The Gene Ratios c-MYC:Cyclin-dependent Kinase (CDK)N2A and CCND1:CDKN2A Correlate with Poor Prognosis in Squamous Cell Carcinoma of the Head and Neck

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ABSTRACT

Purpose: Tumor-Node-Metastasis classification does not fully predict outcome of treatment and prognosis in patients with squamous cell carcinoma of the head and neck. Different biomarkers have been suggested to yield additional prognostic information, but no single marker has thus far been introduced in the clinic. The objective of the present study was to analyze the copy number of the frequently amplified oncogenes CCND1 and c-MYC in relation to the commonly deleted tumor suppressor gene cyclin-dependent kinase (CDK)N2A (p16) to enhance the clinical significance.

Experimental design: Extracted DNA from diagnostic biopsies of 78 untreated patients were analyzed by real-time PCR with specific primers for c-MYC, CCND1, and CDKN2A. Gene copy number ratios were calculated by dividing the copy number of c-MYC or CCND1 with CDKN2A. Ratios > 2 were defined as enhanced. These data were related to disease-free interval and disease-specific survival.

Results: Enhanced gene ratio of c-MYC:CDKN2A was detected in 35 of 78 (45%) and enhanced ratio of CCND1:CDKN2A in 36 of 78 (46%) of the cases. The c-MYC:CCND1 and CCND1:CDKN2A ratios correlated with disease-specific survival with respect to death (P = 0.042 and 0.049, respectively; Log-rank test). Furthermore, enhancement of c-MYC:CDKN2A was associated with a shorter disease-free interval as marked by the development of recurrences or metastases (P = 0.014; Log-rank test).

Conclusions: We conclude that CCND1 and/or c-MYC amplification, when combined with CDKN2A deletion, yield additional prognostic information as compared with analysis of single genetic aberrations. These gene ratios, analyzed by a sensitive method like real-time PCR on diagnostic biopsies, might help clinicians to individualize the treatment of squamous cell carcinoma of the head and neck as they reflect the biological properties of the tumors. This could be used as an adjunct to the Tumor-Node-Metastasis classification system.

INTRODUCTION

The prognosis for patients with SCCHN is still poor today despite tremendous technical advances in surgical treatment and radio and chemotherapy. The TNM classification is the basis for management of this disease. However, it does not provide information about biological features of a tumor. Individualization based on biomarkers could potentially enhance survival as well as minimize treatment-related morbidity, because earlier studies have shown that many of these biological indicators provide prognostic information independent of the TNM classification system.

CCND1 (PRAD-1), located at 11q13, encodes a cell cycle-regulating protein, cyclin D1, which is involved in the G1 to S transition (2). Cyclin D1 forms an activating complex with CDK4/6, which phosphorylates retinoblastoma protein, releases the transcription factor complex DP1/E2F, and initiates transcription of early response genes, e.g., CDKN2A (3). CCND1 is frequently amplified and overexpressed in a variety of human carcinomas, e.g., SCCHN (1). Such dysregulation causes growth advantage and enhances tumorigenesis (4, 5). Previous studies have shown a correlation between poor prognosis in SCCHN and 11q13 amplification (6, 7), CCND1 amplification (8–10), and cyclin D1 overexpression (11–14).

CDKN2A (INK4a/MTS-1/CDKN2A) is located on chromosome 9, band p21, and also involved in G1 to S phase transition of the cell cycle. The gene encodes a CDK inhibitor, p16, which binds to CDK4 and prevents it from forming the above-mentioned kinase complex with cyclin D1. Thus, expression of CDKN2A mediates negative feedback to the retinoblastoma pathway and cyclin D1 activity (15), thereby arresting the cell

The abbreviations used are: SCCHN, squamous cell carcinoma of the head and neck; TNM, Tumor-Node-Metastasis; CDK, cyclin-dependent kinase; LR, Log-rank test; UICC, Unio Internationale Contra Cancrum; pN, lymph node metastases; pT, parameters of tumor size.

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cycle at the G_1-S border. The protein has been shown to down-regulate the cell cycle through induction of G_1 arrest and suppress growth when transfected into carcinoma cells in vitro, and CDKN2A is thus regarded as a putative tumor suppressor gene (16, 17). Additional evidence for CDKN2A being a tumor suppressor gene is its frequently observed inactivation associated with malignant transformation in tumor cell lines and human carcinomas, including SCCHN (18–22). The most frequent genetic event, leading to CDKN2A inactivation, is homozygous deletion, observed in 25–66% of primary SCCHN (18, 20).

The c-MYC oncogene encodes a transcription factor that plays a key role in cell proliferation, differentiation, and apoptosis (23). Recent work suggests that c-MYC may stimulate the activity of cyclin E/CDK2 complexes and antagonize the action of the CDK inhibitor p27KIP1 (24). c-MYC expression can be activated through many different genetic mechanisms, e.g., chromosomal translocations and amplifications. DNA amplification has been reported in many solid tumors, e.g., lung carcinomas (25) and breast carcinomas (26, 27), as well as SCCHN (28).

Because many different cell cycle regulating genes are involved in malignant transformation and cancer development, it is reasonable to believe that a panel of different aberrant biomarkers would yield more prognostic information than each of them individually. Such a panel of biomarkers might identify patients at risk for aggressive biological behavior of their tumors. The aim of the present study was to analyze the combined prognostic value of some of the most frequently dysregulated cell cycle controlling genes in SCCHN, i.e., CCND1, CDKN2A, and c-MYC.

**MATERIALS AND METHODS**

**Patients and Tumor Samples.** The study comprised 78 patients with single primary SCCHN. None of the patients had previous malignancies or received treatment before initial tumor biopsy. They were all treated for cure by surgical removal of the primary carcinoma, along with a neck dissection that was complemented by adjuvant postoperative radiation in advanced stages. All specimens were obtained from surgical resections of the primary tumors at the Department of Otorhinolaryngology, Charité Hospital, Humboldt University, Berlin, Germany, during the period between 1994 and 1996. Operation specimens were transferred to the Institute of Pathology of the Charité University Hospital within 1 h after surgical removal. One aliquot of tumor tissue was frozen in liquid nitrogen and kept at −80°C until DNA extraction. DNA was extracted from several 30-μm cryostat tissue sections by proteinase K digestion and phenol-chloroform extraction that was verified to consist of a minimum of 70% tumor cells in each case. A second aliquot was submitted to formalin fixation and paraffin embedding. The histopathological diagnosis was established in every case according to the WHO guidelines on H&E-stained tissue sections, and the tumors were staged using the TNM classification (International Union Against Cancer). The distribution of TNM status and stage of the 78 patients are summarized in Table 1. In addition to the primary tumors, two cell lines (D36-1 and D6) established from two primaries were also investigated.

**Follow-up of the patients,** performed on an ambulatory basis after completion of therapy, lasted until December 31, 2000. Patients were observed for ≥40 months. The causes of death were determined at autopsy or by clinical examination at the Charité University Hospital. None of the patients had distant metastases (pM = 0) at the time of diagnosis.

**Real-time PCR Primers and Conditions.** Quantitative PCR was performed using the ABI PRISM 7700 Sequence Detector (Applied Systems, Foster City, CA) as described previously (29). The PCR reaction master mix consisted of 5 μl of ×10 buffer, 5.5 μl of 25 mM MgCl_2, 8 μl of 50% Glycerol, 13.2 μl of H_2O, 4 μl of 2.5 mM deoxynucleotide triphosphate, 1.5 μl of 10 pmol of each primer, 0.05 μl of fluorescent probe, 0.25 μl of Taq Polymerase, 0.5 μl of uracil-N-glycosylase, and 1 μl of Rox dye. All reagents were provided by Applied Systems. PCR conditions were as follows: 95°C for 5 min and 40 cycles of 95°C for 15 s and 60°C for 1 min, respectively.

We used CDKN2A tumor suppressor gene as the internal control. The rationale for this was that homozygous deletion of CDKN2A is observed frequently in patients with SCCHN and in SCCHN cell lines. However, the frequency of deletion is much less pronounced in tumor samples because of the contamination of normal cells, e.g., stromal cells. Thus, using CDKN2A as the denominator rather than a normal copy sequence maximized the abnormalities we observed for the gene ratios. We interpreted a target gene:CDKN2A gene ratio ≥ 2 as an abnormality, signifying oncogene amplification and/or CDKN2A deletion. This enabled us to detect abnormalities more sensitively than when we used a nondeleted DNA sequence, i.e., D18S71, as internal control (data not shown). This was in accord with the hypothesis

<table>
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<tr>
<th>Table 1</th>
<th>Clinicopathological data of the study cohort*</th>
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<tr>
<td>Total</td>
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<tr>
<td>No. of patients</td>
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<td>Females</td>
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* A, alive without disease; AWT, alive with tumor; DOD, dead of disease; DID, dead of intercurrent disease.
that the development of SCCHN is multifactorial, and each genetic aberration contributes to biological aggressiveness.

For analysis of **CCND1**, the following probe and primers were used: fluorescent TaqMan probe, 6-FAM-5’-CAAGCGCTTGT-3’-TAMRA; forward primer, 5’-GGACAACGGGCAGTATAGAG-3’; and reverse primer, 5’-CACAGTGATCCAGGGGTATAA-3’. These primers and probe bind to intron 1 in the **CCND1** gene.

For analysis of **CDKN2A**, the following probe and primers were used: fluorescent TaqMan probe, 6-FAM-5’-CTGGCTTCTGGGCACCC-3’-TAMRA; forward primer, 5’-GGAGAGCTGTGACCTGTATAA-3’; and reverse primer, 5’-CCAGCCTGATCCAGGAAGGCT-3’.

For analysis of **c-MYC**, the following probe and primers were used: TaqMan Probe, 6-FAM-5’-AGAAGCCGCCTACAATACAGTCTGAG-3’-TAMRA; forward primer, 5’-GGAGCAAGCAGACGTCTACAA-3’; and reverse primer, 5’-CCAGCTTCTCTGAGGAGGCTG-3’.

**Statistical Methods.** Statistical analysis of the data was performed using the SPSS package (version 9.0; SPSS, Inc., Chicago, IL). All statistical tests were two sided; *P* ≤ 0.05 were considered to be statistically significant. Correlation of gene amplifications with clinicopathological parameters were tested by performing gene amplifications with clinicopathological parameters were tested by performing the Kaplan-Meier product limit estimate (Kaplan and Meier, 1958). Deaths from causes other than the index tumor or recurrence/metastases were not considered treatment failures, and these patients were censored in all analysis involving the length of survival. Survival analysis was performed for the gene amplifications (c-MYC and CCND1), as well as the clinicopathological pT, pN, histopathological grade, and UICC stage with respect to disease-specific survival, disease-free interval, metastasis-free interval, and recurrence-free interval, respectively. The results of the Kaplan-Meier plots were tested for statistical significance with the LR. Multivariate Cox proportional hazards models were used to examine the relative impact of variables demonstrated to be statistically significant in univariate analysis. The stepwise backward/forward procedures provided by the SPSS software were used to further reduce the number of variables in the Cox models. For assessing and comparing the Cox models, a Wald test with a significance level of 0.05 was used for both inclusion and exclusion of variables. An analysis for comorbidities was not performed, because only 3 patients were known to suffer from cardiovascular disease, and all patients were smokers.

**RESULTS**

Real-time PCR Analysis of c-MYC, CCND1, and CDKN2A DNA Abnormalities. An increased gene ratio (>2) between c-MYC and CDKN2A was detected in 35 of 78 (45%) of the cases (Fig. 1). Corresponding findings for CCND1: CDKN2A were observed in 36 of 78 (46%). The ratios showed ranges between 0.6 and 166 for CDKN2A and 0.7 and 47 for c-MYC:CDKN2A, indicating that CDKN2A in no single case was homozygously deleted in all cells in a biopsy.

**Association of Amplification of CCND1 and c-MYC with Clinicopathological Parameters.** The results of the comparison between target gene:CDKN2A ratio and pT, pN, and UICC stage, as well as histopathological grading tested by the *χ*² test, are shown in Table 2. Enhanced gene ratios were found in all classes with no statistically significant differences. However, in the cases with c-MYC:CDKN2A enhancement, there was a tendency toward higher frequency of amplification in those cases with positive lymph nodes and higher pT stages.

**Amplification of CCND1 or c-MYC and Clinical Course.** Clinical outcome for all patients is shown in Table 1. Two patients developed recurrences, and 3 patients showed second primary tumors during the follow-up period but are alive today. Thirty-two patients died of their SCCHN, 14 of them...
attributable to metastases and 18 patients attributable to recurrent disease.

Survival was analyzed with respect to the occurrence of metastasis/recurrence and death from SCCHN, respectively. The results of univariate Kaplan-Meier analysis are shown in Table 3.

The analysis demonstrated that increases of c-MYC:CDKN2A and CCND1:CDKN2A ratio had a statistically significant association with disease-specific survival with respect to death (LR; \( P = 0.042 \) and 0.049, respectively). Increases of the c-MYC:CDKN2A gene ratio were associated with a shorter disease-free interval with respect to the development of recurrences or metastases (LR; \( P = 0.014 \)), whereas there was only a tendency toward significance regarding CCND1:CDKN2A enhancement. Both increased ratios of c-MYC:CDKN2A and CCND1:CDKN2A were significantly associated with the development of asynchronous metastases (LR; \( P = 0.009 \) and 0.006, respectively). Fig. 2 illustrates the correlation of enhanced CCND1:CDKN2A ratio with disease-specific survival. Of 29 cases with enhanced CCND1:CDKN2A ratios, 16 (55%) died because of tumor, compared with 16 of 51 (31%) patients with normal gene ratios. Fig. 3 illustrates the correlation of enhanced c-MYC:CDKN2A ratio with disease-free interval. Of 30 cases with enhanced c-MYC:CDKN2A ratios, 18 (60%) presented with recurrence or metastases during follow-up, compared with 15 of 50 (30%) in the normal gene ratio group.

As the only clinicopathological parameter, Kaplan-Meier analysis demonstrates statistical significance for pT stage with respect to each disease-specific survival and disease- and metastasis-free intervals. The best prognosis was associated with the pT1 tumors, followed by pT3, pT2, and pT4 carcinomas. Surprisingly, the pT2 carcinomas had a worse outcome than pT3 tumors. This can be explained by the composition of both subgroups regarding the location of the primary tumor and nodal status. The pT2 group consisted mainly of pharyngeal carcinomas (21 pharyngeal carcinomas versus 1 pN0 laryngeal carcinoma) that have a worse prognosis than laryngeal carcinomas. Moreover, 11 of the pharyngeal carcinomas carried nodal metastases, compared with 5 node-positive laryngeal tumors.

Increases of c-MYC:CDKN2A and CCND1:CDKN2A were included in the multivariate analysis together with pT, pN, and UICC stage. The Cox model for disease-specific survival using
the backward and forward procedures showed no independence of any of the variables.

**DISCUSSION**

In the present study, we investigated the prognostic value of aberrations in gene copy number, as measured by real-time PCR, of **CCND1**, **c-MYC**, and **CDKN2A** in 78 cases of SCCHN. Amplifications of the two oncogenes, **CCND1** and **c-MYC**, and homozygous deletion of **CDKN2A** are all frequent genetic changes in SCCHN (10, 18, 20, 28). However, the literature does not convincingly show either of these abnormalities to solely yield independent prognostic information (1). This was the rationale for combining commonly amplified oncogenes with a deleted tumor suppressor gene in a ratio to identify the cases in which both these phenomena are seen. From a biological point of view, **CCND1** and **CDKN2A** are closely linked because cyclin D1 expression results in up-regulation of early response genes, e.g., **CDKN2A**, in the G1 to S phase transition of the cell cycle. Expressed p16 has an inhibiting effect on the activity of cyclin D1 in a feedback manner, and deletion of **CDKN2A** diminishes this inhibition. **c-MYC** is a transcription factor that enhances cell proliferation. The gene is frequently activated by DNA amplification in SCCHN.

Gene ratios were calculated, reflecting the combined effect of an amplified oncogene and a deleted tumor suppressor gene. Increased ratio of **c-MYC:CDKN2A** was detected in 35 of 78 (45%) of the cases. Corresponding findings for **CCND1:CDKN2A** were observed in 36 of 78 (46%). These findings agree with the literature, where the genes have been analyzed separately (7, 28). In the tumor biopsies from patients, we did not observe homozygous deletion of **CDKN2A**. This is not unexpected, because there is always contamination from normal cells, e.g., stromal cells and lymphocytes, which each contain two copies of each gene. However, in the cell lines where the normal stromal cells are lost within a few passages, we frequently observed a homozygous loss of **CDKN2A**, resulting in no detectable p16.

The present analysis demonstrated that increases of **c-MYC:CDKN2A** and **CCND1:CDKN2A** ratios were associated with poor disease-specific survival (LR: \( P = 0.042 \) and 0.049, respectively). Furthermore, an increased **c-MYC:CDKN2A** gene ratio correlated with a shorter disease-free interval as assayed by development of recurrences or metastases (LR: \( P = 0.014 \)), whereas there was only a tendency toward significance for increased **CCND1:CDKN2A** gene ratio.

Particularly, the gene ratios showed a highly significant correlation with the development of metachronous metastases, which characterizes a distinct biological behavior of the tumors, i.e., the potential for metastatic spread. If this finding is validated by additional analysis in a more extensive population, these gene ratios could be used as prognostic markers in diagnostic biopsies.

In contrast, none of the investigated clinicopathological parameters, e.g., stage and nodal status, correlated with outcome. However, either of the studied parameters yielded independent prognostic information in the multivariate analysis. This could be explained on the basis of a rather small sample size, and additional studies on larger materials are needed.

The combined ratio, as analyzed by real-time PCR, of an amplified oncogene, i.e., **c-MYC** and/or **CCND1**, and a deleted tumor suppressor gene, i.e., **CDKN2A**, identified patients at risk for aggressive biological behavior of their tumors. We conclude that gene ratios, in which a frequently amplified oncogene and deleted tumor suppressor gene are compared, might be a strategy to achieve more reliable prognostic information about patients with SCCHN. Additional studies should include more patients and homogeneous materials regarding site and treatment and also focus on whether these parameters can predict outcome of treatment. Such an approach might result in individualized treatment protocols based on biological features of the tumors, rather than on TNM classification alone.

**REFERENCES**


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