Cyclin D1 and p16 Alterations in Advanced Premalignant Lesions of the Upper Aerodigestive Tract: Role in Response to Chemoprevention and Cancer Development[1]

Vassiliki A. Papadimitrakopoulou,[2,3] Julie Izzo,[2]
Li Mao, Jamie Keck, David Hamilton,
Dong Moon Shin, Adel El-Naggar,
Petra den Hollander, Diane Liu,
Walter N. Hittelman, and Waun K. Hong

Departments of Thoracic/Head and Neck Medical Oncology (V. A. P.,
J. K., D. H., D. M. S., W. K. H.), Experimental Therapeutics (J. I.,
P. D. H., W. H.), Pathology (A. E. N.) and Biostatistics (D. L.), The
University of Texas M. D. Anderson Cancer Center, Houston, Texas
77030

ABSTRACT

Purpose: To better understand the role of G_1-S transition regulator abnormalities in the pathogenesis of advanced premalignant lesions of the upper aerodigestive tract and the biological effects of chemoprevention, we studied biopsies obtained sequentially from participants in a prospective trial using 13-*cis* retinoic acid, IFN-α, and α-tocopherol for 12 months.

Experimental design: Cyclin D1 and p16 expression were analyzed by immunohistochemistry, loss of heterozygosity by polymerse chain reacting amplification, and then electrophoretic separation of the products, methylation of the *p16* promoter by methylation-specific polymerase chain reacting, and *cyclin D1* gene amplification by fluorescence *in situ* hybridization.

Results: Baseline dysregulation of cyclin D1 expression was found in 50% (14 of 28) and was reversed in 6 of 14 cases, whereas p16 expression was lost in 46% (13 of 28) and regained in 2 of 13 cases. Loss of heterozygosity at 9p21 occurred in 68% and *p16INK4a* promoter methylation occurred in 75% of cases, with increasing frequency from mild to severe dysplasia. C*yclin D1* gene amplification was iden-

tified in two cases. Cyclin D1 protein dysregulation at last follow-up alone and in combination with p16 loss was associated with histological progression and cancer development ($P < 0.01$).

Conclusions: Additional study of these alterations in a larger sample and exploration of the upstream signaling partners of these cell cycle regulators *in vivo* is warranted to identify cancer risk profiles that would be meaningful targets for chemopreventive intervention.

INTRODUCTION

Although tobacco and its derivatives are considered to be leading causes for HNSCC[4] development, their underlying genetic targets are not yet completely clarified. Cell cycle dysregulation is intimately linked to carcinogenesis. In the past decade, scientists have uncovered several important hints to how mechanisms that control the cell cycle play central roles in both the development and the prevention of cancer (1). The G_1-S transition of the cell cycle is important in determining cell fate, because several mitogenic signals converge into activation of cyclin D and related cdk complexes, resulting in commitment for entry into S phase (1). It is not surprising then, that alterations in the molecular machinery that controls transition from G_1 to S phase might represent central events leading to HNSCC development.

The cdk-cyclin D complex can be turned off by the binding of inhibitory subunits from the Cip and INK4 families and by additional phosphorylation. The INK4 family members specifically inhibit cyclin ᴅ-dependent cdks. The prototypic and most prominent member of the INK4 family, *p16INK4a*, located at 9p21–22, is a major tumor suppressor gene that is incapacitated by homozygous deletion, mutation and loss of the other allele, or promoter methylation in a wide variety of cell lines and tumors, including HNSCC (2, 3). Disruption of the regular patterns of expression of cyclin D1, which functions as a cooperating oncogene and confers abnormal proliferative characteristics to the cells when it is overexpressed (4, 5), have been frequently observed in HNSCC and its precursor lesions (6). A range of 35–64% of HNSCCs show cyclin D1 overexpression and/or amplification (7–13), and these alterations are associated with more frequent recurrence, more advanced disease, lymph node involvement, and reduced overall survival (7–11). Several reports have pointed out a difference in the frequency of

[4] The abbreviations used are: HNSCC, head and neck squamous cell carcinoma; cdk, cyclin-dependent kinase; LOH, loss of heterozygosity; UADT, upper aerodigestive tract; IHC, immunohistochemistry; FISH, fluorescence *in situ* hybridization; MSP, methylation-specific PCR; CR, complete response.
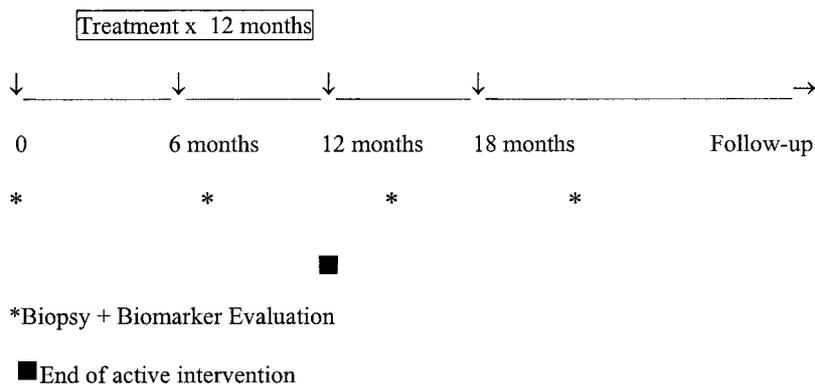
Treatment x 12 months

*Biopsy + Biomarker Evaluation

■ End of active intervention

Fig. 1 Study schema. Biochemoprevention, consisting of 13-*cis* retinoic acid, α-tocopherol, and IFN-α was administered for 12 months or until evidence of histological or clinical progression. Biopsies and biomarker evaluations were performed at baseline, 6 months, 12 months, and 18 months.

CDKN2/p16ink4a genetic alterations between cell lines and primary tumors (14, 15), and although chromosome 9p21 abnormalities are cited as early events in the development of HNSCC, based on LOH (16, 17), few studies of p16 alterations in premalignant lesions have been reported to date (18).

Chemoprevention, the administration of agents to block or reverse carcinogenesis, has been studied extensively by our group and others mainly with the use of retinoids (19). Retinoids as single agents have proven activity in the prevention of second primary tumors and the reversal of oral premalignant lesions (20, 21). However advanced premalignant lesions of the UADT, defined histologically as moderate to severe dysplasia, are usually resistant to single-agent retinoid treatment and harbor a high degree of genetic alterations. To address these lesions that are almost inevitably destined to progress into invasive cancer (22, 23), a new approach, biochemoprevention, using a combination of 13-*cis* retinoic acid, IFN- α, and α-tocopherol was designed and administered for 12 months (Fig. 1). Both traditional clinical end points (*i.e.,* clinical and pathological response evaluations) as well as selected biomarkers, suggested to accurately reflect the degree of alterations harbored in these lesions and thus potential indicators of risk for cancer development or response to chemoprevention, were studied. The study found evidence of activity of the combination with a dramatic difference in terms of response favoring laryngeal lesions, in which the reversal of dysplasia was seen in 60% of cases, whereas oral cavity lesions only partially responded in 27% of cases (24).

Because of the central role of cell cycle dysregulation in carcinogenesis and the high frequency of alterations in this pathway observed in established cancer and precancerous lesions, we chose to examine closely, by immunohistochemical and molecular methods, cyclin D1 and p16 alterations in a series of biopsies obtained from participants in the biochemoprevention trial and correlate the findings with clinicopathological features, response to chemopreventive intervention, and eventual outcome of the patients. The specific aims of this proposal were to define the extent of abnormalities of cell cycle regulators cyclin D1 and p16 in advanced premalignant lesions of the UADT and to correlate the presence of cyclin D1 and p16 abnormalities and their potential reversal with response and resistance to biochemopreventive intervention.

MATERIALS AND METHODS
Patients and Biopsies

Biopsies were obtained at baseline, 6, 12, and 18 months from the participants in this trial. After obtaining a pathological diagnosis, paraffin blocks were cut to obtain 4-μm paraffin sections that were then subjected to IHC, FISH, microdissection, and DNA extraction.

Biopsies from 28 cases were analyzed (22 males and 6 females), 3 with mild, 14 with moderate, and 11 with severe dysplasia in the baseline biopsy. Of these cases, only 25 had informative lesions for LOH.

Pathological changes in tissue in response to the therapy were consistently evaluated according to the criteria described previously (24) by one pathologist (A. E. N.), and they represented the basis for correlation with protein expression.

IHC

For immunohistochemical analysis, the following antibodies were used: DCS-6 mouse monoclonal anti-cyclin D1 antibody (NeoMarkers) and anti-p16 mouse monoclonal antibody (NeoMarkers). All cyclin D1 and p16 analyses were performed in a blinded fashion by two independent reviewers (V. P. and J. I.).

Glass slides with 4-μm tissue sections of formalin-fixed, paraffin-embedded specimens were deparaffinized, rehydrated, and incubated for 15 min with 3% hydrogen peroxide to quench the endogenous peroxidase activity. Antigen retrieval was carried out with the heat-mediated antigen retrieval method by placing the slides in 10 mM citrate buffer (pH 6.0) and microwaving at 630 W for 12–16 min and then blocking nonspecific protein binding by incubating the samples with 5% normal horse serum blocking solution for 20 min at 37°C (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA). The sections were incubated with the anti-cyclin D1 mouse monoclonal antibody at a concentration of 1:100 or anti-p16 mouse monoclonal antibody at a concentration of 1:25–1:50 at 4°C overnight. For negative controls, no primary antibody was placed, and the samples were incubated overnight with the blocking solution. Secondary antimouse antibody at 1:200 dilution in blocking serum was added to the slides for 30 min at 37°C, and slides were incubated with 1:50 dilution of biotin-avidin-peroxidase conjugate (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA) for 30 min at room temperature. Antigen

visualization was performed with 3–3′-diaminobenzidine tetrachloride (Sigma Chemical Co., St. Louis, MO; 1mg/ml in 1 × PBS;) and hydrogen peroxide (30 ng/ml) for 5–8 min, and the signal was monitored under a light microscope. Sections were rinsed under tap water and counterstained with Mayer's hematoxylin. Cytoplasmic reactivity was disregarded, and only nuclear staining above any cytoplasmic background was considered as evidence of expression of the p16 and cyclin D1 proteins.

A four-point semiquantitative scale of nuclear staining intensity was used as follows: 0, no staining; 1, weak; 2, moderate; and 3, strong. Positivity was defined as ≥5% of cells with a score ≥2 for cyclin D1 and ≥1% of cells with a score ≥1 for p16, based on epithelial areas selected by the pathologist as representing the worst histology in each particular biopsy. In the case of p16, stromal cells had to show nuclear reactivity serving as an internal control. The whole epithelial region was counted for all slides. The Saos 2 and MDA 886 cell lines with well-defined p16 and cyclin D1 status, respectively, were used as positive controls by placing tissue sections of cell line pellets on the same slides as the tissue sections. The criteria for scoring were based on previous experience from our own group as well as review of the relevant literature. For cyclin D1, the 5% cutoff was chosen based on the distribution of expression observed in the lesions examined and similar cutoffs chosen previously by us and other investigators.

FISH-DNA Probes

A spectrum green-labeled chromosome 11-specific pericentromeric α-satellite probe (D11Z1; Vysis, Inc., Downer's Grove, IL) combined with a spectrum red-labeled 300-kb bacteria artificial chromosome probe spanning the *Bcl-1* locus, the entire *cyclin D1* locus, and 50 kb of the *fibroblast growth factor-4* locus (Vysis, Inc.) was used for hybridization of all sections described in the manuscript. In addition, a digoxigenin-labeled plasmid probe spanning 16 kb of the *cyclin D1* gene provided by Dr. A. Arnold combined with a chromosome 11-specific pericentromeric α-satellite biotin-labeled probe (D11Z1) obtained from Oncor, Inc. (Gaithersburg, MD) were used in selected cases to confirm amplification of the *cyclin D1* gene. The latter probes were labeled with digoxigenin-11dUTP or biotin-16dUTP (Boehringer Mannheim) by nick translation using optimized conditions to generate a probe size of 300–600 bp. Dual-color FISH technique was carried out as previously described (6).

Fluorescence Microscopy and Scoring Criteria. The fluorescent signals on slides were examined with a Nikon OPTIPHOT epifluorescent microscope equipped with oil-immersion objective and triple band-pass filter for DAPI. A lesion was considered amplified if the average cyclin D1 copy number/centromere 11 signal/scored cell exceeded 2.

DNA Extraction

Genomic DNA was extracted from paraffin-embedded specimens after deparaffinization and microdissection of epithelial areas from serial sections of the biopsy sample as described previously (17). The entirety of the epithelial lesion was dissected in the majority of cases, avoiding stromal contamination as much as possible. For DNA extraction from blood samples, leukocytes are isolated by lysing RBCs and subjected to digestion and DNA purification.

Microsatellite Analysis

DNA from at least 150 nuclei is used for each PCR amplification to avoid possible PCR artifacts caused by a small amount of DNA template. The markers used were *D9S171* and *D9S1747* (9p21; Research Genetics, Huntsville, AL). PCR amplification was carried out as described previously (17), and the PCR products were separated on a 7% polyacrylamide-urea-formamide gel, followed by radiographic exposure for 12–48 h at −80°C. LOH is defined as a >50% reduction in the intensity, by visual inspection, in one of the two alleles as compared with those in normal control (blood-derived) panels.

MSP

MSP was performed after DNA modification. An effort was made to begin the modification procedure with 1 μg of DNA, but in the majority of cases, the starting material was 100 ng of DNA. The CpGenome DNA Modification kit (Intergen Company, Purchase, NY) was used according to the manufacturer's instructions using hydroquinone and sodium bisulfite. Chemical modification and MSP were performed based on the procedure as described previously (25). For MSP the CpG WIZ p16 amplification kit was used, which included a U, M, and W primer set; U, M, and W control DNA; and Universal 10× PCR buffer [where U stands for unmethylated, and the primer set will anneal to unmethylated DNA that has undergone chemical modification; M stands for methylated, and the primer set will anneal to methylated DNA that has undergone a chemical modification; and W stands for wild-type, and the primer sets serve as a control for the efficiency of chemical modification and will anneal to any DNA (unmethylated or methylated) that has not undergone chemical modification].

"Hot Start" PCR was performed using AmpliTaq Gold (PE Biosystems, Foster City, CA), with the following amounts of reagents for each reaction: 2.5 μl of 10× Universal PCR buffer; 2.5 μl of 2.5 mM dNTP mix; 1.0 μl of U, M, or W primers; 0.2 μl (1 unit) of AmpliTaq Gold; 16.8 μl of dH$_2$O; and 2.0 μl of template DNA (50 ng/μl) for a total volume of 25.0 μl and under the following conditions: 95°C for 15 min, then 35 cycles of 95°C for 45 s, 60°C for 45 s, and 72°C for 60 s. PCR products were run on a 2% agarose gel, and the gel was stained with ethidium bromide with the expected PCR products being 154 bp for unmethylated DNA, 145 bp for methylated DNA, and 142 bp for wild-type.

Statistical Analysis

Statistical analysis was based on two-sided Fisher's exact test for the effect of clinicopathological variables on the expression of the proteins (2 × 3 matrix for effect of histology and smoking status on protein expression; Table 1), effect of histology on molecular alterations (2 × 3 matrix; used in Table 4), correlation between protein expression and molecular alterations, and association of protein expression with outcome. McNemar's test was used for modulation of protein expression with treatment. The computations were carried out using the SAS software package. The statistical result was considered significant if P was <0.05.

Table 1 Baseline cyclin D1 and p16 protein expression by patient characteristics

| | DYP1[a] (n = 3) | DYP2 (n = 14) | DYP3 (n = 11) | Smoking status | | | Site | |
				Current (n = 10)	Former (n = 12)	Never (n = 6)	Larynx (n = 17)	Oral cavity (n = 11)
p16 absent or low	1	6	6	3	7	3	9	4
			P = 0.87			P = 0.44		P = 0.46
Cyclin D1 dysregulation	2	7	5	5	7	2	7	7
			P = 1.00			P = 0.72		P = 0.44
Both	0	3	2	1	2	2	3	2
			P = 1.00			P = 0.68		P = 1

[a] DYP1, mild dysplasia; DYP2, moderate dysplasia; DYP3, severe dysplasia.
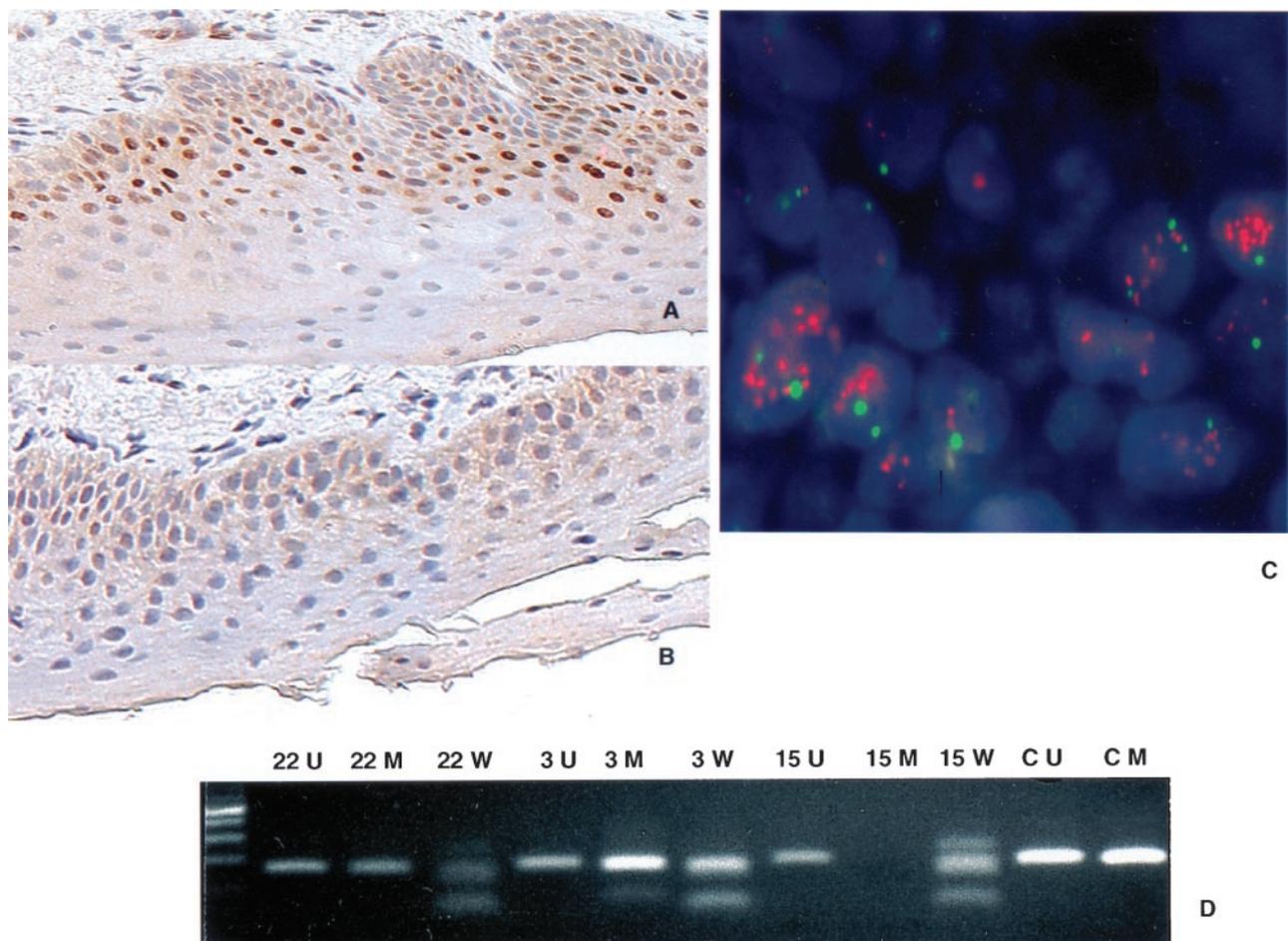

Fig. 2 Analysis of cyclin D1 (*A*) and p16 (*B*) by IHC, FISH for the *cyclin D1* gene (C), and MSP for p16 (*D*) in dysplastic lesions of the UADT. *A,* characteristic pattern of staining with cyclin D1, demonstrating dysregulation of protein expression. *B,* lesion harboring histological characteristics of mild dysplasia with absent p16 expression. Lesions are counterstained with hematoxylin. *C,* demonstration of gene amplification in a severely dysplastic lesion. *D,* primer sets used for amplification are designated as unmethylated (*U*), methylated (*M*), or unmodified/wild-type (*W*). The *first lane* of the gel is occupied by the molecular weight marker. The smaller molecular weight fragments seen in *U lanes* represent primer dimers.

RESULTS

Cyclin D1 and p16 Expression

Baseline. Twenty-eight patients had adequate baseline and 27 follow-up biopsies to assess protein expression. Of these 28 subjects, 22 were male and 6 were female with a median age of 56.5 (range, 32–79) years. The worst histology present at

each time point from the same index site as the baseline biopsy was selected for scoring, except in cases of appearance of new lesions with worsening histology.

Cyclin D1 dysregulated expression (defined as >5% of the cells demonstrating moderate or strong staining) was observed in 14 (50%) of 28 cases at baseline. Nuclear staining involved

predominantly basal and parabasal cells, with the majority of cells in the superficial layers being negative (Fig. 2*A*). Decreased or absent p16 expression (Fig. 2*B*) was observed in 13 (46%) of 28 cases at baseline. The expression of the two proteins at baseline was not correlated, because coincident aberrant expression of p16 and cyclin D1 was observed in only 5 of 14 cases with baseline aberrant cyclin D1 expression ($P = 0.44$; Fisher's exact test).

Cyclin D1 and p16 Expression after Intervention. Cyclin D1 dysregulated expression was seen in 12 (44%) of 27 cases at completion of active intervention. Reversal of dysregulation was observed in 6 (46%) of 13 cases (one case missing follow-up), and new development of dysregulation in 5 (36%) of 14 cases with normal expression at baseline. Reversal of dysregulation was defined as the reduction of expression to a percentage of cells below the cutoff value of 5% (mean reduction, 10.67%; range, 4.8–14.7%). Cyclin D1 expression did not change significantly with intervention ($P = 0.76$; McNemar's test).

p16 loss was seen in 15 (54%) of 28 cases at the end of intervention. Two (15%) of 13 cases regained expression of the protein, whereas 4 (27%) of the initial 15 with retained expression lost expression of the protein at follow-up. Regaining of expression was defined as an increase in the percentage of cells above the predefined cutoff value of 1%. p16 expression did not significantly change with intervention ($P = 0.41$; McNemar's test).

Eight cases (29%) had concurrent aberrant expression at study completion, including two of the baseline aberrant cases and six with concomitant aberrant expression that developed at follow-up, whereas three of the initially aberrant cases had reversal of the abnormalities at follow-up. The presence of aberrant expression of any of the two proteins at baseline occurred in 22 (79%) of 28 cases and in 19 (68%) cases at study completion ($P = 0.74$; McNemar's test).

Relationship of Cyclin D1, p16 Expression with Clinicopathological Parameters

The relationship of protein expression at baseline with smoking habits, histology, and disease site are summarized in Table 1. Among the characteristics studied, smoking status made no difference in the degree of expression of any of the two proteins, and neither did histology. p16 loss was seen more frequently in laryngeal lesions (9 of 17 *versus* 4 of 11 oral cavity lesions; $P = 0.46$; Fisher's exact test) and cyclin D1 dysregulation more frequently in oral cavity lesions (7 of 11 *versus* 7 of 17 laryngeal lesions; $P = 0.44$).

Patterns of Expression in Correlation with Response to Chemoprevention and Outcome

We examined histology as a determinant of response and found that it did not predict CR to the intervention, because 2 of 3 cases with mild dysplasia, 3 of 14 cases with moderate dysplasia, and 3 of 11 cases with severe dysplasia achieved CR ($P = 0.41$; Fisher's exact test). Similarly, histology did not predict progression, which occurred in none of 3 cases with mild dysplasia, in 5 of 14 cases with moderate dysplasia, and 2 of 11 cases with severe dysplasia ($P = 0.47$). Smoking status was not

Table 2 Association of protein expression with response

	Cyclin D1 dysregulation— baseline ($n = 14$)	No cyclin D1 dysregulation— baseline ($n = 14$)	p16 loss— baseline ($n = 13$)	No p16 loss— baseline ($n = 15$)
Response				
CR	4	4	5	3
<CR	10	10	8	12
Fisher's test		$P = 1.00$		$P = 0.4$

a determinant of response to the intervention or progression. Four of 10 current smokers, 3 of 12 former smokers, and 1 of 6 never-smokers achieved CR ($P = 0.65$; Fisher's exact test), and 1 of 10 current smokers, 5 of 12 former, and 3 of 6 never-smokers progressed ($P = 0.19$; Fisher's exact test). Because histology did not predict for response to chemoprevention, the relationship between expression of the two proteins and patient outcome was examined. Cyclin D1 dysregulation at baseline had no impact on the probability of response, because 4 of 14 cases with dysregulation at baseline and 4 of 14 cases with normal expression sustained a CR ($P = 1.0$; Fisher's exact test). Similarly, aberrant expression of cyclin D1 at baseline did not predict for progression to worse histology or cancer during the intervention; 4 of 14 cases with aberrant expression progressed *versus* 3 of 14 with normal expression ($P = 1.00$; Fisher's exact test). No significant association between p16 loss at baseline and progression (4 of 13 cases *versus* 3 of 15 cases with retained expression; $P = 0.67$; Fisher's exact test) or p16 expression retention and CR (3 of 15 cases *versus* 5 of 13 cases with loss; $P = 0.40$; Fisher's exact test) was seen. When either or both of the proteins demonstrated aberrant expression at baseline, 7 of 22 cases achieved CR *versus* 1 of 6 with retained expression, demonstrating that their expression had no impact on response.

Cyclin D1 dysregulation detected at last follow-up, however (either persisting from baseline or developing in follow-up biopsies and persisting through the end of intervention), was strongly associated with adverse outcome. Eight of 12 cases with dysregulated expression at the end of the intervention showed concomitant progression either to a worse degree of dysplasia or to cancer *versus* 1 of 15 nondysregulated cases ($P = 0.003$; Fisher's exact test). It should be noted that of the 4 cases with dysregulated cyclin D1 expression at last follow-up that did not show progression or cancer during the last follow-up biopsy, one developed cancer with longer follow-up, whereas the other 3 had persistence of dysplasia. Association of p16 loss at last follow-up with adverse outcome was also observed; 7 of 15 cases with p16 loss showed progression or cancer in the last follow-up biopsy *versus* 2 of 13 cases with retained expression; but this association did not reach statistical significance ($P = 0.11$; Fisher's exact test). Furthermore, the simultaneous presence of both alterations at last follow-up was significantly associated with histological progression or cancer ($P = 0.005$; Fisher's exact test).

These results are summarized in Tables 2 and 3.

FISH of the *Cyclin D1* Gene

On the basis of the fact that abnormalities at the cyclin D1 protein level seemed to be reversible in some cases and not in

Table 3 Association of protein expression with outcome

	Cyclin D1 dysregulation at last follow-up ($n = 12$)	No cyclin D1 dysregulation at last follow-up ($n = 15$)	p16 loss at last follow-up ($n = 15$)	No p16 loss at last follow-up ($n = 13$)	Both alterations present at last follow-up ($n = 8$)	Any or none ($n = 20$)
Outcome						
PD or Ca	8	1	7	2	6	3
No PD or Ca	4	14	8	11	2	17
Fisher's exact test	$P = 0.003$		$P = 0.11$		$P = 0.005$	

Table 4 LOH at 9p21 and p16 methylation by degree of dysplasia

	Mild dysplasia	Moderate dysplasia	Severe dysplasia	Total	Fisher's exact test
9p21 LOH	2 (100%)	8 (66%)	7 (64%)	17 (68%)	$P = 0.83$
9p21 retention	0	4	4	8	
Methylation	1 (33%)	11 (79%)	9 (82%)	21 (75%)	$P = 0.24$
No methylation	2	3	2	7	

others, we set out to determine whether the presence of *cyclin D1* gene amplification was responsible for persistent dysregulated protein expression. Dual-color FISH was selectively performed in cases with dysregulated cyclin D1 protein expression at baseline (14 cases) or in follow-up biopsies, a total of 35 biopsies corresponding to 19 cases, using the chromosome 11-specific probe combined with the 300-kb bacteria activating chromosome probe spanning the *Bcl-1* locus, the entire *cyclin D1* locus, and 50 kb of the *fibroblast growth factor-4* locus. Amplification of *cyclin D1* was identified in two cases (Fig. 2*C*). The amplification was confirmed by using a digoxigenin-labeled plasmid probe spanning 16 kb of the *cyclin D1* gene combined with a chromosome 11 α-satellite biotin-labeled probe (D11Z1). Both biopsies with gene amplification harbored severe dysplasia, and both cases went on to develop cancer, and the amplification was maintained in the cancer specimen.

MSP for p16 LOH at 9p21

Because mutations of the gene are considered a relatively uncommon mechanism of *p16* inactivation in all tumors and in HNSCC in particular (2), we chose to investigate the status of the gene, by performing LOH studies with two markers at 9p21, D9S1717, and D9S1747, flanking the *p16* locus and MSP. The MSP results were analyzed and compared with the patterns of LOH at 9p21 obtained through PCR-based microsatellite analysis and with protein expression. p16 methylation at baseline was detected in 21 (75%) cases examined with a trend for the event to increase in frequency during disease progression from mild (33%) to severe dysplasia (82%). Of these 21 cases, only 8 showed reversal of methylation in follow-up biopsies. LOH at 9p21 was seen in 17 (68%) of 25 cases (Table 4). The comparison of genetic and protein expression analysis is shown in Table 5. No significant correlation was found between either methylation or LOH at 9p21 and loss of p16 expression, accounted for by the fact that selected regional epithelial analysis was reported for protein expression, as compared with whole-epithelium DNA analysis for MSP and LOH.

Table 5 Correlation of p16 expression with LOH of 9p and p16 promoter methylation

	p16 loss	p16 retention	Total	Fisher's exact test
9p21 LOH	9	9	18	
9p21 retention	3	4	7	$P = 1.00$
Methylation	9	12	21	
No methylation	4	3	7	$P = 0.67$

DISCUSSION

The recognition of the events that contribute to malignant transformation, their prognostic significance, and their role in determining resistance or sensitivity to potential interventions is of the utmost importance for the development of additional therapeutic and preventive strategies in head and neck cancer. Alterations of the critical regulators of the G_1-S cell cycle transition are frequent and potentially important. In this study, we hypothesized that altered patterns of cyclin D1 and p16 expression may serve as biomarkers of carcinogenesis in the UADT and as risk markers for cancer development. A second hypothesis was that reversal of these alterations may underlie the reversal of certain advanced premalignant lesions, and thus, that they could be used as surrogate markers for response to chemoprevention. We found frequent alterations of expression of these two proteins but no significant correlation between these alterations and clinicopathological characteristics, although cyclin D1 alterations tended to occur more frequently in oral cavity lesions and p16 alterations in laryngeal lesions.

No significant modulation by chemopreventive intervention in the expression of either protein was seen. Dysregulated cyclin D1 expression persisted or developed during the intervention in 12 (44%) of 27 cases. Gene amplification was not a frequent underlying cause for this persistent dysregulation, suggesting alternative mechanisms. Previous *in vitro* work in human bronchial epithelial cells has shown that retinoid-induced G_0-G_1 arrest occurs through activation of RAR-dependent path-

ways and is associated with suppressed cyclin D1, cyclin E, cdk2, and cdk4 expression and increased expression of p27 (26–29). Ubiquitin-mediated cyclin D1 proteolysis is involved in this suppression (30). Retinoid refractoriness in transformed human bronchial epithelium is associated with the lack of down-regulation of cyclin D1 (26), which parallels the findings in our study. Possible mechanisms for the persistent cyclin D1 dysregulation are failure of the ubiquitin-proteasome-induced degradation, uncontrolled gene transcription caused by low-level gene amplification or translocation involving the *cyclin D1* locus or by alterations in upstream signaling pathways such as β catenin/LEF-1 (31, 32).

In our study, the majority of cases (8 of 12) with a dysregulated expression of cyclin D1 at last follow-up developed cancer or histological progression concomitantly to the detection of dysregulated expression during the intervention period or at follow-up. Our study, therefore, is in accordance with the previously described, poor prognostic significance of cyclin D1 expression in laryngeal lesions (33), and it shows that the adverse prognostic value of dysregulated expression is not altered by chemoprevention, dictating the need for more effective and possibly targeted intervention focusing on the specific defect in the future.

Frequent *p16* gene and protein alterations were observed. p16 protein absence despite the apparent lack of genetic alterations in some cases might be attributable to homozygous deletions that were not specifically investigated because of a lack of sufficient material. Sequence analysis was not performed purposely, because *p16* mutations have only rarely been reported in premalignant lesions by us or others (3, 18). We chose to systematically investigate methylation of the *p16* promoter in these premalignant lesions, previously only reported for premalignant lesions of the lungs and cervix (34, 35), and have demonstrated that *p16* promoter methylation is an early epigenetic alteration in head and neck carcinogenesis. The apparent retention of p16 protein expression in lesions with 9p21 LOH or methylation can be explained by the fact that LOH and MSP studies examined the bulk of epithelial lesion DNA, whereas, for protein quantitation, certain areas representing the worst histology were selected. Moreover, in most such cases, a mix of both unmethylated and methylated DNA was detected, justifying p16 expression by areas. We have to emphasize that IHC in the case of p16 sets out to find reductions in the already-reduced levels of p16 generally present in primary tissues, such that defining criteria for the presence of the protein in tissue becomes difficult and often arbitrary. We have taken the diverse criteria for positivity in the literature (3, 36–38) under careful consideration when scoring the lesions in our study. p16 loss was not strongly associated with the presence of histological progression or cancer at last follow-up.

Regarding the value of these two protein alterations as risk markers for cancer development, although baseline expression did not predict subsequent cancer development, there was a strong association between dysregulated cyclin D1 expression at last follow-up (alone and in combination with p16 loss) and the presence of histological progression or cancer. The lack of predictive power for baseline cyclin D1 dysregulation as opposed to cyclin D1 dysregulation at last follow-up could be attributed to either statistical artifact or the effect of the chemo-

preventive intervention on protein expression. Obviously, because the sample is small in this study, and sampling error in follow-up biopsies is a potential inherent flaw of all translational studies, the validity of statistical observations is limited, and a larger sample would be required to establish a more clear-cut predictive role for cyclin D1 and p16 alterations. Continued efforts to investigate other determinants of cell cycle dysregulation as well as their signaling partners is warranted to establish validated biomarkers of risk and response to chemoprevention.

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