Tumor Response to Combination Celecoxib and Erlotinib Therapy in Non-small Cell Lung Cancer Is Associated with a Low Baseline Matrix Metalloproteinase-9 and a Decline in Serum-Soluble E-Cadherin

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Introduction: Cyclooxygenase-2 overexpression may mediate resistance to epidermal growth factor receptor tyrosine kinase inhibition through prostaglandin E2-dependent promotion of epithelial to mesenchymal transition (EMT). Suppression of epithelial markers, such as E-cadherin, can lead to resistance to erlotinib. Prostaglandin E2 down-regulates E-cadherin expression by up-regulating transcriptional repressors, including ZEB1 and Snail. Furthermore, E-cadherin can be modulated by matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs), promoting tumor invasion and metastasis. Markers of EMT and tumor invasion were evaluated in patient serum from a phase I clinical trial investigating the combination of celecoxib and erlotinib in non-small cell lung cancer (NSCLC) patients.

Methods: Samples from 22 subjects were evaluated. Soluble E-cadherin (sEC) was evaluated by enzyme linked immunosorbent assay in patient serum at baseline, week 4, and week 8 of treatment. Other markers of EMT and angiogenesis were evaluated by enzyme linked immunosorbent assay, including MMP-9, TIMP-1, and CCL15.

Results: Serum sEC, MMP-9, TIMP-1, and CCL15 levels were determined at baseline and week 8. Patients with a partial response to therapy had a significant decrease in sEC, TIMP-1, and CCL15 at week 8. In patients who responded to the combination therapy, baseline MMP-9 was significantly lower compared with nonresponders ($p = 0.006$).

Conclusions: sEC, MMP-9, TIMP-1, and CCL15 levels correlate with response to combination therapy with erlotinib and celecoxib in patients with NSCLC. A randomized phase II trial is planned comparing erlotinib and celecoxib with erlotinib plus placebo in advanced NSCLC. This study will prospectively assess these and other biomarkers in serum and tumor tissue.

Key Words: Non-small cell lung cancer, Epidermal growth factor receptor, Cyclooxygenase-2, Biomarkers.

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Lung cancer is the leading cause of cancer death in the United States, and for all stages, the 5-year survival for non-small cell lung cancer (NSCLC) is approximately 15%.[1] Targeted therapy with an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI) has been shown to prolong survival in advanced non-small cell lung cancer.[2] Although many tumors are insensitive to this treatment, combination therapy targeting multiple pathways may improve clinical outcome. Combination therapies might delay the onset of resistance in patients who show an initial response to EGFR inhibition, or block competing tumor growth pathways in patients who do not respond to single-agent treatment.[3] An understanding of the mechanisms of action in these combination-targeted approaches will help us define the mechanisms of resistance.[4,5] EGFR and cyclooxygenase-2 (COX-2) signaling pathways interact to promote tumor proliferation, invasion, angiogenesis, and resistance to apoptosis.[6–9]

A potential mechanism of resistance to EGFR TKI in NSCLC is mediated through an EGFR-independent activation of the MAPK/Erk signaling pathway by prostaglandin E2 (PGE2), a COX-2 metabolite.[6] This pathway involves PGE2-mediated, protein kinase C-dependent Erk activation that is not inhibited by otherwise effective doses of the EGFR inhibitor erlotinib. COX-2 overexpression can also mediate resistance to EGFR TK inhibition through a mechanism relating to the PGE2-dependent promotion of epithelial to mesenchymal transition (EMT).[10] Thomson et al. reported that the suppression of epithelial markers such as E-cadherin led to resistance to erlotinib.[11] In addition, PGE2 down-regulates E-cadherin expression by up-regulating transcriptional repressors, including ZEB1 and Snail.[10] The down-

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regulation of E-cadherin and overexpression of matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs) in multiple tumor types have been associated with increased tumor progression and metastatic potential.[12–14]

Cadherins are Ca^{2+}-dependent cell-cell adhesion molecules which interact with catenins.[15] The E-cadherin–catenin complex plays an integral role in the maintenance of tissue architecture and cell-cell adhesion.[12,15,16] The loss of E-cadherin has been shown to be associated with increased tumor invasiveness, metastasis, and poor prognosis in lung cancer.[12,16] Furthermore, MMPs have the ability to cleave E-cadherin at the cell surface, resulting in the release of a soluble 80-kDa fragment of E-cadherin.[17] Elevated levels of soluble E-cadherin (sEC) have been described in the blood and urine of patients with cancer, and increased levels of sEC have been correlated with decreased survival in NSCLC.[18–24] Moreover, both MMP-9 and MMP-2 can be induced by sEC in lung cancer cells.[25]

MMPs are proteolytic enzymes that are important in promoting invasion through the extracellular matrix, and are involved in tumor invasion and progression.[26–28] They can be induced by PGE2 in NSCLC.[29] TIMPs are found in the extracellular matrix and bind MMPs to inhibit their activation.[30] The balance between MMPs and TIMPs in tumor tissue plays an important role in tumorigenesis, although elevated levels of TIMP-1 have been associated with tumor progression in NSCLC.[14,31–33] MMP-2 and MMP-9 are secreted molecules that mediate tumor invasion and metastasis through the degradation of collagen IV.[26] Overexpression of MMP-9 has been associated with a more aggressive tumor phenotype in NSCLC.[31,34–36] Although the importance of MMP-9 in tumor progression has been well established, the prognostic significance of MMP-2 in lung cancer has been variable.[37,38]

Chemokines are a family of cytokines, subdivided on the basis of the position of the N-terminus cysteine residue. They are involved in leukocyte chemotaxis and activation, and have been associated with the regulation of angiogenesis.[39–41] Chemokines can be produced by tumor cells, leukocytes, and endothelial cells.[42,43] Through interactions with stromal cells and neoplastic cells, chemokines can potentiate tumor growth, metastasis, angiogenesis, and immune evasion.[44,45] CCL15 is a CC chemokine that induces the recruitment of monocytes and lymphocytes to sites of inflammation through the chemokine receptors, CCR1 and CCR3.[46,47] Furthermore, signaling through CCR1 can modulate MMP expression and promote tumor proliferation and invasion.[48] CCL15 has also been shown to induce angiogenesis and is elevated in malignancy.[49]

A phase I clinical trial evaluated the combination of erlotinib (an EGFR TKI) and celecoxib (a COX-2 inhibitor) in advanced NSCLC.[50] This study established the optimal biologic dose of celecoxib as 600 mg twice daily, and demonstrated clinical responses without significant toxicity. In the patient samples from this trial, we evaluated serum markers of COX-2 gene expression, EMT, and angiogenesis to further define a population of patients who are most likely to benefit from this combination treatment.

TABLE 1. Patient Responses

Celecoxib Dose (mg)	Time to Progression (wk)	Mutation Analysis[50]
Partial response		
300 bid	95	Exon 18 2105C → T
400 bid	36	Exon 18 2156G → C
400 bid	27	wt
600 bid	34	wt
800 bid	33	del exon 19
800 → 400	72	del exon 19
	48	del exon 19
Stable disease		
200 bid	19	n/a
200 bid	84	wt
600 bid	47	wt
800 bid	9	wt
800 → 400	16	wt
Progressive disease		
200 bid	10	wt
300 bid	7	wt
300 bid	6	n/a
400 bid	13	wt
600 bid	9	wt
800 bid	9	n/a
800 → 400	7	wt
	9	n/a
	5	wt

bid, twice daily; wt, wildtype; del, deletion; n/a, not available.

PATIENTS AND METHODS

Clinical Study

A phase I, dose escalation trial was conducted in patients who had disease progression after standard chemotherapy for advanced NSCLC at the University of California, Los Angeles (UCLA) Medical Center.[50] Three subjects were assigned to each cohort and received erlotinib at a fixed dose of 150-mg orally daily for two 4-week cycles. In addition, they received celecoxib in escalating doses per cohort, starting with 200-mg orally twice daily and increasing by 100-mg doses to 400-mg orally twice daily, and then increasing by 200-mg doses to 800-mg orally twice daily. The primary endpoints were evaluation of the optimal biologic dose of the combination and assessment of toxicity. Secondary endpoints included biomarker assessment and evaluation of response as determined by Response Evaluation Criteria in Solid Tumors (RECIST) Guidelines by computed tomography (CT) at week 8 after initiation of treatment, and compared with baseline. All responses were confirmed by repeat CT scans no less than 4 weeks later. The UCLA institutional review board approved this study protocol, and all patients provided written informed consent.

Protein Quantification

Biomarker analysis was blinded with respect to demographic information and clinical response. Serum and plasma

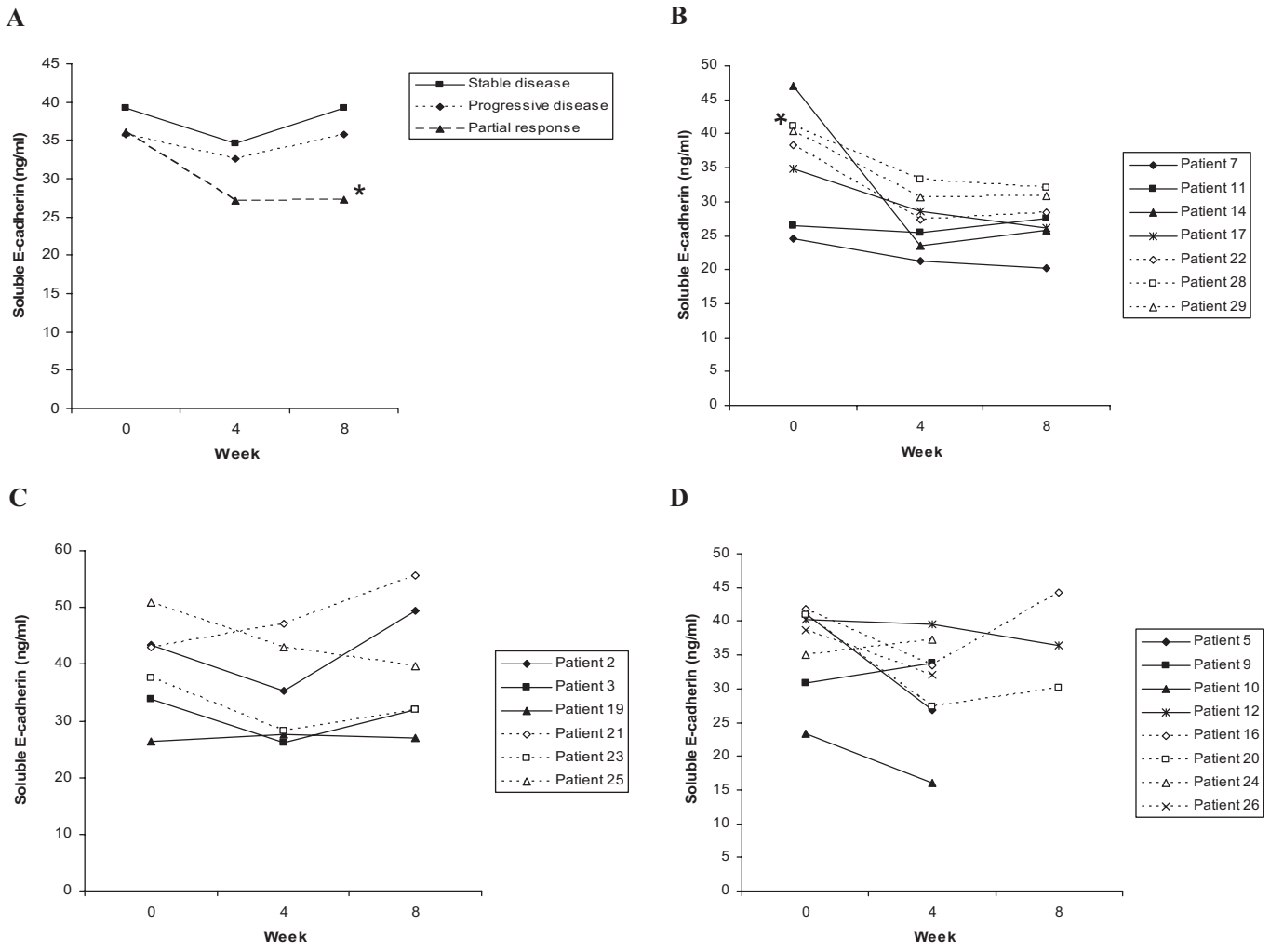

FIGURE 1. Soluble E-cadherin (sEC) decreases in patients treated with celecoxib and erlotinib who achieve partial response. sEC was evaluated by enzyme linked immunosorbent assay in patient serum at baseline and at week 8 of treatment. *A,* All patients at weeks 0, 4, and 8, *$p = 0.021$—decline in patients with PR compared with those with SD and PD. Individual patient values for (*B*) patients with PR; (*C*) patients with SD; and (*D*) patients with PD.

were prepared from peripheral venous blood obtained from patients at baseline and 4 and 8 weeks after the initiation of study treatment. Serum was allowed to clot for 1 hour at room temperature and then centrifuged at 3000 rpm for 10 minutes. The resulting serum was stored in aliquots at −80°C until assayed. Plasma tubes were inverted to mix, and were centrifuged at 3000 rpm for 10 minutes. The resulting supernatant was collected and stored in aliquots at −80°C until assayed.

Soluble E-cadherin

Soluble E-cadherin was determined in serum in triplicate by enzyme linked immunosorbent assay using a commercially available kit (R&D Systems, Minneapolis, MN) following the manufacturer's instructions.

MMP-9 and TIMP-1

MMP-9 and TIMP-1 were determined in heparin-plasma using commercially available kits (R&D Systems) following the manufacturer's instructions.

CCL15

A Bio-Plex assay was developed to determine the concentration of CCL15 in plasma. Commercially available mouse anti-human CCL15 antibody was covalently linked to a Bio-Plex bead region 63 with a Bio-Plex amine coupling kit following the manufacturer's instructions (Bio-Rad, Hercules, CA). A biotinylated goat anti-human CCL15 was used as the detection antibody. CCL15 was determined in EDTA plasma. The assay was read in a Bio-Plex 100 (Bio-Rad).

Statistical Analysis

Marker levels were compared between time points (0 versus 4 or 8 weeks) with the paired *t* test. Markers levels at individual time points or marker change scores were compared between pairs of response groups with the two-sample *t* test and were compared between the three response groups with one-way analysis of variance models. All analyses were performed with S-plus version 6 (Insightful Corp., Seattle, WA), and *p* values less than 0.05 were considered significant.

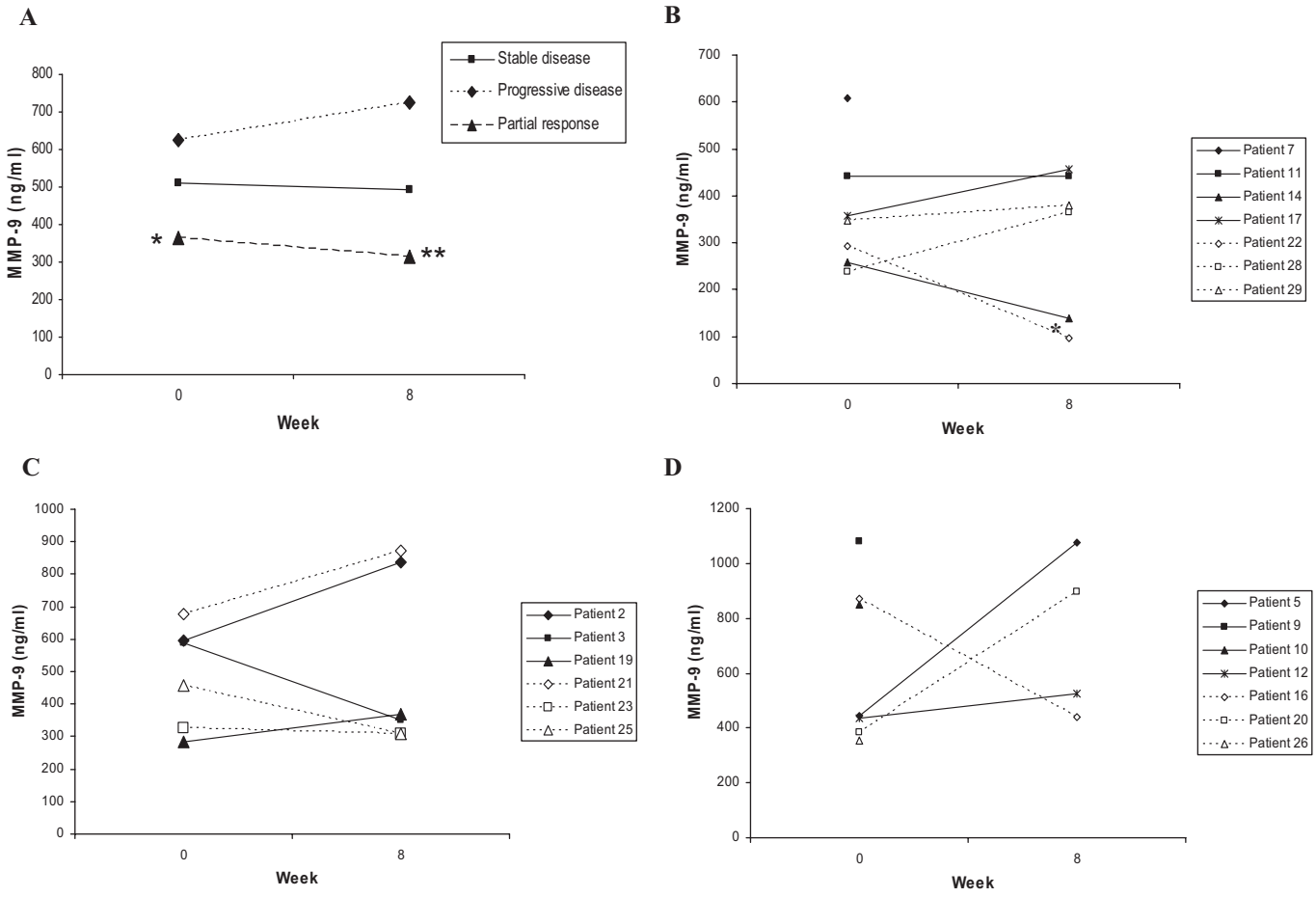

FIGURE 2. MMP-9 is lower at baseline and after 8 weeks in patients treated with celecoxib and erlotinib who achieve partial response. MMP-9 was evaluated by enzyme linked immunosorbent assay in patient serum at baseline and at week 8 of treatment. *A*, All patients at weeks 0 and 8, $*p = 0.006$—baseline values of patients with PR compared with those with SD and PD; $**p = 0.048$—decreased values at week 8 in patients with PR compared with those with SD and PD. Individual patient values for (*B*) patients with PR; (*C*) patients with SD; and (*D*) patients with PD.

TABLE 2. Patient ELISA Data by Response (Average ± SD)

	Partial Response			Stable Disease		Progressive Disease	
Biomarker (ng/ml)	**Week 0**	**Week 8**	***p***	**Week 0**	**Week 8**	**Week 0**	**Week 8**
Soluble E-cadherin	36 ± 8	27 ± 4	0.021[a]	39 ± 9	39 ± 11	36 ± 7	36 ± 8
MMP-9	363 ± 143	315 ± 158	0.048[a] 0.006[b]	510 ± 180	495 ± 248	627 ± 291	724 ± 295
TIMP-1	402 ± 101	314 ± 38	0.047[a]	412 ± 79	459 ± 138	415 ± 53	402 ± 47
CCL15	1.7 ± 0.8	1.0 ± 0.4	0.016[a]	1.7 ± 1.2	2.1 ± 1.8	2.0 ± 1.2	2.0 ± 1.1

[a] Decline at week 8 in patients with partial response compared with those with stable or progressive disease.
[b] Baseline value in patients with partial response compared with those with stable or progressive disease.

RESULTS

Patients

Between August 2003 and June 2005, 22 patients were enrolled in the phase I clinical trial of combination erlotinib and celecoxib.[50] Serum samples were available from all patients. Table 1 lists the patients with the clinical response at the respective dose level.

Decline in Soluble E-Cadherin Correlates with Response

Based on the ability of COX-2 inhibition to decrease PGE2-dependent suppression of E-cadherin levels and in-

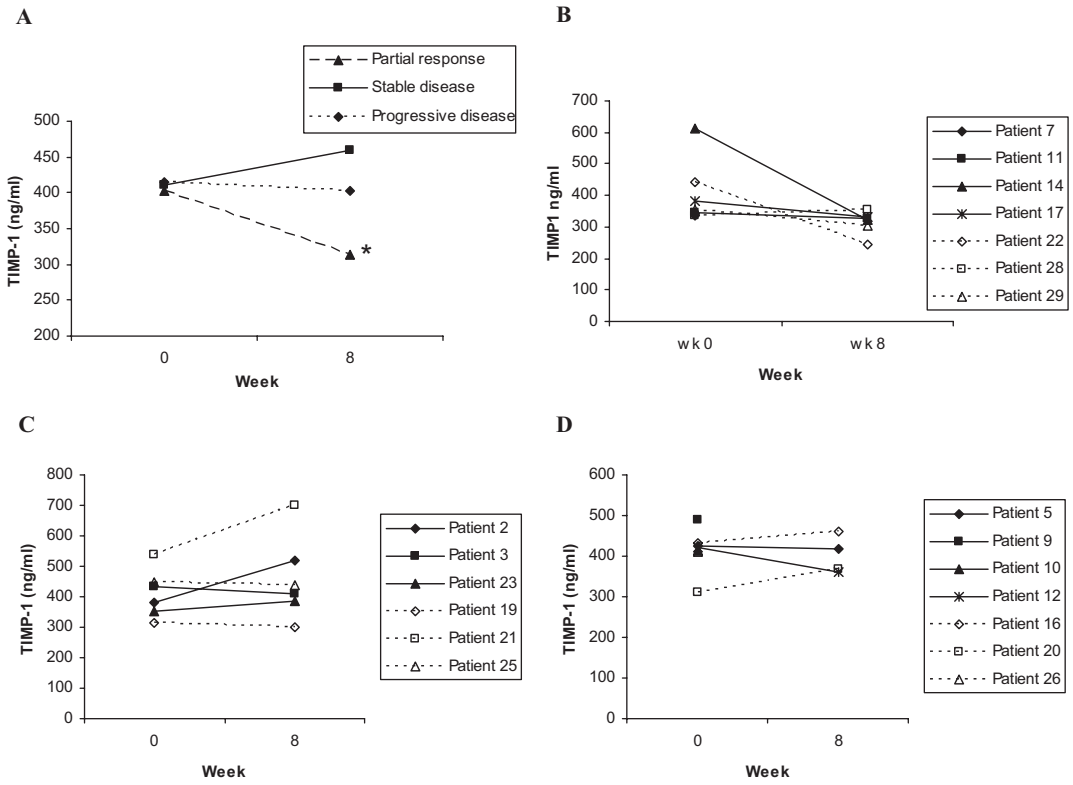

FIGURE 3. TIMP-1 is lower in patients treated with celecoxib and erlotinib who achieve partial response. TIMP-1 was evaluated by enzyme linked immunosorbent assay in patient serum at baseline and at week 8 of treatment. *A,* All patients at weeks 0 and 8, *$p = 0.047$—decreased levels in patients with PR compared with those with SD and PD. Individual patient values for (*B*) patients with PR; (*C*) patients with SD; and (*D*) patients with PD.

crease MMPs in NSCLC,[10] we measured sEC in patient serum at baseline and at weeks 4 and 8 of study treatment. Baseline levels of sEC in patient serum were not significantly different between groups with PR, SD, or PD. The sEC levels at week 8 were significantly lower in patients who achieved a PR when compared with those with SD and PD ($p = 0.017$; Figure 1*A*). In addition, the decline in sEC from baseline to week 8 was significant in patients with PR compared with SD and PD ($p = 0.021$; Figure 1*A*).

Baseline MMP-9 and Change in TIMP-1 Predict Patient Response

The importance of COX-2 regulation of MMPs and TIMPs[28,29,51] and their role in tumor invasion and metastasis led us to investigate MMP-2, MMP-9, and TIMP-1 in patient samples. MMP-9 was lower at baseline in NSCLC patients treated with celecoxib and erlotinib who had a partial response ($p = 0.006$; Figure 2*A*; Table 2). MMP-9 was also lower at week 8 in those patients with PR ($p = 0.048$; Figure 2*A*). In addition, baseline levels of MMP-2 were decreased in patients who achieved a PR, although the p value was not significant (data not shown). In patients who had a partial response, TIMP-1 showed significant reduction at week 8 when compared with patients with SD and PD ($p = 0.047$; Figure 3*A*).

CCL15 Reduction Is a Marker for Partial Response

The capacity of CCR1 and its ligands to modulate MMPs and promote tumor angiogenesis and invasion[45,48] prompted our evaluation of CCL15. CCL15 did not show significant differences in patients at baseline. In patients with PR to combination treatment, CCL15 demonstrated a significant decline from baseline to week 8 compared with patients with SD and PD ($p = 0.003$; Figure 4*A*).

DISCUSSION

Evidence that EGFR and COX-2 have related signaling pathways that can interact to regulate cellular proliferation, migration, and invasion[6–9] has triggered interest in evaluating the combination of COX-2 inhibition and EGFR inhibition in multiple malignancies, including NSCLC. The co-expression of EGFR and COX-2 in human cervical cancer specimens portended a poor prognosis with increased recurrences.[52] When combined COX and EGFR inhibition was evaluated in a familial adenomatous polyposis (FAP) mouse model, treatment resulted in a 95 to 97% reduction in the incidence of colonic polyps.[53] Furthermore, the combination of an EGFR TKI with celecoxib either additively or synergistically inhibited growth of squamous cell carcinoma of the head and neck (SCCHN), induced G1 arrest and apoptosis, and suppressed

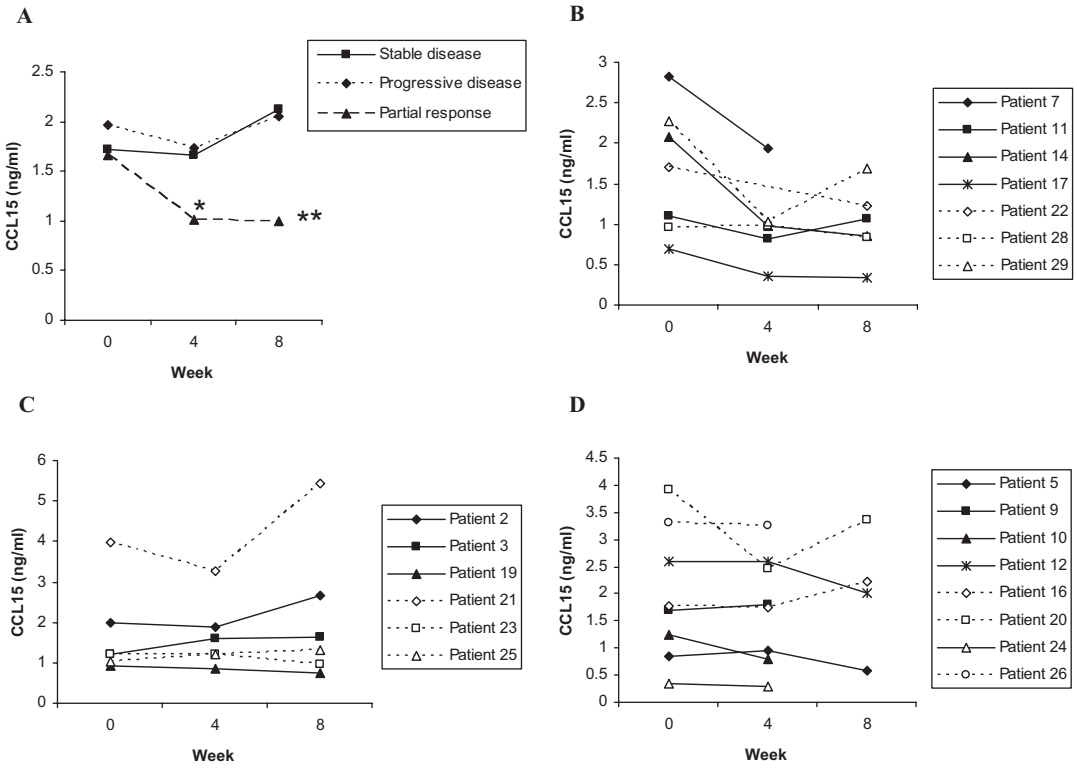

FIGURE 4. CCL15 decreases in patients treated with celecoxib and erlotinib who achieve partial response. CCL15 was evaluated by enzyme linked immunosorbent assay in patient serum at baseline and at week 8 of treatment. *A,* All patients at weeks 0, 4, and 8, *$p = 0.016$—decline in patients with PR compared with those with SD and PD at week 4; **$p = 0.003$—decline in patients with PR compared with those with SD and PD at week 8. Individual patient values for (*B*) patients with PR; (*C*) patients with SD; and (*D*) patients with PD.

endothelial capillary formation.[54] The combination of gefitinib and celecoxib at 400-mg twice daily did not improve responses when compared with gefitinib alone in patients with NSCLC.[55] In addition, the combination of gefitinib and rofecoxib demonstrated similar disease control to expected outcomes with gefitinib alone.[56] Despite these results, the combination of erlotinib and celecoxib in escalating doses with an optimal biologic dose of 600-mg twice daily resulted in a 33% response rate without significant toxicity.[50]

Modulation of EMT in NSCLC has been associated with the sensitivity of tumors to EGFR TKI in NSCLC.[11,57] COX-2 plays an important role in EMT through PGE2-mediated interactions with E-cadherin, MMPs, TIMPs and chemokines.[10,29,51,58] MMP-9 has been shown to be elevated in lung cancer, and overexpression has been associated with poor prognosis.[31,34–37] Although the evaluation of MMP-9 as a predictive marker has been limited, in this study, we found that a low baseline MMP-9 is associated with tumor response to combined erlotinib and celecoxib therapy in NSCLC. Decreased posttreatment levels also correlated with patient response to the combination therapy. In addition, TIMPs have been associated with a decrease in metastatic potential,[28] although multiple studies have identified TIMP-1 as a marker of adverse outcomes in NSCLC.[14,31–33] Our results are consistent with these data. We found that patients with a partial response to combination erlotinib and celecoxib therapy had significantly reduced TIMP-1 levels after 8 weeks on therapy. A decline in markers of EMT and tumor angiogenesis is associated with improved responses to this combination therapy.

Elevated levels of circulating sEC have been associated with poor prognosis in various tumors.[18–24] In a recent study, patients with gastric cancer demonstrated increased levels of sEC when compared with normal control subjects, and treatment of patients with resectable gastric carcinoma with celecoxib resulted in decreased levels of sEC.[59] Celecoxib intervention was associated with increased apoptosis and inhibition of angiogenesis.[59] It is hypothesized that a decrease in sEC in the serum may occur secondary to treatment with celecoxib, and may be a marker for increased sensitivity to EGFR TKI therapy. CCL15 is an additional biomarker associated with tumor progression and angiogenesis, which demonstrated a decline in our patients with a partial response to this combination therapy. Our findings that sEC and CCL15 significantly decline in those patients who achieve a partial response when compared with those with stable or progressive disease suggest that the addition of celecoxib to erlotinib may increase sensitivity to this therapy.

In this study, we demonstrate that baseline MMP-9 levels correlate with tumor response to the combination of erlotinib and celecoxib, and this may be useful as a predictive marker. Other markers of EMT and angiogenesis demon-

strated a change with treatment, which was associated with tumor response in patients. Furthermore, we acknowledge that the associations between patient outcome and tumor biomarkers in the context of this phase I trial are considered hypothesis generating. COX-2 inhibition may enhance the efficacy of EGFR TKI therapy in NSCLC by increasing tumor sensitivity to this therapy. Additional knowledge of biomarkers that have baseline values that are associated with improved outcomes may be useful to identify patients with a greater likelihood of benefiting from this combined therapy. A randomized phase II trial investigating the combination of erlotinib and celecoxib in advanced NSCLC will evaluate these markers in a prospective manner.

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