Molecular Determinants in Targeted Therapy for Esophageal Adenocarcinoma

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Hypothesis: Cyclooxygenase 2 (COX-2), vascular endothelial growth factor (VEGF), and epidermal growth factor receptor (EGFR) are useful biological determinants in targeted therapy for esophageal adenocarcinoma.

Design: Prospective analysis.

Setting: University tertiary referral center.

Patients: Sixteen patients with squamous mucosa and normal results of a pH study without mucosal injury (control group), 15 with Barrett esophagus (metaplasia group), and 44 with adenocarcinoma (carcinoma group).

Interventions: Biopsy specimens were obtained 3 cm above the gastroesophageal junction. Dysplastic tissue was additionally isolated from 9 of the patients in the carcinoma group. After laser-capture microdissection, quantitative real-time polymerase chain reaction was used to measure gene expression across the spectrum of the metaplasia-dysplasia-carcinoma sequence.

Main Outcome Measures: Expression of COX-2, VEGF, and EGFR in each patient group.

Results: Expression of both COX-2 and VEGF was significantly up-regulated in patients with metaplasia, dysplasia, and cancer compared with controls (P<.01). Expression levels of both were significantly higher in cancer than in the metaplasia group (P<.05) and increased sequentially from metaplasia to dysplasia to cancer. Expression of VEGF was significantly higher in the dysplastic tissue than in nondysplastic Barrett epithelium (P<.05). No change in expression levels of EGFR was seen in the histologic progression to esophageal adenocarcinoma.

Conclusion: Gene expression data suggest that pharmacologic inhibition of COX-2 and VEGF may be useful adjuncts in targeted therapy for esophageal adenocarcinoma.

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During the past 20 years, there has been an exponential growth in our understanding of the cellular and molecular events associated with cell cycle regulation, programmed cell death, angiogenesis, and tumor growth. The discovery and characterization of growth factor receptors, agents that stimulate or inhibit angiogenesis, cell cycle regulatory proteins, and other compounds that regulate cellular function led, early on, to the possibilities that biological therapy could be used to prevent or treat neoplasia. Slow, steady progress in the understanding of molecular biology has led to the recent introduction of a new class of antineoplastic agents called biologics, with widespread enthusiasm for their potential.

Current available agents can inhibit or modulate signal transduction pathways that are functionally important in the growth of solid tumors. These include inhibitors of angiogenesis (bevacizumab [Avastin; Genentech, Inc, South San Francisco, Calif]), epidermal growth factor receptor (EGFR) (cetuximab [Erbitux; ImClone systems Inc, Branchburg, NJ], erlotinib [Tarceva; Schwarz Pharma Manufacturing, Seymour, Ind]), and cyclooxygenase 2 (COX-2) (celecoxib [Celebrex; Pfizer, New York, NY]). Phase 1 and 2 clinical trials of these agents, generally in combination with irinotecan hydrochloride–, oxaliplatin–, or fluorouracil-based chemotherapy, have shown promise in patients with colorectal and lung cancer.

The quest for effective adjuvant therapy for esophageal cancer has been ongoing for more than 50 years. As the biological characteristics of esophageal cancer changed to include an increasing proportion of adenocarcinoma, the effectiveness of the agents and techniques for administration...
of adjuvant therapy have also improved. The introduction of biological agents has provided further possibilities for improving adjuvant treatment strategies and has created a greater need to understand the biological correlates of their efficacy. Our aim was to investigate expression levels of COX-2, EGFR, and vascular endothelial growth factor (VEGF) at different stages of the development of esophageal adenocarcinoma by using 2 recent technological methods: laser-capture microdissection and quantitative real-time polymerase chain reaction (PCR).

### METHODS

#### TISSUE SAMPLES

Tissue samples (n = 91) of Barrett epithelium or adenocarcinoma for quantitative real-time PCR were obtained at endoscopy or from surgical specimens from 59 patients with foregut symptoms and were immediately snap-frozen in liquid nitrogen. In all patients, treatment with acid suppression medications was stopped before endoscopic evaluation (2 days for histamine, blockers, 2 weeks for proton pump inhibitors). Samples containing Barrett metaplasia, cancer, or dysplasia (high- or low-grade) were separated into 3 different groups based on findings on routine histologic examination as follows: (1) Barrett metaplasia group: 22 samples from 15 patients showing intestinal metaplasia; (2) carcinoma group: 54 samples from 44 patients showing esophageal adenocarcinoma; and (3) dysplasia group: 15 samples from 9 of the patients in the carcinoma group showing intestinal metaplasia and either low- or high-grade dysplasia. Gene expression levels in patients with multiple biopsy specimens of the same histologic tissue were averaged.

The squamous control group consisted of tissue samples of squamous epithelium collected at the time of endoscopy, taken 3 cm above the gastroesophageal junction, from 16 patients with normal results of a pH study and no histologic evidence of mucosal injury. None of the patients had had previous foregut surgery.

Approval for this study was obtained from the institutional review board of the University of Southern California Keck School of Medicine, Los Angeles, and written informed consent was obtained from participating patients.

#### HISTOLOGIC DEFINITIONS OF TISSUE SAMPLES

All histologic examinations were performed by a single pathologist (P.T.C.) with specialized expertise in gastrointestinal abnormalities and adenocarcinoma associated with Barrett esophagus. The following criteria were used to define each tissue type: (1) Normal squamous epithelium was defined by the absence of criteria for the diagnosis of reflux esophagitis, infection, dysplasia, or malignancy. (2) Intestinal metaplasia was defined by the presence of a cardiac mucosa that contained well-defined goblet cells on a hematoxylin-eosin–stained section. (3) Dysplastic Barrett epithelium was defined by the presence of cytologic abnormalities of dysplasia in the columnar epithelium involving both surface and foveolar-glandular regions. For low-grade dysplasia, the cytologic changes exceeded those associated with repair but did not satisfy criteria for high-grade dysplasia. High-grade dysplasia was defined by the presence of severe cytologic abnormality associated with either gland complexity or loss of nuclear polarity. (4) Adenocarcinoma was defined by the presence of invasive cells with malignant characteristics attempting to form glands.

#### RNA ISOLATION AND COMPLEMENTARY DNA SYNTHESIS

Tissue samples to be extracted were placed in a 0.5-ml, thin-walled tube containing 400 µL of 4M dithiothreitol with guanidine isothiocyanate solution (4M guanidine isothiocyanate, 30mM Tris hydrochloride [pH 7.5], and 25mM EDTA) (Invitrogen Corp, Carlsbad, Calif; No. 15577-018). The samples were homogenized, and an additional 60 µL of guanidine isothiocyanate solution was added. Fifty microliters of 2M sodium acetate was added at pH 4.0, followed by 600 µL of freshly prepared phenol-chloroform–isoamyl alcohol (250:50:1). The tubes were vortexed for 15 seconds, placed on ice for 15 minutes, and then centrifuged at 13,000 rpm for 8 minutes in a chilled (8°C) centrifuge. The upper aqueous phase (250-350 µL) was carefully removed and placed in a 1.5-ml centrifuge tube. Glycogen (10 µL) and 300 to 400 µL of isopropanol were added, and the samples were vortexed for 10 to 15 seconds. The tubes were placed at −20°C for 30 to 45 minutes to precipitate the RNA. The samples were then centrifuged at 13,000 rpm for 7 minutes in a chilled (8°C) centrifuge. The supernatant was poured off, and 500 µL of 75% ethanol was added. The tubes were centrifuged at 13,000 rpm for 6 minutes in a chilled (8°C) centrifuge. The supernatant was carefully poured off so as not to disturb the RNA pellet, and the samples were quick-spun for 15 seconds at 13,000 rpm. The remaining ethanol was removed with a 20-µL pipette, and the samples were air dried for 15 minutes. The pellet was resuspended in 50 µL of 5mM Tris. (The RNA isolation technique described is a proprietary procedure of Response Genetics Inc, Los Angeles, Calif; US patent No. 6248535.) Afterward the complementary DNA was prepared.

#### REAL-TIME PCR QUANTIFICATION OF MESSENGER RNA EXPRESSION

Expression of COX-2, EGFR, and VEGF and an internal reference gene (β-actin) was quantified with a fluorescence-based real-time detection method (ABI PRISM 7900 Sequence Detection System [TaqMan]; Perkin-Elmer Applied Biosystems, Foster City, Calif). The PCR reaction mixture consisted of 1200nM of each primer; 200nM probe; 0.4 U of DNA polymerase (AmpliTaq Gold Polymerase; Perkin-Elmer Applied Biosystems); 200nM each deoxyadenosine triphosphate, deoxyguanosine triphosphate,
deoxothymidine triphosphate; 3.5mM magnesium chloride; and 1× sequence detection system buffer A, containing a reference dye, to a final volume of 20 µL (all reagents from PerkinElmer Applied Biosystems). Cycling conditions were 50°C for 2 minutes and 95°C for 10 minutes, followed by 46 cycles at 95°C for 15 seconds and 60°C for 1 minute. The primers and probes used are listed in Table 1.

The detection system measurements yield cycle threshold (Ct) values that are inversely proportional to the amount of complementary DNA in the tube; that is, a higher Ct value means that more PCR cycles are required to reach a certain level of detection. Gene expression values (relative messenger RNA [mRNA] levels) are expressed as ratios (differences between the Ct values) between the gene of interest and an internal reference gene (β-actin) that provides a normalization factor for the amount of RNA isolated from a specimen.

**STATISTICAL ANALYSIS**

Gene expression values are expressed as ratios between 2 absolute measurements: the gene of interest and the internal reference gene, β-actin. The COX-2, EGFR, and VEGF mRNA levels were log-transformed to render the data compatible with the assumptions of the normal distribution before the analysis. The differences in gene expression levels across tissue groups (control, Barrett metaplasia, dysplasia, and carcinoma) were evaluated with both a nonparametric method, the Kruskal-Wallis test, and a parametric method, multivariate analysis of variance. The Student-Newman-Keuls method was used for the pairwise comparisons of 3 tissue groups. Medians and ranges were used to summarize gene expression levels of COX-2, EGFR, and VEGF within groups of patients.

All tests of statistical significance were 2 sided. The analyses were performed with the SAS statistical package, version 9.0 (SAS Institute Inc, Cary, NC).

The study population included 23 women and 52 men. Median age was 58 years (range, 23-86 years). The median value and range of expression levels of COX-2, EGFR, and VEGF determined with quantitative reverse transcription PCR analysis of the 4 tissue groups are listed in Table 2.

**COX-2 GENE EXPRESSION**

The COX-2 mRNA levels were lowest in squamous epithelium from control patients (median, 0.12) and highest in patients with adenocarcinoma (median, 1.76) (Figure 1). When compared with that in normal squamous epithelium, COX-2 expression was significantly higher in Barrett metaplasia, dysplasia, and cancer tissue (P < .001). There was a stepwise increase in COX-2 expression throughout the metaplasia-dysplasia-
adenocarcinoma sequence in that expression levels in intestinal metaplasia were significantly lower than in adenocarcinoma (P<.05).

EGFR GENE EXPRESSION

The EGFR mRNA levels were lowest in squamous epithelium from control patients (median, 4.4) and highest in dysplastic tissue (median, 5.89) (Figure 2). No significant difference was detected among the tissue groups.

VEGF GENE EXPRESSION

The VEGF mRNA levels were lowest in squamous epithelium from control patients (median, 4.13) and highest in high-grade dysplasia (median, 20.34) and adenocarcinoma (median, 4.4) when all-patient-combined groups were analyzed (Spearman correlation coefficient, +0.46; P<.001). No correlation was detected when the groups were analyzed separately.

CORRELATION OF GENE EXPRESSION LEVELS

The only significant correlation was detected for COX-2 and VEGF when all-patient-combined groups were analyzed (Spearman correlation coefficient, +0.46; P<.001). No correlation was found when the groups were analyzed separately.

COMMENT

The incidence of esophageal adenocarcinoma is currently rising faster than that of any other cancer in the United States.11-13 With improvements in local disease control by en bloc resection, systemic metastasis is now the major cause for failure.14 This emphasizes the need for new systemic therapies. Recently, “targeted” or “biological” therapies aimed at inhibiting specific molecular signaling pathways have created widespread enthusiasm for a new class of effective antineoplastic agents.

The relationship between protein-gene expression and efficacy of targeted therapy remains only partially understood. In women with metastatic breast cancer, patients overexpressing human growth factor receptor 2 benefit from treatment with trastuzumab (a monoclonal antibody against human growth factor receptor 2) significantly more than patients with lower human growth factor receptor 2 expression.15,16 Recently, Burtness et al17 showed a significant correlation between intratumoral EGFR expression and response to cisplatin and cetuximab in patients with head and neck cancer. In contrast, clinical trials showing cetuximab to have promising effects in patients with advanced or metastatic colorectal cancer have failed to demonstrate a significant correlation between EGFR expression and response to treatment with cetuximab.5,18 At this point, we can only speculate as to the significance of these findings. How genetic events, including homozygous deletion, intragenic mutation, rearrangement of chromosomal material, loss of heterozygosity, and aberrant methylation of CpG islands may be involved needs to be determined in the future. In the current study, we measured gene expression levels of enzymes involved in the signaling pathways relevant to 3 identified targets for which therapeutic agents have been developed, namely, COX-2, EGFR, and VEGF.

Our studies have shown a stepwise increase in expression levels of VEGF and COX-2, but not of EGFR, in the metaplasia-dysplasia-adenocarcinoma sequence. This indicates that therapeutic inhibition of angiogenic and COX-2 but not EGFR signaling may be useful in the treatment of esophageal adenocarcinoma.

Vascular endothelial growth factor is up-regulated in dysplasia-dysplasia adenocarcinoma sequence in that expression levels in intestinal metaplasia were significantly lower than in adenocarcinoma (P<.05).

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VEGF GENE EXPRESSION

The VEGF mRNA levels were lowest in squamous epithelium from control patients (median, 4.13) and highest in patients with adenocarcinoma (median, 20.34) (Figure 3). When compared with that in normal squamous epithelium, VEGF expression was significantly higher in Barrett metaplasia, dysplasia, and cancer tissue (P<.001). There was a stepwise increase in VEGF expression throughout the metaplasia-dysplasia-adenocarcinoma sequence in that expression levels in intestinal metaplasia were significantly lower than in dysplasia and adenocarcinoma (P<.05).

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toward cancer better than all other genes we have tested. Further, VEGF has been shown to be the most potent angiogenic factor and a powerful mediator of tumor angiogenesis. The importance of its role in carcinogenesis is reflected in its association with the metastatic potential and prognosis of gastrointestinal tract cancers. Evidence of its oncogenic role is the correlation of tumor microvessel density with VEGF expression. Of interest, the goblet cells in Barrett epithelial mucosa stain intensely for VEGF, suggesting that they may supply the factor on the pathway leading to cancer. Clinical studies using bevacizumab, a humanized anti-VEGF monoclonal antibody, have shown encouraging results in patients with metastatic gastrointestinal tract tumors.4,22 Clinical evidence of selective interference with VEGF signaling in patients with esophageal cancer is sparse. We found only 2 reports,23 consisting of preliminary data from a phase II study. This study showed a partial response in 75% of patients with measurable disease. While this result is encouraging, further clinical trials are needed. We as well as others have shown that COX-2 expression levels, a rate-limiting enzyme in the conversion of arachidonic acid to prostaglandins, are sequentially increased in the metaplastic-dysplastic sequence leading to esophageal adenocarcinoma. These findings are consistent with recent studies implicating COX-2 in the development of esophageal adenocarcinoma associated with Barrett esophagus.24-26 Cyclooxygenase 2 participates in the regulation of a broad range of cellular processes including proliferation, angiogenesis, and resistance to apoptosis. Epidemiologic studies have shown that regular aspirin use is associated with a decreased risk of esophageal cancer, an effect that was also observed with selective COX-2 inhibitors in a case-control study.27,28 The combination of these clinical studies and the observation of the sequential elevation of COX-2 expression in Barrett epithelial mucosa and cancer tissue implies that the inhibition of COX-2 is an effective strategy in the prevention and treatment of esophageal adenocarcinoma. In regard to prevention, we have recently shown that elevated COX-2 expression in squamous mucosa of patients with gastroesophageal reflux disease is reduced to normal after antireflux surgery.29 The addition of COX-2 inhibitors to chemotherapy in the treatment of patients with esophageal cancer is currently under investigation.6 In contrast to VEGF and COX-2, we found virtually no difference in EGFR expression over the reflux-metaplasia-dysplasia-adenocarcinoma sequence. This is in contrast to the overexpression of EGFR in squamous cell cancer of the esophagus. Small retrospective studies have reported increased EGFR expression with the semiquantitative method of immunohistochemistry.31,32 There may be a generalized EGFR overexpression in gastroesophageal reflux disease that does not vary with tissue changes showing progression to cancer. Interestingly, in a phase 2 multicenter trial by Lenz et al30 assessing the efficacy of single-agent cetuximab treatment in patients with EGFR-expressing metastatic colorectal cancer, 1 of 9 patients with undetectable levels of EGFR protein expression responded to cetuximab therapy. Activation of EGFR may be more important than EGFR levels. Indeed, in a limited phase 2 trial, patients with advanced esophageal adenocarcinoma have shown responses to gefitinib (Iressa), an EGFR tyrosine kinase inhibitor.34 We also found a significant correlation between the expression levels of COX-2 and VEGF, evidence in support of the hypothesis that COX-2 may induce its known angiogenic effects by the activation of VEGF. von Rahden et al35 using esophageal cancer cell lines, recently showed that 3 COX-inhibiting substances resulted in significant reduction in VEGF-A and VEGF-C expression. We were not able to demonstrate a significant correlation of gene expression levels of either COX-2 or VEGF with EGFR, however. The foregoing observations suggest that a combination of drugs targeted against COX-2 and VEGF may be even more effective than either alone. In conclusion, by using laser-capture microdissection and real-time reverse-transcriptase PCR, we were able to show that COX-2 and VEGF, but not EGFR, are up-regulated not only in adenocarcinoma associated with Barrett esophagus, but in the metaplasia-dysplasia-adenocarcinoma sequence, indicating that COX-2 and VEGF may be useful factors in targeted therapy for esophageal adenocarcinoma. The role of EGFR in the pathogenesis of esophageal adenocarcinoma and its status in the targeted therapy of this type of cancer remains to be determined. Accepted for Publication: January 5, 2006. Correspondence: Jeffrey H. Peters, MD, Department of Surgery, University of Rochester School of Medicine and Dentistry, 601 Elmwood Ave, Rochester, NY 14642 (jeffrey_peters@urmc.rochester.edu). Funding/Support: This study was supported by grant RO1-CA84424-02 from the National Institutes of Health (Dr Peters). Previous Presentation: This paper was presented at the 113th Scientific Session of the Western Surgical Association; November 7, 2005; Rancho Mirage, Calif; and is published after peer review and revision. The discussions that follow this article are based on the originally submitted manuscript and not the revised manuscript.

REFERENCES
This page contains a discussion on the clinical significance of angiogenesis in gastrointestinal malignancies. It references studies by various authors and institutions, including the Mayo Clinic and the University of Rochester.

The discussion begins with a question from the audience, asking why certain targets were selected for study. The speaker responds by explaining that the selection was based on the 3 most common clinically available targets as of the time the study was conducted.

The discussion then turns to the role of angiogenesis in the esophageal cancer sequence and expands on the sequential expression of COX-2 and VEGF, but not EGFR. The importance of EGFR seems to have been discounted because the trend did not follow the metaplasia-dysplasia-carcinoma sequence. The authors presented data on the expression of EGFR and its activation in esophageal cancer.

The discussion also touches on the potential for targeted agents to improve outcomes in esophageal cancer. The speaker mentions the use of monoclonal antibodies against HER2 for metastatic breast cancer that overexpresses HER2, and the role of overdiagnosis and reclassification in the marked increase of esophageal adenocarcinoma incidence.

The speaker concludes by acknowledging the role of EGFR in esophageal cancer and suggesting that further research is needed to understand the clinical significance of angiogenesis in gastrointestinal malignancies.
considered that there was an anti-EGFR trial ongoing with esophageal cancer. That is really very little biologic background for its rationale. Are there others that we should look at? Absolutely. TK [tyrosine kinase] inhibitors in general should be studied. Estrogen receptors have been reported in esophageal cancer and probably should be looked at, HER2 perhaps, and other inhibitors of angiogenesis all need to be surveyed and evaluated more clearly than we have shown here.

You went on to ask whether the findings that we observed with regard to EGFR could perhaps be a phenomenon of reflux disease or a more general phenomenon than a baseline elevation. You are quite correct in pointing out that a stepwise increase over the course of the pathogenesis, or the lack of one, doesn’t necessarily mean that an EGFR inhibitor wouldn’t work in esophageal cancer. We did find relatively high levels of EGFR receptor activity in all the tissues. There was no difference, however, between squamous mucosa in patients who were symptomatic but pH negative and any of the other metaplastic and neoplastic tissues. This was as quiescent a control group as we could come up with and still be able to take biopsies. The data suggest that it is probably not involved in the pathogenesis and gives me pause as to whether an anti-EGFR antagonist will work in esophageal cancer.

Finally, you asked if we have plans to test these in vitro. It would be nice to do so if we had a model. Most in vitro tests, of course, would require cancer cell lines. Of course, these inhibitors would inhibit most cancer cell lines. What we really would like to do is have tissue, not cells, that is, dysplastic tissue, and nondysplastic Barrett’s tissue, to test these inhibitors on. I remind all that Barrett’s is a tissue, not a cell line. There are experiments that have been done in the short term on Barrett’s tissue, although they are difficult experiments to do. It would be interesting to see, for example, if these inhibitors would change the proliferation pattern of Barrett’s in vitro. That has not been done.

Fiemu Nwariaku, MD, Dallas, Tex: One of the issues in esophageal cancer is the fact that it is advanced at the time of diagnosis, so the determinants of outcome tend to be recurrence and metastasis. So, did you find a relationship between gene expression and disease stage in the subgroup of cancer patients?

My other question is, with the 3 genes, in which you identified differential expression, were there any mutations? One lesson we learned from lung cancer, especially with EGFRs, suggests that even though there may be differences in gene expression, the determinant drug efficacy may be receptor mutations.

Dr Peters: First, the presentation of esophageal cancer is changing. At USC, remarkably, 50% of the patients who we saw over the last 5 or 10 years had relatively early, stage 1 or 2 disease. We are seeing more and more of that as surveillance endoscopy is used.

Is there a relationship between the levels of these genes and the stage of the disease? Possibly so. We are only beginning to explore that. That begs the question of whether gene expression of these or any gene can be used as a prognostic factor in addition to a therapeutic target. There has been a little bit of work done in that regard that would suggest that some of these gene expressions may be indeed prognostic, but not enough really to hang your hat on any one of them as yet.

And your final question, is there a difference in mutations. Good question. We do not know the answer to that. We have not sequenced any of these receptors to find out if they are indeed mutated and if there are specific mutations promoting activity as has been found in lung cancer.

Fabrizio Michelassi, MD, New York, NY: I want to congratulate you and Dr DeMeester on this very interesting study and your suggestion that pharmacologic inhibition of COX-2 and VEGF may be used in the treatment of adenocarcinoma. Over the past 30 years, we have learned that in fields of chronic inflammation, whether it is Barrett or ulcerative colitis, progression may occur from normal mucosa to low-grade dysplasia, high-grade dysplasia, and then adenocarcinoma. I wonder, therefore, whether this inhibition could be better used as chemoprevention in the treatment of low-grade dysplasia to attempt to either prevent or delay its transformation into high-grade dysplasia and adenocarcinoma.

Dr Peters: Of course that would be the holy grail. A cancer prevented is better than a cancer treated. There was actually a multicenter US trial that you may be aware of investigating the possibility that COX-2 inhibition would prevent cancer development in patients with high-grade dysplasia. Unfortunately, that trial was stopped prematurely and closed because of the issue of COX-2 inhibitors causing cardiac events. Whether we can find an inhibitor with a low enough side effect profile to treat an otherwise asymptomatic population with a relatively low risk for cancer remains to be seen. It would certainly be nice to do so.