Image Cytometry of Cyclin D1: A Prognostic Marker for Head and Neck Squamous Cell Carcinomas

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Abstract
CCND1 gene amplification and cyclin D1 protein overexpression are indicators for poor prognosis in invasive head and neck carcinomas. Increased CCND1 gene dosage is a more sensitive prognostic factor than protein overexpression as evaluated by conventional immunohistochemical techniques. Qualitative immunohistochemistry cannot distinguish cyclin D1 overexpression accompanied by amplification of the CCND1 gene from overexpression associated with normal CCND1 gene copy number. To improve the sensitivity of cyclin D1 protein determination, we applied quantitative techniques of image analysis to evaluate cyclin D1 in 54 head and neck carcinomas. There was a significantly higher rate of occurrence of adverse events ($P = 0.043$) among patients with CCND1 gene amplification than among those without gene amplification. There was a strong association between CCND1 gene amplification (as detected by Southern blot analysis) and the highest nuclear score (by image cytometry of the immunostained tumor sections). The predominance of cells in the lowest nuclear score category was significantly associated with normal copy number ($P = 0.005$). Conversely, the highest nuclear score was a significant predictor of gene dosage ($P = 0.02$). Similarly, high nuclear score was a good predictor of death as the final outcome of the disease ($P = 0.01$). Although somewhat less accurate than Southern blotting, image cytometry of immunohistochemical cyclin D1 stain appears to be a promising tool that could be useful for other tumor marker expression studies.

Introduction
Cyclin D1 has been thoroughly investigated in head and neck SCCs and is one of the genes most frequently altered during upper aerodigestive tract carcinogenesis. Several investigators have found that cyclin D1 is overexpressed in precursor lesions and in invasive SCCs (1–9). The percentage of SCCs exhibiting overexpression has been reported to range from 30 to 68% of tumors (4, 10–12). In addition, a lower percentage of these tumors also contains CCND1 gene amplification. The reported levels of gene amplification are between 17 and 36% of SCCs (10, 13–16).

CCND1 gene amplification has been recognized as an indicator of poor prognosis in invasive head and neck carcinomas (14, 15, 17). Immunohistochemical studies also have found a correlation between overexpression and poor prognosis (3, 5, 6, 10, 18–20). Nevertheless, the sensitivity of the latter approach is not as accurate as CCND1 gene dosage determinations (15). Unfortunately, standard immunohistochemistry cannot distinguish between overexpression of cyclin D1 that is accompanied by amplification of the CCND1 gene from cyclin D1 overexpression that is not associated with higher copy number. The intensity and number of cyclin D1 stained cells vary markedly from tumor to tumor (8, 9, 21). Thus, it is possible that the high expressers are those tumors that harbor CCND1 gene amplifications, whereas the low-expressing tumors could be the ones without or with marginal levels of CCND1 gene amplification.

Immunohistochemical methods for detecting cyclin D1 have been widely used as markers for genetic abnormalities that accompany premalignant and malignant progression. Both paraffin-embedded and frozen tissues are amenable to analysis (22–28). These features are especially appealing for retrospective studies of archival materials, for screening people at risk, or in chemoprevention trials. Image analysis of immunohistochemical stains provides objective and quantitative measurements of tissue and cell features that are otherwise interpreted subjectively. One quantitative study of p53 expression in head and neck lesions (29) has been reported. To our knowledge, no other studies have attempted to evaluate immunohistochemical markers of head and neck SCCs using image analysis techniques.

With the advent of affordable high-resolution color video camera systems and the development of commercial computer software, it is possible for most pathology laboratories to quantitatively evaluate immunohistochemical stains. In a previous publication we demonstrated the feasibility of cyclin D1 image cytometry in precursor lesions of the oral cavity (21). Thus, by quantifying cyclin D1 immunohistochemistry, it should be possible to distinguish between tumors that overexpress cyclin D1 protein because of increased gene dosage and those that overexpress without detectable gene amplification. Hence, we evaluated CCND1 gene dosage in 54 invasive SCCs of the head and neck together with image analysis determinations of the cyclin D1 protein expression in tissue sections of the same tumors.

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3 The abbreviation used is: SCC, squamous cell carcinoma.
Materials and Methods

Tissue Samples. Fifty-four specimens of SCCs of the head and neck were analyzed: 34 (63%) of the cases were men, and 33 (61%) cases were reported to have experienced an adverse event (death, metastasis, or recurrence). The most common sites were oral tongue (n = 15), larynx (n = 12), floor of the mouth (n = 8), and pharynx (n = 6). Other tumors were seen in the gingival, buccal, maxillary, and salivary gland regions. The average age was 62.35 years with a SD of 13.1 years. There were 13% stage I, 19% stage II, 24% stage III, and 44% stage IV tumors. According to histopathological grade, there were 5 grade I SCCs, 33 grade II, and 16 grade III.

The tissues had been fixed overnight in 10% phosphate-buffered formaldehyde and embedded in paraffin. Paraffin sections (5 μm) were cut and placed on poly-L-lysine-coated slides for the immunohistochemical detection of cyclin D1.

Southern Analysis. Genomic DNA was isolated from snap-frozen samples as described previously (30). Ten μg of DNA were digested with the restriction endonuclease EcoRI, electrophoresed through 1% agarose gels, blotted on Hybond-XL membrane (Amersham Pharmacia Biotech), and hybridized with cDNA probes labeled by the random-primer technique with [α-32P]dCTP. The probe for human cyclin D1 was a 1.4-kb cDNA fragment from plpL8 (31). Human β-actin cDNA probe (Clontech, Palo Alto, CA) was used as DNA loading control. The hybridization signal was quantified using NIH Image 1.61 software. Intensities of cyclin D1 bands on the Southern blots were normalized to their corresponding β-actin control bands. The degree of cyclin D1 amplification was calculated from those normalized values. Only values of 4 or above were considered positive.

Immunohistochemistry. The tissues were fixed overnight in 10% phosphate-buffered formaldehyde and embedded in paraffin. Paraffin sections (5 μm) were cut and placed on poly-L-lysine-coated slides for the immunohistochemical detection of cyclin D1. All paraffin sections were subjected to antigen retrieval for 10 min in 1 mM EDTA. The primary antibody used was an anticyclin D1 (mouse monoclonal, NCL-cyclin D1-GM; Novocastra). An avidin-biotin-peroxidase kit (Vectastain Elite, Burlingame, CA) was then used, followed by the chromogen 3’,3’-diaminobenzidine to develop the immunostain. As negative controls, sections were incubated with normal control serum. All sections were counterstained with hematoxylin and mounted. Nuclear immunostain was evaluated in the tumor tissues. Qualitative visual estimation of cyclin D1 immunostain was performed by two pathologists (A. K. S. and S. C. L.) and categorized as (a) intensely stained (++++), (b) moderately stained (+++), (c) slightly or marginally stained (++), and (d) not stained (−). A minimum of 20 stained cells per slide was required to include the sample in any of the first three categories.

Image Analysis and Statistical Analysis. The image analysis system (Roche Pathology Workstation; Roche Image Analysis Systems/Autocyte, Elon College, NC), which basically consists of a microscope (Zeiss, Oberkochen, Germany), a camera, and a computer, was used to determine the intensity of the immunohistochemical staining. In each slide, the hematoxylin counterstain differentiated the positive cells (brown) from the negative cells (blue), and the microscopic field was divided into very small, equal-sized elements (pixels) in a two-dimensional array, each associated with a value. With true color analysis systems, the contributions of the red, green, and blue components to each pixel define its exact color. Image analysis of cyclin D1 immunostain was done by establishing nuclear scores that indicated the percentage of positive brown staining. These nuclear or cyclin D1 scores were used to estimate the intensity of the positive immunohistochemical reactions.

The nuclear score is defined as the product of the relative positive area and an index. The index value is the difference between the maximum brightness and the mean gray value of the positive area. This index represents the intensity of the immunostain (20, 28). The cyclin D1 staining patterns were classified into four categories: score 0–40, 40–80, 80–120, and 120–200, which represent negative, marginal, moderately positive, and intensely positive staining, respectively. For each sample, we evaluated the percentage of cells that fell in each of the nuclear score categories of cyclin D1 staining.

Stained tissue sections were viewed under the microscope with a ×40 objective. Each stain was evaluated in the same areas on successive sections. We counted 200–1000 cells in each specimen, using 5–10 random fields separated by 0.5-mm distance in the periphery of the tumor. The periphery of the tumor nests were selected to avoid necrotic or necrobiriotic areas. The nuclei were thresholded and counted separately, avoiding overlapping or fragmented nuclei. The quantitative system automatically displayed the distribution of nuclear scores for each sample.

Logistic regression was implemented to identify factors that were predictive of either gene amplification or the occurrence of an adverse event. Patients were partitioned into groups according to gender, CCND1 copy number, tumor stage, or tumor grade. Fisher’s exact test was used to compare the patient groups with respect to the incidence of adverse events and the percentage of patients having cells in each of the nuclear score classifications. Differences between the groups in terms of the percentage of cells in each of the nuclear score classifications were assessed via the Kruskal-Wallis test.

Results

CCND1 Gene Amplification in Head and Neck Tumors. Six SCCs of the head and neck showed an increase in CCND1 gene copy number. This represented 11% of the 54 specimens analyzed. All of the amplified cases had a 4–5-fold increase in gene copy number with respect to normal tissues of the head and neck region and with respect to the other 48 nonamplified tumor cases (Fig. 1). There was a significantly higher rate of occurrence of adverse events (P = 0.043) among patients with CCND1 gene amplification than among those without gene amplification. Specifically, all 6 cases with increased CCND1 copy number had experienced metastasis, recurrence, or death in the 5 years following diagnosis, whereas only 27 of 48 (56%)...
patients without gene amplification experienced an adverse effect. Four of the six patients died (75%) within 5 years. In contrast, only 7 of 31 evaluable patients (22%) without **CCND1** gene amplification died within 5 years.

**Immunohistochemical Expression of Cyclin D1.** To identify levels of protein expression of cyclin D1 in head and neck tumors, we performed immunohistochemical staining of paraffin-sections using an anticyclin D1 antibody. Of the 54 tumor samples evaluated, 23 tumors (42%) exhibited intense to moderate nuclear stain and an additional 10 tumors (18%) showed mild to marginal nuclear stain. All cases that exhibited **CCND1** gene amplification were found to be moderately to intensely positive by qualitative immunohistochemistry. Only 35% of the cases without gene amplification were moderately to intensely positive. The nuclei of the SCC cells showed a wide range of staining intensities for cyclin D1, i.e., not all cases stained with the same intensity. Some specimens stained homogeneously with moderate to high intensity, whereas others showed mild to moderate nuclear stain (Fig. 2). This qualitative estimation of the staining intensity was difficult to correlate with the more solid quantitative data on gene amplification. Thus, a more objective quantitative measurement of cyclin D1 expression in tumor cell populations was attempted, using image cytometry.

**Image Cytometry of Cyclin D1 Expression.** We evaluated the accumulation of cyclin D1 in SCCs of the head and neck, using image analysis, and classified the nuclei of each tumor according to four categories of nuclear score (see Fig. 3A). The lowest category included nuclear scores 0–40, which were considered equivalent to negative staining. The nuclear score category 40–80 included cells that had marginal or mild staining. The cells with nuclear score 80–120 were considered moderately stained, and those with nuclear score >120 were intensely stained. The average percentage of cells falling in each nuclear score category is shown for patients with and without **CCND1** gene amplification in Fig. 3.

Five of six cases of SCCs with amplified **CCND1** (83%), had a subpopulation of cells that fell in the highest nuclear score category, and all cases had cell subpopulations that fell within the two highest nuclear score categories. Conversely, a significantly smaller percentage of specimens without **CCND1** gene amplification (23%; *P* = 0.002) contained cells in the highest nuclear score category as well as in the two highest nuclear score categories (48%; *P* = 0.016).

There were no statistically significant difference between genders (*P* > 0.6), stages (*P* > 0.15), or grades (*P* > 0.25) with respect to the percentage of cells in any of the nuclear score categories. Logistic regression was implemented to determine whether any combination of the protein expression variables was predictive of amplification. When considered separately, only the nuclear score categories 0–40 (*P* = 0.0054) and 120–200 (*P* = 0.025) were significant indicators of copy number.

Using data from 38 patients known to have either died or survived the duration of the study, we found that both gene dosage (*P* = 0.031) and the highest nuclear score category (*P* = 0.016) were significant predictors of death as outcome of the disease process.
Discussion

Qualitative analysis of immunohistochemical staining cannot distinguish between overexpression of cyclin D1 that is accompanied by amplification of the CCND1 gene and overexpression that is not associated with gene amplification. In this report, we show findings indicating that higher nuclear scores, as determined by image cytometry, are associated with gene amplification. Several investigators have found that overexpression correlates with poor prognosis (3, 5, 6, 18–20, 32). These observations were based on conventional qualitative immunohistochemistry. In another report using a quantitative approach, Kyomoto et al. (15) showed that CCND1 gene amplification is associated with a high labeling index (percentage of cells stained) for cyclin D1 nuclear immunostaining in invasive SCCs. Nevertheless, that work quantified the percentage of positively stained nuclei and not the staining intensity or content of cyclin D1 per nucleus.

CCND1 gene amplification is a well-recognized indicator of poor prognosis in invasive head and neck carcinomas (14, 15, 17; 33). Although overexpression is a good indicator of primary prognosis in this type of neoplasm, Kyomoto et al. (15) have established that gene dosage determinations are more valuable than protein expression as a prognostic tool. This study confirms our observation. We also detected a significant association between image cytometry of cyclin D1 and CCND1 gene dosage. The highest nuclear score category (120–200) of intense cyclin D1 protein expression was an excellent predictor of increased CCND1 gene copy number. In addition, the proportion of cells in the highest two nuclear score categories were significantly higher in the specimens with CCND1 amplification than in those without CCND1 gene amplification. Hence, quantitative evaluation of cyclin D1 expression by image cytometry could suggest elevated CCND1 dosage and promote discussion. Although we did not observe a significant association between all adverse events and high cyclin D1 nuclear scores, the highest nuclear score category was a significant predictor of death as a final outcome of disease. It is probable that with future accrual of a larger sample size, the association with recurrence and metastasis will become significant.

Although the present approach is not free from imperfections (such as those that could be introduced by different tissue section thicknesses and the controversial stoichiometry of some immunohistochemical reaction conditions), the procedure is accurate and devoid of subjectivity. We believe that this quantitative approach can help in establishing a more reliable and reproducible system to evaluate immunohistochemical stains. With respect to cyclin D1 expression in human head and neck cancers, this approach has clearly been useful in detecting cases that harbor CCND1 amplifications and have suffered adverse events such as lower survival rates.

Combined analyses using conventional and quantitative immunohistochemistry of markers such as cyclin D1 could be used in many different studies in which variations of protein expression in tissue sections could yield prognostic information or could be useful in determining subtle effects of curative or prevention therapies.

References


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