A Pilot Surrogate Endpoint Biomarker Study of Celecoxib in Oral Premalignant Lesions

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Abstract This study evaluated changes in prostaglandin E2 (PGE2) levels and related biomarkers in oral premalignant lesions (OPL) in response to celecoxib treatment. Twenty-two subjects were enrolled and treated with celecoxib. Pretreatment and 12-week biopsies were done. Subjects whose biopsy showed ≥30% decrease in PGE2 remained on celecoxib for a total of 12 months when repeat biopsy was done. Biopsies were examined to assess degree of dysplasia, DNA ploidy, and immunohistochemical expression of BCL2, pAKT-Ser473, Ki-67, and CD31 (microvessel density). In 18 paired biopsies available at baseline and 12 weeks, mean normalized PGE2 levels decreased by 38% (P = 0.002). After 12 months, PGE2 decreased by 31% (P = 0.340). Twelve biopsies (67%; P = 0.0129) showed improvement in degree of dysplasia after 12 weeks, and 8 of 11 biopsies (73%; P = 0.0703) continued to show an improvement in the degree of dysplasia after 12 months. Trends suggested down-modulation of cyclooxygenase-2 and Ki-67 in some tissues, increased pAKT-Ser473 expression, and an inverse relationship between PGE2 and BCL2 expression. This study documents the feasibility of measuring potential surrogate endpoint biomarkers of chemopreventive agent response in OPLs. Treatment with celecoxib in subjects with OPLs favorably modulates the primary mediator of cyclooxygenase-2 activity, PGE2, after 12 weeks.

Oral premalignant lesions (OPL), including leukoplakia and erythroplakia, indicate a high risk of malignancy. As many as 15% to 36% of patients with OPLs will develop squamous cell carcinoma of the head and neck (SCCHN) over 5 to 10 years (1–3). For those who do develop SCCHN, the rate of 5-year survival approximates 60%, and optimizing current treatment has produced an improvement in survival of only 3% over the past 25 years (4, 5). Ideally, successful identification and treatment of high-risk OPLs would prevent a significant number of deaths and morbidity from SCCHN. When limited in size and number, OPLs can be readily detected by oral cavity examination and treated by excision. Unfortunately, these lesions typically arise in a background of field cancerization and are therefore subject to multifocal recurrence, limiting the ability of surgery to address the underlying problem (3, 6–11). Tobacco cessation can reduce the risk of SCCHN; however, treatments for tobacco addiction are not always successful, and former smokers remain at risk for cancer long after quitting (12, 13).

Given the limitations of local therapy for OPLs, chemoprevention is an appealing notion. In patients with a history of SCCHN, chemoprevention with high-dose 13-cis-retinoic acid reduced the recurrence rate of OPLs and decreased the risk of second primary tumors (14). Retinoids have not become standard therapy, however, because effective doses are not well tolerated over a meaningful period of time (14–16). No other systemic agents, including vitamin A and β-carotene, have shown both tolerability and efficacy in reducing the risk of cancer in patients with OPLs (17, 18).

The study of SCCHN carcinogenesis has identified therapeutic targets for cancer treatment and prevention that are now under investigation in the treatment setting (19). One promising prognostic and therapeutic target is cyclooxygenase-2 (COX-2), an enzyme responsible for elevated prostaglandin levels in chronic inflammation and cancer (20, 21). COX-2 is expressed in the majority of human epithelial tumors (22) and preclinical studies show that COX-2–associated prostaglandins suppress tumor cell apoptosis, invasion, and angiogenesis and also activate epidermal growth factor receptor (23–26). A direct role for COX-2 in tumor promotion was shown in studies showing reduced spontaneous intestinal tumor formation in a murine model by targeted deletion of the COX-2 gene (27). Human chemoprevention clinical trials confirmed the effectiveness of selective COX-2 inhibitors...
against colorectal neoplasia (28–31). Unfortunately, recent placebo-controlled trials in patients with sporadic colorectal adenomas also found that use of selective COX-2 inhibitors was associated with a small but significant increase in the incidence of cardiovascular complications (32, 33). These results illustrate the importance of balancing the risks and benefits of chemoprevention regimens to achieve optimal disease prevention.

Developing safe and effective chemoprevention requires an understanding of the natural history of the premalignant condition so that treatment is focused on individuals at highest cancer risk. There is presently no reliable marker that can differentiate those who will be diagnosed with a future SCCHN from those with more innocent lesions. Another challenge is the substantial length of time involved in progression of premalignant lesions to invasive malignancy. Consequently, definitive phase III cancer prevention trials take many years, many subjects, and substantial financial resources. One strategy to facilitate progress is to use surrogate endpoint biomarkers in smaller-scale studies to identify agents with potential chemopreventive activity (34).

We undertook a pilot study of patients with OPLs to determine whether treatment with the selective COX-2 inhibitor celecoxib decreased intralesional levels of prostaglandin E$_2$ (PGE$_2$). Our goal was to document drug-associated reduction in PGE$_2$ levels, indicating in vivo activity against the primary target of celecoxib activity. We also evaluated the effect of celecoxib on a panel of disease progression markers, including degree of dysplasia, BCL2, phosphorylated AKT (pAKT-Ser473), Ki-67, and microvessel density by CD31. The overall purpose of this study was to assess the feasibility of measuring these biomarkers in patients at high risk for SCCHN, anticipating that the data would be used to design future chemoprevention clinical trials.

Material and Methods

Study design

Patients with OPLs referred to the Dana-Farber Cancer Institute Head and Neck Oncology Program were enrolled in an open-label study of orally administered celecoxib (400 mg bd). The presence of OPL with dysplasia was confirmed by pretreatment tissue biopsy. Other enrollment criteria included adequate bone marrow, renal and liver function, Eastern Cooperative Oncology Group performance status ≤2, life expectancy ≥12 months, no prior SCCHN within ≥9 months, and no significant comorbid illness, such as active coronary artery disease, congestive heart failure, severe chronic obstructive pulmonary disease, other malignancy, bleeding diathesis, or history of gastrointestinal ulcer. Tobacco users were required to have quit tobacco ≥1 month before enrollment. This requirement resulted in enriching the cohort for those who were never smokers but was necessary because all of our patients undergo rigorous tobacco cessation programs, and it is likely that tobacco cessation during the study would be a significant confounder for the biomarker measurements. Although not precluded by entry criteria, there were no users of smokeless tobacco enrolled. Alcohol consumption was strongly discouraged. Subjects were required to refrain from use of nonsteroidal anti-inflammatory drugs for the duration of the study, except for ≤81 mg daily aspirin.

The protocol was approved by the Dana-Farber Office for the Protection of Research Subjects and all subjects provided written informed consent.

Biopsies of the OPLs were obtained at baseline and after 12 weeks of celecoxib use. In a subset of subjects (44%), a single dominant lesion was identified. When this was the case, the maximal diameter of this target lesion was recorded, and a threshold of 30% was chosen to approximate the definition of partial response by Response Evaluation Criteria in Solid Tumors (i.e., ≥30% decrease in the sum of the maximal diameter of the target lesion). Subjects whose biopsy results at 12 weeks showed ≥30% decrease in PGE$_2$ levels compared with baseline remained on celecoxib to complete a 12-month treatment period, at which time repeat biopsy was done. Because of possible clinical benefit, subjects who experienced a partial response by Response Evaluation Criteria in Solid Tumors also remained on celecoxib to complete a 12-month treatment period. Clinical response, however, was not an endpoint of this feasibility study. Clinical examinations were done every 3 months until the completion of protocol therapy and for an additional 12 months thereafter. This clinical trial was halted prematurely when data from other cancer prevention studies revealed an increased risk of cardiovascular events in patients taking COX-2 inhibitors compared with placebo (32, 33).

The primary outcome was reduction in PGE$_2$ levels in OPL biopsy specimens after 12 weeks of celecoxib use. Secondary assessments included change in tissue dysplasia and expression of COX-2, BCL2, Ki-67, and pAKT-Ser473 in OPL biopsies after 12 weeks of celecoxib use. Pretreatment and posttreatment microvessel density was assessed by CD31 immunohistochemistry, and DNA ploidy was determined by flow cytometry. Dysplasia and marker expression was also determined after 12 months of treatment. We hypothesized that celecoxib would reduce intralesional levels of PGE$_2$ and that antitumor activity of celecoxib would be associated with decreased dysplasia, reduction in COX-2, Ki-67, and pAKT-Ser473 expression, and increased levels of BCL2. DNA ploidy was evaluated as an exploratory endpoint in the subset of patients for whom there was sufficient material remaining after analysis of the primary and secondary biomarkers. Finally, we predicted that the chemopreventive activities of celecoxib observed after 12 weeks of treatment would still be present after 12 months of continuous celecoxib treatment.

Analysis of biopsy specimens

OPL biopsies were done at baseline, after 12 weeks of celecoxib use, and on completion of study drug if treatment continued after 12 weeks. The technique used was punch biopsy (3 or 4 mm), unless another technique was considered more appropriate by the surgeon doing the procedure. Biopsies were done after local anesthesia with 2% lidocaine with epinephrine. Two adjacent specimens were obtained at each research-associated biopsy. Biopsies done at the two later time points were done by the surgeon who did the baseline procedure in the same lesion and location as the baseline biopsy. If there was no residual OPL on subsequent biopsy, the procedure was done in the same anatomic location, recorded in the biopsy procedure notes. One specimen was placed in formalin overnight (8-24 h) and then paraffin embedded for histologic analysis, and the other was immediately frozen at...
Levels of PGE2 and expression of COX-2, BCL2, pAKT, Ki-67, and CD31

PGE2 enzyme immunoassay monoclonal kits and reagents were purchased from Cayman Chemical Co., indomethacin was obtained from Sigma-Aldrich, and the MicroBCA Protein Determination kit was purchased from Pierce. Samples were lysed in 1 mL of lysis buffer [50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 4 mmol/L EDTA, 1% Triton X-100, 0.1% SDS, 10 μmol/L indomethacin], each specimen was homogenized for 50 strokes, and lysates were then spun at 1,500 × g to precipitate any unlysed cells. Supernatant was removed and aliquots were taken for a protein concentration determination. Lysates were purified for 1 h with PGE2 affinity sorbent beads. Purified samples were diluted with enzyme immunoassay buffer at 1:8, 1:12, and 1:16, incubated overnight at 4°C, developed according to the manufacturer’s protocol, and read at 410 nm using SoftMax Pro software.

Immunohistochemistry was done using Envision Plus/HRP secondary antibody kit and mouse monoclonal antibodies to Ki-67 (clone MIB-1; 1:200; DAKOCytomation), CD31 (clone JC70A; 1:40; DAKOCytomation), pAKT-Ser473 (phosphorylated AKT (Ser473) antibody; Cell Signaling), and BCL2 (clone 124; 1:30; DAKOCytomation). Antigen retrieval was done by 20-min protease digestion for the CD31 antibody and by heating slides immersed in 10 mmol/L citrate buffer (pH 6.0) in a pressure cooker for 2 min for BCL2, MIB-1, COX-2, and pAKT-Ser473. Appropriate positive and negative controls, using normal mouse serum, were stained in parallel. Ki-67 antibody was evaluated for nuclear staining, whereas CD31 antibody was assessed for cytoplasmic reactivity. A semiquantitative scoring system was used to grade intensity of staining: COX-2, BCL2, and pAKT-Ser473 were scored from 0 to 2 (0 corresponds to no staining, 1 to focal immunoreactivity, and 2 to extensive immunoreactivity), Ki-67 was scored from 1 to 4 (1 corresponds to the normal pattern of expression, with 2, 3, and 4 being slight, moderate, and extensive increases in immunoreactivity), and CD31 was measured as the average microvessel density per three high-power fields counted.

Cellular DNA content by flow analysis

DNA ploidy status was evaluated using three sections (50 μm thickness) of formalin-fixed, paraffin-embedded tissue by EdU flow cytometry technique (35, 36). Briefly, tissues were deparaffinized, rehydrated, and pepsin digested, then stained with propidium iodide, and analyzed on a FACSCalibur flow cytometer (Becton Dickinson). Normal oral mucosa cells served as internal diploid control cells. Histograms were generated by analysis of 2,500 to 10,000 nuclei and displayed as linear fluorescence. The DNA index was calculated as the ratio of the abnormal population G0-G1 peak channel to that of normal oral mucosa cells. A DNA index of 1.00 was assigned if only a single G0-G1 peak was identified.

Statistical analysis

The primary endpoint of this study was change in PGE2 levels from the pretreatment biopsy specimen to that taken at 12 weeks. Secondary endpoints included change in PGE2 levels from the pretreatment biopsy specimen to that taken at 12 months in those subjects completing 12 months of celecoxib. Change in size of OPLs was not considered as a study endpoint because of difficulty in measuring change in size. Many subjects presented with small lesions that, after two 4-mm punch biopsies, were no longer measurable, whereas other subjects presented with diffuse, heterogeneous OPLs throughout the oral cavity that could not be measured accurately on repeat visits.

The PGE2 data were log transformed to achieve approximate normality. The log-transformed data were used throughout the data analysis. Paired t tests were done to compare PGE2 levels at baseline and 12 weeks, baseline and 12 months, and 12 weeks and 12 months. The Bonferroni procedure was adopted to control for multiple comparisons given the three tests done simultaneously. That is, a test would be declared significant only if the nominal P value is less than 0.05/3 = 0.017, which controls the overall type I error at the

Table 1. Subject characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, median (range)</td>
<td>57 (42–77)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>8 (36%)</td>
</tr>
<tr>
<td>Female</td>
<td>14 (64%)</td>
</tr>
<tr>
<td>Ethnicity</td>
<td></td>
</tr>
<tr>
<td>Non-Hispanic white</td>
<td>18 (82%)</td>
</tr>
<tr>
<td>Non-Hispanic black</td>
<td>2 (9%)</td>
</tr>
<tr>
<td>Hispanic</td>
<td>2 (9%)</td>
</tr>
<tr>
<td>Baseline histology</td>
<td></td>
</tr>
<tr>
<td>Mild dysplasia</td>
<td>11 (50%)</td>
</tr>
<tr>
<td>Mild-moderate dysplasia</td>
<td>2 (9%)</td>
</tr>
<tr>
<td>Moderate dysplasia</td>
<td>2 (9%)</td>
</tr>
<tr>
<td>Moderate-severe dysplasia</td>
<td>1 (5%)</td>
</tr>
<tr>
<td>Severe dysplasia</td>
<td>1 (5%)</td>
</tr>
<tr>
<td>CIS</td>
<td>5 (23%)</td>
</tr>
<tr>
<td>Smoking history</td>
<td></td>
</tr>
<tr>
<td>Former smoker</td>
<td>12 (55%)</td>
</tr>
<tr>
<td>Never smoker</td>
<td>10 (45%)</td>
</tr>
<tr>
<td>Median number pack-years</td>
<td>23</td>
</tr>
<tr>
<td>Alcohol history</td>
<td></td>
</tr>
<tr>
<td>Former alcohol user (any amount)</td>
<td>2 (9%)</td>
</tr>
<tr>
<td>Current alcohol user (any amount)</td>
<td>17 (77%)</td>
</tr>
<tr>
<td>Never used alcohol</td>
<td>3 (14%)</td>
</tr>
</tbody>
</table>
A series of linear mixed models, which account for within-individual correlations, were run to examine the relationship between PGE2 levels and the covariates (degree of dysplasia, COX-2, BCL2, pAKT-Ser473, Ki-67, and CD31) over time. Statistics were analyzed using Statistical Analysis System software, version 9. Descriptive statistics were used to characterize study subjects, including age, gender, and smoking history.

**Results**

**Study population**

The characteristics of the study population are listed in Table 1. A total of 22 subjects were enrolled, successfully completed baseline biopsies, and began celecoxib treatment (see Fig. 1). Four subjects withdrew from the study before completing 12 weeks of treatment. One subject was removed from study due to grade 2 allergic skin eruption attributed to celecoxib and three subjects withdrew for personal reasons. All 18 subjects who were available for evaluation at 12 weeks completed their second biopsy. Of these, two subjects were withdrawn from study treatment: one due to an increased PGE2 level and one who was found to have CIS for which surgery was indicated. Of the 16 patients who continued celecoxib treatment beyond 12 weeks, 5 did not complete the additional 9 months of therapy. Of these, one subject was withdrawn due to the development of grade 3 dyspepsia. The other four subjects discontinued study treatment at 5 to 9 months when the study was closed early because results from other clinical trials indicated an increased risk of cardiovascular events in patients using 400 mg bd celecoxib. A total of 11 subjects completed 12 months of study treatment and underwent a third biopsy. A total of three adverse events occurred in the 22 patients who received at least one dose of celecoxib (13.6%). Two of these, involving a grade 2 skin reaction and grade 3 dyspepsia, have been described above. The third was development of CIS in a patient who had mild dysplasia at baseline. All three patients were removed from the study on discovery of the adverse event.

**Modulation of PGE2 levels in OPLs treated with celecoxib**

Eighteen paired biopsies were available for evaluation of PGE2 levels at baseline and after 12 weeks of celecoxib treatment. At this first assessment, mean normalized PGE2 levels declined from 77.6 pg/3 mg total protein to 48.4 pg/3 mg total protein (mean decrease of 38%; \( P = 0.002 \)). A \( \geq 30\% \) decrease in PGE2 levels was seen in 11 subjects (61%). Five subjects (28%) showed a decrease in mean normalized PGE2 of <30% (range, 7-22%), and two subjects (11%) showed an increase of 21% and 82% (see Fig. 2). Of the 11 subjects who completed a total of 12 months of treatment, normalized PGE2 levels decreased to 53.5 pg/3 mg total protein (mean decrease of 31% compared with baseline; \( P = 0.340 \)). Between 3 and 12 months, PGE2 increased from 48.4 to 53.5 pg/3 mg total protein (mean increase of 11%; \( P = 0.179 \)). Five of the 11 subjects on celecoxib for 12 months (46%) experienced a sustained decrease in mean normalized PGE2 of \( \geq 30\% \) compared with baseline. One
subject with a PGE$_2$ decrease of only 8% at 12 weeks, who re-
mained on celecoxib because of a partial response by Re-
ponse Evaluation Criteria in Solid Tumors in the target
lesion, showed a 73% decrease in mean normalized PGE$_2$ at
12 months. Four subjects experienced an increase in mean nor-
malized PGE$_2$ levels after 12 months of celecoxib, and one sub-
ject who initially experienced an 86% reduction had a 15%
increase in mean normalized PGE$_2$ at 12 months.

**Tissue histology and immunohistochemistry**

The degree of dysplasia present in OPL biopsies was
scored from 0 (i.e., no dysplasia) to 6 (i.e., CIS). Of the 18
subjects with paired biopsies at baseline and after 12 weeks
of treatment, 12 (67%) showed improvement in the degree of
dysplasia, 3 (17%) showed no change, and 2 (11%) showed
more severe dysplasia than that found at baseline (Table 2;
Fig. 3). After 12 months of treatment, 8 of 11 biopsies (73%)
continued to show an improvement in the degree of dyspla-
sia compared with baseline, 2 (18%) showed no change, and
1 (10%) showed more severe dysplasia. Seven patients had
biopsies indicating CIS at some point during the study. Five
patients (23%) showed CIS in their baseline biopsy. All five
of these patients had undergone multiple prior OPL exci-
sions, had the gross OPL excised in the baseline biopsy,
but had dysplasia present at the margins. Of these, four
showed a decrease in severity during celecoxib treatment.
The other patient continued to have CIS at the 12-week
biopsy and was referred for surgery (Fig. 3A). Two patients
developed CIS while on the study. One of these had grade 5
dysplasia on baseline biopsy, no dysplasia found at 12
weeks, and CIS at 12 months. The second patient had mild
dysplasia at baseline, but CIS was diagnosed at the
12-week biopsy. Because of this, this patient was withdrawn
from the study after 12 weeks of treatment.

Mean normalized PGE$_2$ levels were compared with the
numerical grade of OPL dysplasia. At baseline, lesions with
a greater degree of dysplasia tended to have higher PGE$_2$
levels such that for every unit increase in dysplasia the
PGE$_2$ level was increased by 17% [95% confidence interval
(95% CI), 6-31%]. This relationship diminished over time. At
12 weeks of treatment, PGE$_2$ was increased by 3% (95% CI,
−6% to 14%) for every unit increase in dysplasia. At 12
months, an inverse of this relationship was seen; every unit
increase in dysplasia was associated with 31% less PGE$_2$
(95% CI, −49% to −6%). Table 3 describes the linear mixed
models used to determine these relationships (e.g., formula-
tion, regression coefficients, and SEs). When change in his-
tology was analyzed in relation to PGE$_2$ modulation, no
significant correlation was found (i.e., the “histologic re-
spenders” did not have a greater degree of improvement
in PGE$_2$ levels compared with “histologic nonresponders”).
There were no other statistically significant correlations be-
tween lesion histology and other biomarker measurements
found.

Changes in the OPL expression of COX-2, BCL2, pAKT-
Ser473, and Ki-67 by immunohistochemistry are reported in
Table 2 and represented in Fig. 4. To summarize, after 12
weeks of celecoxib, COX-2 expression was down-modulated
in 46% of biopsies ($P = 0.0312$), and this trend persisted at
12 months, with 57% of biopsies showing less COX-2 expres-
sion than present at baseline ($P = 0.375$). Ki-67 was similarly
down-modulated, with 69% of biopsies showing lessKi-67 ex-
pression at 12 weeks compared with baseline ($P = 0.0215$)
and 71% showing less Ki-67 at 12 months ($P = 0.0625$). On the
other hand, a trend of increasing pAKT-Ser473 expression
was seen over time, with 54% of biopsies showing an increase
at 12 weeks ($P = 0.0703$) and 57% showing an increase at 12
months ($P = 0.125$). No consistent trend in change in BCL2 ex-
pression was seen. Average microvessel density per three
high-power fields measured by CD31 immunohistochemistry
is reported in Table 4. No significant change in microvessel
density was observed.

**Table 2. Change in markers over time**

<table>
<thead>
<tr>
<th>Marker</th>
<th>Change from baseline to 12 wks</th>
<th>Change from baseline to 12 mo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% increased</td>
<td>% decreased</td>
</tr>
<tr>
<td>History</td>
<td>11</td>
<td>67</td>
</tr>
<tr>
<td>COX-2</td>
<td>0</td>
<td>46</td>
</tr>
<tr>
<td>BCL2</td>
<td>33</td>
<td>25</td>
</tr>
<tr>
<td>pAKT</td>
<td>54</td>
<td>8</td>
</tr>
<tr>
<td>Ki-67</td>
<td>8</td>
<td>69</td>
</tr>
</tbody>
</table>
The relationships between PGE2 levels over time and expression of COX-2, BCL2, pAKT-Ser473, and Ki-67 were also examined. A positive correlation was seen between COX-2 and normalized PGE2 levels. For each unit increase in the score for COX-2 expression, PGE2 was increased by 110% (95% CI, 50-192%). In keeping with its role as an antiapoptotic protein, BCL2 expression was inversely related to PGE2 level. For each unit increase in BCL2, PGE2 changed by $-60\%$ (95% CI, $-55\%$ to $-65\%$). pAKT-Ser473 and Ki-67 levels were not associated with statistically significant changes in

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![Fig. 3. Change in lesion histology over time. Bottom, representative lesions stained with H&E at pretreatment and posttreatment time points.](image)

### Table 3. Linear mixed models used to determine marker relationships

<table>
<thead>
<tr>
<th>Covariate</th>
<th>Variable estimate</th>
<th>SE</th>
<th>Model used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time</td>
<td>$-0.12$</td>
<td>$0.70$</td>
<td>$\ln(\text{PGE2}) = 3.80 - 0.12 \cdot \text{Time} + 0.014 \cdot \text{Time}^2 + 0.16 \cdot \text{Dysplasia} - 0.044 \cdot \text{Dysplasia} \cdot \text{Time}$</td>
</tr>
<tr>
<td>Time$^2$</td>
<td>$0.014$</td>
<td>$0.0058$</td>
<td></td>
</tr>
<tr>
<td>OPL dysplasia</td>
<td>$0.16$</td>
<td>$0.050$</td>
<td></td>
</tr>
<tr>
<td>OPL dysplasia-time</td>
<td>$-0.044$</td>
<td>$0.013$</td>
<td></td>
</tr>
<tr>
<td>COX-2</td>
<td>$0.74$</td>
<td>$0.16$</td>
<td>$\ln(\text{PGE2}) = 3.12 + 0.74 \cdot \text{COX-2}$</td>
</tr>
<tr>
<td>BCL2</td>
<td>$-0.51$</td>
<td>$0.039$</td>
<td>$\ln(\text{PGE2}) = 4.48 - 0.51 \cdot \text{BCL2}$</td>
</tr>
<tr>
<td>pAKT</td>
<td>$0.35$</td>
<td>$0.20$</td>
<td>$\ln(\text{PGE2}) = 3.74 + 0.35 \cdot \text{pAKT}$</td>
</tr>
</tbody>
</table>
PGE₂, although there was a trend in correlation between pAKT-Ser473 and PGE₂, where each unit increase in pAKT-Ser473 score was associated with a 42% increase in normalized PGE₂ (95% CI, −8% to 116%). Microvessel density was not significantly related to PGE₂ levels. There were no statistically significant differences in marker measurement or modulation found between subjects with and without a history of tobacco use.

Fig. 4. Marker expression by immunohistochemistry in one patient at baseline and after 12 wks of celecoxib: baseline and 12-wk COX-2 (A₁ and A₂), BCL2 (B₁ and B₂), pAKT-Ser473 (C₁ and C₂), Ki-67 (D₁ and D₂), and CD31 (E₁ and E₂).
**Clinical response**

Clinical response was evaluable by Response Evaluation Criteria in Solid Tumors in only three subjects, as many subjects had lesions that were subcentimeter or less following baseline biopsy or had diffuse multifocal disease throughout the oral cavity. These three subjects all had stable lesions at 12 weeks. None of these continued celecoxib beyond 12 weeks. Five more subjects had a dominant OPL <1 cm following baseline biopsy. Two of these small lesions resolved by 12 weeks. One of these subjects completed 12 months of celecoxib and did not experience a recurrence, and one subject did not continue celecoxib beyond 12 weeks.

**DNA ploidy and correlation with PGE2 levels**

To explore the feasibility of measuring DNA ploidy in OPL biopsies, flow cytometry was done on a limited number of samples. The DNA index was measured from 12 biopsies, with histologies ranging from mild dysplasia to CIS. DNA content was successfully determined in 10 samples, with two biopsies yielding too few cells for analysis. Seven biopsies showed normal DNA content, two were near triploid, with DNA indices of 1.50 (Fig. 5) and 1.9, and one was near tetraploid, with a DNA index of 2.15. The mean normalized PGE2 levels in the specimens with normal versus abnormal DNA content were 70.4 and 70.8 pg/3 mg total protein, respectively (P = 1.0). Two of the three cases of abnormal DNA content were seen in biopsies with mild dysplasia, and the third biopsy showed mild-moderate dysplasia.

**Discussion**

Abundant and compelling data show that COX-2 activity contributes to epithelial carcinogenesis by modulating key aspects of this process, including proliferation, apoptosis, angiogenesis, invasion, metastasis, and immune response (19). COX-2 is induced by proinflammatory and mitogenic stimuli, and its expression in the oral cavity has been associated with tobacco use (37, 38). In the oral cavity, as in other epithelial tumors, COX-2 is overexpressed at all stages of tumorigenesis and is predictive of prognosis (39–43). Consequently, COX-2 is an attractive therapeutic target for prevention of head and neck cancer.

COX-2 inhibitors, including aspirin and other nonspecific nonsteroidal anti-inflammatory drugs, have been studied extensively for prevention of other epithelial malignancies, including colorectal, breast, and lung cancers. Studies of colorectal cancer provide unequivocal support for the chemopreventive benefits of these agents (28, 33, 44, 45). For example, an ~30% level of regression of existing disease, in the form of premalignant colorectal adenomas, was reported in patients with familial adenomatous polyposis treated with 6 months of celecoxib at 400 mg bd compared with placebo (28). In addition, celecoxib prevented the recurrence of sporadic colorectal adenomas, as shown in the APC and PreSAP trials, achieving a significant reduction in lesions with advanced histology (30, 33). Unfortunately, the APC Trial also found that celecoxib was associated with an increased incidence of serious cardiovascular adverse events. In particular, the dose used in this OPL study, 400 mg bd, was associated with a 3.4-fold increase in one or more serious cardiovascular complications, including myocardial infarction, stroke, congestive heart failure, or death due to cardiovascular disease (29). At the present time, available data do not allow clinicians to make optimal decisions concerning the use of celecoxib for cancer prevention. In particular, the relative high dose of celecoxib used in this current pilot study is considered unacceptable for chemoprevention use.

It is clear that the success of chemoprevention resides in the ability to predict both the risk of disease progression and the risk of adverse drug response. Because of the strong link between COX-2, its effector, PGE2, and tumor promotion, we hypothesize that measurement of lesional PGE2 is an effective method to assess both risk of tumor genesis and effectiveness of chemoprevention. In addition, by providing rapid evaluation of nonsteroidal anti-inflammatory drug response, a surrogate endpoint biomarker such as PGE2 could be used to limit drug exposure in patients who do not derive benefit, thereby optimizing efficacy and limiting treatment risk. This is the case both for the selective COX-2 inhibitors, which carry a risk of cardiovascular toxicity, and for the nonselective nonsteroidal anti-inflammatory drugs, which are associated with significant gastrointestinal adverse events.

This pilot study indicates that determination of lesional PGE2 levels may be an effective surrogate endpoint biomarker of chemopreventive agent response in patients with OPLs. The current standard, change in degree of dysplasia, is difficult to apply reliably due to interobserver variability and sampling variation. In comparison, quantitation of PGE2 levels provides a direct measurement of an enzyme associated with epithelial tumorigenesis that is also the target of chemopreventive agent activity. In small biopsies obtained at prespecified time points, we documented a 38% decrease in PGE2 levels after 12 weeks of celecoxib. As expected, expression of the inhibitor of apoptosis, BCL2, was also inversely related to PGE2 level. Although this study was not designed or powered to assess clinical response to treatment, we also observed a significant correlation between lesional PGE2 levels and degree of dysplasia. Interestingly, after 12 months of celecoxib, PGE2 was suppressed more in OPLs with greater dysplasia, which could indicate a stronger preventative potential in more advanced lesions. Changes in pAKT-Ser473, as a downstream effector of COX-2, would be expected to parallel those of PGE2. Although such confirmatory correlation was not statistically significant, there was a trend of increased pAKT-Ser473 associated with increased PGE2.

There is an obvious limitation to this technique of serial tissue biopsy for surrogate endpoint biomarker measurement, particularly in small OPLs, which is that even a small punch biopsy can remove a large part of the visible lesion. We therefore cannot definitively rule out the possibility that the change

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**Table 4. Change in microvessel density over time**

<table>
<thead>
<tr>
<th>Microvessel density per 3 high-power fields by CD31 staining</th>
<th>Baseline</th>
<th>12 wks</th>
<th>Median change</th>
<th>P</th>
<th>12 mo</th>
<th>Median change</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>22.5</td>
<td>21.0</td>
<td>−1.0</td>
<td>1</td>
<td>35.5</td>
<td>6</td>
<td>0.4375</td>
<td></td>
</tr>
</tbody>
</table>


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in PGE2 levels and other marker changes were not variations due to differences in tissue sampling. That said, the small biopsies done were not excisional biopsies, and even if a significant portion of the gross OPL was removed, residual premalignant tissue representative of the tissue of interest remained.

The effect of celecoxib on PGE2 levels was not sustained over 12 months for this small cohort. Although it is possible that this effect is present only because of small patient numbers, it could also be due to compensatory mechanisms limiting the efficacy of COX-2 inhibition in the long term. Eicosanoids such as PGE2 are "rapid response" molecules, immediately elaborated on activation by appropriate stimuli and then rapidly degraded to prevent sustained effect. Consequently, tissue PGE2 levels can be very dynamic. To minimize sampling error, we used a twice daily celecoxib dosing regimen for this study, and subjects were instructed to take one dose each morning and one each evening (12 h apart) with food. Following a single oral celecoxib dose in healthy volunteers, maximal plasma concentration occurs in ~90 min, and the mean half-life of the drug is 11.5 h (46). The 800 mg daily dose is approximately four times the dose generally required for arthritis treatment; therefore, it is unlikely that biopsies taken at any time of the day for the subjects in this study would vary greatly in PGE2 content.

Celecoxib directly inhibits COX-2 activity, but its downstream effects can also influence enzyme expression. COX-2 gene expression is influenced by a wide variety of inflammatory mediators, including tumor necrosis factor-α, IFN-γ, and interleukins. Transcriptional up-regulation by these stimuli occurs via multiple transcription factor binding sites within the COX-2 gene promoter, which contains binding sites for cyclic AMP, Myb, nuclear factor-interleukin-6, CCAAT/enhancer binding proteins, nuclear factor-κB, polynora virus enhances activator 3, and activator protein 1 (47, 48). The decreased COX-2 expression observed in OPL following celecoxib treatment is consistent with reduced COX-2 transcription resulting from suppression of the inflammatory cascade.

In summary, this pilot study shows that it is possible to assess modulation of the primary therapeutic target of nonsteroidal anti-inflammatory drug chemoprevention by measuring PGE2 levels in small OPL biopsies. The data also suggest that selective COX-2 inhibitor, celecoxib, decreases the severity of dysplasia in patients with OPLs. This study indicates that intralesional PGE2 levels is an accurate early biomarker of SCCHN development, one warranting further study in a definitive clinical trial.

 Disclosure of Potential Conflicts of Interest

M.M. Bertagnolli: Pfizer, Inc. Commercial research grant; Pfizer, Inc. and Metamark, Inc. speakers bureau. The other authors disclosed no potential conflicts of interest.

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Oral Premalignant Lesions: A Pilot Surrogate Endpoint Biomarker Study of Celecoxib in Oral Premalignant Lesions

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