Cyclooxygenase-2 Expression in Pretreatment Biopsy as a Predictor of Tumor Responses After Preoperative Chemoradiation in Rectal Cancer

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Objective: To determine whether cyclooxygenase-2 (COX-2) expression in pretreatment biopsy specimens is a useful predictive marker of tumor response to preoperative chemoradiation (CRT) in rectal cancer.

Design: Case series.

Setting: Colorectal cancer clinic.

Patients: Thirty patients with locally advanced rectal cancer were given preoperative CRT of 5040 cGy for 6 weeks with concurrent administration of 5-fluorouracil and leucovorin.

Main Outcome Measures: Immunohistochemical staining for COX-2 and angiogenesis markers (vascular endothelial growth factor, thymidine phosphorylase, and CD34) were performed on biopsy specimens obtained before preoperative CRT. The responses to preoperative CRT were assessed by radiologic downsizing (measured using magnetic resonance imaging volumetry), histopathologic downstaging, and a 3-point tumor regression grade (TRG) evaluation, based on the ratio of residual cancer to fibrosis.

Results: Tumor downstaging was seen in 15 patients (50.0%) and nodal downstaging was noted in 14 patients (46.7%). Tumor regression grade 1 (good response) was shown by 7 patients (23.3%); TRG2 (moderate response) in 15 patients (50.0%); and TRG3 (poor response) in 8 patients (26.7%). Patients with COX-2 overexpression were more likely to show a poor TRG (P = .003) and were less likely to achieve histopathologic nodal downstaging (P = .03) than those with normal COX-2 expression. Vascular endothelial growth factor overexpression was found to be associated with COX-2 overexpression (P = .02).

Conclusions: Overexpression of COX-2 in pretreatment biopsies might be predictive of poor tumor regression after preoperative CRT. Administration of COX-2 inhibitors to patients with COX-2 overexpression, in an attempt to improve response rate to preoperative CRT, warrants assessment in clinical trials.

with radiation, can significantly increase tumor susceptibility to radiation by blocking prostaglandin release.\textsuperscript{15,16} In addition, clinical studies in patients with laryngeal, cervical, or rectal cancer have explored whether COX-2 expression, in pretreatment biopsies, might predict how the tumors would respond to radiation and chemotherapy.\textsuperscript{17-19} These studies found that high COX-2 expression was associated with poor responses to treatment and unfavorable prognoses.

The aim of this study was to explore the use of COX-2 overexpression as a predictive marker for tumor response to preoperative CRT. Three distinct aspects of tumor response were measured, namely radiologic downsizing by magnetic resonance imaging (MRI) volumetry, histopathologic tumor downsizing, and tumor regression grade (TRG). In addition, the relationship between COX-2 expression and tumor angiogenesis characteristics, measured by expression of the angiogenic markers vascular endothelial growth factor (VEGF), thymidine phosphorylase (TP), and CD34, was assessed.

## METHODS

### PATIENT SELECTION, PREOPERATIVE CRT, AND TOTAL MESORECTAL EXCISION

Thirty patients with biopsy-proven rectal cancer were enrolled prospectively into the study. Informed written consent was obtained from each patient. Cancer was staged using transrectal ultrasonography and pelvic MRI to determine the extent of local disease. Computed tomographic scans and chest radiographs were used to determine the extent of extrapelvic involvement.

Chemoradiotherapy was administered intravenously and was composed of 425-mg/m\textsuperscript{2} per day 5-fluorouracil and 20-mg/m\textsuperscript{2}/d leucovorin during the first and fifth weeks of radiotherapy. Radiation was administered using a 6-MV/10-MV dual-photon linear accelerator and a 4-filed box technique. The total radiation dosage was 5040 cGy divided across 25 fractions during 5 weeks.

Chemotherapy was administered intravenously and was composed of 425-mg/m\textsuperscript{2} per day 5-fluorouracil and 20-mg/m\textsuperscript{2}/d leucovorin during the first and fifth weeks of radiotherapy. Radiation was administered using a 6-MV/10-MV dual-photon linear accelerator and a 4-filed box technique. The total radiation dosage was 5040 cGy divided across 25 fractions during 5 weeks. The upper margin of the radiation field was 1.5 cm above the sacral promontory (L5 level); the lateral margin was up to 1.5 cm lateral from the pelvic wall; and the lower margin was 3 cm below the lower margin of the tumor. Total mesorectal excision with autonomic nerve preservation was performed 4 to 6 weeks after the completion of CRT by experienced surgeons on all cases.

### MRI VOLUMETRIC ANALYSIS AND RADILOGIC TUMOR DOWNSIZING

Radiologic downsizing was measured using MRI volumetric analysis. Magnetic resonance imaging was performed on all cases before and 4 to 6 weeks after the completion of preoperative CRT using phased-array coils. Cross-sectional areas of lesions were measured by tracing lesion boundaries with axial T2-weighted images. Tumor volumes were reconstructed automatically in 3-dimensional format and calculated by summing the volumes of all tumor cross-sections. The volume reduction rate was defined as \( \frac{(\text{preoperative CRT volume} - \text{postoperative CRT volume})}{\text{preoperative CRT volume}} \times 100 \). (Preoperative CRT volume was the tumor volume measured by MRI before preoperative CRT, and postoperative CRT volume was the tumor volume measured by MRI after preoperative CRT.) We decided that the radiologic tumor downsizing cut-off value would be achieved when the tumor volume reduction was greater than 50%.

### ASSESSMENT OF TUMOR DOWNSIZING AND REGRESSION

The resected specimens were staged according to the sixth International Union Against Cancer ypTNM staging system.\textsuperscript{20} Tumor downsizing was assessed by comparing the clinical stages (cT and cN stages) before preoperative CRT with postoperative histopathologic stages (ypT and ypN stages). Tumor downsizing occurred when ypT was lower than cT. Nodal downsizing occurred when cN(+) became ypN0.

We used the 3-point TRG scale proposed by Ryan et al.\textsuperscript{21} A single pathologist (H.K.) reviewed and scored all specimens. Regressive changes—including cytologic changes, such as cytoplasmic vacuolization and/or eosinophilia, nuclear pyknosis, and necrosis, and stromal changes, such as fibrosis (with or without inflammatory infiltration)—were all documented. The 3 TRG grades were defined as follows: TRG1, either cancer was absent or single cancer cells or small groups of cancer cells, with marked fibrosis, were noted; TRG2, residual cancers were outgrown by fibrosis; and TRG3, either fibrosis was outgrown by no fibrosis was observed, but extensive residual cancers were present.

### IMMUNOHISTOCHEMICAL STAINING

Paraffin-embedded tissue was prepared and sectioned. Four-micrometer sections were pretreated with fresh xylene for 3 to 5 minutes and then rehydrated using graded alcohol solutions (100%, 95%, and 75%, all vol/vol). Endogenous peroxidase activity was blocked by incubation with hydrogen peroxide (3% vol/vol) for 20 minutes, followed by a phosphate-buffered saline wash. Non-specific binding of the primary antibody was prevented by incubating specimens in blocking serum for 20 minutes. Specimens were incubated with the primary antibody overnight at 4°C. Next, specimens were placed in citrate buffer (pH, 7.6) and treated with microwave radiation for 25 minutes. The secondary antibody was added and, after 15 minutes at room temperature, the biotin-streptavidin complex was added. Incubation continued for an additional 15 minutes at room temperature. The chromogen diaminobenzidine was used for the immunoperoxidase reaction. The antibodies used were anti-COX-2 (1:100 dilution; Zymed, San Francisco, California), anti-VEGF (1:50 dilution; Santa Cruz Biochemicals, Santa Cruz, California), anti-TP (1:200 dilution; Oncogene Corp, Seattle, Washington), and anti-CD34 (1:75 dilution; DAKO Corp, Carpinteria, California).

### HISTOLOGIC SCORING

A semiquantitative method was used to evaluate the intensity and distribution of COX-2, VEGF, and TP (Figure 1). To eliminate the possibility of individual bias, an experienced investigator who was blind to clinical information microscopically examined the sections (Y.J.C.); the same investigator re-examined all slides after 1 month. Discrepancies in assessment were less than 5% and were resolved in favor of the pathologist’s final decisions.

#### Cyclooxygenase-2

Intensity and distribution were classified and scored. Intensity of staining was classified by the following criteria: 0, none; 1, weak; 2, moderate; and 3, strong. The extent of staining was graded according to the following criteria: 0, 0%; 1, less than...
The intensity and the extent scores were multiplied to give a raw score for each case. On the basis of this result, patients with strong expression of COX-2 were classified as showing COX-2 overexpression, as suggested in previous studies.22-24

**Vascular Endothelial Growth Factor**

The percentage of positive tumor cells (the extent of staining) was determined as follows: 0 indicates that a tumor is negative for VEGF; 1, that fewer than 20% of cells are positive; 2, that 20% to 50% of cells are positive; and 3, that greater than 50% of cells are positive. For our purposes, however, only those patients with scores of 3 were considered to show VEGF overexpression.25

**Thymidine Phosphorylase**

The intensity and distribution of TP were classified and scored as in a previous article.26 The intensity of staining was classified as follows: 0, none; 1, weak; 2, strong; and 3, very strong. The extent of staining was graded as 0, 0%; 1, less than 10%; 2, 10% to less than 25%; 3, 25% to 50%; and 4, greater than 50%. Both the intensity and the extent scores were multiplied to give a raw score for each case. For our purposes, however, only those patients with a score greater than 8 were classified as showing TP overexpression.

**CD34**

For the scoring of CD34+ microvessels, specimens were scanned at ×200 magnification and analyzed for the extent of microvessel staining. The extent of angiogenesis was based on the mean number of CD34+ microvessels counted in 3 slides.

**STATISTICAL ANALYSIS**

Data were analyzed using the SPSS package, version 11.5 (SPSS Inc, Chicago, Illinois). When comparing ordinal categorical variables with nominal variables, the χ² test for trend was used. For comparison of nominal categories, the ordinary χ² test was used. The nonparametric test was applied for the analysis of continuous variables owing to the small sample size. For multivariate analyses, multiple logistic regression tests with a backward elimination method were performed. \( P < .05 \) was considered statistically significant.

**RESULTS**

**PATIENT CHARACTERISTICS**

A total of 30 patients (26 men and 4 women) were enrolled. The median age was 48.0 years (range, 31-69 years). Thirteen patients underwent abdominoperineal resection, 8 underwent low anterior resection, 8 underwent ultralow anterior resection with coloanal anastomosis, and 1 underwent the Hartmann operation. The median serum carcinoembryonic antigen level was 7.7 ng/mL (range, 0.98-138.6 ng/mL) before preoperative CRT. After the completion of preoperative CRT, this level decreased to 2.88 ng/mL (range, 0.43-18.41 ng/mL) and further dropped to 1.62 ng/mL (range, 0.06-14.00 ng/mL) when measured 7 days after the operations. Three patients had stage II cancer before CRT and 27 patients had stage III cancer. By the criteria of cellular differentiation, moderately differentiated tumors were the most common (n = 19). There were also 4 poorly differentiated tumors, 2 well-differentiated tumors, 4 mucinous cell tumors, and 1 signet ring cell tumor.

**TUMOR RESPONSE TO PREOPERATIVE CRT**

Radiologic tumor downsizing was achieved in 19 patients (63.3%) and pathologic complete response was achieved in 5 patients (16.7%). Histopathologic tumor downstaging was seen in 15 patients (50.0%) and histopathologic nodal downstaging was seen in 14 patients (46.7%). Tumor regression grade 1 was recorded for 7 patients (23.3%), TRG2 for 15 patients (50.0%), and TRG3 for 8 patients (26.7%).

We performed analyses to determine associations between tumor response and clinicopathologic variables,
including initial (before preoperative CRT) cT and cN stages, lymphovascular invasion, histologic grade, and initial tumor size by MRI. None of these variables were significantly associated with tumor response.

COX-2 IMMUNOHISTOCHEMICAL STAINING AND TUMOR RESPONSE

Most tumors (93.3%) expressed COX-2 to some degree, showing diffuse, granular, staining patterns (Figure 1). Cyclooxygenase-2 was cytoplasmic. No patient with COX-2 overexpression attained TRG1 (complete regression of primary tumor), whereas 6 of 8 patients (75.0%) who were scored as TRG3 (poor regression) had COX-2 overexpression (Figure 2). Statistical analysis revealed a significant relationship between COX-2 overexpression and poor regression after preoperative CRT ($P = .003$). Of 14 patients who showed histopathologic nodal downstaging, only 2 patients (14.3%) had COX-2 overexpression, whereas 12 patients (85.7%) did not show COX-2 overexpression (Figure 3). Pretreatment biopsies that showed COX-2 overexpression were from patients less likely to achieve histopathologic nodal downstaging ($P = .03$). No association was found, however, between COX-2 overexpression and either histopathologic tumor downstaging or radiologic tumor downsizing (Table).

We also performed multivariate analyses to determine the independence or dependence of the variables studied. The variables analyzed included clinical and histopathologic factors, such as initial (before preoperative CRT) cT and cN stages, lymphovascular invasion, histologic grade, initial tumor size measured by MRI, and immunohistochemical staining of COX-2, TP, VEGF, and CD34. We found that COX-2 overexpression measured by immunohistochemical staining was independently associated with nodal downstaging (odds ratio, 12.857; 95% confidence interval, 1.314-125.778; $P = .03$). Cyclooxygenase-2 overexpression was the most strongly associated with TRG among the variables analyzed, but this association failed to reach statistical significance (odds ratio, 8.713; 95% confidence interval, 0.614-123.594; $P = .11$).

ANGIOGENIC MARKERS AND TUMOR RESPONSE

No significant relationships were found between angiogenic markers (VEGF expression, TP expression, or microvessel density as measured by CD34 immunohistochemical staining) and tumor response (radiologic tumor downsizing, histopathologic tumor or nodal downstaging, or TRG).

INTERRELATION BETWEEN COX-2 AND ANGIOGENIC MARKERS

To determine whether COX-2 overexpression influenced tumor angiogenesis, statistical analysis was performed. Eight of 11 patients (72.7%) who had COX-2 overexpression also showed VEGF overexpression, whereas only 5 of 14 patients (26.3%) who did not have COX-2 overexpression showed VEGF overexpression ($P = .02$) (Figure 4). No significant association was found between COX-2 expression and TP expression ($P = .61$). Microvessel density as measured by CD34 immunohistochemical staining was analyzed. The mean CD34 scores were 60.5 in the COX-2 overexpression group and 67.1 in the group without COX-2 overexpression ($P = .59$).

COMMENT

Preoperative CRT has been proven to be beneficial in the treatment of locally advanced rectal cancer. Dramatic tumor responses, such as pathologic complete response, correlate well with positive oncologic outcomes, increased resectability, and anal sphincter preservation. Tumor responses range, however, from complete response to progressive disease. The molecular response to chemotherapy and/or radiotherapy has gained considerable attention in recent years, and such detailed work may identify clinically relevant predictive markers. To date, most research has focused on constituents of the p53 path-
Table. Tumor Response and Expression of Angiogenetic Markers According to COX-2 Expression

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>COX-2 Overexpression, No. (%)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No (n = 19)</td>
<td></td>
</tr>
<tr>
<td>cT category&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>cN category&lt;sup&gt;a&lt;/sup&gt;</td>
<td>−</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>17</td>
</tr>
<tr>
<td>Histologic grade&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1/2</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>3/4</td>
<td>7</td>
</tr>
<tr>
<td>Mean tumor size, cm&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.3 versatile</td>
<td>3.20</td>
</tr>
<tr>
<td>Tumor downstaging</td>
<td>No</td>
<td>8 (42.1)</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>11 (57.9)</td>
</tr>
<tr>
<td>Nodal downstaging</td>
<td>No</td>
<td>7 (36.8)</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>12 (63.2)</td>
</tr>
<tr>
<td>Radiologic downsizing</td>
<td>No</td>
<td>7 (36.8)</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>12 (63.2)</td>
</tr>
<tr>
<td>TRG</td>
<td>1</td>
<td>7 (36.8)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>10 (52.6)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2 (10.5)</td>
</tr>
</tbody>
</table>

Abbreviations: COX-2, cyclooxygenase-2; TRG, tumor regression grade.
<sup>a</sup>Initial (before preoperative chemoradiation) clinical stages.
<sup>b</sup>Histologic tumor grade by World Health Organization criteria.
<sup>c</sup>Initial (before preoperative chemoradiation) tumor size measured by magnetic resonance imaging.

Cyclooxygenase-2 is a molecular marker that represents alternative pathways and its expression is related to increased invasiveness, resistance to apoptosis, and increased angiogenesis. Kandioler et al<sup>10</sup> found a significant association between the radioreponse of rectal cancer and p53. Recent studies suggested that the tumor suppressor gene, p53, and its downstream effector, p21 WAF/CIP1, play major roles in tumor responses to DNA-damaging agents. Kim et al<sup>14</sup> reported, however, that p53 and BCL2 expression did not correlate with tumor response after preoperative CRT, but that Ki67 expression did. Marijnen et al<sup>27</sup> observed that VEGF expression in nonresponsive tumors was significantly greater than in completely responsive tumors. Overexpression of TP, also known as platelet-derived endothelial cell growth factor, correlates with unfavorable prognoses. In our study, VEGF, TP, and CD34 were significant predictors of tumor response to radiation. Our data also suggest that COX-2 expression may be valuable for predicting tumor responses to preoperative CRT in rectal cancer.

We studied overexpression of angiogenesis effectors, such as VEGF and TP. We also analyzed microvessel density using CD34 expression. Vascular endothelial growth factor is a potent mediator of tumor angiogenesis and can be activated in tumor cells. Elevated VEGF messenger RNA is associated with poor clinical outcomes and greater tumor aggressiveness. In a study of 59 preirradiation rectal cancer biopsy specimens, Zlobec et al<sup>25</sup> observed that VEGF expression in nonresponsive tumors was significantly greater than in completely responsive tumors. Overexpression of TP, also known as platelet-derived endothelial cell growth factor, correlates with unfavorable prognoses. In our study, VEGF, TP, and CD34 showed no significant correlations with tumor responses, such as histopathologic downstaging, radiologic tumor downsizing, or TRG.

Smith et al<sup>19</sup> showed a significant correlation between COX-2 overexpression and poor response to CRT. These authors also found that poor responses were more likely when pretreatment apoptotic rates were low, but they failed to show a correlation between COX-2 overexpression and a low level of apoptosis. They cautiously suggested that the protection of tumors, after COX-2 overexpression, from radiation damage might be independent of radiation-induced apoptosis and that angiogenesis or tumor neovasculature might be important in the cellular response to radiation. Our results do not differ from those of Smith et al<sup>19</sup>; we observed an association between high expression of COX-2 and poor tumor re-
gression. In the present work, however, the validity of COX-2 expression as a predictive marker was evaluated using 3 different measures of tumor response: tumor downsizing, downstaging, and regression. While the TRG reflected the responses of the primary tumors, nodal downstaging reflected the responses of lymph nodes, and these were found to be associated with COX-2 expression levels. Moreover, our data showed a significant relationship between COX-2 and VEGF expression, which may support the hypothesis that neovascularization is a radioprotective mechanism, as suggested by Smith and colleagues.

There has been a paradigm shift in the search for predictive markers for tumor responses to preoperative CRT. Most previous works relied on an old paradigm experimental approach of a single-marker predictive model. With recent advances in biotechnologies, however, identification of gene expression differences between responders and nonresponders, by screening tens of thousands of genes probed using commercially available chips, has become possible. Noninvasive proteomic techniques, using serum rather than tissue samples, have also emerged recently. These state-of-the-art techniques will allow better tumor fingerprinting and will affect prognoses and individualized treatment planning. The use of such new techniques in routine clinical practice remains difficult at present. After thorough validation in large-scale prospective studies, it will be desirable to introduce the best predictive techniques to front-line clinical situations.

On the basis of our results, we conclude that COX-2 may be a useful predictive marker of tumor response to preoperative CRT in patients with locally advanced rectal cancer. Tumor regression grade and histopathologic nodal downstaging are valuable measures of tumor response. The significant relationship between COX-2 expression and VEGF expression observed in our study may imply that high COX-2 expression protects the tumor from radiation damage. Administration of COX-2 inhibitors to patients with COX-2 overexpression, in an attempt to improve response rates to preoperative CRT, warrants assessment in clinical trials.

Accepted for Publication: August 3, 2007.

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Author Contributions: Dr N. K. Kim had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Min, Pyo, Rha, and N. K. Kim. Acquisition of data: Min, Choi, Pyo, H. Kim, Seong, and Chung. Analysis and interpretation of data: Min and N. K. Kim. Drafting of the manuscript: Min and Pyo. Critical revision of the manuscript for important intellectual content: Min, Choi, H. Kim, Seong, Chung, Rha, and N. K. Kim. Statistical analysis: Min and Pyo. Obtained funding: Seong and N. K. Kim. Administrative, technical, and material support: Choi and Pyo. Study supervision: Choi, H. Kim, Chung, Rha, and N. K. Kim.

Financial Disclosure: None reported.

Funding/Support: This study was supported by grants 0405-BC01-0604-00002 from the Korean Health 21 R&D Project, Ministry of Health & Welfare, Republic of Korea, and A04-0151-B50704-06A3-00050B from the Clinical Research for the Cure of Solid Tumors in Adults, National Cancer Center, Republic of Korea.

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M
in et al from Seoul nicely show that the expression of COX-2 protein in colon cancer specimens inversely correlates with the tumor’s response to adjuvant CRT. While I cannot tell if this is really a prospective study or a retrospective analysis of 30 consecutive patients with stage II and III disease, it is work that pushes the envelope of diagnostic tests to the next—molecular—level.

It has been 10 years since the landmark Swedish study demonstrated that preoperative adjuvant CRT improved survival in rectal cancer. The authors are currently asking which patients will really benefit from the therapy. The answer will be at the molecular level.

The hunt for molecular markers in colon cancer has yielded many candidate genes and protein products. Last year in the journal Science, Sjoblom et al identified 69 mutated protein-coding genes in colorectal cancer. In fact, each individual cancer that was studied had an average of 9 mutations, suggesting that each tumor has its own molecular signature. The study did not address the newer types of mutations recently described, such as epigenetics (inhibition of normal gene expression by DNA methylation) or microRNA (miRNA) inhibition of genetic expression, both of which can inhibit tumor suppressor genes and cause cancers. The hunt is on, we are getting inundated with potential markers and processes, and now they tell us each tumor may be unique!

Min et al used basic physiologic and functional knowledge of COX-2 to map their study. Cyclooxygenase-2 is associated with tumor angiogenesis, and they postulated that more blood vessels may make the tumors less responsive to CRT. They indeed found that COX-2 overexpression was a negative predictor of response, and it correlated with presence of VEGF and other angiogenic markers.

But, the study has its drawbacks. The authors used immunohistochemistry, not molecular techniques, to measure COX-2 protein, not genetic expression. They showed that COX-2 overexpression was associated with a decreased response of TRG (a microscopic scoring of cells versus fibrosis) and with nodal downstaging, but not with volume. Finally, when measured independently, the angiogenic markers were not predictive at all.

The next step for us surgical scientists is to look for molecular markers with molecular probes. Using immunohistochemistry to look for changes at the molecular level is like chasing a Maserati with a Studebaker. We have the technology to measure messenger RNA levels in small specimens using quantitative real-time polymerase chain reaction and gene chips of entire molecular pathways, and it is time to start!

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Financial Disclosure: None reported.

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