Cyclin D3 Immunoreactivity Is an Independent Predictor of Survival in Laryngeal Squamous Cell Carcinoma

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ABSTRACT

Purpose: To analyze the prevalence and clinical relevance of cyclin D3 abnormalities in laryngeal squamous cell carcinoma (LSCC).

Experimental Design: Cyclin D3 immunoreactivity was evaluated in 223 formalin-fixed and paraffin-embedded samples of LSCC patients with a mean follow-up of 62.8 ± 43.2 months. The occurrence of cyclin D3 extra signals was analyzed by fluorescence in situ hybridization in 47 randomly selected cases collected in a tissue microarray. Cyclin D1 immunoreactivity had been previously investigated in 133 cases.

Results: Cyclin D3 immunoreactivity and gene extra signals were found in 39.5% and 42.6% of the cases, respectively, and the concordance between immunohistochemical and fluorescence in situ hybridization results was 70.2% (P = 0.0085). Cyclin D3 immunoreactivity was significantly associated with a high risk of death. Multivariate analysis showed that high tumor grade, exophytic/ulcerating tumor type, low performance status, and cyclin D3 immunoreactivity were the only independent predictors of poor overall survival. In the 133 cases analyzed for both cyclin D1 and cyclin D3, patients with cyclin D1+/cyclin D3+ tumors experienced the worst prognosis, patients with cyclin D1+/cyclin D3− exhibited the most prolonged survival, and with cyclin D1−/cyclin D3+ or cyclin D1+/cyclin D3− tumors an intermediate course was associated.

Conclusions: Our data suggest that cyclin D3 immunoreactivity, possibly due to the occurrence of gene extra copies, may represent an adjunct in LSCC patients’ prognostication and contribute to identify D-type cyclins as potential targets of newly developed therapies.

INTRODUCTION

Cyclin D3 plays a pivotal role in tightly controlling the physiologic progression from the G1 to the S phase of the cell cycle. After assembling with cyclin-dependent kinases 4 and 6, cyclin D3 is capable of phosphorylating the retinoblastoma gene product, eventually promoting the entry of the cell into the S phase (1).

Similarly to cyclin D1, cyclin D3 is likely to be a specific target of genetic abnormalities, such as the t(6;14)(p21.1;q32.3) translocation in a fraction of plasma cell myelomas (2) and non-Hodgkin’s lymphomas (3) and gains of the 6p21 locus in several different malignancies, including ovarian (4) and gastric (5) adenocarcinoma. It has been shown that cyclin D3 overexpression renders cells prone to malignant transformation (6). Accordingly, a deregulated expression of cyclin D3 due to genetic abnormalities (2, 3) or posttranscriptional mechanisms (7) has been ascertained by immunohistochemistry or Western blot in a variety of human malignancies (8–11) and is significantly associated with a worse prognosis in patients with malignant melanoma (8), breast carcinoma (9), and non-Hodgkin’s lymphoma (12).

Although data about cyclin D3 abnormalities in head and neck cancer patients are not available thus far, experimental findings suggest that cyclin D3 deregulation may be specifically involved in the pathogenesis and progression of squamous cell carcinoma. At variance with cyclin D1-MMTV transgenic mice, which are usually affected by mammary adenocarcinoma, cyclin D3-MMTV mice exclusively develop mammary squamous cell carcinoma (13). Moreover, UCN-01, a protein kinase C and cyclin-dependent kinase modulator, is capable of blocking G1→S progression in head and neck squamous cell carcinoma cell lines by down-regulating cyclin D3 expression (14).

In the present study, we evaluated for the first time the prevalence and clinical relevance of cyclin D3 abnormalities in a well-characterized series of 223 patients with laryngeal squamous cell carcinomas (LSCC) by immunohistochemistry and fluorescence in situ hybridization (FISH). Overall, our results provide evidence that cyclin D3 immunoreactivity is an independent predictor of poor survival, thus suggesting that it may be a useful adjunct in identifying patients with a more aggressive disease who may benefit from cyclin D3–targeted therapies.

PATIENTS AND METHODS

Patients and Samples. The original slides and blocks of formalin-fixed, paraffin-embedded tissues and the clinical records of 223 consecutive patients with LSCC were retrieved from the files of the Otorhinolaryngology Clinic of the
University of Milan School of Medicine. Paired samples of normal and/or dysplastic mucosa adjacent to infiltrating carcinoma were evaluated for cyclin D1 immunoreactivity and the occurrence of cyclin D3 gene extra signals in 34 (15.2%) and 22 (9.8%) cases, respectively. In 133 cases, cyclin D1 immunoreactivity had been previously investigated using a cutoff of 10% (15).

Eighty-seven (39%) randomly selected cases were collected in a tissue microarray using a custom-built precision instrument (Tissue Arrayer-Beecher Instruments, Silver Spring, MD), as previously described by Kononen et al. (16) Representative tumor (Tissue Arrayer-Beecher Instruments, Silver Spring, MD), as in a tissue microarray using a custom-built precision instrument. The cutoff of 10% (15).

immunoreactivity had been previously investigated using a 22 (9.8%) cases, respectively. In 133 cases, cyclin D1 the occurrence of carcinomas were evaluated for cyclin D3 immunoreactivity and normal and/or dysplastic mucosa adjacent to infiltrating University of Milan School of Medicine. Paired samples of carcinomas were evaluated for cyclin D3 immunoreactivity and normal and/or dysplastic mucosa adjacent to infiltrating University of Milan School of Medicine. Paired samples of carcinomas were evaluated for cyclin D3 immunoreactivity and normal and/or dysplastic mucosa adjacent to infiltrating

<table>
<thead>
<tr>
<th>Table 1 Correlation between cyclin D3 immunoreactivity and extra signals and clinicopathologic variables</th>
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Immunohistochemistry. One 3-μm-thick section was cut from each of the 223 tumor blocks and from the tissue microarray. Sections were pretreated with an antigen retrieval solution (0.01 mol/L EDTA buffer, pH 8) at 99°C for three cycles of 4 minutes each in a microwave oven operating at 780 W and were then incubated with the anti-cyclin D3 monoclonal antibody DC5-22 (Novocastra, Newcastle upon Tyne, United Kingdom) at a working dilution of 1:40 in TBS for 1 hour at room temperature (10, 11). Detection steps were done using a commercially ria of Shameugratnam and Sobin (17), there were 79 well-differentiated (G1, 35.4%), 104 moderately differentiated (G2, 46.6%), and 40 poorly differentiated (G3, 18%) squamous cell carcinomas. The follow-up of the patients ranged from 2 to 177 months (median, 52 months; mean, 62.8 ± 43.2 months). During the follow-up period, 71 (31.8%) tumors relapsed. Sixty-eight (30.5%) patients died of disease and 14 (6.3%) died of other causes; of the 141 (63.2%) surviving patients, 140 (99.3%) were disease free, and 1 (0.7%) was alive with disease.

Clinical Cancer Research.
available kit (EnVision Plus-HRP, Dako, Glostrup, Denmark) according to the manufacturer’s instructions. Peroxidase activity was developed with 3,3-diaminobenzidine-copper sulfate (Sigma Chemical Co., St. Louis, MO) to obtain a brown-black end product.

Three hundred neoplastic cells from each of the 223 whole sections and all the tumor cells (range, 120-850 cells; mean, 379 cells) of each punch included in the tissue microarray were evaluated at ×400 magnification and the percentage of cells with definite nuclear immunoreactivity was recorded. Only cases showing ≥10% of immunoreactive neoplastic cells were considered positive. All the 223 cases were evaluated by one of us without knowledge of the genetic and clinicopathologic data. Thirty-six (16.1%) randomly selected cases were blindly reviewed by a second investigator. The interobserver agreement was 92% [Cohen’s κ = 0.78, 95% confidence intervals (95% CI), 0.56-1.00; McNemar’s test, P = 1.000]. Positive control tissues were obtained from a gastric diffuse large B-cell lymphoma known to be cyclin D3 immunoreactive (11). Built-in positive controls were endothelial cells of peri- and intratumoral vessels. Staining of serial sections of the test cases with an unrelated isotypic mouse monoclonal antibody provided the negative controls.

**FISH Analysis.** FISH analysis of the cyclin D3 locus was done on 2-µm-thick sections obtained from the tissue microarray with a specific probe (PAC clone RP5-973N23) labeled with the Cy3 fluorochrome (Amersham, Little Chalfont, United Kingdom) by nick translation (10, 11). The analysis of the cyclin D3 locus in normal and dysplastic mucosa was done in 2-µm-thick sections obtained from the whole samples. Briefly, 600 ng of labeled probe were used for each experiment, and hybridization was done at 37°C in 2× SSC, 50% (v/v) formamide, 10% (w/v) dextran sulfate, 7.5 µg Cot1 DNA (Boehringer Mannheim, Germany), and 3 µg of sonicated salmon sperm DNA in a final volume of 5 µL. Posthybridization washing was at 71°C in 2× SSC (thrice). Cy3-labeled probes were detected directly as a red signal. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) staining. Digital images were obtained using a DMR epifluorescence microscope (Leica Imaging Systems Ltd, Cambridge, United Kingdom) equipped with a charged-coupled device camera (Cohu Inc., San Diego, CA). Cy3 and DAPI fluorescent signals were detected using specific filters. The images were recorded, pseudocolored and merged using the QFISH software (Leica), and finally edited using Photoshop, version 6.0 (Adobe Systems, Mountain View, CA). One hundred nuclei were examined for each punch.

Negative controls were samples of laryngeal mucosa from 10 patients with nonneoplastic disease (laryngeal nodule and inflammation). One hundred nuclei were also analyzed from each of these samples, and the mean percentage of nuclei bearing three cyclin D3 signals was 1.5 ± 1.57%. Accordingly, the cutoff value for assessing the occurrence of extra signals in the tumor samples was settled at 6.21% (mean + 3 SDs).

**Statistical Analysis.** Associations between the clinicopathologic features and cyclin D3 immunoreactivity were evaluated by Fisher’s exact test. Survival estimates were calculated by Kaplan-Meier’s method and compared by the log rank test. The follow-up was truncated at 120 months with 31 (14%) of the starting sample cases still at risk to obtain sufficiently precise probability estimates. The prognostic implications of cyclin D3 immunoreactivity and gene extra signals were assessed after dichotomizing the tumor samples in two classes: <10 and ≥10% of immunoreactive neoplastic cells and presence and absence, respectively. The other investigated prognostic factors included sex, age at diagnosis (<60 and ≥60 years), performance status (≤70, 80, and ≥90), smoking exposure (0-19, 20-45, and >45 pack-years), alcohol consumption (≥7 and <7 drinks per day/non drinkers), histologic grade (G1/G2 and G3), anatomic site (glottic and supraglottic), presence and absence of lymph node metastasis, tumor extension (pT1/T2 and pT3/T4), tumor type (infiltrating and exophytic/ulcerating), and clinical stage (I-II and III-IV). Multivariate Cox proportional hazard regression model was used to evaluate the simultaneous effect of explanatory variables on survival time. All analyses were carried out using statistical software (SAS Institute, Inc., Cary, NC). All P values were based on two-sided testing.

**RESULTS**

**Immunohistochemistry.** Eighty-eight (39.5%) of the 223 tumors analyzed showed nuclear cyclin D3 immunoreactivity in ≥10% of the neoplastic cells (Figs. 1 and 2). Of the remaining 135 cases, 70 (52%) showed cyclin D3 immunoreactivity in 1% to 9% of the neoplastic cells (Figs. 1 and 2) and 65 (48%) were unreactive. The intensity of immunoreactivity was variable, ranging from weak to strong in the same tumor, albeit such variability was not taken into account in evaluating the results.

The concordance in cyclin D3 immunoreactivity between whole sections and tissue microarray punches of the same cases was 81.6%. In particular, cyclin D3 immunoreactivity in tissue microarray punches was <10% in 32 (96.9%) of 33 cases that scored negative in the whole sections and ≥10% in 39 (72.2%) of 54 cases that scored positive (P < 0.0001).

Cyclin D3 immunoreactivity in more than 10% of cells was detectable in 3 (8.8%) of 34 and in 5 (22.7%) of 22 normal and dysplastic mucosa samples, respectively, with a higher prevalence in the suprabasal layers (Fig. 1). The concordance with infiltrating carcinoma was 71% for normal mucosa (P = 1.00) and 100% for dysplastic mucosa (P < 0.0001).

**Fluorescence In situ Hybridization.** Technically satisfactory results were obtained in 47 (54%) of 87 cases analyzed. Twenty (42.6%) cases showed three cyclin D3 signals in 8% to 33% of the nuclei analyzed (mean, 16.5 ± 6.8%; Fig. 2). Two (10%) of the positive cases also showed four cyclin D3 gene signals in 10% and 18% of the nuclei analyzed (Fig. 2). None of the 56 samples of normal and dysplastic mucosa samples analyzed showed cyclin D3 gene extra signals.

**Comparison between Immunohistochemistry and FISH.** The concordance between immunohistochemical and FISH results in the 47 cases with both data available was 70.2%. In particular, cyclin D3 immunoreactivity was detectable in 14 (70%) of 20 and 8 (29.6%) of 27 cases with and without cyclin D3 gene extra signals, respectively (P = 0.0085; Fig. 2).

**Comparison between Immunohistochemical and FISH Data and Clinicopathologic Characteristic.** Cyclin D3 immunoreactivity and the occurrence of cyclin D3 extra signals
were not significantly associated with any of the clinicopathologic characteristics evaluated (Table 1).

**Survival Analyses.** The overall survival of the patients at 5 years was 0.67 (95% CI, 0.60-0.73). Univariate analysis showed that advanced (III/IV) clinical stage ($P < 0.0001$), low performance status ($P < 0.0001$), high tumor grade ($P = 0.0010$), the occurrence of nodal metastases ($P = 0.0061$), supraglottic site ($P = 0.0069$) and cyclin D3 immunoreactivity ($P = 0.0435$) were significantly associated with reduced overall survival (Table 2). In particular, the 5-year overall survival probability for patients with LSCC showing $\geq 10\%$ and $<10\%$ of immunoreactive neoplastic cells was 0.61 (95% CI, 0.50-0.72) and 0.70 (95% CI, 0.62-0.78; log rank $P = 0.0427$; Fig. 3). At multivariate analysis, low performance status ($P < 0.0001$), cyclin D3 immunoreactivity ($P = 0.0189$), exophytic/ulcerating tumor type ($P = 0.0351$), and high tumor grade ($P = 0.0450$) were independent predictors of higher mortality (Table 2).

The progression-free survival of the patients at 5 years was 0.66 (95% CI, 0.59-0.72). Univariate analysis showed that advanced clinical stage ($P < 0.0001$), low performance status ($P < 0.0001$), high tumor grade ($P = 0.0010$), the occurrence of nodal metastases ($P = 0.0043$), supraglottic site ($P = 0.0065$), and, at a borderline level of statistical significance, cyclin D3 immunoreactivity ($P = 0.0545$) were associated with reduced progression-free survival (Table 2). In particular, the 5-year progression-free survival probability for patients with LSCC showing $\geq 10\%$ and $<10\%$ of immunoreactive neoplastic cells was 0.61 (95% CI, 0.50-0.71) and 0.69 (95% CI, 0.61-0.77; log rank $P = 0.0535$). At multivariate analysis, performance status ($P < 0.0001$), cyclin D3 immunoreactivity ($P = 0.0217$), high tumor grade ($P = 0.0223$), and exophytic/ulcerating tumor type ($P = 0.0336$) were independent predictors of reduced progression-free survival (Table 2).

Data about cyclin D1 immunoreactivity were available in 133 cases (15). Patients with cyclin D1+/cyclin D3− tumors experienced the poorest overall survival, patients with cyclin D1+/cyclin D3− or cyclin D1+/cyclin D3− tumors an intermediate course, and patients with cyclin D1−/cyclin D3− tumors the most favorable outcome (log rank $P = 0.0002$; Table 3; Fig. 4). In a multivariate model adjusted for gender and clinical stage, patients with cyclin D1+/cyclin D3− or cyclin D1+/cyclin D3− tumors had a risk of death of 2.71 (95% CI, 1.42-5.18), and patients with cyclin D1−/cyclin D3− tumors of 4.11 (95% CI, 1.96-8.63) compared with patients with cyclin D1−/cyclin D3− tumors (Table 3). Similar data were observed for progression-free survival (Table 3).

The occurrence of cyclin D3 gene extra signals was not associated with patients’ overall ($P = 0.5415$) and progression-free (0.4287) survival.

**Fig. 1** Examples of the immunohistochemical results. Normal (A) and dysplastic (B) mucosa samples showing cyclin D3 immunoreactivity in the suprabasal layers. Different cases of LSCC with $\geq 10\%$ (C-E) and $<10\%$ (F-H, arrows) of cyclin D3 immunoreactive cells. H&E counterstain, original magnification $\times 20$.

**Fig. 2** Examples of the immunohistochemical (left) and FISH (right) results in paired samples of LSCC. Top, a case with $<10\%$ of cyclin D3 immunoreactive neoplastic cells and a normal FISH pattern (two signals). Middle and bottom, two cases with $>10\%$ cyclin D3 immunoreactivity and cyclin D3 gene extra signals (one, middle, and either one or two, bottom).
DISCUSSION

In this study, we first analyzed the prevalence and clinical correlates of cyclin D3 abnormalities in LSSC patients. The prevalence of cyclin D3 extra signals was assessed by FISH in formalin-fixed and paraffin-embedded tissue microarray punches with technically satisfactory results in more than 50% of the cases. Collectively, these data suggest that in keeping with previous studies (18), FISH analysis on tissue microarray may be a fast, high-throughput approach to simultaneously investigate a high number of cases. Interestingly, cyclin D3 extra signals were found in approximately 43% of the cases and were significantly associated with immunoreactivity for the encoded protein, thus suggesting that the occurrence of gene extra copies may be a mechanism involved in cyclin D3 deregulation in head and neck cancer. Nevertheless, we also found that approximately one third of the cases showed cyclin D3 immunoreactivity despite the lack of gene extra signals. Although the possibility of false-negative FISH results cannot be completely ruled out, the most straightforward explanation for this finding is that cyclin D3 deregulation may also occur independently of genetic abnormalities, as documented in a subset of infiltrating tumors.

This study provides the first evidence that cyclin D3 immunoreactivity is an independent predictor of poor progression-free and overall survival in patients with LSSC. Of note, there was a significant correlation between results obtained with the standard immunophenotypic characterization in tumor whole sections and those stemming from tissue microarray analysis, thus further confirming the usefulness of the latter experimental approach for high-throughput screenings. On the other hand, the occurrence of false negative results in approximately 30% of tissue microarray cases, possibly due to technical reasons or to a heterogeneous pattern of immunoreactivity, suggests that using whole sections is the preferred approach.

Table 2  Univariate and multivariate overall and progression-free survival analysis for the whole series (223 patients)

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<td>Cyclin D3 ≥10%</td>
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<td>G3 vs. G1-G2</td>
<td>2.25 (1.37-3.69)</td>
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<td>1.73 (1.01-2.95)</td>
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<td>Supraglottic vs. glottic</td>
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<td>N+ vs. N0</td>
<td>1.84 (1.18-2.86)</td>
<td>0.0061</td>
<td>0.58 (0.32-1.05)</td>
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<td>Infiltrating vs. other</td>
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<td>&lt;80% vs. ≥90%</td>
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<td>1.33 (0.46-3.87)</td>
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*Hazards ratio (HR) and 95% CIs obtained from a Cox proportional hazards regression model with all the variables fitted simultaneously.  
†P for the score χ² statistic.

Fig. 3 Overall survival according to cyclin D3 immunoreactivity.
gold standard for correctly assessing the clinical relevance of cyclin D3 immunoreactivity in patients with LSCC. Cyclin D3 immunoreactivity has been previously reported to be an independent predictor of survival in patients with malignant melanoma (8) and non-Hodgkin’s lymphoma (12). Furthermore, high levels of cyclin D3, as measured by Western blot, were significantly associated with poor survival in patients with breast cancer (9). Because cyclin D3 is a positive regulator of the G1→S phase transition (1), it is tempting to speculate that its overexpression may alter the physiologic balance between inhibitors and promoters of cell cycle progression, eventually conferring on the tumor a growth advantage. Along this line, we have recently found in non-Hodgkin’s follicular lymphomas that cyclin D3 is positively correlated with a high Ki-67 labeling index (19). We provide evidence that the variable of clinical stage, which was significantly associated with patient prognosis at univariate analysis, was no longer associated with survival at a multivariate analysis adjusted for performance status. This is not surprising because clinical stage and performance status are highly correlated, and performance status proved to be the most powerful prognostic factor in our series.

We had previously reported that cyclin D1 was also a marker of poor prognosis in a smaller series of patients with LSCC, independently of traditional clinicopathologic characteristics (15), a finding subsequently confirmed by different groups (20, 21). In the present study, we provide evidence that patients with tumors immunoreactive for one or both D-type cyclins show a progressively higher risk of progression and death. In particular, deregulation of both cyclins D1 and D3 represents an even more powerful predictor of dismal prognosis than nodal status and clinical stage. These data further confirm that abnormalities in the pathway regulated by D-type cyclins play a crucial role in the progression of LSCC and reinforce the concept that cyclin D1 and cyclin D3 may be potential therapeutic targets. Flavopiridol and UCN-01 are two recently identified drugs that exert their antitumor activity by functional subversion in the pathways regulated by D-type cyclins and their cyclin-dependent kinase partners (22). Interestingly, both these compounds have been shown to be effective in reducing tumor size in head and neck squamous cell carcinoma of mouse models (14, 23). Accordingly, phase I trials of flavopiridol and UCN-01 combined with chemotherapy in several tumor types including head and neck squamous cell carcinoma have been already designed (24).

Table 3  Univariate and multivariate overall and progression-free survival analysis for the 133 patients with evaluated cyclin D1 and cyclin D3 immunoreactivity

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<td>Cyclin D3+ or cyclin D1+</td>
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<td>N+ vs. N0</td>
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<td>Infiltrating vs. others</td>
<td>1.05 (0.60-1.81)</td>
<td>0.8763</td>
</tr>
<tr>
<td>Performance status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>80% vs. ≥90%</td>
<td>3.72 (1.94-7.12)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>&lt;80% vs. ≥90%</td>
<td>8.91 (3.85-20.6)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥60 vs. &lt;60</td>
<td>1.01 (0.59-1.74)</td>
<td>0.9771</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Women vs. men</td>
<td>2.80 (0.68-11.6)</td>
<td>0.1556</td>
</tr>
</tbody>
</table>

*HR and 95% CIs obtained from a Cox proportional hazards regression model with all the variables fitted simultaneously.
†P for the score χ² statistic.

Fig. 4 Overall survival according to cyclin D1 and cyclin D3 immunoreactivity (133 patients).
REFERENCES

Cyclin D3 Immunoreactivity Is an Independent Predictor of Survival in Laryngeal Squamous Cell Carcinoma

Giancarlo Pruner, Lorenzo Pignataro, Stefano Valentini, et al.


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