Can Determination of Circulating Endothelial Cells and Serum Caspase-Cleaved CK18 Predict for Response and Survival in Patients with Advanced Non–Small-Cell Lung Cancer Receiving Endostatin and Paclitaxel–Carboplatin Chemotherapy? A Retrospective Study

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Introduction: Early prediction of the efficacy of a combination of an antiangiogenic drug with cytotoxic chemotherapy is a significant challenge. In that regard, circulating endothelial cells (CECs) and cytokeratins (CKs) seem to reflect their roles in both tumor angiogenesis and tumor cell death.

Methods: Patients with advanced, previously untreated non–small-cell lung cancer were randomly assigned to an endostatin treatment group (paclitaxel + carboplatin + endostatin) and a control group (paclitaxel + carboplatin + placebo). A total of 122 patients were evaluated, of whom 107 had measurements of blood CECs, CK8, caspase-cleaved CK18 (ccCK18), and uncleaved CK18 (CK18) before and at weeks 3 and 6 of treatment, respectively.

Results: Higher baseline CECs in patients with a tumor response (partial remission + stable disease, $p = 0.002$ for the entire group; $p = 0.000$ for the treatment group) were observed. The number of CECs decreased significantly after endostatin treatment ($p = 0.000$), whereas CK levels increased. Increased levels of ccCK18 and CK18, but not CK8, reached significance ($p = 0.001$ and $p = 0.048$, respectively) when compared with the baseline. Tumor response showed a strong correlation with reduction of CECs ($p = 0.000$) and increase of ccCK18 ($p = 0.040$) after endostatin therapy. Cutoff values of changes of CECs and ccCK18 for prediction of survival were 0.58/μl and 19.6 ng/ml, respectively. Reduction of CECs and increase of ccCK18 significantly correlated with longer median survival ($p = 0.013$ and $p = 0.016$ for progression-free survival; $p = 0.009$ and $p = 0.012$ for overall survival, respectively).

Conclusions: CECs and CKs could be biomarkers for selecting patients with non–small-cell lung cancer who will benefit from treatment with endostatin in combination with paclitaxel plus carboplatin.

Key Words: Circulating endothelial cells, Cytokeratins, Endostatin, Biomarker, Non–small-cell lung cancer.

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Non–small-cell lung cancer (NSCLC) accounts for the most cancer deaths worldwide, along with a 5-year survival of only 5% to 15%.[1] Chemotherapy remains an important modality for treatment of this tumor, in particular, for those with advanced disease. However, response rates to chemotherapy are only approximately 30% and have only slightly improved results in recent years, making its treatment a challenge.[2] Antiangiogenic drugs, usually in combination with chemotherapy, are sometimes used. Increasing clinical evidence indicates that such drugs can reduce tumor angiogenesis and inhibit growth of solid tumors.[3–5]

Endostatin, a peptide identified in 1996, specifically acts against tumor-related neovascular endothelial cells (ECs), inducing cancer cell apoptosis.[6] Clinical trials have positively evaluated its application in NSCLC.[7] A previously multicenter, randomized, phase II, double-blind, placebo-controlled study in China evaluated it as first-line therapy for advanced NSCLC and found that paclitaxel–carboplatin with endostatin significantly improved the objective response rate, although neither longer progression-free survival (PFS) nor better overall survival (OS) was achieved.[8]

Paclitaxel, an active agent in NSCLC, not only induces tumor cell apoptosis and necrosis but also has antiangiogenic activity, thereby reducing the number of circulating endothelial cells (CECs).[9] However, it has been difficult to better assess and predict the efficacy of combined antiangiogenesis and paclitaxel-based chemotherapy.[10] Thus, biomarkers may be useful.

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Neovascularization in tumor vessels is generated by mature ECs. Thus, the role of CECs and circulating progenitor (CEP) cells in cancer growth and their measurement as biomarkers in antiangiogenic cancer therapy is important. It has been demonstrated that CEC levels are increased in various cancers at diagnosis and subsequently reduced, even close to normal, in patients achieving complete remission.[11] In patients with metastatic breast cancer, for example, treated with low-dose metronomic chemotherapy for 2 months with continuous therapy, CEC levels were a predictor of disease-free survival (DFS) and OS after a 2-year follow-up.[12] Previous investigations showed that levels of CECs and CEPs were strongly elevated in patients with advanced NSCLC. CEC count at baseline was significantly higher in patients with NSCLC than in healthy volunteers. Pretreatment CEC values were significantly higher in patients experiencing a clinical benefit (partial response, stable disease) than in patients with progressive disease. A statistically significant decrease in CECs on day 22 was observed only in patients with a partial response.[13] Significantly increased CEP levels were noted before the first and second cycles of chemotherapy. Although there was a decrease of CEPs noted on the eighth day after treatment, this did not predict an improvement in PFS.[14]

Serum levels of various cytokeratins (CKs) have been proposed as biomarkers of chemotherapy-induced tumor cell death.[15,16] Reported NSCLC CKs have mainly been CK7, CK8, CK18, and CK19.[17] Increased CK8 and CK18 have also been associated with tumor progression and decreased survival.[18,19] The latter two CKs are coexpressed and constitute the primary keratin pair on one-layered epithelial cells.[20] They are released into the circulation, remaining in a relatively stable state in either their intact or caspase-cleaved forms during necrotic and apoptotic cell death. Extracellular release of CK8 in NSCLC caused by apoptosis is full length. Measurement of molecular subtypes of CK18 can identify epithelial-derived cell death. For example, caspase-cleaved CK18 fragments (ccCK18), a result of apoptosis, and uncleaved CK18 (CK18) are released from necrotic cells. Thus, CK18s are potentially both quantitative and qualitative biomarkers for cell death.[21] In fact, plasma CK18 levels have been used as markers of response to chemotherapy in patients with gastrointestinal adenocarcinomas.[22]

To investigate whether there are biomarkers for selecting patients with NSCLC who might benefit from continued endostatin in combination with paclitaxel plus carboplatin,[8] we evaluated serum CK8, ccCK18, CK18, and CECs in patients with advanced NSCLC receiving one of two randomized treatments. Serum levels of CKs and CECs at baseline, week 3, and week 6 after therapy were measured to assess their predictive or monitoring for tumor response and possible correlation with survival.

MATERIALS AND METHODS

Patient Selection

This was a randomized, double-blind, placebo-controlled, multicenter phase II trial. Inclusion criteria, study design, and informed consent and ethics were described previously[8] One hundred and twenty-six patients with previously untreated and advanced NSCLC were enrolled and randomly assigned to the treatment group ($n = 63$) and the control group ($n = 63$). One patient in each group withdrew before treatment, and one patient in each group was excluded because of paclitaxel allergy. Thus, by intent to treat, 122 patients were included in the efficacy analysis, 61 in each group.[8]

Therapy Strategy

Patients enrolled were randomly divided into endostatin group (paclitaxel plus carboplatin plus endostatin) and control group (paclitaxel plus carboplatin plus placebo). Recombinant human endostatin was provided by Shandong Simcere-Medgenn Bio-Pharmaceuticals (National Medicine Permit No. S20050088).[8]

Treatment schemes were described previously.[8] Paclitaxel (175 mg/m^2, d1) and carboplatin (area under the curve [AUC] = 5, d1) were administrated on the first day of each cycle with endostatin or placebo by intravenous infusion on days 8 to 21, with 21 days per cycle. Treatment was continued until progressive disease (PD) or unacceptable toxicity to a maximum of four cycles. Tumor size was determined by computed tomography scans at baseline and after each cycle of chemotherapy. Patient response was evaluated according to Response Evaluation Criteria in Solid Tumors criteria: complete response (CR), partial remission (PR), stable disease (SD), and PD.[23] Toxicity was assessed once a week and recorded according to World Health Organization toxicity criteria.[24]

Blood Collection and Evaluation of CECs and CKs

Five patients in the treatment group and four in the control group declined to provide blood samples. Blood samples before or after treatment from two patients in the endostatin group and four in the control group could not be successfully obtained. Therefore, a total of 107 patients were included in the final analysis, 54 in the endostatin, and 53 in the control groups. Peripheral blood was taken from patients on three occasions: at baseline and at weeks 3 and 6, for CK and CEC determination.

Blood was collected in ethylenediaminetetraacetic acid tubes, with circulating CECs being measured using a full blood-flow cytometric method.[12] CECs were defined: negative for hematopoietic marker CD45, positive for endothelial marker P1H12, negative for progenitor marker CD133.[12] A volume of 1.5 ml full blood was used for flow cytometry, and appropriate IgG isotypes were used as control. Cell suspensions were evaluated after red cell lysis with lysing solution (Beckman Coulter). Antibodies were from Becton Dickinson (USA), with anti-CD45 used to exclude hematopoietic cells and anti-CD133 to exclude progenitor cells P1H12 (CD146, an EC marker). Using FL-3/SSC gating strategy, acquisition was performed by flow cytometry (Becton Dickinson, FACSCalibur) equipped with a 488-nm argon-ion laser. Software-Cellqust (Becton Dickinson) was used to analyze sample data (Fig. 1).

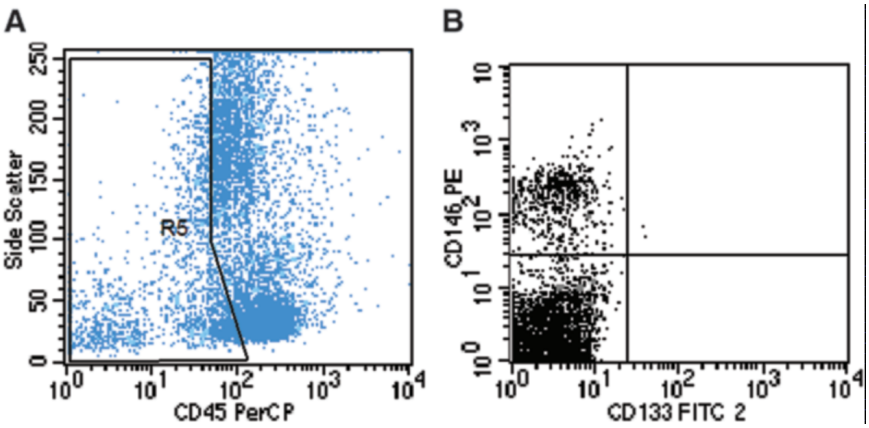

FIGURE 1. Representative CEC enumeration by flow cytometry. *A,* Panels showing the gate used to exclude CD45– positive cells. *B,* Panels showing the gate used to count CECs (defined as CD45– CD146+ CD133–). CEC, circulating endothelial cell.

Sera were collected at baseline and at weeks 3 and 6. Samples were stored at −80°C until analysis. Serum CK8, ccCK18, and CK18 levels were measured with enzyme-linked immunosorbent assay kit (R&D, China). Concentration of CK antigens was expressed as ng/ml.

Statistical Analysis

Statistical analysis was performed using SPSS13.0 version. Nonparametric statistics were used because of nonnormal distribution of the study parameters. Mann–Whitney *U* test was used to test for correlations of biomarkers and tumor response between the two groups. Wilcoxon matched-pair signed ranks test was used to examine two related samples represented by CECs and CK antigens before and after chemotherapy. Pearson's correlation test was used when determining an association between changes of CECs and CK antigens. To evaluate values of serum level of CK antigens and CECs in the prediction of patient survival, receiver operating characteristic (ROC) curve analysis was performed. Correlations with PFS and OS were determined with the Kaplan–Meier method and log-rank statistic. All tests were used to assess the prognostic significance of parameters taken in association, using a two-sided level of 0.05.

RESULTS

Patient Characteristics

Patient characteristics are shown in Table 1. No patients died during the course of the 6 weeks. All tested individuals had blood samples taken at the various sampling stages, except one patient who showed PD at week 3. His blood samples were taken at baseline and week 3 and included in the analysis.

Serum CK8, ccCK18, CK18, and CECs before Treatment

Serum baseline CK8, ccCK18, and CK18 levels demonstrated median values for CK8 of 30.28 ng/ml (range, 4.43–120.43 ng/ml), ccCK18 of 64.60 ng/ml (range, 7.02–170.15 ng/ml), and CK18 of 417.48 ng/ml (range, 104.47–2164.62 ng/ml). Pretreatment CECs ranged from 0.15 to 2.50/μl (median 1.07/μl).

There was no significant association of serum CK8, ccCK18, CK18, or CEC levels at baseline with patient demographic factors, including age, sex, histology, staging, smoking history, or Eastern Cooperative Oncology Group status in either the entire group or the two treatment groups (data not shown).

No association between tumor response and the various CK levels at baseline was observed. Median CECs at baseline for the entire group, however, were statistically higher (1.12/μl versus 0.33/μl) in patients who subsequently developed PR/SD (n = 90) than in the patients with PD (n = 17, p = 0.002). This was because of the endostatin group, where there was a significant relationship between pretreatment CEC levels and tumor response (n = 47 for PR/SD, n = 7 for PD, p = 0.000), whereas in the control group, a significant relationship between tumor response and baseline CEC levels was not noted. (n = 45 for PR/SD, n = 8 for PD, p = 0.066).

Changes of Serum CK8, ccCK18, CK18, and CECs during Treatment

In the control group, CK levels after chemotherapy were stable or lower than at baseline, whereas in the endostatin group, CK levels gradually increased with therapy. Of the three CKs subtypes, ccCK18 and CK18 increases at week 6 reached significance (p = 0.001 and p = 0.048, respectively). Furthermore, when comparing CK levels between endostatin and control groups, CK levels at two different time points, 3 and 6 weeks after treatment, were significantly higher in the endostatin group (week 3: p = 0.003 for CK8; p = 0.008 for ccCK18; p = 0.356 for CK18 [not significant]. Week 6: p = 0.006 for CK8; p = 0.007 for ccCK18; p = 0.028 for CK18 [see Fig. 2A]).

The CEC counts of endostatin group decreased significantly at both 3 weeks (p = 0.015) and 6 weeks (p = 0.000). Similarly, when also compared with control group, CECs in the endostatin group showed a significant decrease at week 6 (p = 0.054 at week 3, p = 0.000 at week 6, Fig. 2*B*).

Correlation of Change of Serum CK8, ccCK18, CK18 and CEC Levels with Tumor Response

Objective tumor response data were available from all 107 patients, including 22 with PR, 25 with SD, and 7 with PD in the endostatin group (Table 1). ccCK18 increase from baseline to week 6 was significant in treatment patients who achieved PR/SD when compared with PD (p = 0.040, Fig. 3A). However, in contrast, no statistical response correlations were

TABLE 1. Clinical Characteristics of the Patients with NSCLC

Characteristics	Paclitaxel/Carboplatin + Endostatin(n = 54)	Paclitaxel/Carboplatin + Placebo(n = 53)
Age, range(yr)	57.35 (39–79)	57.4 (29–75)
Sex, *n* (%)		
Male	37 (68.5)	41 (77.4)
Female	17 (31.5)	12 (22.6)
Smoking		
Yes	27 (50.0)	29 (54.7)
No	27 (50.0)	24 (45.3)
Baseline ECOG performance status, *n* (%)		
0	7 (13.0)	6 (11.3)
1	47 (87.0)	47 (88.7)
Pathological subtype, n (%)		
Adenocarcinoma	37 (68.5)	41 (77.4)
Squamous-cell carcinoma	17 (31.5)	12 (22.6)
Clinical stage, *n* (%)		
IIIb	23 (42.6)	20 (37.7)
IV	31 (57.4)	33 (62.3)
Chemotherapy outcome, *n* (%)		
PR	22 (40.7)	12 (22.6)
SD	25 (46.3)	33 (62.3)
PD	7 (13.0)	8 (15.1)
Median PFS, range (m)	6.5 (0.7–31.6)	6.0 (1.0–24.6)
Median OS, range (m)	17.6 (4.5–31.8)	18.7 (2.3–33.4)

NSCLC, non–small-cell lung cancer; PR, partial remission; SD, stable disease; PD, progressive disease; ECOG, Eastern Cooperative Oncology Group; PFS, progression-free survival time;OS, overall survival time.

observed in CK8, CK18, or the control group after two cycles of therapy. Tumor response in the endostatin group, however, showed a strong correlation with reduced CEC at week 6 (p = 0.000, Fig. 3*B*).

Association between Change of ccCK18, CK18, and CECs and Patient Survival

In our previous report,[8] paclitaxel–carboplatin plus endostatin showed an improvement in overall response rate (p = 0.078) but failed to reach a survival benefit. In the current study, however, whether biomarker changes could predict patient survival was addressed.

The relationship between increased ccCK18 and patient survival (PFS and OS) was analyzed by ROC in the endostatin group. The optimal cutoff value for prediction of survival was 19.6 ng/ml, where AUC = 0.704 (95% CI = 0.559–0.849). Sensitivity and specificity of the ccCK18 endostatin-induced increase were calculated using ROC analysis for survival. The result was 70% for sensitivity and 61% for specificity (Fig. 4A).

Median PFS and OS for the patients treated with endostatin were 6.5 and 17.6 months, respectively. The relationship between ccCK18 and survival was analyzed by the Kaplan–Meier analysis. With a cutoff of 19.6 ng/ml, patients with a lesser ccCK18 increase (n = 26) had a median survival (MS) of 5.5 months for PFS and 14.9 months for OS. However, those with a greater increase (n = 27) survived for medians of 9.1 and 22.6 months for PFS and OS, respectively. This translated into a significant survival benefit for those with the higher ccCK18 increase (p = 0.016 for PFS; p = 0.012 for OS) (Fig. 4*B* and *C*).

Similarly, the relationship between decreased CEC and patient survival (PFS and OS) was analyzed. ROC analysis revealed the optimal cutoff value for prediction of survival as 0.58/μl, where AUC = 0.759 (95% CI = 0.629–0.889, Fig. 5A). Sensitivity and specificity of the endostatin-induced CEC decrease were calculated using ROC analysis for survival. They were 60% and 88%, respectively. With a cutoff value of 0.58/μl, patients with a less CEC reduction (n = 34) had a 5.0-month PFS and 10.6-month median OS, whereas those with a greater CEC reduction (n = 19) had a 9.8-month PFS and 23.9-month median OS. This demonstrated that MS with the greater reduction of CECs after endostatin treatment was significantly longer (p = 0.013 for PFS, p = 0.009 for OS) (Fig. 5*B* and *C*). However, in the control group, changes in CEC and CK18 levels failed to predict PFS or OS (data not shown).

Correlation of Serum ccCK18 and CK18 Levels and CECs during Therapy for the Entire Group

Correlation of dynamic changes of CKs and CECs during therapy in the 107 patients was evaluated. In the entire group, strong correlation was observed between the increase of ccCK18 levels and the reduction of CECs (r = −0.203, p = 0.048; Fig. 6A). The increase of ccCK18 and CK18

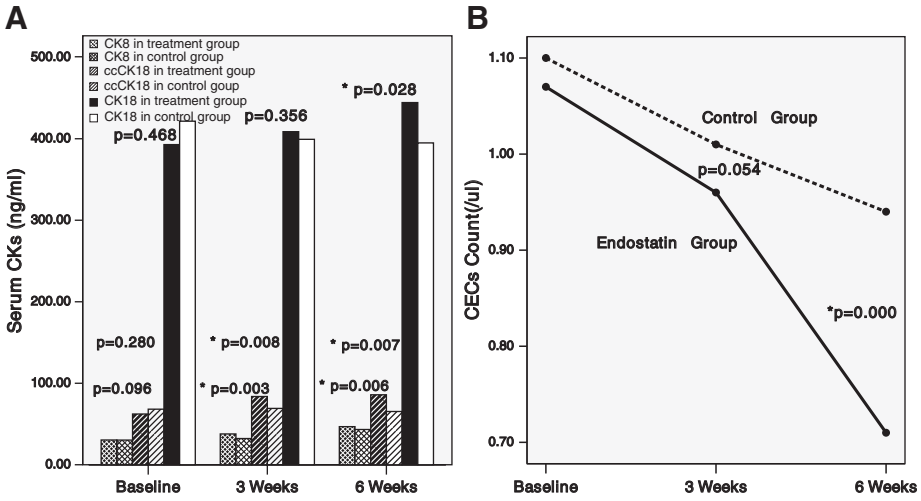

FIGURE 2. Dynamic changes of serum CK and CEC counts before and during treatment course at weeks 3 and 6. *A*, No significant difference observed in CKs between two groups. After endostatin therapy, serum CK levels showed gradual increase. Of the three CKs, increased ccCK18 and CK18 levels at week 6 reached significance in endostatin group ($p = 0.001$ and $p = 0.048$, respectively). Furthermore, when comparing serum CK changes between therapy courses of endostatin group and control groups, CK levels at weeks 3 and 6 were significantly increased ($p = 0.003$ for CK8, $p = 0.008$ for ccCK18, $p = 0.356$ for CK18 at week 3; $p = 0.006$ for CK8, $p = 0.007$ for ccCK18, $p = 0.028$ for CK18 at week 6, respectively). *B*, CECs decreased significantly after 6 weeks of endostatin therapy when compared with baseline ($p = 0.000$). Similarly, when compared with control group, CECs in the endostatin group showed significant decrease at week 6 ($p = 0.000$). CEC, circulating endothelial cell; CKs, cytokeratins; ccCK18, caspase-cleaved cytokeratins.

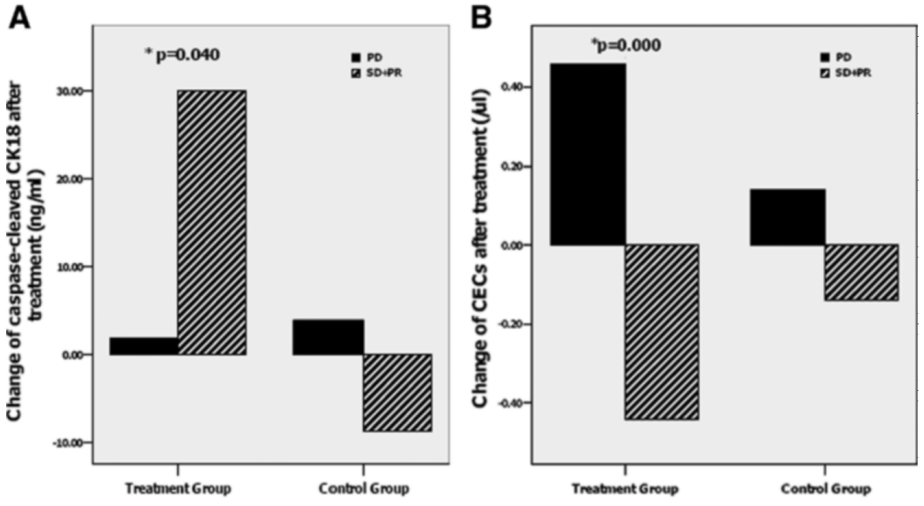

FIGURE 3. Correlation of changes of serum ccCK18 and CECs with tumor response. *A*, Increase of caspase-cleaved CK18 level at week 6 was significant in patients treated with endostatin achieving PR/SD when compared with PD ($p = 0.04$). *B*, Strong correlation of tumor response with CEC reduction at 6 weeks treatment with endostatin ($p = 0.000$). CEC, circulating endothelial cell; CKs, cytokeratins; ccCK18, caspase-cleaved cytokeratins; PR, partial remission; SD, stable disease; PD, progressive disease.

synchronously occurred during therapy and the correlation between them was significant ($r = 0.260$, $p = 0.010$; Fig. 6*B*).

DISCUSSION

With increasing use of antiangiogenic therapy for cancer, conventional measurement tools or biomarkers might not be sufficient to evaluate clinical efficacy of these targeted drugs. New surrogates for pharmacodynamic markers reflecting drug activity are much needed.

In this study, we measured and compared serum levels of CECs, CK8, and two different subtypes of CK18 (caspase-cleaved CK18 and uncleaved CK18) in patients with advanced NSCLC treated with paclitaxel plus carboplatin combined with or without the antiangiogenic agent, endostatin. The specificity of changes in serum CECs and CK levels with endostatin treatment and their relationship to tumor response and survival was also evaluated.

The data showed that baseline CECs seemed to predict treatment outcome in the 107 patients with advanced NSCLC. Patients presenting with lower CEC levels tended to have PD in both treatment arms. One can only speculate about the basis of this finding. Baseline CEC levels might then be an indication for the general use, or not, of antiangiogenic agents. It was also found that the level of CECs decreased significantly after 6 weeks in the endostatin-treated group compared with the control. Reduction of CECs during endostatin therapy

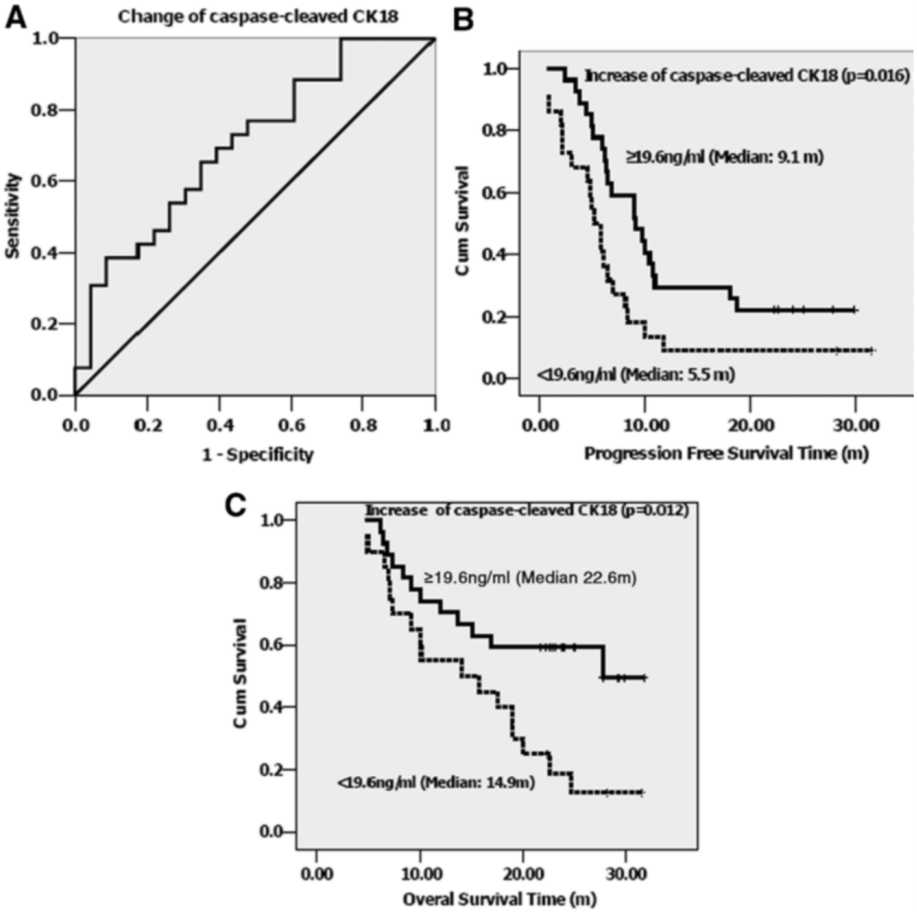

FIGURE 4. Association between increase of ccCK18 and patient survival in the endostatin group. *A*, Best cutoff value of ccCK18 increase for prediction of survival was 19.6 ng/ml where AUC = 0.704 (95% CI = 0.559–0.849). Sensitivity and specificity of ccCK18 increase after endostatin therapy calculated using ROC analysis for survival were 70% and 61%, respectively. *B* and *C*, When cutoff value of 19.6 ng/ml was used, patients whose ccCK18 levels increased less (n = 26) presented shorter median survival (5.5 months for PFS, 14.90 months for OS). Those with significantly increased ccCK18 antigen levels (n = 28) survived 9.1 months for PFS and 22.6 months for OS. Median survival of patients with increased ccCK18 after endostatin treatment was statistically longer than those with less increase ccCK18 (p = 0.016 for PFS, p = 0.012 for OS). ccCK18, caspase-cleaved cytokeratins; ROC, receiver operating characteristic; PFS, progression-free survival; OS, overall survival; AUC, area under the curve.

might thus be a predictor of continued therapy, outcome, and survival. Moreover, it was found that a greater CEC reduction indicated that patients treated with endostatin were more likely to have PR/SD (p = 0.000). Specifically, analysis revealed that patients with CECs reduction of more than 0.58/μl during endostatin therapy had better PFS and OS (9.8 versus 5 months, p = 0.013 for PFS; 23.9 versus 10.6 months, p = 0.009 for OS).

It has been unclear whether serum biomarkers can assist in determining treatment response. The question remains whether the parameters of drug-induced cell death can show a significant correlation with tumor response. In patients with locally advanced breast cancer, a significant correlation was seen between an increase in apoptosis index and clinical response to neoadjuvant chemotherapy.[25] However, in one lung cancer study, no association was found between postchemotherapy ccCK18 levels and survival.[18] The current study failed to prove outcome predictability of baseline CKs. However, it was demonstrated that the degree of increase of ccCK18 at week 6 of endostatin therapy seemed to reflect a drug-induced tumor response (p = 0.040). Although CK8, ccCK18, and CK18 levels increased significantly after 6 weeks treatment with endostatin compared with controls, only ccCK18 in the treatment group had a statistically significant increase in PR/SD (p = 0.04). Moreover, patients with levels of ccCK18 that increased more than19.6 ng/ml with endostatin therapy at 6 weeks showed significantly better PFS and OS (9.1 versus 5.5 months, p = 0.016 for PFS; 22.6 versus 14.9 months, p = 0.012 for OS). This suggests that the increased ccCK18 in patients with a clinical benefit was caused by an increase in endostatin-induced apoptotic-derived ccCk18.

Our study showed that CEC reduction during therapy significantly correlated with an increase in ccCK18 (r =−0.203, p = 0.048). In addition, the increases of ccCK18 and CK18 were synchronous during therapy, with their correlation being significant for the entire group (r = 0.260, p = 0.01). Finally, this seems to demonstrate that chemotherapy, both with and without endostatin, leads to ccCK18 and CK18 release from tumor cell death, and that CECs are reduced accordingly.

Serum CK8 and CK18 assays have also recently been found to be clinically useful biomarkers because of features such as epithelial specificity and their abundant expression in epithelial cells and complexes.[15] Caspase-cleaved CK18 can be easily identified by use of monoclonal antibody M30, which recognizes a neoepitope of CK18 generated during apoptosis.[26] Therefore, measurement of such CKs may also facilitate both prediction and prognosis in patients being treated for cancer. With regard to the use of CK18 as a tumor response biomarker, there is a concern as to whether circulating CK18 is a real reflection of its release from tumor cells, instead of other cells. Observations from previous studies, however, suggest that CKs are, in fact, derived from tumor cells. For example,

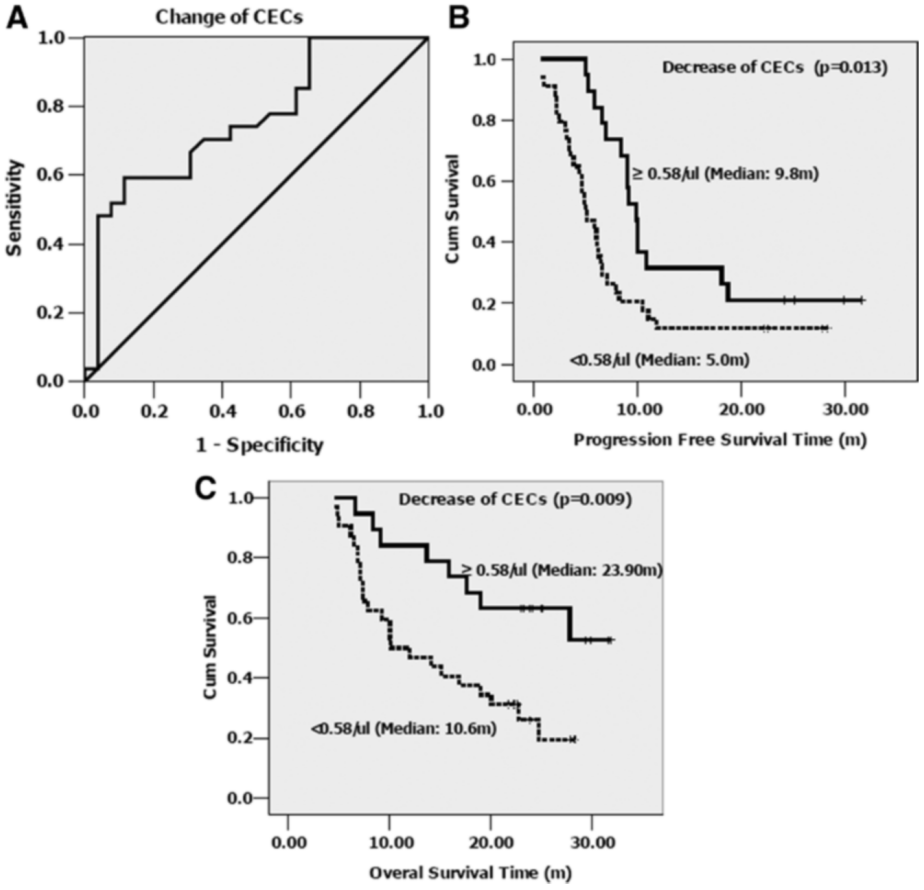

FIGURE 5. Association between CEC reduction and patient survival in the endostatin group. *A,* ROC analyses revealed best cutoff value for prediction of survival of 0.58/μl, where AUC = 0.759 (95% CI = 0.629–0.889). Sensitivity and specificity of CEC reduction after endostatin therapy calculated using ROC analysis for survival were 60% and 88%, respectively. *B* and *C,* When cutoff value of 0.58/μl was used, patients with CEC reduction less than 0.58/μl ($n = 34$) had a PFS of 5 months and OS of 10.6 months; those with CEC reduction more than 0.58/μl ($n = 20$) had a PFS of 9.8 months and an OS of 23.9 months. Median survival of patients with greater CEC reduction endostatin significantly longer than those with fewer reduction ($p = 0.013$ for PFS, $p = 0.009$ for OS). CEC, circulating endothelial cells; ROC, receiver operating characteristic; CI, confidence interval; AUC, area under the curve.

CK18 levels generally decreased after surgical removal of tumors.[27] Higher serum CK18 levels were detected in local tumor veins compared with peripheral blood in the same patients with endometrial carcinoma.[15] A strong association between the amplitude of docetaxel-induced increases in ccCK18/CK18 and tumor load in patients with prostate cancer suggested that CK18 originated from the tumor de novo.[28] An association between CK18 markers and number of circulating tumor cells in patients treated with platinum-based therapy has been demonstrated.[29] It is, therefore, likely that increased serum CK18 levels in patients are due to release by the tumors. Moreover, the relationship between CK18 increases and tumor response observed in various studies also indicates that CK18 is derived from tumor cells.[22,30,31]

However, it has also been noted that circulating CK18 increases might be derived from drug overexposure and thus could represent toxicity. Hepatocytes express CK18, and liver toxicity induced by cancer therapeutics may release CK18. Thus, it may be advisable to determine whether an agent can induce liver toxicity before clinical studies if CK18 markers are being used to monitor treatment response. Alternatively, it is beneficial to measure liver enzymes, such as aspartate aminotransferase (AST)/alanine aminotransferase, in parallel with CK18 levels, to observe liver toxicity in clinical studies.

In this study, liver toxicity was not a common side effect induced by endostatin. AST/alanine aminotransferase was measured at baseline and at weeks 3 and 6 in parallel with CK18 levels. No grade 3 or grade 4 liver toxicity was observed. Only two patients, one in the endostatin group, the other in the control group, had a slight increase of AST (grade 1) after the first cycle of treatment. Using compound glycyrrhizin, enzyme levels in the two were close to normal within 1 week.

CKs are expressed in cells and released when cell membrane integrity is severely damaged, thereby leading to apoptosis or secondary necrosis. It has been noted that peak levels of ccCK18 and CK18 were often found within 1 to 3 days after administration of chemotherapy.[28,31] In fact, during subsequent courses, an overall decrease was observed, with the superposition of peaks for both ccCK18 and CK18.[16] Therefore, to emphasize the cumulative effect of the antiangiogenic agent in combination with chemotherapy and to focus on the predictive or monitoring value of CEC and CK level for tumor response and potential correlation with survival, we only observed CEC and CK levels before and after each cycle instead of shortly after treatment in this study. Our data showed that this assay protocols would be more feasible and hold clinically efficient value.

In conclusion, there seems to be a significant response and survival correlation between serum ccCK18 and CEC levels in patients with advanced NSCLC treated with paclitaxel/carboplatin plus the antiangiogenic agent, endostatin. Higher baseline CECs, a greater increase of ccCK18 and a reduction of CECs with such treatment seems to represent

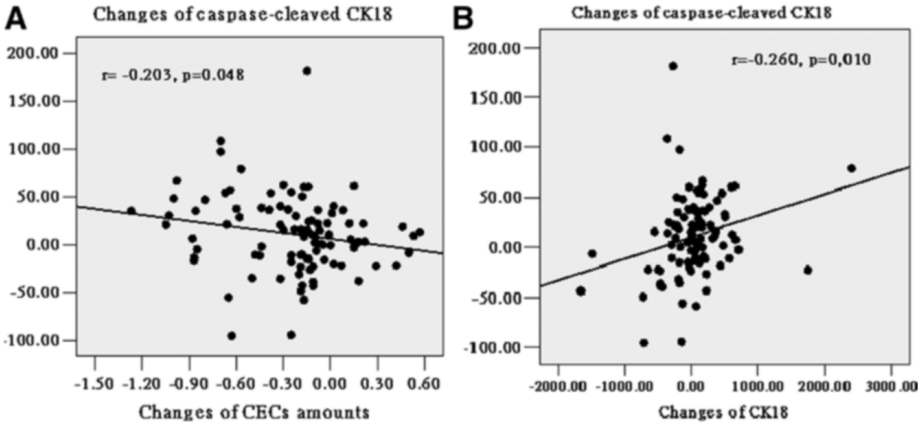

FIGURE 6. Correlation of serum CK levels and CECs during therapy. In the entire group, strong correlation was observed between the increase value of serum ccCK18 levels and the reduction amount of CECs ($r = -0.203$, $p = 0.048$, Fig. 5*A*). However, the increase of ccCK18 and CK18 levels presented to be synchronal during therapy courses, and the correlation between them also reached significance ($r = 0.260$, $p = 0.010$, Fig. 5*B*). CEC, circulating endothelial cell; CKs, cytokeratins.

an indirect indicator of tumor cell death and predictor of a better response and longer survival. Selecting patients with high CECs could prove helpful for decision-making to determine therapy modality. However, prospective studies will be required to confirm the value of these biomarkers during anti-angiogenic therapy.

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