Cyclin D1 Genotype, Response to Biochemoprevention, and Progression Rate to Upper Aerodigestive Tract Cancer


Background: Altered cyclin D1 expression in advanced preinvasive lesions of the upper aerodigestive tract (UADT) is associated with an increased risk of developing cancer and histologic progression during and after combination biochemopreventive therapy (13-cis-retinoic acid, α-interferon, and α-tocopherol). Both alleles of the adenine (A)/guanine (G) cyclin D1 polymorphism located at nucleotide 870 encode two alternatively spliced transcripts, but the A allele preferentially encodes a protein with an extended half-life. We investigated whether the cyclin D1 genotype at nucleotide 870 was associated with baseline levels of cyclin D1 protein, post-treatment modulation of cyclin D1 protein levels, histologic response to treatment, and the outcome for subjects with preinvasive UADT lesions after biochemopreventive therapy. Methods: UADT tissue biopsy samples were obtained before and 6 and 12 months after biochemopreventive treatment from 31 individuals with advanced preinvasive UADT lesions. Tissues were examined for cyclin D1 genotype (by DNA single-strand conformation polymorphism analysis), for cyclin D1 protein expression (by immunohistochemistry), and for cyclin D1 gene copy number (by fluorescence in situ hybridization). Associations of cyclin D1 genotype with histologic response to therapy and time to progression to a higher degree of dysplasia or invasive cancer were investigated. All statistical tests were two-sided. Results: The A allele was associated with increased baseline cyclin D1 expression in the parabasal epithelial layer (16 of 18 AA/AG subjects versus four of nine GG subjects; \( P = .02 \)), decreased histologic response to biochemopreventive treatment (six of 21 AA/AG subjects versus four of 10 GG subjects; \( P = .70 \)), decreased favorable modulation of cyclin D1 expression by the treatment (seven of 18 AA/AG subjects versus eight of nine GG subjects; \( P = .02 \)), and shorter progression-free survival (\( P = .05 \)). Conclusions: The cyclin D1 A allele was associated with a diminished modulation of normal physiologic and treatment-induced decreased expression of cyclin D1, a decreased likelihood of response to biochemopreventive intervention, and an increased rate of progression to cancer development, findings that require validation in a larger cohort. [J Natl Cancer Inst 2003;95:198–205]

Head and neck squamous cell carcinoma (HNSCC) develops as a multistep process driven by genetic instability (1–4). Cyto- genetic and molecular analyses of invasive HNSCC and its adjacent preinvasive lesions have identified common genotypic and epigenetic abnormalities in genes important for cell cycle regulation (5–10). Similar changes have been reported in altered epithelial tissues of patients at risk for the development of cancer (11–13).

Cyclin D1 is a key cell cycle regulatory protein for the mammalian G1–S phase transition and is involved in the regulation of proliferation and differentiation. The expression of cyclin D1 is regulated by a coordinated cascade of intracellular events in response to extracellular signaling (14). In organized epithelia, the decreased expression of cyclin D1 after proliferation is necessary for ordered differentiation (15). The proper balance of proliferation and differentiation is crucial for the maintenance of tissue homeostasis. Deregulated cyclin D1 expression promotes genetic instability in vitro and tumorigenesis in vivo (16,17). Overexpression and/or amplification of the cyclin D1 gene, located at chromosome 11q13 (18), is reported in 35%–65% of patients with HNSCC and is associated with poor prognosis (19–25).

We have reported that amplification of 11q13 (including the cyclin D1 gene) is an early event during HNSCC tumorigenesis (26,27). Cyclin D1 expression is deregulated in preinvasive lesions adjacent to invasive tumors and is associated with increased chromosomal instability and the likelihood of subsequent gene amplification (26,28). Moreover, cyclin D1 deregulation in preinvasive lesions of the upper aerodigestive tract (UADT) is associated with an increased risk for the development of cancer and with histologic progression during and after chemopreventive intervention (29,30).

The mechanisms underlying deregulated cyclin D1 expression in head and neck preinvasive lesions are not well understood. Recently, an adenine (A)/guanine (G) polymorphism at position 870 in exon 4 of the cyclin D1 gene was associated with a splice-site variant encoding for two alternative transcripts (31). The normally spliced variant includes exon 5, which carries the destruction box sequence, important for ubiquitin-mediated pro-

Affiliation of authors: J. G. Izzo, P. L. C. den Hollander, I. M. Babenko, W. N. Hittelman (Department of Experimental Therapeutics), V. A. Papadimitrakopoulou, J. Keck, D. M. Shin, W. K. Hong (Department of Thoracic/Head and Neck Medical Oncology), A. K. El-Naggar (Department of Pathology), D. D. Liu, J. Jack Lee (Department of Biostatistics), The University of Texas M. D. Anderson Cancer Center, Houston.

Correspondence to: Julie G. Izzo, M.D., Department of Experimental Therapeutics, The University of Texas M. D. Anderson Cancer Center, Box 19, 1515 Holcombe Blvd., Houston, TX 77030 (e-mail: jizzo@mdanderson.org).

See “Notes” following “References.”

Journal of the National Cancer Institute, Vol. 95, No. 3, February 5, 2003

198 ARTICLES
teolysis (14,31,32). The alternatively spliced transcript encodes a protein lacking the destruction box, leading to an increased half-life for cyclin D1 protein (33–35). Both the A and G alleles can encode the two transcripts; however, the A allele preferentially encodes an altered transcript leading to a state of increased CD1 levels even in the heterozygous state (31,36). Few studies have addressed the relationship of cyclin D1 genotype, tumor development, and clinical outcome (37–42). In one HNSCC study (41), the GG genotype was associated with an unfavorable outcome, whereas in another study, the A allele was associated with an increased risk for early-onset HNSCC (42).

We recently completed a biochemoprevention trial in subjects with advanced preinvasive UADT lesions who received the combination treatment of 13-cis-retinoic acid, α-interferon, and α-tocopherol (43). Biomarker analyses of tissue biopsies from these subjects demonstrated that continued cyclin D1 protein deregulation was associated with an increased risk for histologic progression of HNSCC (30). To better understand the cellular mechanisms underlying response to biochemoprevention and progression to cancer in these subjects, the cyclin D1 genotype was determined and analyzed for associations with cyclin D1 protein modulation and the outcome after treatment.

Subjects and Methods

Subjects and Biopsies

From June 15, 1994, through August 15, 1995, 36 individuals with advanced preinvasive lesions of the larynx and/or oral cavity (i.e., histologically confirmed mild to severe dysplasia) were enrolled in a prospective nonrandomized phase II chemoprevention study. Thirty-one subjects were assessable for response and cyclin D1 genotype analysis. The patients received high-dose 13-cis-retinoic acid (100 mg/m²/day), α-interferon (3 million U/m², twice a week), and α-tocopherol (1200 IU/day) for 12 months, unless the lesions progressed to a higher degree of dysplasia or invasive cancer. Clinical and histologic responses were assessed at 6 and 12 months, and a final biopsy specimen was obtained at 18 months (6 months after the completion of treatment). Although the design of the clinical study did not provide for mandatory follow-up after 18 months, the majority of the subjects were routinely followed in the clinic after the end of the trial. In the remaining subjects, attempts to obtain follow-up information were made every 6 months. The results of the clinical trial were previously reported (43). The Institutional Review Board approved the study, and all patients provided written informed consent. After pathologic diagnosis from hematoxylin–eosin-stained slides, unstained slides made from paraffin-embedded blocks of the biopsies were cut into 4-μm-thick sections and used for biomarker assessment by immunohistochemistry and fluorescence in situ hybridization (FISH) (30,44). Histopathologic changes in tissue in response to the therapy were evaluated by one pathologist (A. El-Naggar) according to previously described criteria (43,44). The results of biomarker analyses, such as cyclin D1 genotype and protein levels, were examined for associations with histopathologic parameters. Complete response was defined as complete reversal to nondysplastic squamous epithelium after treatment.

Cyclin D1 Genotype Assessment

Polymerase chain reaction (PCR) and DNA single-strand conformation polymorphism (SSCP) analyses were performed to determine the cyclin D1 genotype at nucleotide 870. Briefly, genomic DNA was isolated from whole blood samples (10 mL) from each study subject as previously described (12). Subsequently, the fragment spanning the A870G polymorphism was generated by PCR from 50 ng of genomic DNA in a 25-μL reaction mixture containing 50 mM KCl, 20 mM Tris–HCl (pH 8.0), 1.5 mM MgCl₂, 0.2 mM deoxyadenosine 5′-triphosphate, 0.2 mM deoxyguanosine 5′-triphosphate, and 0.2 mM deoxythymidine 5′-triphosphate, 0.1 mM deoxycytidine 5′-triphosphate (dCTP), 1 μCi of [α-32P]dCTP (3000 Ci/mmol; ICN Radiochemicals, Irvine, CA), and 2.5 U of Taq polymerase (Gibco BRL, Grand Island, NY). The PCR was carried out in a Perkin Elmer 9700 thermocycler (PE Biosystems, Foster City, CA) as follows: 8 minutes at 94°C, followed by 30 cycles at 94°C for 30 seconds, 65°C for 30 seconds, and 72°C for 1 minute, with a final extension step at 72°C for 10 minutes. The PCR primer sequences used were those previously described (40). For SSCP analysis, 4 μL of the PCR product mixture was mixed with an equal volume of formamide-based loading buffer (PE Biosystems), denatured at 95°C for 5 minutes, quickly chilled on ice for 10 minutes, and then separated in a 6% mutation enhancement gel, according to the manufacturer’s instructions (FMC BioProducts, Rockland, ME). The gels were vacuum dried and subjected to autoradiography for 12–24 hours at ~80°C. Human placental genomic DNA from a known heterozygote (AG) was included as a positive control, whereas DNA was omitted in negative control samples. All cyclin D1 genotypes were confirmed by DNA sequencing with a 5′-GTGAAGT-TCATTCCAATCC-3′ primer as previously described (45). Two investigators (J. G. Izzo and V. A. Papadimitrakopoulou) independently determined the CCND1 (cyclin D1) genotype (i.e., AA, AG, or GG).

Immunohistochemistry

Epithelial regions representing the most advanced histology were identified by the pathologist on the hematoxylin–eosin-stained slides, and the corresponding regions were evaluated for cyclin D1 expression on adjacent slides. Immunohistochemical staining for cyclin D1 was performed on formalin-fixed sections with the DCS-6 mouse monoclonal antibody (NeoMarkers/Lab Vision Corp., Fremont, CA) as previously described (26). This antibody recognizes an epitope common to both cyclin D1 protein variants (36,46). Basal, parabasal, and superficial epithelial layers were identified and evaluated separately. The intensity of nuclear cyclin D1 staining was evaluated with a four-point semiquantitative staining intensity scale: 0 = no staining, 1 = weak, 2 = moderate, or 3 = strong. For comparison with patient characteristics and response to treatment, the results were expressed as the labeling index, that is, the fraction of cells expressing staining intensity levels 2 and 3 in the epithelium. Deregulated cyclin D1 expression was defined as a labeling index of 5% or more, from our previous experience in this preinvasive setting (30). Cyclin D1 protein levels were evaluated with the weighted mean index, which is calculated as the sum of the intensity scale values (0–3) of each cell divided by the total number of evaluated cells.

Lymphocytes residing in each biopsy specimen served as a negative control for cyclin D1 expression. Paraffin-embedded pellets of MDA886Ln cells (27), which strongly express cyclin D1, were sectioned and placed on each biopsy slide to serve as positive controls for normalizing cyclin D1 staining.
FISH Analysis

To assess the copy number of cyclin D1 genes, dual-color FISH was carried out on paraffin-embedded biopsy tissue sections as previously described (26). The 11q13 region copy number was determined with a spectrum red-labeled, 300-kb bacterial artificial chromosome probe spanning the BCL1 locus, the CCND1/PRAD1 gene, and the first exon of the FGF4 gene (Vysis Inc., Downers Grove, IL). Chromosome 11 copy number was determined with a spectrum green-labeled, pericentromeric ɑ-satellite probe specific to chromosome 11 (D11Z1; Vysis Inc.). To confirm cyclin D1 gene amplification in selected cases, a plasmid probe spanning 16 kb of the cyclin D1 gene [a gift from Dr. A. Arnold, University of Connecticut, (47)] was used in combination with the chromosome 11 pericentromeric probe. The cyclin D1 plasmid was labeled with spectrum red-coupled deoxyuridine 5′-triphosphate (Vysis Inc.) by nick translation under conditions optimized to generate a 300-base-pair to 600-base-pair probe, as previously described (26).

FISH-generated fluorescent signals were visualized with a Nikon OPTIPHOT epifluorescent microscope (Nikon Inc., Melville, NY). Digitized images were captured with a high-resolution image analysis system (IP Lab Spectrum, Spectra Services, Webster, NY) with a cooled charge-coupled device camera. Two investigators (J. G. Izzo and V. A. Papadimitra-kopoulou) independently evaluated the FISH signals without knowledge of the clinical response. The cyclin D1 gene was considered amplified if the average cyclin D1 gene copy number per centromere 11 signal exceeded a value of 2 (26, 48).

Statistical Analysis

Fisher’s exact test, Wilcoxon rank sum test, and Cochran–Armitage trend test were performed to determine associations between categorical variables, such as cyclin D1 genotype, cyclin D1 protein staining levels, clinicopathologic variables, and clinical outcome. The association between progression-free survival and cyclin D1 genotype was tested by comparing the Kaplan–Meier survival curves by genotype (AA/AG versus GG). \( P \) values of .05 or less from two-sided statistical tests were considered to be statistically significant. Computations were carried out with a SAS software package (version 6.12; SAS Institute Inc., Cary, NC). All statistical tests were two-sided.

RESULTS

A total of 31 subjects were analyzed for cyclin D1 genotype. As illustrated in Fig. 1, three different genotypes (i.e., AA, AG, and GG) were distinguishable. Of the 31 study subjects examined, three (9.7%) carried the AA genotype, 18 (58.1%) carried the AG genotype, and 10 (32.2%) carried the GG genotype. The calculated allele frequency distributions were 39% for the A allele and 61% for the G allele. The observed allele and genotype frequencies were similar to those found in head and neck cancers. The patients’ clinicopathologic characteristics are summarized in Table 1. Because only three subjects showed an AA genotype and because the A allele has a dominant effect on the half-life of the cyclin D1 protein, the AA and AG genotype groups were combined for statistical analysis. The cyclin D1 genotype was not statistically significantly associated with sex, degree of dysplasia, lesion site, or smoking status of the subjects (Table 1).

To determine whether the levels of cyclin D1 protein were associated with a specific cyclin D1 genotype, protein levels were immunocytochemically examined in samples from 27 subjects with adequate biopsy material (i.e., both baseline and follow-up biopsy samples). Taking the epithelium as a whole, deregulated cyclin D1 expression (defined as a labeling index of >5%) was observed in 14 (51.9%) of the 27 subjects at baseline including 10 (55.6%) of the 18 AA/AG subjects and four (44.4%) of the nine GG subjects (\( P = .70 \), Fisher’s exact test) (Table 2).

Because cyclin D1 regulation may be different in different epithelial layers (i.e., increased expression versus increased turnover), we examined cyclin D1 expression on a layer-by-layer basis. The labeling indices in each layer appeared similar for both AA/AG groups (median [interquartile range]: basal = 0.01 [0–0.13]; parabasal = 0.09 [0.02–0.3]; superficial = 0.01 [0–0.09]) and GG groups (basal = 0.01 [0–0.01]; parabasal = 0.03 [0.02–0.29]; superficial = 0.01 [0–0.02]) (\( P = .70 \), \( P = 1.0 \), and \( P = 1.0 \), respectively, Wilcoxon test). As illustrated in Fig. 2, A and B, and Fig. 3, A, the AA/AG subjects showed a slightly increased weighted mean index compared with the GG subjects in both parabasal and superficial layers. However, for a cutoff parabasal weighted mean index of 0.12 (the baseline median of the GG group), 16 (88.9%) of the 18 AA/AG subjects showed high expression compared with four (44.4%) of the nine GG subjects (\( P = .02 \), Fisher’s exact test).
We previously reported that biochemoprevention treatment in this population of 36 individuals was associated with decreased expression of cyclin D1 in 28 individuals who were assessable for biomarker expression (30). Because retinoids have been shown to decrease the expression of cyclin D1 in vitro (49,50), we examined whether the cyclin D1 genotype influenced protein modulation during treatment. Of the 10 AA/AG subjects with a high pretreatment cyclin D1 labeling index, only two (20%) showed decreased expression after treatment. Moreover, two (25%) of the eight AA/AG subjects with a low pretreatment cyclin D1 labeling index showed protein deregulation after treatment. In contrast, three (75%) of the four GG subjects with a low pretreatment cyclin D1 labeling index showed protein deregulation after treatment, and one (20%) of five showed in-

To better understand the pathophysiology of cyclin D1 regulation during the biochemopreventive intervention, changes in cyclin D1 expression were also examined on a layer-by-layer basis. Although post-treatment labeling indices were similar in the basal layers (median [interquartile range]: 0.01 [0.00–0.02] for AA/AG subjects versus 0 [0.00–0.01] for GG subjects, \( P = .2 \), Wilcoxon test), the parabasal and superficial post-treatment labeling indices were statistically significantly higher in the AA/AG group than in the GG group (parabasal = 0.13 [0.05–0.21] versus 0 [0.00–0.03], \( P = .04 \); superficial = 0.05 [0.00–0.11] versus 0 [0.00–0.02], respectively, \( P = .09 \), Wilcoxon test). Moreover, the post-treatment cyclin D1 weighted mean index values were higher in all layers of the AA/AG subjects than in those of the GG group (Fig. 3, B). This difference was statistically significant for the parabasal layer (\( P = .04 \), Wilcoxon test). Using a cutoff value of 0.29 (i.e., baseline mean of the GG group), only four (33.3%) of 12 AA/AG subjects with high baseline weighted mean index showed decreased expression compared with three (75%) of four GG subjects. In addition, three (50%) of six AA/AG subjects with low baseline expression showed increased expression after treatment compared with zero (0%) of five GG subjects. Thus, only seven (38.9%) of 18 AA/AG subjects showed a favorable cyclin D1 response (i.e., low-to-low or high-to-low changes) compared with eight (88.9%) of nine GG subjects (\( P = .02 \)). This statistically significant trend was observed for all cutoff values ranging from 0.01 to 0.09.

Of the 21 AA/AG subjects, only six (28.6%) showed histologic response to treatment compared with four (40%) of the 10 GG subjects (\( P = .70 \); Fisher’s exact test). For subjects evaluated for cyclin D1 expression (Table 2), the pretreatment cyclin D1 labeling index was not predictive for histologic response. Three (23.1%) of the 13 patients with low pretreatment cyclin D1 labeling indices showed complete response compared with four (28.6%) of the 14 patients with high pretreatment cyclin D1 labeling indices (\( P = 1.0 \), Fisher’s exact test). In contrast, seven (46.7%) of the 15 patients with low post-treatment cyclin D1 labeling indices showed a complete response compared with zero (0%) of 12 patients with high post-treatment expression (\( P = .008 \), Fisher’s exact test).

In a previous study of HNSCC specimens containing both tumor and adjacent preinvasive lesions, we demonstrated that cyclin D1 gene amplification could be detected in some of the preinvasive lesions and that deregulated cyclin D1 expression preceded gene amplification (27). In addition, unpublished pilot studies from our group on HNSCC cell lines suggest that gene amplification occurs preferentially in AA/AG specimens. To examine the relationship of the cyclin D1 genotype, protein levels, and gene amplification, FISH was performed on specimens from 19 subjects with baseline (14 subjects) or follow-up (five subjects) cyclin D1 deregulation. Cyclin D1 gene amplification was identified in two (10.5%) of the 19 subjects (Fig. 2, D). Both showed severe dysplasia of the oral cavity, and both had the AA genotype. To the best of our knowledge, this is the first observation in a clinical setting of cyclin D1 gene amplification occurring in a preinvasive lesion before the development of cancer.

The presence of the A allele was statistically significantly associated with shortened progression-free survival compared with that observed in GG subjects (Fig. 4). Although the numbers are small, GG subjects showed a relative lag in progression to cancer during the 12-month biochemopreventive intervention, and AA/AG subjects progressed to cancer at a constant rate despite treatment. The 2-year progression-free survival for AA/AG subjects was 50.6% (95% confidence interval [CI] = 31.8% to 78.0%) compared with 87.5% (95% CI = 67.3% to 100%) for GG subjects. At a median follow-up of 27 months, 12 (57.1%) of 21 AA/AG subjects progressed compared with only two (20%) of 10 GG subjects (\( P = .05 \), Kaplan–Meier

### Table 1. Pretreatment patient clinicopathologic characteristics and cyclin D1 genotype

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Total (n = 31)</th>
<th>AA (n = 3)</th>
<th>AG (n = 18)</th>
<th>GG (n = 10)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, No. (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>22 (71)</td>
<td>3 (100)</td>
<td>11 (61)</td>
<td>8 (80)</td>
<td>.68*</td>
</tr>
<tr>
<td>Female</td>
<td>9 (29)</td>
<td>0 (0)</td>
<td>7 (39)</td>
<td>2 (20)</td>
<td></td>
</tr>
<tr>
<td>Age, y†</td>
<td>55.5 ± 11.3</td>
<td>60 ± 14.2</td>
<td>56.2 ± 10.2</td>
<td>53 ± 12.9</td>
<td>.67†</td>
</tr>
<tr>
<td>Histology (dysplasia), No. (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mild</td>
<td>3 (10)</td>
<td>0 (0)</td>
<td>3 (16.6)</td>
<td>0 (0)</td>
<td>.06§</td>
</tr>
<tr>
<td>Moderate</td>
<td>16 (52)</td>
<td>3 (100)</td>
<td>9 (50)</td>
<td>4 (40)</td>
<td></td>
</tr>
<tr>
<td>Severe</td>
<td>12 (39)</td>
<td>0 (0)</td>
<td>6 (33.4)</td>
<td>6 (60)</td>
<td></td>
</tr>
<tr>
<td>Site, No. (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>.70*</td>
</tr>
<tr>
<td>Oral cavity</td>
<td>13 (42)</td>
<td>2 (66.6)</td>
<td>6 (33.4)</td>
<td>5 (50)</td>
<td></td>
</tr>
<tr>
<td>Larynx</td>
<td>18 (58)</td>
<td>1 (33.3)</td>
<td>12 (66.6)</td>
<td>5 (50)</td>
<td></td>
</tr>
<tr>
<td>Smoking status, No. (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>.51§</td>
</tr>
<tr>
<td>Never</td>
<td>7 (23)</td>
<td>1 (33.3)</td>
<td>3 (16.7)</td>
<td>3 (30)</td>
<td></td>
</tr>
<tr>
<td>Former</td>
<td>13 (42)</td>
<td>1 (33.3)</td>
<td>8 (44.4)</td>
<td>4 (40)</td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>11 (35)</td>
<td>1 (33.3)</td>
<td>7 (38.9)</td>
<td>3 (30)</td>
<td></td>
</tr>
</tbody>
</table>

*Two-sided Fisher’s exact test comparing the AA/AG and GG groups. †Data are the mean ± standard deviation. §Two-sided Wilcoxon rank sum test comparing the AA/AG and GG groups. ¶Cochran–Armitage trend test comparing the AA/AG and GG groups.

### Table 2. Cyclin D1 genotype, changes in cyclin D1 protein levels, and response to treatment

<table>
<thead>
<tr>
<th>Cyclin D1 level change from pretreatment to post-treatment</th>
<th>AA/AG (n = 18)</th>
<th>GG (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pretreatment cyclin D1 labeling index</td>
<td>No. CR (n = 4)</td>
<td>No. CR (n = 3)</td>
</tr>
<tr>
<td>Low → Low</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Low → High</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>High → Low</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>High → High</td>
<td>8</td>
<td>0</td>
</tr>
</tbody>
</table>

*CR = complete pathologic response.
DISCUSSION

The frequent finding of cyclin D1 alterations in HNSCC tumors indicates that cyclin D1 may play a critical role during head and neck tumorigenesis and thus could be a target for preventive intervention. An ongoing clinical biochemoprevention trial involving subjects with advanced preinvasive UADT lesions provided a unique opportunity to study cyclin D1 expression and gene amplification in preinvasive lesions before treatment (i.e., complete reversal to nondysplastic squamous epithelium or regression of dysplasia to a lower grade), although this factor was not statistically significant, and 4) an increased rate of cancer development.

The allele and genotype frequencies detected in these subjects were similar to those previously reported for both normal control subjects and patients with cancer (37–42,51). A previous molecular epidemiologic study (42) suggested that, although AA/AG individuals were not over-represented in HNSCC populations, their age at the onset of cancer was statistically significantly younger than that of GG individuals, and no association was observed between the cyclin D1 genotype and clinical-pathologic characteristics, suggesting that the cyclin D1 genotype does not predispose individuals to the development of preinvasive UADT lesions. However, the intensity of cyclin D1 protein labeling was higher in AA/AG subjects. The increased expression of cyclin D1 in these lesions is likely associated with a physiologic wound-healing process after tissue injury (e.g., smoking and alcohol exposure), perhaps downstream of activated growth factors and/or integrin signaling pathways (52–54). These results suggest that there was little difference between the allele groups in terms of initiation of cyclin D1 expression. However, the finding of continued high levels of cyclin D1 into the parabasal and superficial layers in AA/AG subjects suggests that the polymorphism status influences the subsequent protein turnover rate in response to signals associated with cessation of proliferation (55).

In vitro studies have suggested that retinoids and interferon can lead to the decreased expression of cyclin D1 through multiple mechanisms (50–60). In this biochemoprevention trial, cyclin D1 levels are likely influenced by a balance between inducers of continued expression (e.g., continued tobacco and alcohol exposure) and treatments that decrease the expression of cyclin D1 and/or enhance its turnover. Our results show that the cyclin D1 A allele was associated with the continued expression of cyclin D1 after treatment. This observation suggests that the A allele may make epithelial cells resistant to multiple retinoid-mediated, α-tocopherol-mediated, and α-interferon-mediated mechanisms. However, the relative roles of retinoids, α-interferon, and α-tocopherol could not be determined from our data in this in vivo setting.

A decreased histologic response was also observed in the AA/AG group, suggesting that regulation of the expression and turnover of cyclin D1 is an important component of the retinoid-based chemopreventive response. These findings underscore the need to identify treatment strategies that also target cyclin D1 expression at the transcriptional level. In this context, we expect that the elimination of factors that induce tissue damage (e.g., smoking and alcohol cessation) should decrease the expression of cyclin D1 and thus enhance responsiveness to these agents. At the same time, not all subjects with low levels of cyclin D1 expression after treatment showed a histologic response, suggesting that the decreased expression of cyclin D1 is necessary but not sufficient for a response to biochemoprevention.
The results of this study also indicate that AA/AG subjects progressed to cancer at a higher rate than GG subjects. The finding that the cyclin D1 A allele was associated with continued high levels of cyclin D1 expression despite treatment suggests that deregulated cyclin D1 contributes to tumorigenesis. This hypothesis is mechanistically supported by studies showing that the overexpression of cyclin D1 is associated with increased genomic instability (26,28). In this study and in prior leukoplakia studies, the presence of high degrees of chromosome polysomy was statistically significantly associated with increased likelihood of subsequent cancer development (11,61,62). Increased cyclin D1 expression is also associated with increased likelihood of gene amplification, both in vitro and in vivo (16,28). In fact, the two subjects in our study who exhibited cyclin D1 gene amplification in their preinvasive lesions carried the AA genotype, showed deregulated cyclin D1 expression, and developed cancer within 1 year, despite treatment.

Subjects with the GG genotype showed an increased response rate and a lack of cancer development during the 12-month treatment period in association with the decreased expression of cyclin D1. However, after discontinuation of treatment, two of the 10 GG subjects subsequently developed cancer. This result highlights the potential benefit of a prolonged chemopreventive intervention. Such a study of individuals with advanced preinvasive lesions of the larynx is currently underway.

It should be noted that these conclusions are derived from correlative biomarker analyses of a phase II biochemoprevention trial whose original target sample size was based on a primary end point of response. This design limited the power of correlative analyses. Thus, our findings need to be verified in larger cohort studies. Nevertheless, our findings suggest that the cyclin D1 A allele was associated with a diminished modulation of normal physiologic and treatment-induced decreased expression of cyclin D1, a decreased likelihood of response to biochemo-
preventive intervention, and an increased rate of progression to cancer development. Although elimination of factors that increase cyclin D1 expression would benefit all genotype groups, subjects carrying the A allele might require additional treatment strategies that modulate the expression of cyclin D1 through other mechanisms.

**REFERENCES**

(24) Michalides RJ, van Veelen NM, Kristel PM, Hart AA, Loots BM, Hilgers FJ, et al. Overexpression of cyclin D1 indicates a poor prognosis in squa-


**NOTES**

Supported by Public Health Service grants DE-13157 from the National Institute of Dental Research and Craniofacial Research (to W. N. Hittelman); CA-68089 and CA-52051 (to W. K. Hong), CA-86390 from the Early Detection Research Network (EDRN), and Cancer Center Core Grant CA-16672 (all from the National Cancer Institute), National Institutes of Health, Department of Health and Human Services; and the American Society of Clinical Oncology Career Development Award (to V. A. Papadimitrakopoulou). W. N. Hittelman is a Sophie Caroline Steves Professor in Cancer Research, and W. K. Hong is an American Cancer Society Clinical Research Professor.

We thank Drs. Jeffrey Myers, Gary Clayman, Ann Gillenwater, and Eduardo Diaz for excellent patient care, Kristie Lathrom for patient enrollment and nursing care, and Susan Cweren for tissue preparation.