Original article

Soluble CD40 in plasma and malignant pleural effusion with non-small cell lung cancer: A potential marker of prognosis

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Abstract

Objective: Soluble CD40 (sCD40) is a potential modulator for both antitumor responses and CD40-based immunotherapy; however the levels and significance of sCD40 in non-small cell lung cancer (NSCLC) patients with malignant pleural effusion are unknown.
Methods: Forty-eight patients with lung cancer were treated in our institutions from January 2008 to January 2010. Peripheral blood and pleural effusion samples were collected from each subject. sCD40 levels in plasma and malignant pleural effusions supernatant were measured. The CD40L expression on CD3t T-cells was confirmed by flow cytometric direct immunofluorescence analysis. All patients were followed up after the study ended on January 1, 2010.
Results: Patients with malignant pleural effusion of NSCLC had elevated circulating and pleural effusion levels of sCD40, and these elevated sCD40 levels were associated with advanced diseases and a poor prognosis.
Conclusions: These findings indicate that elevated sCD40 may have a role in modulating antitumor responses and may also be a useful prognostic marker.

Introduction

Malignant pleural effusions (MPE) worsen the clinical course of patients with lung cancer.[1,2] The majority of the MPEs develop when tumor cells directly infiltrate the pleura. Due to mediastinal lymph node or bronchial obstruction, pulmonary embolism or superior vena cava syndrome, pleural effusion may also occur in patients with indirect malignant diseases.[3] CD40 is best appreciated as a critical regulator

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of cellular and humoral immunity via its expression on B lymphocytes, dendritic cells, and monocytes.[4,5] CD40 is also expressed on the surface of many other normal cells. The global physiologic effect of the CD40 signaling pathway is profound.[4] CD40 ligand (CD40L), which is also known as CD154, is the chief ligand described for CD40 and is expressed primarily by T lymphocytes and platelets. Atherosclerosis, graft rejection, coagulation, infection control, and autoimmunity are all regulated by CD40-CD40L interactions.[5,6] In the present study, we provide data about sCD40 levels in NSCLC patients with malignant pleural effusion and analyze the relationship between the levels of sCD40 and the prognosis of patients.

Materials and methods

Patients and study design

Forty-eight patients with lung cancer were treated in our institutions from January 2008 to January 2010. All patients (28 males, 20 females with an average age of 64.4 years, ranging from 35 to 82 years old) were histologically diagnosed with lung cancer and pathologically staged according to the Tumor-Node-Metastasis (TNM) classification.[7] Among them, 13 patients were stage I and II, and the other 35 patients were stage III and IV. No patients had chemotherapy or immunotherapy within the six weeks preceding the study. Out of 48 patients, 29 of them with MPE (when malignant cells were detected in the pleural fluid or in the pleura with a cytopathological examination) were enrolled in this study, 17 patients had Performance Status (PS) = 0–1, 12 patients had PS ≥2. Two samples of peripheral blood were successfully obtained from 26 patients before and after two cycles of chemotherapy. Eighteen patients experienced progressive disease (PD) and eight patients experienced stable disease and partial remission (SD + PR). The same procedure was also performed in 15 peripheral blood samples from healthy volunteers.

Enzyme immunoassays and flow cytometric immunofluorescence analysis

Peripheral blood and pleural effusion samples were withdrawn from each subject after they signed an informed consent, and anticoagulated in Natrium Citrate. Samples were immediately centrifuged at 3000 r/min for 10 min. sCD40 levels in plasma and MPE supernatant were measured with an sCD40 enzyme-linked immunosorbent assay kit (eBioscience,

USA) according to the manufacturers' instructions. Peripheral blood mononuclear cells were obtained by Hypaque-Ficoll gradient centrifugation and then washed. The CD40L expression on $CD3^+$ T-cells was confirmed by flow cytometric direct immunofluorescence analysis, using CD40L-polyethylene (PE) and CD3-fluorescein isothiocyanate (FITC) monoclonal antibodies (BioLegend, USA). All samples were assayed twice.

Follow-up

All patients were followed up after the study ended on January 1, 2010 for at least three months unless deceased occurred. Before the end of the study, the patients (or their families) were contacted by telephone to assess the survival status or the date of death.

Statistical analysis

All analyses were performed using GraphPad Prism 5.0 (GraphPad Software, USA); Pearson's correlation, the t-test, one-way Analysis of Variance (ANOVA), and Log-rank (Mantel–Cox) Test were used. Statistical significance was assumed if a P-value (two tailed) was less than 0.05. Data are expressed as mean ± standard deviation (SD).

Results

sCD40 levels in patients' plasma with different TNM stages and PS score

Levels of sCD40 in the plasma of patients with lung cancer were higher than in healthy volunteers (249.60 ± 40.37 pg/ml vs. 128.70 ± 16.49 pg/ml, $P = 0.002$). Different sCD40 levels were detected in the patients' plasma with different TNM stages. The sCD40 levels in plasma from patients who at stage III–IV, 310.00 ± 51.55 pg/ml ($n = 35$), were higher than the levels in patients with stage I–II disease, 87.08 ± 16.74 pg/ml ($n = 13$) (Fig. 1A). The sCD40 levels from patients with the most advanced lung cancer stage IV ($n = 29$) whose PS ≥2 was 521.7 ± 124.7 pg/ml ($n = 12$), higher than in patients with PS = 0–1, 216.00 ± 30.76 pg/ml ($n = 17$) (Fig. 1B).

Different sCD40 levels pre-chemotherapy and post-chemotherapy

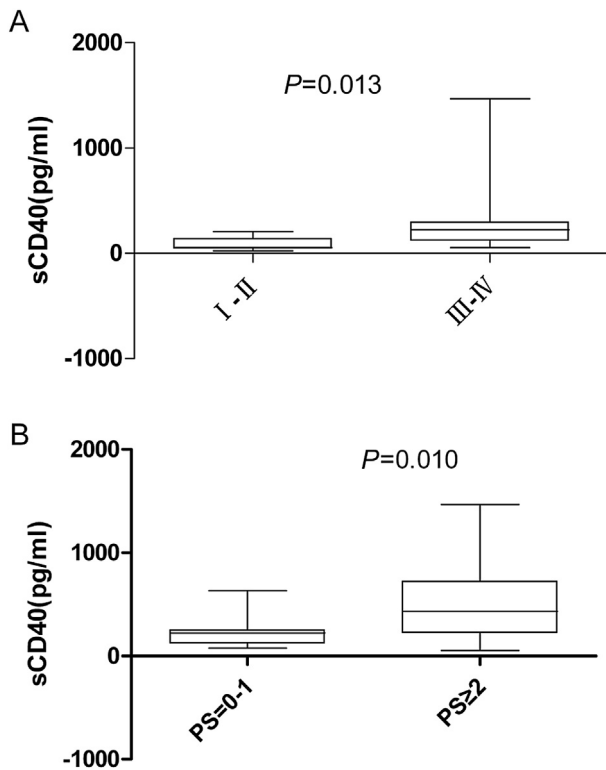The sCD40 level of patients after two cycles of chemotherapy was higher, 193.70 ± 13.17 pg/ml

Fig. 1. Comparison of levels of sCD40 in patients with different TNM stages (A) and PS scores (B).

Fig. 2. Different sCD40 levels pre-chemotherapy and post-chemotherapy (A), PD and SD + PR (B).

(n = 26) than before chemotherapy, 142.00 ± 14.13 pg/ml ($n = 26$) (Fig. 2A). The sCD40 levels in patients who experienced PD before chemotherapy were higher, 161.40 ± 5.05 pg/ml ($n = 18$) than in patients who experienced SD + PR, 98.44 ± 26.21 pg/ml ($n = 8$) (Fig. 2B).

Relationship among sCD40 levels, CD40L$^+$/CD3$^+$ T-cell ratios, and ADA levels in pleural fluid

The sCD40 levels in pleural fluid were negatively correlated with CD40L$^+$/CD3$^+$ T-cell ratios, $R^2 = 0.6588$, $P = 0.001$, (Fig. 3B) and ADA levels, $R^2 = 0.4196$, $P = 0.023$, (Fig. 3C) in pleural fluid. The ADA levels were positively correlated to CD40L$^+$/CD3$^+$T cell ratios, R2 = 0.5663, $P = 0.0047$ (Fig. 3D). There was no significant correlation between sCD40 and ADA the levels in plasma (results not shown).

Survival analysis

Median survival time of patients with high sCD40 levels in pleural fluid, more than 200 pg/ml, was significantly lower compared with those having lower sCD40 levels, 3.3 months vs. 6.6 months, Chi
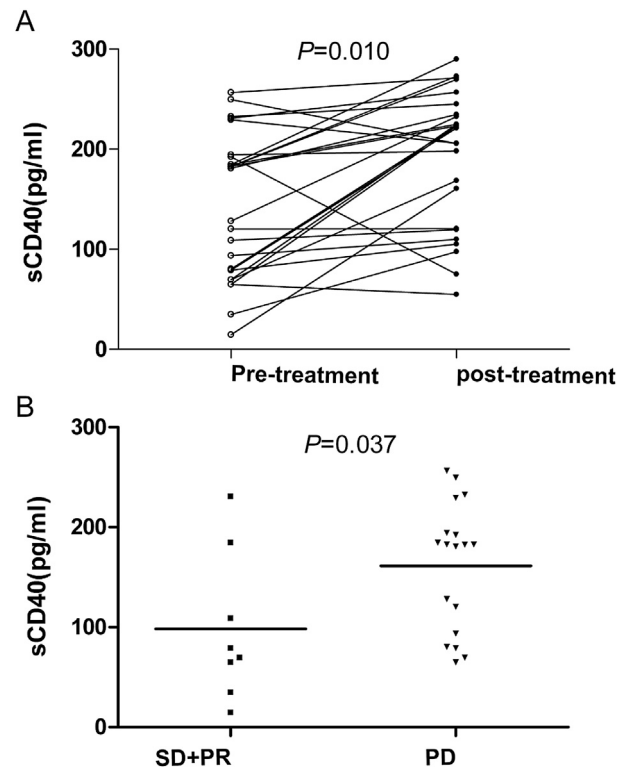
square = 4.004, Hazard Ratio = 0.4643, $P = 0.045$ (Fig. 4).

Discussion

The CD40-CD40L interaction was first shown to play critical roles in B-cell activation and differentiation. In a recent investigation, it was found that the physiologic consequences of CD40 signaling were multifaceted, and even biologically opposed, depending on the type of cell expressing CD40 and the microenvironment in which the CD40 signal is provided. For example, CD40-CD40L engagement induces activation and proliferation of B lymphocytes but triggers apoptosis of carcinoma cells. The CD40 molecules are found in two forms, membrane-type (membrane-anchored CD40, mCD40) and soluble (soluble CD40, sCD40). Fanslow in 1992 was the first who described sCD40 protein and confirmed sCD40 had the ability to bind CD40L.[8] Our study independently confirmed the findings of Fanslow that sCD40 could be detected in blood and pleural effusion from lung cancer patients; we also found that sCD40 levels from advanced stages of lung cancer were significantly higher than in early stage disease. After chemotherapy,
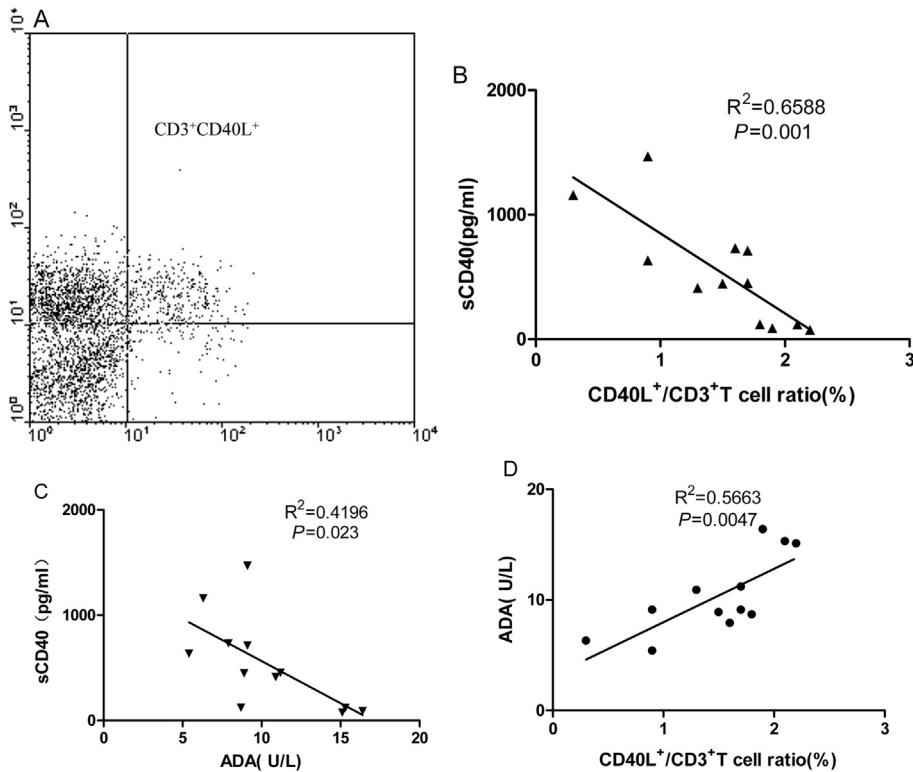
Fig. 3. Expression of CD3$^+$CD40L$^+$ T cells was detected by flow cytometry (A). The levels of sCD40 was negatively correlated with CD40L$^+$/CD3$^+$ T-cell ratios (B) and ADA levels (C). ADA levels were positively correlated with CD40$^+$/CD3$^+$ T-cell ratios (D).

sCD40 levels in plasma were higher than before chemotherapy, especially in patients who experienced PD after two cycles of chemotherapy. We postulate that chemotherapy may elevate sCD40 levels of plasma by irritating tumor cells and promoting sCD40 secretion. For those who were not sensitive to chemotherapy, increased sCD40 levels can act as one of the important pathways of immune escape for tumor cells.
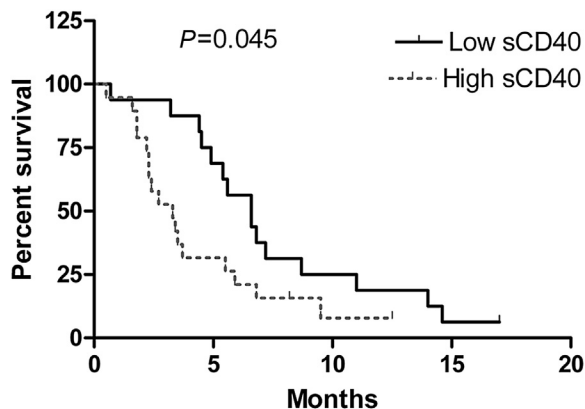

Fig. 4. Kaplan Meier progression free survival curves for patients with MPE according to sCD40 levels.

Studies have indicated that the interaction of the CD40-L$^+$ T lymphocytes augments the antigen presenting function of CD40$^+$ professional antigen-presenting cells (APCs), which in turn stimulates interacting CD4$^+$ and CD8$^+$ T-cells.[9−11] As expected, CD40 expressed by carcinoma cells has been shown to serve a similar costimulatory role; CD40$^+$ tumor cells promote dendritic cell survival and proliferation and differentiation of CD40L$^+$ cytotoxic T lymphocytes (CTLs).[12,13] Our data showed that sCD40 levels and CD40L$^+$ T-cell ratios in pleural fluid were negatively correlated to ADA and that ADA is one of the indicators of T cell activation. We suggest that sCD40 secreted by tumor cells could compete for binding and blocking of CD40L$^+$ on T-cells and modulate the anti-tumor immune function, resulting in increased tumor cell ability to escape anti-tumor immune function. We have also observed that there is no significant difference between sCD40 levels and ADA in plasma, indicating that malignant pleural effusion, as a relatively typical tumor micro-environment, has more value than plasma in reflecting the immune status of lung cancer patients.

Many tumor cells express CD40, including nearly 100% of B-cell malignancies and up to 70% of solid

tumors, and CD40 expression has diverse functions.[14−19] In primary cutaneous melanoma, CD40 expression has been reported to be a negative prognostic factor, and the expression of CD40 in metastatic melanoma in situ is far weaker than in primary melanoma.[20,21] Our study found that the median survival time of patients with high levels of sCD40, more than 200 pg/ml, was significantly less than for those who had lower levels, indicating sCD40 could be a prognostic indicator for patients with lung cancer. Other studies have found that engagement of CD40 *in vitro* can inhibit the growth of solid tumor cells, and the combination of an anti-CD40 agonist antibody and gemcitabine cured most mice with established implanted tumors, and the cured mice were resistant to tumor rechallenge. This effect is absolutely dependent on $CD8^+$ T-cells and independent of $CD4^+$ T-cells.[22−24] These analyses indicate that immune activation and direct tumor cytotoxicity after systemic CD40 activation can have synergistic antitumor effects.

In summary, on lung carcinoma cells, CD40 is presented in a non-immunogenic context and may contribute to T-cell unresponsiveness, providing a thus far unrecognized mechanism to evade anti-tumor immune activity. The reversal of this CD40 dependent inactivation may be important for future immunotherapeutic approaches.

Acknowledgments

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