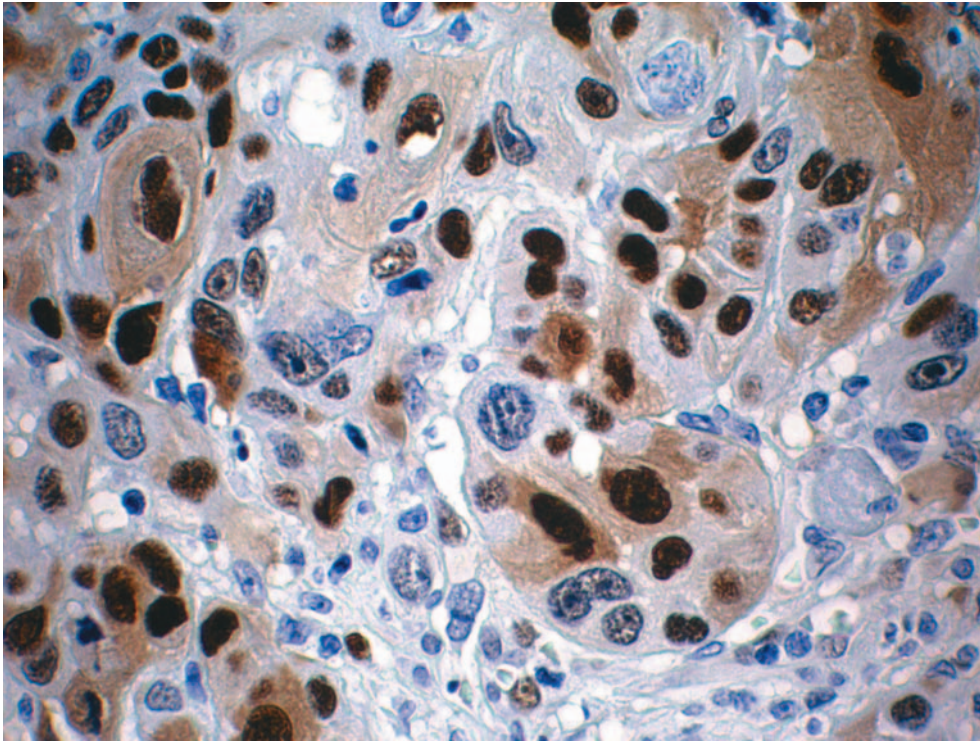


Figure 1. *Strong cyclin D1-positive nuclear staining in poorly-differentiated SCC (x400).*

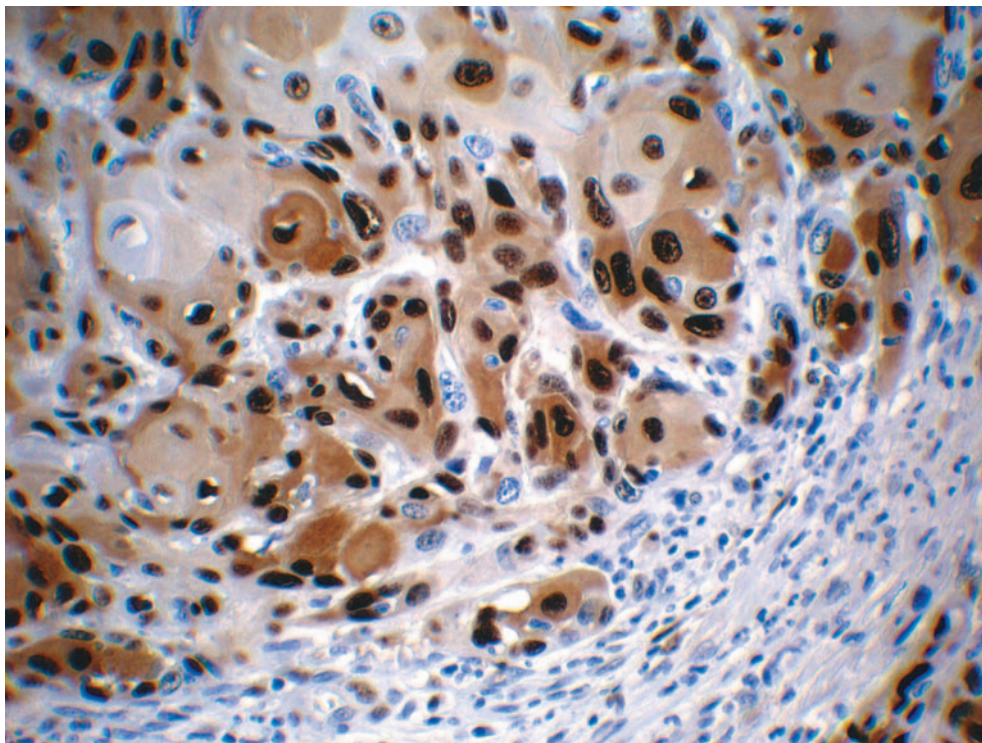


Figure 2. *Strong cyclin D1-positive nuclear staining in well-differentiated SCC (x400).*

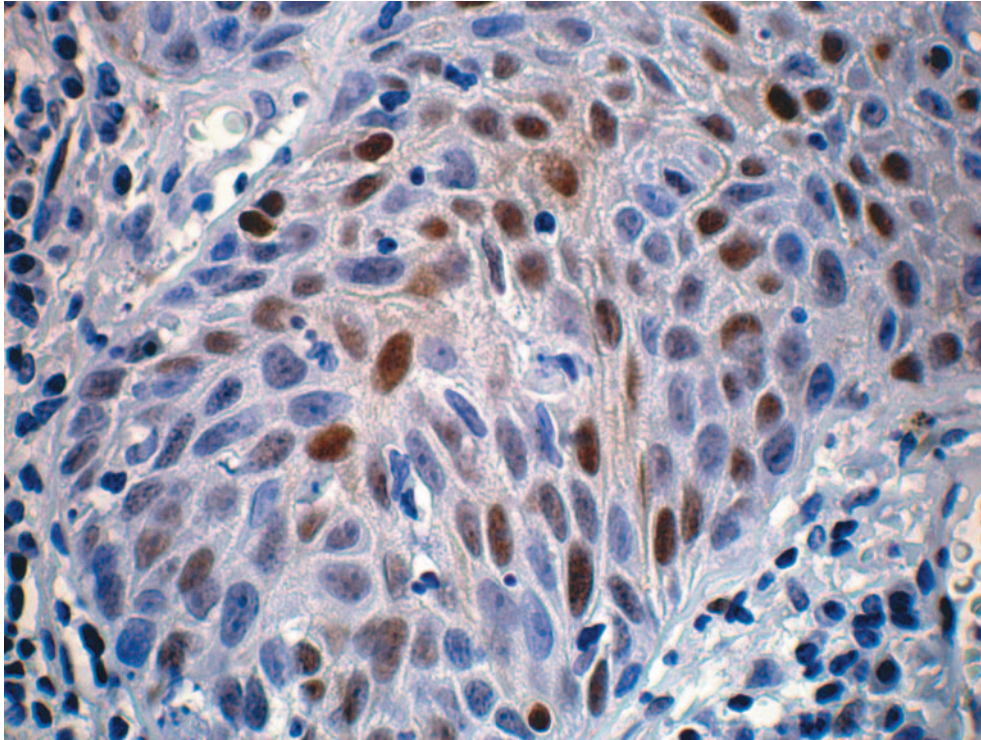


Figure 3. *Cyclin D1* expression in erythroplasia of Queyrat (x400).

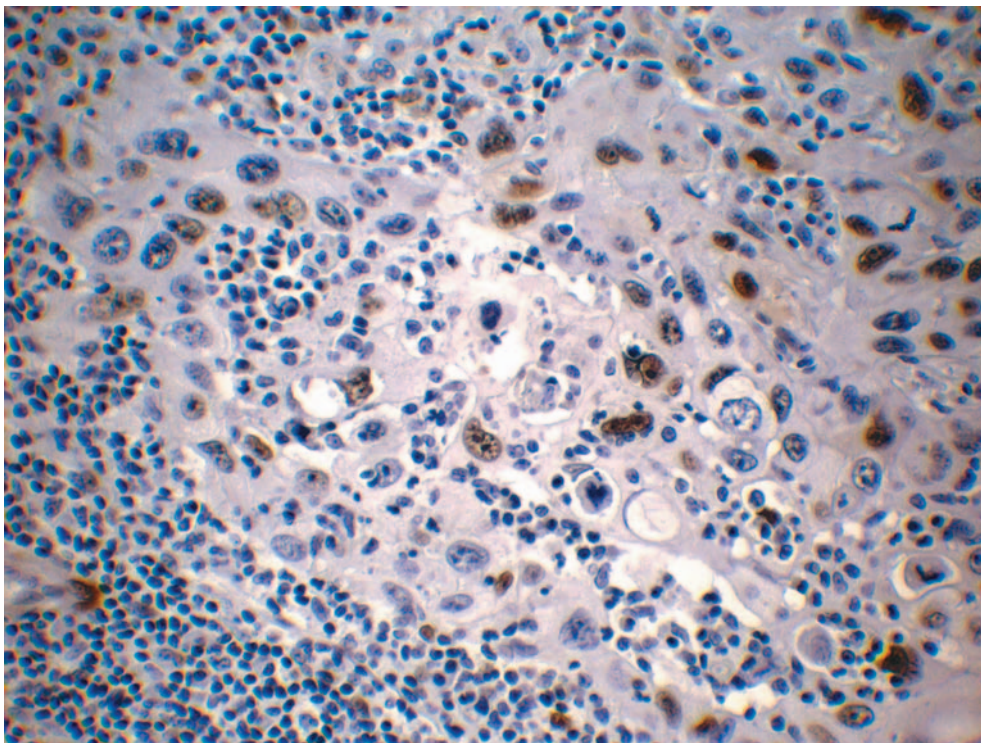


Figure 4. *Low cyclin D1*-positive nuclear staining in poorly-differentiated SCC (x200).

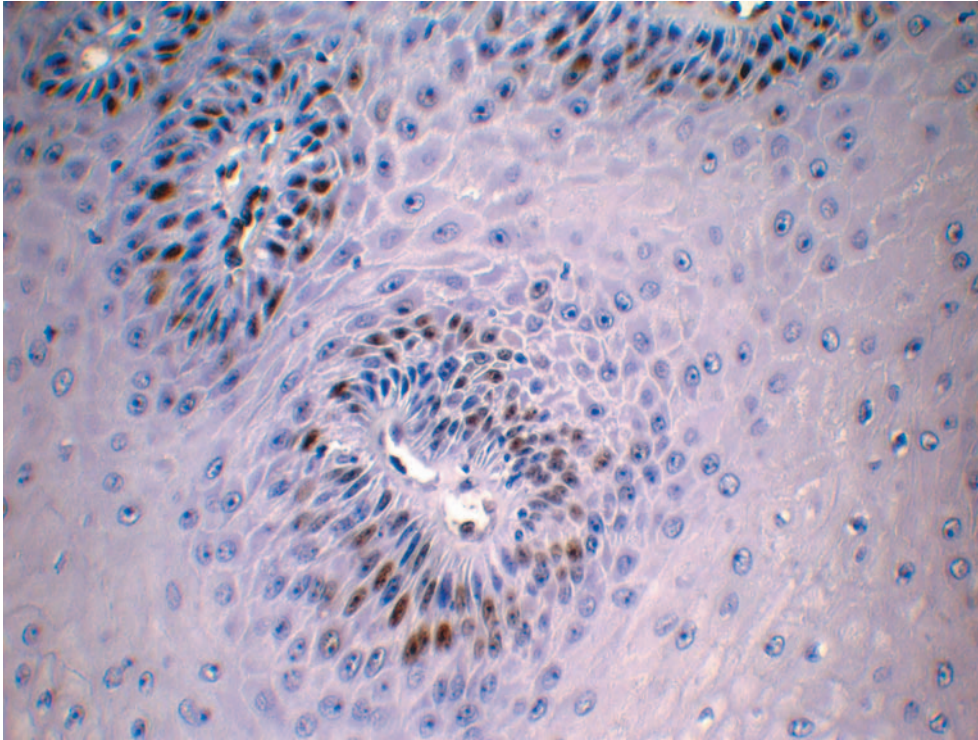


Figure 5. *Cyclin D1* expression in *condyloma acuminatum* (x200).

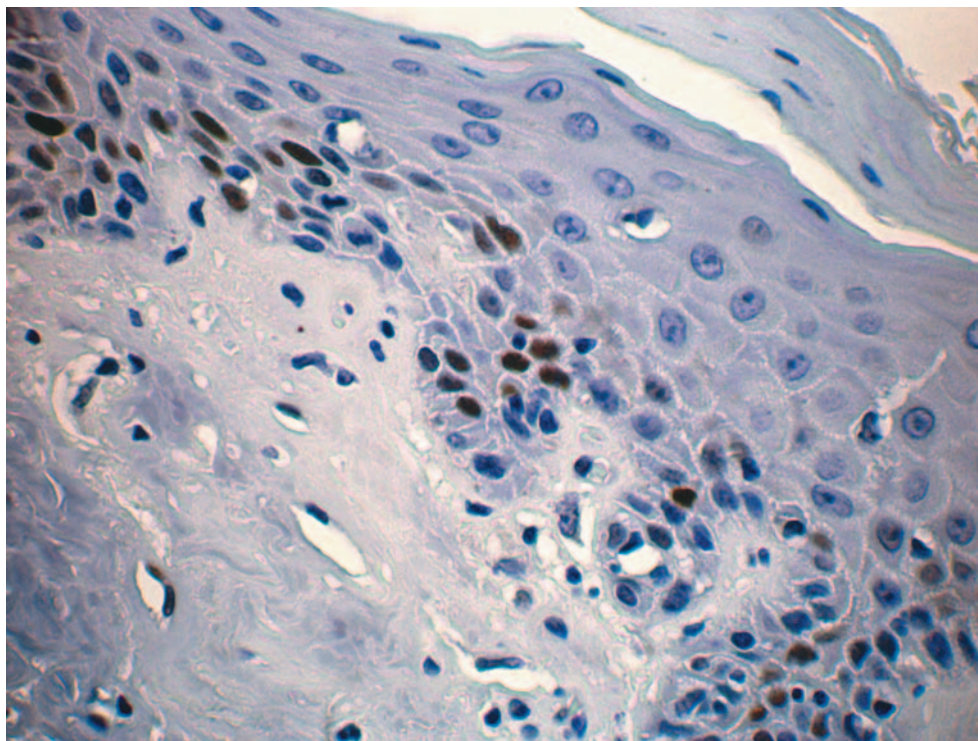


Figure 6. *Cyclin D1* expression in *lichen sclerosus* (x200).

considered as a plausible explanation for this observation (22). Another hypothesis is that while a moderate increase in the expression of cyclin D1 can enhance cell growth, a high level of expression can have an inhibitory effect (42).

Lichen sclerosis, condyloma and erythroplasia of Queyrat had a higher mean percentage of cyclin D1-positive cells than normal skin adjacent to benign lesions, but lower than SCC. Overexpression of cyclin D1 in premalignant lesions has been reported in murine skin carcinogenesis (43). The observation of cyclin D1 protein in premalignant and pre-invasive lesions suggests that this alteration is an early event in skin carcinogenesis.

The percentage of cyclin D1-positive cells decreased with increasing age, a finding that is not in accordance with other studies (42). Experiments *in vitro*, however, revealed that senescent human diploid fibroblasts expressed cyclin D1 at much higher levels than did young counterparts (44).

It was not possible to investigate the relationship between the expression levels of cyclin D1 and Ki-67 and overall survival in our series of patients, since the number of events (deaths or recurrences) was low. However, it would be of interest to evaluate a possible association by increasing the sample size or lengthening the follow-up.

In conclusion, our results suggest that cyclin D1 seems to function mainly in promoting cell proliferation and indicate its potential role in the pathogenesis of SCC.

Further studies are necessary to elucidate the prognosis of cyclin D1 expression in SCC of the penis.

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