Biologic Correlates of a Biochemoprevention Trial in Advanced Upper Aerodigestive Tract Premalignant Lesions

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Abstract
Objective: To identify tissue biomarkers that might be used to assess an individual’s cancer risk and response to chemoprevention, we studied dysplastic lesions of the larynx and the p.o. cavity, a series of biomarkers extensively used in previous chemoprevention trials, including chromosome polysomy (CP), proliferative status, p53 expression and gene mutations, and loss of heterozygosity at 9p, 3p, and 17p.

Method: Biopsies from 32 participants in a prospective chemoprevention trial using 13-cis-retinoic acid, IFN-α, and α-tocopherol for 12 months (20 with vocal cord and 12 with p.o. cavity lesions) were analyzed for p53 and Ki-67 expression by immunohistochemistry, loss of heterozygosity by PCR amplification, p53 mutations by PCR-based direct sequencing, and CP by in situ hybridization.

Results: High CP (≥4% cells with more than three chromosome copies per nucleus) was more common in p.o. (8 of 10) than laryngeal lesions (4 of 16; P = 0.01), and so was a combination of CP ≥ 4% and parabasal layer p53 labeling index ≥ 0.2 (P = 0.02). Low CP was a significant predictor of complete histological response (8 of 14 cases with low versus 1 of 12 cases with high CP; P = 0.01). A trend for histological progression or cancer development was observed in cases with high CP and parabasal layer p53 labeling index.

Conclusion: Low CP, more frequently observed in laryngeal lesions, appears to be a predictor of response to chemoprevention and could be used as a screening tool to identify suitable candidates for such approaches. Further investigation of biological parameters of response and cancer risk was warranted.

Introduction
It is now well understood that epithelial carcinogenesis is a multistep process with multiple genotypic and phenotypic alterations contributing to malignant transformation. Chemoprevention, the use of pharmacologic or natural agents to reduce or reverse the carcinogenic process in individuals with a high risk of developing cancer, is a promising field of research. The identification of biomarkers that can: (a) accurately predict for cancer risk and thus lead to selection of appropriate candidates for intervention trials; and (b) serve as intermediate end points for evaluation of novel chemopreventive agents is crucial in chemoprevention research.

Single agent retinoid chemoprevention has shown activity in prevention of second primary tumors and reversal of p.o. premalignant lesions (1–3). However, advanced premalignant lesions of the UADT,4 defined histologically as moderate to severe dysplasia, typically harbor a high degree of genetic alterations, are associated with high risk of malignant transformation (4, 5), and are usually resistant to single agent retinoid treatment. A prospective trial addressing these lesions with a combination of 13-cis-retinoic acid, IFN-α, and α-tocopherol for 12 months with both clinical end points and biomarker studies performed on biopsy specimens performed at baseline, 6, 12, and 18 months was completed by our group, and the clinical results were reported (6). Eleven of 20 cases with laryngeal lesions achieved CR, whereas only 1 of 12 p.o. cavity cases did (P = 0.01), and demonstrated a longer time to PD or cancer development (P = 0.04; Log-rank). The choice of biomarkers was based on previous findings from our p.o. premalignancy studies. In these studies, CP was detected in histologically normal epithelium adjacent to tumors and shown to increase in frequency from histologically normal to hyperplasia to dysplasia and ultimately cancer, supporting the concept of multistep tumorigenesis (7), and in a limited sample of early p.o. premalignant lesions, high CP was associated with subsequent cancer development (8). We had also found that in p.o. premalignancy, p53 protein levels were significantly higher in lesions resistant to high-dose 13-cis-retinoic acid intervention (9, 10). Therefore, our study set out to characterize the extent of biomarker abnormalities in advanced premalignant lesions of the UADT and correlate that with response and resistance to biochemopreventive intervention, cancer risk, and time to cancer development and also to answer questions generated from our previous studies and the clinical findings of the biochemo-

4 The abbreviations used are: UADT, upper aerodigestive tract; CR, complete histological response; LOH, loss of heterozygosity; LI, labeling index; CISH, chromosome in situ hybridization; CP, chromosome polysomy; IHC, immunohistochemistry; PB, parabasal; PD, histological progression.
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 pathologic diagnosis, paraffin blocks were cut to obtain 4 μm paraffin sections that were then subjected to IHC, in situ hybridization, microdissection, and DNA extraction.

Biopsies from 32 cases were analyzed, 24 males and 8 females, 20 with vocal cord and 12 with p.o. cavity lesions, 4 with mild, 16 with moderate, and 12 with severe dysplasia in the baseline biopsy.

Pathologic changes in tissue in response to the therapy were consistently evaluated according to the criteria described previously (6) by one pathologist (A.E.-N.), and they represented the basis for correlation with protein expression. Specifically, the definition of CR was complete reversal to non-dysplastic squamous epithelium, and partial defined response was because regression of dysplasia to a lower grade rescored the one with the worst biomarker expression was chosen as representative of each lesion at each time point from the baseline biopsy.

Multiple regions, designated by the pathologist, were scored per biopsy, but the worst histologically regions were selected as representative of each lesion at each time point from the same index site as the baseline biopsy (except in cases of appearance of new lesions with worsening histology). In case of multiple regions of the same histological degree of dysplasia, the one with the worst biomarker expression was chosen as representative.

IHC. For immunohistochemical analysis, the following antibodies were used: (a) monoclonal anti-p53 antibody (clone DO7; Biogenex, Inc., San Ramon, CA); and (b) mouse monoclonal anti-Ki-67 antibody (clone MIB-1; Zymed Laboratories, Inc., San Francisco, CA). IHC was performed as described previously (9).

Protein levels (p53 and Ki-67) were expressed as LI (number of cells with nuclear staining divided by the total number of cells counted). Maximum value of each marker was used for statistical correlations and reporting. p53 positivity was defined as high or low based on a cutoff point of LI (versus p.o. cavity). Apart from genetic instability in the form of CP and p53 expression, other markers studied previously by our investigators were (larynx versus p.o. cavity). p53 positivity was defined previously (9).

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 (13). For DNA extraction from blood samples, leukocytes are isolated by lysing RBCs and subjected to digestion and DNA purification as mentioned above.

Microsatellite Analysis. DNA from ≥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, D9S1747 (9p21), D3S1285, and D3S1234 at 3p14 and TP53 at 17p13 (Research Genetics, Huntsville, AL). PCR amplification and visualization of products were carried out as described previously (13). 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.

p53 DNA Sequencing. Three separate fragments of the p53 gene encompassing exons 5–9 were amplified from the extracted DNA from these microdissections and directly sequenced, as described previously (16).

Statistical Analysis. The Wilcoxon’s rank-sum test was used to test for equal median of variables (i.e., age and Ki-67) between two levels of categories (i.e., site and response). The Kruskal-Wallis test was used to test for equal median of markers among various histologies (i.e., mild, moderate, and severe dysplasia). The two-sided Fisher’s exact test was used to test equal proportion between groups in two-way contingency tables. The computations were carried out using the SAS Institute, Inc. (Cary, NC) software package. The time-to-cancer development curves were estimated and plotted by the Kaplan-Meier method using the S-Plus software package, and the Log-rank test was used for statistical significance. The statistical difference was considered significant if the P ≤ 0.05.

To overcome the limitations of the small sample size and occasional missing biomarker values, we devised combined scores to assess the impact of multiple biomarkers on probability of response and long-term outcome, as well as their correlation with clinicopathological characteristics. These were defined as follows: (a) CP.p53LI, defined as 1 for the presence of either PB p53 LI ≥ 0.2 or CP ≥ 4% and 2 for the presence of both; and (b) CP.p53.LOH, defined as the sum of all three biomarkers, each one being assigned a value of either 0 (low risk) or 1 (high risk). The risk was assigned based on the previously described cutoffs of ≥4% for CP and ≥0.2 for p53

Chromosome 9 was chosen because polysomy for chromosome 9 correlates with polysomy for chromosome 7 (15).

Two types of DNA probes for the centromeric region of chromosome 9 were used during the course of these studies, giving identical results. Early studies used the commercially available biotin-labeled satellite DNA specific for the centromeric region of chromosome 9 (Oncor, Gaithersburg, MD). Later studies used a DNA probe for the same centromeric region derived from PCR amplification of the human DNA clone pMR9A (GenBank accession no. M64320) using two sets of primers (set 1: pEBR-25 and pEB9-170; set two: pEB9-151 and pEB9-317).

CISH was carried out as described previously (14). The criteria for the scoring of CISH signals were as described previously (14). The CP index was defined as the percentage of scored nuclei exhibiting three or more chromosome copies. A cutoff point of 4% was used for dividing lesions in high and low CP groups, based on the distribution of indices observed in this population, the median being 3.5 (range 0–23) and the mean +1/– SD being 6.65 +/– 7.09.

DNA from ≥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, D9S1747 (9p21), D3S1285, and D3S1234 at 3p14 and TP53 at 17p13 (Research Genetics, Huntsville, AL). PCR amplification and visualization of products were carried out as described previously (13). 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.

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of treatment, and baseline marker values for each case were plotted over the entire course of treatment. For the impact of biomarker modulation on outcome, biomarker values were noted at end-of-treatment values were taken into consideration for definitions of persistently high or increasing value and persistently low or decreasing value (with the above-mentioned cutoffs). Cox regression was used to model the predictive effect of biomarkers and site on survival time with adjustment for clinical and histopathological parameters (age, sex, smoking status, and histology).

**Results**

**Clinical Characteristics and Histopathological Features.** The analysis included 32 patients that were evaluable for response assessment and had adequate baseline and follow-up biopsies to assess biomarker expression. There was a difference in site distribution by sex, in that females had predominantly p.o. cavity lesions (six of eight), and males mostly had vocal cord lesions (18 of 24). No correlation was evident between site and biomarker expression. Discrete hybridization signals were obtained in the three chosen chromosomal regions had no correlation with the above-mentioned cutoffs. Cox regression was used to model the predictive effect of biomarkers and site on survival time with adjustment for clinical and histopathological parameters (age, sex, smoking status, and histology).

**Baseline Biomarker Expression by Site and Other Clinicopathological Characteristics.** Of the 32 cases, all had available tissue at baseline for p53 and Ki-67 IHC, for LOH and p53 sequencing. Discrete hybridization signals were obtained in combination with the retention of a proper nuclear and tissue morphology in 26 cases for CP. As shown in Table 1, the only significant correlation between site and biomarker expression was seen for CP. Eight (80%) of 10 p.o. cavity cases and 4 (25%) of 16 laryngeal cases had high CP (cutoff 4%, \( P = 0.01 \); Fisher’s exact test). Eight of 12 p.o. cavity lesions and 10 of 20 vocal cord lesions had high p53 expression (cutoff 0.2 for CP, \( P = 0.47 \); Fisher’s exact test). We observed mutations in the p53 gene in six of the cases examined at baseline, three in vocal cord, and three in p.o. cavity lesions (\( P = 0.65 \)). LOH at any of the three chosen chromosomal regions had no correlation with site; 10 of 12 p.o. cavity lesions and 14 of 20 vocal cord lesions demonstrated LOH at baseline (\( P = 0.68 \)). With a cutoff of 1.5 for the CPp53LI composite score, 5 of 12 p.o. cavity lesions carried a high score versus only 1 of 20 vocal cord lesions (\( P = 0.02 \); Fisher’s exact test), and similarly, 10 of 12 p.o. cavity lesions and 9 of 20 laryngeal lesions carried a high CP.p53.LOH score (cutoff of 1.5; \( P = 0.06 \)).

When smoking status was examined, high CP was seen in 4 of 5 never-, 6 of 13 former, and 2 of 8 current smokers (\( P = 0.17 \); Fisher’s exact test), whereas LOH was present in 6 of 7 never-, 9 of 14 former, and 9 of 11 current smokers (\( P = 0.58 \); Fisher’s exact test). High p53 was observed with the same frequency among the groups of never- (three of seven), former (7 of 14), and current smokers (5 of 11; \( P = 1 \)). Smoking status approached significance in its correlation with high CP.p53 composite score, in that 3 of 7 (43%) never-smokers had a score >1, whereas only 3 of 14 former and none of the 11 current did (\( P = 0.06 \)).

CP, p53 protein expression and mutations, and LOH were not significantly different by histology. The median percentage of cells carrying more than three copies was 3.05 (range 0.3–16.7%) in mild, 3.45 (range 0.5–23%) in moderate, and 5.7 (range 0–20.8%) in severe dysplasia (\( P = 0.7 \), Kruskal-Wallis test). p53 PB LI was 0.07 (range 0–0.24) in mild, 0.37 (range 0–0.94) in moderate, and 0.13 (0–0.98) in severe dysplasia (\( P = 0.36 \)). p53 mutations were detected in none of 4 mild...
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Dysplasia cases, 3 (19%) of 16 moderate dysplasia cases, and 3 (25%) of 12 severe dysplasia cases (P = 0.3; Cochran-Armitage trend test). LOH was observed in 2 (50%) of 4 mild dysplasia cases, 11 (69%) of 16 moderate dysplasia cases, and 11 (92%) of 12 severe dysplasia cases (P = 0.06 trend test).

The median LI for Ki-67 in the basal layer was 0.64 in mild, 0.37 in moderate, and 0.44 in severe dysplasia (P = 0.22; Kruskal-Wallis test), whereas in the PB layer, the respective LI 0.25 for mild, 0.12 for moderate, and 0.06 for severe dysplasia were statistically significant (P = 0.02; Kruskal-Wallis test).

Correlations between Different Biomarkers. There was no correlation between presence of LOH and high p53 LI in the PB layer or between LOH and presence of p53 mutations, although LOH tended to occur more frequently in lesions harboring p53 mutations (6 of 6 versus 18 of 26; P = 0.3). There was also a trend for correlation between high CP and presence of LOH (10 of 12 versus 8 of 14; P = 0.21). High CP was not correlated with p53 mutations (2 of 6 versus 10 of 20) or with high p53 PB LI. Among the cases with p53 gene mutations, three had high p53 protein expression in the baseline biopsy as defined by a PB LI ≥ 0.2, and three did not (two of these demonstrated complete lack of p53 expression); therefore, there was no direct association between p53 expression and protein mutations.

Impact of Pretreatment Biomarkers on Response. To simplify our analysis, best response to treatment was analyzed for each case in correlation with clinical pathological parameters and biomarker expression. Gender, smoking status, and degree of dysplasia had no significant impact on response to biochemoprevention. Three of 4 cases with mild dysplasia, 5 of 16 with moderate, and 4 of 12 with severe dysplasia achieved CR (P = 0.27; Cochran-Armitage test). Only site (larynx) was a predictor of favorable response; 11 of 20 cases with vocal cord lesions achieved a CR, whereas only 1 of 12 cases with p.o. cavity lesions did (P = 0.01; Fisher’s exact test).

No difference in the median pretreatment Ki-67 LI was seen between responders and nonresponders to the intervention, and as such, it was not further analyzed as a predictor of long-term outcome.

Patients with low CP achieved CR in 8 of 14 cases (57%), whereas only 1 of 12 (8%) patients with high CP did (P = 0.01). All of the other biomarkers, including p53 PB LI, p53 mutation, p53 composite score (p53 PB LI and p53 mutations), and LOH were not statistically significant predictors of response, although the trend was in the right direction for p53 PB LI (P = 0.29) and LOH (P = 0.12). A low CP,p53 composite score (cutoff 1.5) predicted for CR (12 of 26 cases with low, versus 0 of 6 with high score, P = 0.06), and so did a low CP,p53,LOH score (cutoff 1.5; CR in 8 of 13 cases with low score versus 4 of 19 with high score, P = 0.03; Fisher’s exact test). These results are summarized in Table 2.

Table 3 Biomarker modulation with treatment in correlation with response

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Larynx (n = 19)</th>
<th>p.o. cavity (n = 12)</th>
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<tbody>
<tr>
<td>CR</td>
<td>Non-CR</td>
<td>Total</td>
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<tr>
<td>p53 PB LI</td>
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<tr>
<td>↓ or low</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>↑ or high</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>CP</td>
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<td>↓ or low</td>
<td>5</td>
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<td>↑ or high</td>
<td>0</td>
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<tr>
<td>Ki-67 PB LI*</td>
<td></td>
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<tr>
<td>↓ or low</td>
<td>2</td>
<td>0</td>
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<tr>
<td>↑ or high</td>
<td>5</td>
<td>12</td>
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* Maximum Ki-67 PB LI taken into consideration and 0.2 cutoff used.

Biomarker Changes in Correlation with Response. Cp and p53 PB LI were overall lower at baseline in laryngeal lesions and higher in p.o. cavity lesions, and responses were mainly observed in laryngeal lesions. Low or decreasing biomarker was defined as either persistently low expression or decrease below cutoff in the last biopsy obtained, and conversely high or increasing biomarker was defined as persistently high or increasing above the cutoff value. Of 13 laryngeal lesions with low or decreasing p53 PB LI, 7 achieved CR, and of the 6 lesions with high or increasing p53 PB LI over time, none did (P = 0.04; Table 3). None of the six p.o. cavity cases with high or increasing-over-time p53 PB LI responded. A similar trend was found in analyzing CP, but because of the small number of available data, the result was not statistically significant (P = 0.1). Ki-67 PB LI decrease or low expression was associated with response to treatment in 2 of 2 laryngeal cases and 1 of 2 p.o. cavity cases, whereas high or increasing expression was associated with CR in 5 of 17 laryngeal cases and 0 of the 10 p.o. cavity cases (Table 3). Overall, low-risk or improved biomarker values correlated with response at least in laryngeal sites, but this correlation was only significant for p53 PB LI.

Biomarkers as Predictors of Cancer Development. With long-term follow-up (median 30 months), 14 cases developed either PD or cancer (six of the cancers occurred within the period of intervention). Neither sex, nor histology (2 of 4, 8 of 16, and 4 of 12, respectively, for mild, moderate, and severe dysplasia) predicted for adverse outcome. Never-smokers fared the poorest in terms of adverse outcome development, but the trend was not significant (P = 0.18; Log-rank test; Fig. 1A).

The only significant clinical parameter affecting long-term outcome was the site of the original lesions; cases with laryngeal lesions had significantly longer time to progression cancer development than cases with p.o. cavity lesions (P = 0.04; Log-rank test; Fig. 1B). High CP cases had a shorter time to
progression cancer development, but the difference with the low CP group was not significant (8 of 12 cases versus 5 of 14 with low CP, P = 0.3; Log-rank test; Fig. 1C). High p53 PB LI was also associated with worse outcome (8 of 15 cases versus 6 of 17 with low p53 PB LI, P = 0.34; Log-rank test), whereas p53 mutation had no impact (2 of 6 with p53 mutations versus 12 of 26 without, P = 0.65; Log-rank test). LOH at baseline was associated with better outcome (adverse outcome in only 9 of 24 cases with LOH versus 5 of 8 cases without LOH, P = 0.16). A high CP.p53 score (cutoff 1.5; Fig. 1D) was associated with adverse outcome, but the trend was not significant (0.36, Log-rank test).

Multivariate analyses using the Cox model indicate that the site is the only independent predictor of progression-free survival among clinical and histological parameters and biomarkers examined in this study.

Discussion
On the basis of the promising results obtained in dysplastic laryngeal lesions with the biochemoprevention combination and the relatively disappointing results for p.o. lesions harboring the same histological characteristics (6), we studied the effect of phenotypic and genotypic alterations on chemoprevention outcome and eventual malignant transformation. The choice of these alterations was based on previous findings from earlier studies in p.o. premalignant lesions (9–13).

In our study, 14 of 32 cases (43.7%) developed PD to cancer in 13 of the cases (40.6%) with a median follow-up of 30 months. Although histology and smoking have been considered important determinants of outcome, none of the two appear to be significant in our study. Histology distribution was similar between the two groups of laryngeal and p.o. cavity lesions, but there was a higher prevalence of never-smokers in the p.o. cavity group. A possible explanation for the lack of correlation of histological severity with biomarker abnormalities in our study is that the spectrum of histologies examined was narrow (from mild to severe dysplasia). However, this also emphasizes the need to identify biomarkers that could represent better surrogates for prediction of outcome within this group of premalignant lesions.

In accordance with the knowledge that aneuploidy denoting accumulation of genetic damage and genetic instability (17) is crucial for carcinogenesis, and the previous findings of adverse prognostic value for high CP (8), in the current study, low CP was significantly associated with favorable response to the intervention, and cases with high CP developed PD cancer more frequently. Therefore, CP is a useful predictor of response to retinoid intervention. This is also in line with previous observations that chromosome instability (aneuploidy and CP) could be used as an indicator of malignant progression in laryngeal mucosa (18) and p.o. cavity (19). It is possible that with a larger sample or with longer follow-up, CP could also prove to be a predictor of malignant transformation in the current study. The choice of chromosome 9 was based on the knowledge from our previous studies that generalized CP can be detected with any chromosome probe (15).

Presence of LOH did not independently predict for lack of response to chemoprevention; however, there were more responders in the group with retention compared with the group with LOH. We have shown previously persistence of these alterations in biopsy specimens despite histological response (20). Presence of LOH at any of the three loci studied appeared to be associated with better long-term outcome (in terms of PD and cancer development), in contrast to our previous report in early premalignant lesions (13). It is possible that the small sample size accounts for these contradictory findings.

Baseline p53 protein expression or gene alterations were not predictors of response or adverse outcome in this study, as opposed to previous studies in p.o. premalignancy where high p53 protein levels were associated with resistance to 13-cis-
retinoic acid intervention (9, 10) and resistance to the current combination of 13-cis-retinoic acid and IFN-α (21), as well as a higher incidence of cancer development. We observed, however, a nonsignificant trend in the same direction for response prediction, because only 4 of 15 patients with high p53 expression (LR = 0.2) achieved CR versus 8 of 17 with low p53, and in the subgroup of laryngeal dysplasia, low or decreasing p53 PB LI was associated with CR (P = 0.04). When responses were compared in the high p53 subgroup with our previous p.o. premalignancy study, 7 (47%) of 15 lesions with high p53 achieved CR or PR in the current study as compared with 2 (18%) of 11 lesions in our previous study (9), possibly reflecting the positive outcome of laryngeal lesions. High p53 did not predict for cancer development (8 of 15 cases with high p53 developed cancer or PD versus 6 of 17 cases with low p53), possibly because in these advanced lesions, p53 alterations are not the main determinant of outcome.

Surprisingly, smoking status revealed an unexpected trend in our sample. Never-smokers appeared to do worse both in terms of response and long-term outcome, followed by former smokers. This points to the possibility that lesions that arise in individuals without the traditional carcinogenic risk factors or with long-term abstinence from carcinogen exposure harbor advanced degrees of biological abnormality that is not reversible and have a higher risk for resistance to chemopreventive intervention and cancer development.

In conclusion, high CP independently and the combination of CP and p53 altered expression appear to have an impact on response to chemoprevention. None of these abnormalities nor their combination, in contrast to previous reports in p.o. leukoplakia and laryngeal premalignancy (12, 18, 19), was an independent strong predictor of malignant transformation, possibly attributable to small sample size. Therefore, we propose continued study of genetic and phenotypic alterations, such as p16 (22), epidermal growth factor receptor, cyclin D1 (23, 24), and others specifically involved in pathways of carcinogenic progression to identify and validate biomarkers that could serve as surrogate end points. The identification of such biomarkers should substantially enhance the conduct of short-term chemoprevention trials with new targeted agents by revealing early responses based on their modulation or by revealing patterns of resistance, long before the ultimate outcome of decrease in cancer incidence can be proven.

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