

**Cyclin D1 Gene Amplification in Human Laryngeal Squamous Cell Carcinomas: Prognostic Significance and Clinical Implications**

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**ABSTRACT**

The *cyclin D1 (CCND1)* gene is amplified, rearranged, and overexpressed frequently in human cancer, including squamous cell carcinoma. The gene dosage of *CCND1* was examined in 51 primary laryngeal squamous cell carcinomas, and amplification of the gene was found in 9 (17.6%) cases. *CCND1* amplification did not correlate with age, tumor localization and extension, histopathological grading, and epidermal growth factor receptor levels. In a univariate analysis, *CCND1* amplification, tumor extension, lymph node involvement, poor histological differentiation, and high epidermal growth factor receptor levels were correlated significantly with shorter overall survival. In a median follow-up period of 29 months, the overall survival rate was 71.4% for patients affected with tumors displaying a normal *CCND1* dosage and only 25.0% for patients affected with tumors carrying amplified *CCND1* (*P* = 0.0288). In a multivariate analysis, only *CCND1* and tumor extension retained statistically significant prognostic values (*P* = 0.037 and 0.041, respectively).

This is the first report in which *CCND1* amplification is identified as a significant independent prognostic factor in laryngeal carcinoma. Evaluation of *CCND1* amplification could be applicable to the clinical management of laryngeal cancer, allowing identification of patients with poor prognoses.

**INTRODUCTION**

Laryngeal SCC accounts for approximately 2% of all cancers in the United States and Southern Europe, is more prevalent in males than in females, and usually develops in the sixth and seventh decades of life (1).

The predicted mortality is 32%, with an overall survival rate of approximately 70% at 5 years (2). The TNM classification is a reliable prognostic determinant of survival. However, prognosis is far from being determined accurately in laryngeal cancer, and it is influenced by many host and tumor factors (3, 4). In recent years, considerable efforts have been made in the identification of biological factors that could have prognostic value, such as DNA index and ploidy, *ras* and *p53* mutations, and *EGFR* expression or amplification (5–9).

Amplification of the chromosome 11q13 region is a frequent genetic alteration in SCCs of several tissues (10), including esophagus (11, 12) and lung (13). This region is also amplified in breast (14–16), bladder (17), and liver (18) carcinomas and rearranged in parathyroid adenomas at the *PRADI* locus (19, 20) and in centrocytic lymphomas at the *BCL1* locus (21). The 11q13 region harbors several genes that could play a role in tumorigenesis, including the proto-oncogenes *INT2* and *HST1* (10) and *EMS1*, which encodes a putative src kinase substrate (22). However, the single most important molecular target and driving force of amplifications and rearrangements of this region seems to be the *CCND1* gene, which is overexpressed frequently and consistently following those genetic alterations (15, 19, 23–25). *CCND1* corresponds to the *PRADI* gene and is the most likely candidate for the *BCL1* proto-oncogene (19–21). Cyclin D1 is known to regulate cell cycle progression at the G1-S checkpoint, and its overexpression, as a result of amplifications and rearrangements, is expected to drive the cells through the G1-S transition, thus contributing to oncogenesis (26).

*CCND1* and 11q13 amplification also has been detected in laryngeal SCC (27–30), but a precise estimate of the frequency of this phenomenon is lacking. In fact, amplification has been evaluated usually in the context of the head and neck tumor group, which includes carcinomas of the mouth, tonsils, tongue, pharynx, larynx, salivary glands, and upper esophagus. Only a single study has investigated the amplification of the *CCND1* gene in a collection of laryngeal tumors (25). In this study, amplification and overexpression correlated with advanced-stage laryngeal carcinomas, suggesting that *CCND1* gene alterations might identify aggressive tumors.
In the present study, we analyzed CCND1 gene amplification in a large, single-institution, homogeneous series of laryngeal SCC to evaluate its correlation with clinical outcome. CCND1 gene amplification was found to be a significant independent prognostic indicator in laryngeal SCC.

MATERIALS AND METHODS

Patients and Tumor Specimens. Laryngeal carcinoma specimens were obtained during 1988–1993 from patients undergoing surgery at the “A. Gemelli” Catholic University Hospital of Rome. In many cases, normal laryngeal mucosa adjacent to the tumor was also removed during surgery. Tissue samples were snap-frozen in liquid nitrogen and stored at −80°C until further processing. We evaluated a total of 51 primary neoplastic specimens from 51 patients (median age, 64; range, 37–85 years), 48 males and 3 females. According to location, tumors were defined as supraglottic, glottic, or transglottic (when extension of the tumor did not allow identification of the original site), and they were staged following the International Union Against Cancer TNM classification (31). All tumors were epidermoid SCC, and they were graded as well (G1), moderately (G2), or poorly (G3) differentiated. Clinicopathological characteristics of the patients are summarized in Table 2.

Surgical treatment was aimed at the complete removal of the tumor mass and, therefore, differed in different patients according to the extension of the tumor. Radical laryngectomy was performed on 27 patients. Twenty-four patients underwent conservative surgery, i.e., cordectomy (n = 3), horizontal supraglottic laryngectomy (n = 18), and hemilaryngectomy (n = 3). In all cases, the margins of resection were judged to be free of disease both macroscopically and microscopically. At the time of surgery, 12 patients underwent neck dissection due to lymph node involvement. Eight of 12 lymphadenectomies revealed nonspecific reactive lymphadenitis, whereas the remaining 4 cases displayed infiltration of cancer cells. Two of these 4 cases had evidence of extracapsular spread, one of which was positive for CCND1 amplification. The number of involved lymph nodes ranged from two to five. Stage IV patients with positive lymph nodes (5 patients) received postoperative radiotherapy, following the standard protocol of treatment at our institution. Of the patients receiving radiotherapy, one had CCND1 amplification. None of the patients received chemotherapy.

Survival analysis was conducted on 50 patients. (One patient of the series, carrying an amplified CCND1, died of intercurrent disease and was not enrolled in the analysis.) The median follow-up period was 29 (range, 2–60) months; for those still alive, 37.5 months.

Molecular Probes. The probe used for the Southern blot analysis of CCND1 amplification was the 1.4-kb EcoRI insert of the plasmid pPL-8, containing the partial cDNA of the PRAD-1/CCND1 gene (kindly provided by Dr. Andrew Arnold (Massachusetts General Hospital, Boston, MA); Ref. 20). Densitometric signals were normalized using two probes: a 1.2-kb cDNA fragment of the proto-oncogene BCL2, which is located at 19q13.1-q13.2 (32); and a probe from the upstream genomic region of the human proto-oncogene ETS1, which maps on chromosome 11q23. The latter probe was obtained by screening a human genomic DNA library prepared in λ-phage with the rat proto-oncogene Ets-1/Tpl-1 pSB1 probe (33), and it corresponds to the 6.4-kb EcoRI fragment located 3 kb 5′ to the first exon of ETS1 (34). The use of the telomeric 11q23 ETS1 control probe allowed us to rule out those cases in which an increased CCND1 signal was due to polysomy of the whole chromosome 11 and not to actual amplification of the gene.

DNA Extraction and Southern Blot Analysis. Nuclear pellets remaining from previous cell fractionations for determination of EGFR were used for DNA preparation. Genomic DNA isolation from nuclei of normal laryngeal mucosa and tumor specimens was carried out by standard procedures (35). DNAs were digested with EcoRI (New England Biolabs), following the manufacturer’s recommendations, and, on careful spectrophotometric determination of DNA concentration, were electrophoresed in 0.7% agarose gels, transferred to nylon membranes (Hybond N+; Amersham), and hybridized under high-stringency conditions to the 32P-labeled PRAD-1/CCND1 probe. The final high-stringency wash was at 65°C for 40 min in 0.1× SSC (1× SSC = 0.15 m NaCl, 0.015 m sodium citrate) and 0.1% SDS. Membranes were exposed in the linear range to X-ray films (Kodak X-omat AR). Membranes were then stripped of the PRAD-1/CCND1 probe and hybridized to the ETS1 and BCL3 control probes. Each blot included, as internal control, normal laryngeal mucosa DNA from the same patient or, when not available, normal placenta DNA. To evaluate the level of gene amplification, the intensity of the bands was quantitated with a laser scanning densitometer (UltraScan XL; Pharmacia LKB). A 2-fold amplification level relative to placenta or normal laryngeal mucosa DNA was considered as the cutoff value for a sample to be scored positive (36).

EGFR Assay. EGFR was determined by a radioreceptor method, as described previously (8). Briefly, tumor specimens were homogenized in TEN buffer (25 mM Tris-HCl, 1.5 mM EDTA, 5 mM Na3VO4, and 20% glycerol) supplemented with 0.1% monooctanoylglycerol. Following an initial centrifugation at 7,000 × g for 20 min, the supernatant was spun at 105,000 × g for 75 min. The resulting membrane pellet was resuspended in TEN buffer containing 10 mM MgCl2, and 100-μL aliquots (300–500 μg protein) were incubated with 125I-labeled EGF (2.6 nM) in the presence or absence of unlabeled EGF (1 μM) at room temperature for 16 h in a 400-μL volume. Following a centrifugation at 2,000 × g for 20 min, the pellets were counted in a gamma counter. An EGFR level of 16 fmol/mg protein was shown to be the best discriminating value for prognostic assessment and was chosen as the cutoff value to define EGFR status.

Statistical Analysis. The χ2 and two-tailed Fisher’s exact tests were used to evaluate the correlations between amplification of CCND1 and clinicopathological parameters. The distribution of EGFR levels according to CCND1 status was analyzed by the Wilcoxon rank-sum test. For disease-free and overall survival analysis, all medians and life tables were computed using the product-limit estimate by Kaplan and Meier, and the curves were examined by log-rank test (37, 38). Multivariate analysis was performed with BMDP statistical software. A
backward stepwise approach was used for the identification of the major prognostic factors, and statistical significance was ascertained by the Cox-Mantel proportional hazards method (39).

RESULTS

**CCND1 Is Amplified in Laryngeal Carcinomas.** In Southern blots of EcoRI-digested tumor DNAs, three bands of 4.0, 2.2, and 2.0 kb were detected by the CCND1 probe. The control ETS1 probe hybridized to a PRAD1/CCND1 probe (upper panel) and to an ETS1 control probe to normalize densitometric signals (lower panel). The size (in kb) of the CCND1 and ETS1 bands is indicated. Laryngeal carcinoma samples 7019, 7049, and 7091 display CCND1 amplification. The figure is a composite derived from a single Southern blot.

observed a trend toward positive correlations between amplification of CCND1 and high histological grade (G3; P = 0.10) and high EGFR levels (P = 0.09). The latter potential correlation was explored further by evaluating the distribution of EGFR levels according to CCND1 status. The results, although not statistically significant, confirmed that EGFR levels tend to be higher in tumors with CCND1 amplification than in tumors with normal CCND1 (median, 22.58; range, 4.02–49.90 fmol/mg protein; versus median, 6.06; range, 0–169.90 fmol/mg protein; P = 0.0962).

It should be noted that, although CCND1 amplification and TNM classification both correlated with clinical outcome (see below), they did not correlate with each other in a statistically significant manner.

**CCND1 Amplification and Risk of Progression.** To determine whether the CCND1 status is associated with unfavorable clinical outcome, the disease-free and overall survival of patients with or without CCND1 amplification were examined. Disease-free survival was defined as the time interval from surgery to local recurrence or cervical lymph node involvement. Complete follow-up data were available for 50 patients. Higher survival rates were found in patients with the normal CCND1 gene dosage [30 (71.4%) of 42 surviving] than in patients with amplification [2 (25.0%) of 8 alive]. Analysis of the overall survival curves revealed that CCND1 amplification is associated significantly with a shorter overall survival (P = 0.0288). The association with disease-free survival, although showing the same trend, was not statistically significant (P = 0.12; Fig. 2). The disease-free survival rate was 59.5% for patients with the normal CCND1 copy number and 25% for patients with amplification.

In the univariate analysis, shorter overall survival was associated significantly not only with CCND1 amplification but also with T1–T4 tumors, lymph node involvement, poor histological differentiation, and high EGFR levels. However, when all the above parameters were submitted to multivariate analysis using a backward stepwise procedure, only CCND1 gene amplification and tumor classification retained statistically significant prognostic values (P = 0.037 and 0.041, respectively; Table 3).

DISCUSSION

CCND1 and 11q13 amplification has been detected previously in laryngeal SCC, yet the analysis has been confined to cell lines or small series of primary laryngeal neoplasms, evaluated in the general context of head and neck carcinomas (27–30). In the present study, evaluation of the CCND1 copy number was performed on a large series of primary laryngeal carcinomas, and the frequency of amplification was estimated around 17.6%. In a recent survey, which also examined a large number of primary laryngeal carcinomas (n = 46), the reported frequency of CCND1 amplification was 37% (25). It is conceivable that this discrepancy might reflect differences in the populations studied. We also used more stringent criteria to assess gene amplification. In fact, by using a control probe mapping on chromosome 11 (ETS1), polyomysy of this chromosome was not scored as amplification. Chromosome 11 polynosy has been reported in head and neck carcinomas (40) and was detected,
Table 1  Clinicopathological characteristics of laryngeal carcinomas with CCND1 amplification

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age at diagnosis (yr)</th>
<th>Sex</th>
<th>Site</th>
<th>TNM</th>
<th>Stage</th>
<th>Grade</th>
<th>EGFR (fmol/mg protein)</th>
<th>CCND1 amplification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7010</td>
<td>61</td>
<td>Male</td>
<td>Supraglottic</td>
<td>T2N0M0</td>
<td>IV</td>
<td>2</td>
<td>26.48</td>
<td>4</td>
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<tr>
<td>7016</td>
<td>65</td>
<td>Male</td>
<td>Supraglottic</td>
<td>T2N0M0</td>
<td>IV</td>
<td>3</td>
<td>18.68</td>
<td>3</td>
</tr>
<tr>
<td>7032</td>
<td>58</td>
<td>Male</td>
<td>Transglottic</td>
<td>T2N0M0</td>
<td>IV</td>
<td>3</td>
<td>31.39</td>
<td>7</td>
</tr>
<tr>
<td>7045</td>
<td>59</td>
<td>Male</td>
<td>Supraglottic</td>
<td>T2N0M0</td>
<td>IV</td>
<td>3</td>
<td>NA*</td>
<td>5</td>
</tr>
<tr>
<td>7049</td>
<td>55</td>
<td>Male</td>
<td>Transglottic</td>
<td>T2N0M0</td>
<td>IV</td>
<td>3</td>
<td>5.00</td>
<td>18</td>
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<tr>
<td>7057</td>
<td>49</td>
<td>Male</td>
<td>Supraglottic</td>
<td>T2N0M0</td>
<td>IV</td>
<td>3</td>
<td>4.02</td>
<td>3.5</td>
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<tr>
<td>7084</td>
<td>56</td>
<td>Male</td>
<td>Transglottic</td>
<td>T2N0M0</td>
<td>IV</td>
<td>3</td>
<td>49.90</td>
<td>4.5</td>
</tr>
<tr>
<td>7085</td>
<td>71</td>
<td>Female</td>
<td>Transglottic</td>
<td>T2N0M0</td>
<td>IV</td>
<td>3</td>
<td>35.40</td>
<td>6</td>
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</table>

*NA, not available.

Table 2  Correlation between CCND1 amplification and clinicopathological parameters in laryngeal carcinomas

<table>
<thead>
<tr>
<th>Age (yr)</th>
<th>n</th>
<th>Amplification (%)</th>
<th>P*</th>
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<tr>
<td>≤60</td>
<td>21</td>
<td>(23.8)</td>
<td>0.46</td>
</tr>
<tr>
<td>&gt;60</td>
<td>30</td>
<td>(13.3)</td>
<td></td>
</tr>
<tr>
<td>T classification</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>10</td>
<td>1 (10.0)</td>
<td>0.26*</td>
</tr>
<tr>
<td>T2</td>
<td>22</td>
<td>3 (13.6)</td>
<td></td>
</tr>
<tr>
<td>T3</td>
<td>12</td>
<td>1 (8.3)</td>
<td></td>
</tr>
<tr>
<td>T4</td>
<td>7</td>
<td>4 (57.1)</td>
<td></td>
</tr>
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<td>N classification</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>N0</td>
<td>39</td>
<td>6 (15.4)</td>
<td>0.66*</td>
</tr>
<tr>
<td>N1</td>
<td>7</td>
<td>2 (28.5)</td>
<td></td>
</tr>
<tr>
<td>N2</td>
<td>5</td>
<td>1 (20.0)</td>
<td></td>
</tr>
<tr>
<td>Histopathological grading</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>10</td>
<td>0 (0)</td>
<td>0.10*</td>
</tr>
<tr>
<td>G2</td>
<td>26</td>
<td>4 (15.4)</td>
<td></td>
</tr>
<tr>
<td>G3</td>
<td>15</td>
<td>5 (33.3)</td>
<td></td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>10</td>
<td>1 (10.0)</td>
<td>0.46*</td>
</tr>
<tr>
<td>II</td>
<td>20</td>
<td>3 (15.0)</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>12</td>
<td>1 (8.3)</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>9</td>
<td>4 (44.4)</td>
<td></td>
</tr>
<tr>
<td>Localization</td>
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<td></td>
<td></td>
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<tr>
<td>Supraglottic</td>
<td>19</td>
<td>4 (21.0)</td>
<td>0.67*</td>
</tr>
<tr>
<td>Glottic</td>
<td>4</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Transglottic</td>
<td>28</td>
<td>5 (17.8)</td>
<td></td>
</tr>
<tr>
<td>EGFR (fmol/mg protein)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤16</td>
<td>33</td>
<td>3 (9.1)</td>
<td>0.09</td>
</tr>
<tr>
<td>&gt;16</td>
<td>16</td>
<td>5 (31.2)</td>
<td></td>
</tr>
</tbody>
</table>

* By two-tailed Fisher’s exact test.

①T1-T2 versus T3-T4.
②N0 versus N1-N2.
③G1-G2 versus G3.
④Stage I-II versus stage III-IV.
⑤by χ² test (not accurate).

Fig. 2  A, overall survival rates according to CCND1 status. a, normal CCND1 copy number: patients entered, 42; died, 12; b, amplified CCND1: patients entered, 8; died, 6. B, Disease-free survival rates according to CCND1 status. a, normal CCND1 copy number: patients entered, 42; relapsed, 17; b, amplified CCND1: patients entered, 8; relapsed, 6.

indeed, in two specimens when using BCL3 as a control probe. Taken together, both studies are consistent with the hypothesis that CCND1 amplification plays a role in the pathogenesis of laryngeal SCC.

This conclusion is supported further by the novel finding presented here that CCND1 status is a prognostic indicator for laryngeal carcinomas, independent of the generally accepted survival predictor TNM. Previous studies had suggested a link between CCND1 and 11q13 amplification and advanced, poorly differentiated head and neck or laryngeal carcinomas (25, 27–30). However, the present report represents the first instance in which amplification of the CCND1 gene is associated significantly with a subgroup of laryngeal tumors characterized by...
poor prognosis, much like breast (14–16) and esophageal (11, 12) carcinomas.

The mechanisms by which CCND1 amplification might contribute to SCC pathogenesis need further investigation. In recent years, substantial experimental evidence has accumulated, linking deregulation of cell cycle progression to malignant transformation. The progression through the different phases of the cell cycle is regulated at several checkpoints, and the late G1 checkpoint is altered frequently in tumor cells (26). This checkpoint is regulated by D-type cyclins (D1, D2, and D3), which bind to and activate CDK4 (and, to lesser degree, CDK5 and CDK6). The D-type cyclin-CDK complex is likely responsible for the phosphorylation of the retinoblastoma tumor suppressor protein, which in turn allows the cell to enter S-phase (26). It is possible that tumors carrying an amplified CCND1 gene have a high proliferation rate (26), which also could account for the tendency of association between EGFR and EGFR levels (26) and EGFR expression (26) observed in SCC cases with poor histological differentiation observed in this study.

Expression of the short-lived, D-type cyclins is increased by growth factors, and it has been suggested that their function is to sense growth stimuli, thus integrating external signals in the cell cycle machinery (42). Overexpression of cyclin D1, which follows CCND1 amplification in laryngeal cancer consistently (25), is expected to relieve or decrease the dependence of the cell from growth factor stimulation. Remarkably, the two tumors in our series with the highest CCND1 copy numbers had very low levels of EGFR. In those other cases with a lesser degree of amplification, increased levels of EGFR could be interpreted as a synergistic mechanism to stimulate cell proliferation. This would explain the trend in the association between CCND1 amplification and increased EGFR levels. Be that as it may, the interactions between EGF and EGFR and cyclin D1 are likely to be complex and to require further investigation.

The finding that the G1 checkpoint is altered critically in a large fraction of laryngeal SCCs might indicate that some tumors with a normal CCND1 gene could have a functionally equivalent defect in inhibitors of the cyclin D1-CDK complex, such as p16. Interestingly, mutations of the p16 gene and loss of heterozygosity at its locus on chromosome 9p21 have been detected frequently in head and neck SCC (43). Therefore, it will be of interest to assess the prognostic significance of p16 and 9p21 alterations alone or with respect to the CCND1 status.

In previous studies, we showed that high EGFR levels are an independent prognostic variable in predicting disease-free survival (8). The possible correlation between CCND1 and EGFR might explain why, in the present analysis, EGFR levels did not retain independent prognostic significance when challenged with the CCND1 copy number in the multivariate analysis.

Our study suggests that evaluation of CCND1 amplification and/or overexpression might integrate the established prognostic indicators for laryngeal SCC, helping in the identification of patients with poor prognoses, who could be enrolled in protocols including more aggressive surgery and adjuvant radiotherapy or chemotherapy. For this purpose, a rapid and reliable assay, based, for instance, on immunohistochemistry or quantitative PCR, would be ideally suited.

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