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ORIGINAL ARTICLE

Apolipoprotein C1 (APOC1) as a novel diagnostic and prognostic biomarker for lung cancer: A marker phase I trial

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Abstract

Background: Tumor cells continuously evolve over time in response to host pressures. However, explanations as to how tumor cells are influenced by the inflammatory tumor microenvironment over time are, to date, poorly defined. We hypothesized that prognostic biomarkers could be obtained by exploring the expression of inflammation-associated genes between early and late stage lung cancer tumor samples.

Methods: Candidate inflammation-associated genes, apolipoprotein C-1 (APOC1), MMP1, KMO)1, CXCL5, CXCL)7, IL-1α, IL-1β, TNF-α and IL-6 were verified by real-time quantitative polymerase chain reaction. Gene expression profiles and immunofluorescence staining of 30 lung cancer tissues were compared.

Results: Expressions of APOC1 and IL-6 mRNA on tumor tissues in late stage disease were significantly higher than in early stage lung cancer samples. Immunofluorescence staining of tumor samples showed that the expression of APOC1 gradually increased from early to late stage in lung cancer patients. The expression levels of IL-6 and APOC1 in tumor samples were positively correlated; however, no prognostic value of APOC1 can be identified in serum samples.

Conclusions: We found that the level of tumor APOC1 was highly expressed in late stage lung cancer. Further research is warranted to determine the molecular mechanisms underlying the cross talk of APOC1 and IL-6 in tumor progression. An expanded sample size marker phase II study may lead to the discovery of new lung cancer therapeutics targeting APOC1.

Introduction

Lung cancer is one of the most prevalent cancers worldwide. The overall five-year survival rate of lung cancer patients is only 15%.[1] Because lung cancer is highly heterogeneous and tumor cells continuously evolve genetically in response to host pressures, a consistent and specific lung cancer marker has been difficult to identify.

Microarray-based gene expression profiling technology has enabled the simultaneous analysis of a large number of genes, searching for molecular markers to predict outcomes in certain stages and histology of lung cancer patients.[2–6] Wide variation genetic and molecular biomarkers, such as the epidermis growth factor receptor signaling pathway gene, tumor suppressor genes, cell immortalization genes, and ion channel genes have been reported.[7] However, immunohistochemistry (IHC) is the most practical method of assessing protein expression changes on the tumor. Several markers have been reported to be important for prognosis judgment, such as p53, Bcl-2, Ki-67, cyclin E, p16, p27, and β-catenin, but none of them become a target for personal medicine.[8,9] Prognostic markers in lung cancer are most relevant for early stage disease. By including patients with advanced stage tumor into cohorts of early stage disease, the prognostic value may decrease. Some specific markers could have different prognostic significance in different histology.[10]

Most of the discovered markers focus on tumor cell expression; only a few markers relate to the interaction of tumor and stromal cells. We hypothesized that looking for the difference between early versus late stage histology should be more

prone to find out prognostic markers related to the interaction of the tumor and its microenvironment. It has long been reported that an immune suppressive phenotype is closely associated with a tumor burden.[11] We had previously found several proteins were significantly elevated in serum isolated from lung cancer patients by using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS).[12,13] These protein peaks were identified basically as serum amyloid A and many of these are fragments of inflammatory substance.[14] Membrane gene arrays specific for inflammation-related genes further identified apolipoprotein C1 (APOC1), matrix metalloproteinase 1 (MMP1), kynurenine 3-monooxygenase (KMO), CXCL5, CXCL7, interleukin (IL)-1α, IL-1β, tumor necrosis factor (TNF)-α and IL-6 up-regulated in lung cancer tissue (Chi *et al.*, unpublished data). These proteins were relative to inflammatory response.[15–17] This indicated that lung cancer is very similar to inflammatory disease and shares some characteristics.

The link between cancer development and inflammation is generally accepted. Tumors often present with characteristics of inflamed tissues, including immune cell infiltration and activated stroma.[18] Inflammation precedes and promotes tumor development and progression, and, likewise, tumor development and progression promote inflammation. This generates local and systemic inflammatory environments. However, the relationship between inflammatory factor gene expressions on lung cancer cells has not been fully investigated.

APOC1 binds and enhances the inflammatory response to endotoxin[17] and has never been reported to elevate in lung cancer patients. Here, we investigated whether the expression of relatively few, but critically important, inflammation-associated genes measured by real-time polymerase chain reaction (PCR) in tumor samples could potentially predict patients' clinical prognoses. In this small scale marker phase I trial, we found that APOC1 overexpression related to a late stage of lung cancer may serve as a prognostic and therapeutic target. We also proposed an association of APOC1 with IL-6 in lung cancer progression.

Materials and methods

Patients and sample collection

Forty-eight lung cancer patients, 16 pneumonia patients, and eight healthy volunteers were enrolled in this study at the Department of Chest Medicine and the Department of Radiation Therapy and Oncology, Shin Kong Wu Ho-Su Memorial Hospital. The ethics committee/institutional review board approved our study protocol. All patients and healthy volunteers provided informed consent forms. Tissues from bronchoscopic biopsy and blood samples were obtained from 30 lung cancer patients. The others were blood samples only. Patients' tissue samples were categorized by different histologic types: 19 had adenocarcinomas, four had squamous cell cancers, one had undifferentiated cancer, and six had small cell lung cancer. Sixteen of them had a smoking history. Tumor tissue samples for mRNA analysis were collected and directly stored in TRIzol (0.5 mL TRIzol for 1×10^6 cells) at −80°C. The peripheral blood samples were collected in 10 mL ethylenediaminetetraacetic acid (EDTA)-containing vacutainer tubes and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque (density: 1.077, Amersham Pharmacia Biotech, Piscataway, NJ, USA). PBMCs were disrupted in TRIzol and stored at −80°C until mRNA analysis.

mRNA and cDNA isolation using peripheral blood mononuclear cells (PBMCs) and tumor samples

Total RNA was extracted separately from PBMCs and tumors by using TRIzol (Invitrogen), according to manufacturer's recommendations. The total mRNA concentration was determined at 260 nm spectrophotometrically. RNA quality was assessed by electrophoresis using agarose gels. Double-stranded cDNA was synthesized from total RNA using a SuperScript II reverse-transcriptase kit (Invitrogen). mRNA (2 μg) were transcribed into cDNA by using 5.5 mM $MgCl_2$, 0.5 mM of each dNTP, 2.5 μM random hexamers, 0.4 U/μL RNase inhibitor, and 1.25 U/μL multiscribe reverse transcriptase, in a reaction volume of 10 μL. Samples were incubated at 80 °C for 10 minutes followed by transcription at 37 °C for 60 minutes and enzyme inactivation at 95 °C for five minutes.

Primers for real-time quantitative polymerase chain reaction (RT-qPCR)

The primer sequences and the respective human cDNA genes are listed in Table 1. β-Actin was used as the housekeeping gene.

RT-qPCR

Real-time quantitative polymerase chain reaction (RT-qPCR) was performed using a LightCycler rapid thermal-cycler system (Roche Diagnostics Ltd, Lewes, UK), according to the manufacturer's instructions. Reaction mixes were assembled in 20 μL total volume, containing 0.5 μM primers and $MgCl_2$ concentration optimized between 2 to 5 mM. Nucleotides, Taq DNA polymerase, and buffers were included in the mix containing LightCycler DNA Master and SYBR Green I (Roche Diagnostics, Lewes, UK). A typical protocol, which takes ~15 minutes to complete, included a 30-second

Table 1 Primer sequences used for quantitative real-time polymerase chain-reaction

Transcript	Sequence	
APOC1	Forward primer:	TTCTGTCGATCGTCTTGGAA
	Reverse primer:	TCAGCTTATCCAAGGCACTG
KMO1	Forward primer:	CTCTTCATTTCACTTTAAATCTCACTG
	Reverse primer:	AAATACCAAGCAGTCTTCAAAGC
MMP1	Forward primer:	GCTAACCTTTGATGCTATAACTACGA
	Reverse primer:	GGATTTGTGCGCATGTAGAAT
CXCL5	Forward primer:	CCTTTTCTAAAGAAAGTCATCCAGA
	Reverse primer:	TGGGTTCAGAGACCTCCAGA
CXCL7	Forward primer:	TGCTGCTGACTGCTCTGG
	Reverse primer:	GCATACAAGTCACTGTCTAGACTTTCC
IL-1α	Forward primer:	CGCCAATGACTCAGAGGAAGA
	Reverse primer:	AGGGCGTCATTCAGGATGAA
IL-1β	Forward primer:	CTCGCCAGTGAAATGATGGCT
	Reverse primer:	GTCGGAGATTCGTAGCTGGAT
TNF-α	Forward primer:	TCTTCTCGAACCCCGAGTGA
	Reverse primer:	CCTCTGATGGCACCACCAG
IL-6	Forward primer:	AACCTGAACCTTCCAAAGATGG
	Reverse primer:	TCTGGCTTGTTCCTCACTACT
β-Actin	Forward primer:	CATGTACGTTGCTATCCAGGC
	Reverse primer:	CTCCTTAATGTCACGCACGAT

APOC1, apolipoprotein C1; IL, interleukin; KMO, kynurenine 3-monooxygenase; MMP, matrix metalloproteinase; TNF, tumor necrosis factor.

denaturation step, followed by 45 cycles of denaturation at 95°C for 10 seconds, annealing at 55°C for five seconds, and extension at 72°C for 10 seconds. To confirm amplification specificity, the PCR products from each primer pair were subjected to melting curve analysis and a subsequent agarose gel electrophoresis. The baseline for each reaction constituted the mean of the measured lowest five data points, and this was subtracted from each reading point. Background fluorescence was removed by setting a noise band. The number of cycles, at which the log-linear line of best fit for each amplification curve intersected with the noise band, was inversely proportional to the log of copy number.

Serum apolipoprotein C1 (APOC1) determination

APOC1 levels in the patients' serum samples were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits from Invitrogen (Camarillo, CA, USA) according to the manufacturers' instructions. All ELISAs were performed in duplicate.

Immunofluorescence staining

Tissue samples from lung cancer patients were fixed in 4% paraformaldehyde overnight before paraffin embedding and sectioning. Antigen retrieval was performed at 95°C in citrate buffer (6.4 M sodium citrate dihydrate and 1.6 M citric acid monohydrate, pH 6.0) for 40 minutes. Slides were cooled at room temperature for 20 minutes and washed three times for three minutes each in Tris buffer (0.15 M sodium chloride and 0.05 M Trizma HCl, pH 7.6). The tissue peroxidase activity was blocked for five minutes, and the slides were washed, as mentioned above. The slides were incubated overnight with the primary antibody goat-anti-APOC1 (Abnova; 1:500), followed by blocking in 3% normal rabbit serum. Finally, fluorescein isothiocyanate (FITC)-conjugated donkey anti-goat-IgG was used as the secondary antibody for one hour, followed by 4′,6-diamidino-2-phenylindole (DAPI) staining for 15 minutes. Scoring was done in a blinded fashion by two investigators, independent of immunofluorescence staining results. The staining patterns were evaluated using the 0 to 3 + scale: 0 indicated absence of staining; 1+ indicated the lowest level of detectable staining and/or nonhomogeneous weak staining; 2+ indicated moderate homogeneous staining; and 3+ indicated intense homogeneous staining; 0–1+ were interpreted as negative staining and 2+–3+ were interpreted as positive staining.

Statistical analyses

All data were analyzed using the Statistical Package for the Social Sciences software, version 12.0 (SPSS Inc., Chicago, IL, USA). The two-sided χ^2 and the Fisher exact test were used to compare clinicopathological parameters between mRNA marker-positive and mRNA marker-negative between early (stage I, II) and late stage (stage III, IV) lung cancer patients. Statistical significance was analyzed using a two-tailed student's *t*-test with $P < 0.05$ indicating statistical significance. Overall survival (OS) was calculated from the date of cancer diagnosis to the date of death from any cause. OS was estimated as a function of time by the Kaplan-Meier method. Correlations between different variables were assessed by the Pearson correlation coefficient.

Results

Marker gene expression in early and late stage lung cancer patients

Nine genes were found to be elevated by membrane array chip analyses from our previous study (Chi *et al*. unpublished data). The candidate malignancy genes – APOC1, MMP1, KMO1, CXCL5, CXCL7, IL-1α, IL-1β, TNF-α and IL-6 – were verified by RT-qPCR. Expression of APOC1 in late stage lung cancer patients was significantly higher than in early stage patients (Fig 1a, $P < 0.01$). The survival percentage was significantly different between the low (<0.3, relative to the expression of the internal control housekeeping gene β-actin) or high (≥0.3) APOC1 expression groups ($P < 0.05$, Fig 1b).

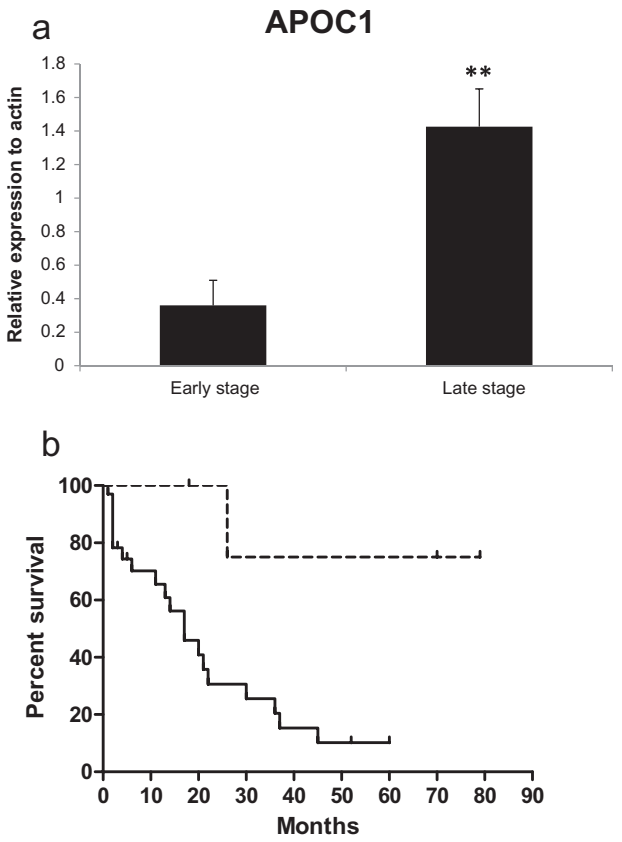

Figure 1 Apolipoprotein C-1 (APOC1) mRNA levels and survival rates were significantly different between early and late stage lung cancer patients. (**a**) Levels of APOC1 mRNA expression in tumor tissue from lung cancer patients were measured by real-time quantitative polymerase chain reaction. The amounts of mRNA are expressed relative to the amount of β-actin mRNA in each sample and are shown as the mean ± standard deviation of three separate experiments. Significant differences between early stage (stage I, II, $n = 16$) and late stage (stage III, IV, $n = 14$) lung cancer patients were found (**$P < 0.01$). (**b**) Overall survival of lung cancer patients with low (<0.3, $n = 9$) or high (≥0.3, $n = 21$) gene expression of APOC1 ($P < 0.05$). - - -, low; ——, high.

The other gene showing significant differential expression was IL-6 (Fig 2h, $P < 0.04$). Other gene expression profiles were not significantly different between early and late stage (Fig 2a–g).

Immunofluorescence staining of APOC1

Immunofluorescence staining of tumor samples showed the variation of APOC1 expression in different stages of lung cancer patients (Fig 3, representing data). Of the 30 tumor specimen samples, overexpression of APOC1 was present in 86% (12 of 14) of late stage tumors, as indicated by scores of 2 + or 3+. Of the early stage, eight samples were scored as 1 + and eight samples scored as 0. The relative mRNA levels showed a high correlation to IHC data. Therefore, our immunofluorescence staining results agreed with mRNA findings (Fig 1) and protein levels.

APOC1 and interleukin (IL)-6 mRNAs were positively correlated

To assess the correlation between APOC1 and IL-6 expression levels, a Pearson correlation scatter plot of IL-6 versus APOC1 levels was generated using data from the 30 tumor samples (Fig 4). The results further indicated that IL-6 and APOC1 expression levels were positively correlated in tumor samples from lung cancer patients (Fig 4).

No prognostic value of serum APOC1 levels in lung cancer patients

As APOC1 is also secreted into the serum, we attempted to determine whether APOC1 serum levels might serve as a disease prognosticator. Serum APOC1 levels showed no significant difference among lung cancer patients, pneumonitis patients, or healthy volunteers (Fig 5a). Serum APOC1 levels failed to show any possible prognostic value in different stages of lung cancer patients (Fig 5b).

Discussion

In this study, we showed, for the first time, that APOC1 was highly expressed in the late, but not in the early stage of lung cancer. The positive correlation between tumor APOC1 levels and tumor stage in lung cancer patients may suggest that APOC1 is likely involved in a progression of the tumor burden.

This study, showing elevated APOC1 and IL-6 levels in advanced stages of lung cancer tumor tissue, suggested a potential cause-and-effect relationship between APOC1 and IL-6. Similarly, previous studies have shown that apolipoproteins modulate inflammatory processes.[19] In this model, because APOC1 was shown to be secreted in an autocrine manner,[20] we postulated that increased intracellular levels of APOC1 might stimulate IL-6 production or vice versa. Increased IL-6 levels may, in turn, stimulate cell proliferation in lung cancers. IL-6 has been previously shown to stimulate proliferation of lung cancer cell lines.[21] Mechanisms leading to IL-6 induction in the serum of cancer patients include IL-6 production and secretion by tumor-associated macrophages, or the tumor cells themselves.[22,23] Tumor-associated macrophages are key orchestrators of the inflammation present in the tumor microenvironment.[24] Because macrophage has been reported as the main cell expressing APOC1,[25] tumor-associated macrophages may also be involved in the activation of APOC1 in lung cancer tumors. Further mechanistic experiments are required to answer these questions.

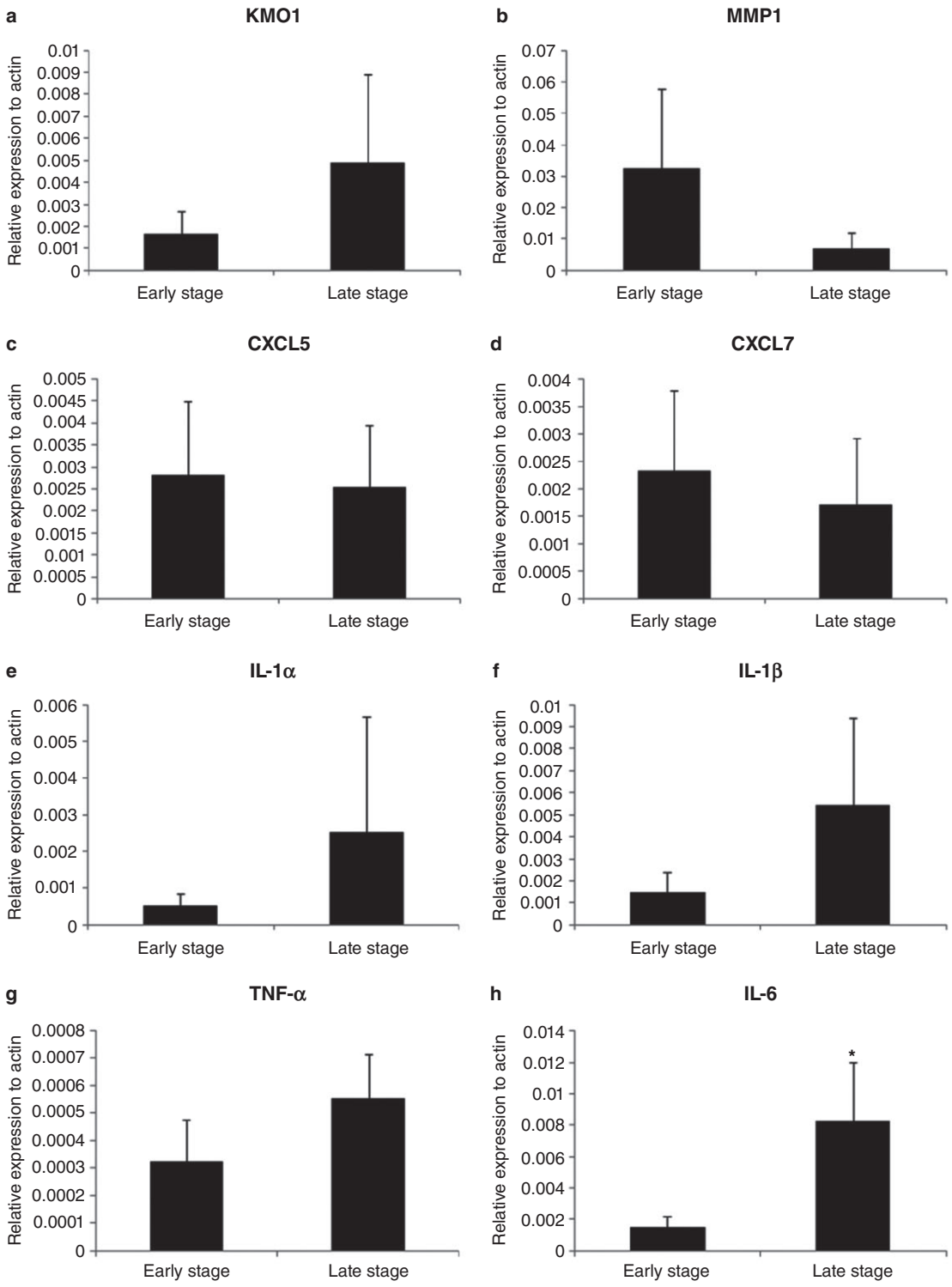

Figure 2 Interleukin (IL)-6 mRNA expression was elevated in late stage lung cancer patients. Levels of KMO1, MMP1, CXCL5, CXCL7, IL-1α, IL-1β, TNF-α and IL-6 mRNA expression in tumor tissue from lung cancer patients were measured by real-time quantitative polymerase chain reaction (**a**, **b**, **c**, **d**, **e**, **f**, **g** and **h**, respectively). The amounts of mRNA are expressed relative to the amount of β-actin mRNA in each sample and are shown as the mean ± standard deviation of three separate experiments. IL-6 mRNA expression showed a significant difference between early stage (stage I, II) and late stage (III, IV) lung cancer patients (*$P < 0.04$). The total patient number was 30 (early stage, $n = 16$; late stage, $n = 14$).

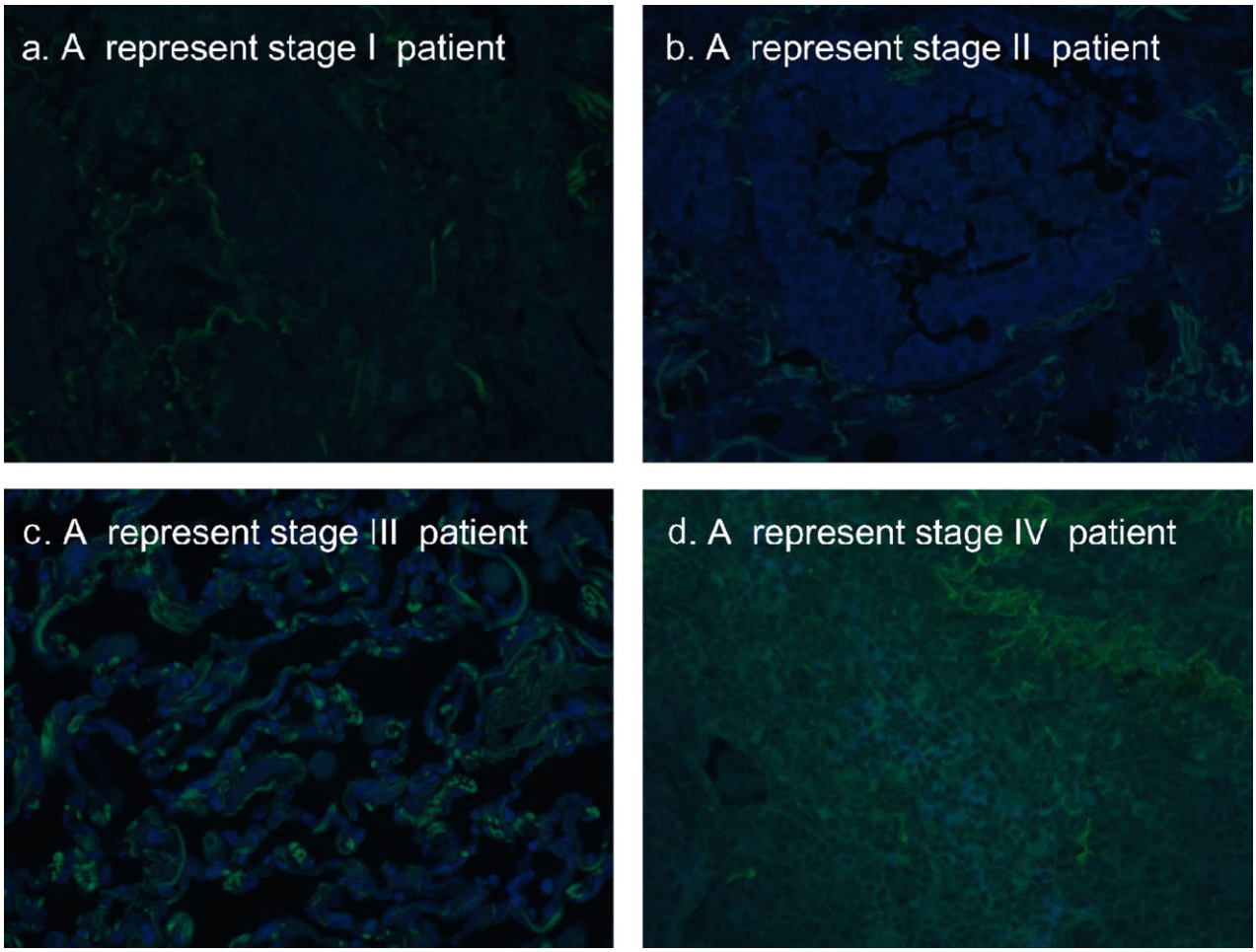

Figure 3 Immunofluorescence staining of apolipoprotein C-1 (APOC1) protein expression in a representative tumor section of lung cancer patients at different stages. Photomicrograph of representative lung cancer tissue sample immunostained by the method used in this study (magnification, ×200). APOC1-positive cells showed a green or blue nuclear signal.

On the other hand, there was no significant difference in the APOC1 serum levels between controls and lung cancer patients. Human APOC1 is known to be produced by several organs, including the liver, lung, skin, testes, and spleen.[26] Among them, the liver is the predominant APOC1 producer. APOC1 is secreted as a 6.6-kDa protein in plasma, where it is found bound to chylomicrons, very low density lipoproteins (VLDL), and high density lipoproteins.[27] Therefore, the serum level of APOC1 is affected by APOC1-producing organs, especially the liver. Thus, serum APOC1 may not represent a good lung cancer prognosticator. The tumor APOC1 level, rather than the serum APOC1 level, as a potential disease marker, may indicate that APOC1 plays an autocrine role which effects cancer in situ. Autocrine production of APOC1 has been proved essential for cancer cell survival and malignant phenotype, likely by precluding apoptosis.[20] Supporting this hypothesis, silencing of another apolipoprotein, apolipoprotein J, in osteosarcoma and prostate cancer cells significantly reduced cell growth while promoting apoptosis.[28] Chen *et al*.[29] also showed that inhibiting apolipoprotein E, which is genetically closely linked to APOC1,[26] in ovarian cancer cells, led to G2 cell cycle arrest and apoptosis. In addition, knockdown of the RELN pathway and its downstream signals, including the VLDL receptor, to which APOC1 is known to bind, increases cell motility and invasiveness in pancreatic cancer.[30] It is tempting to speculate that inhibiting APOC1 expression in lung cancer would suppress tumor progression in vivo; hence, siRNA-mediated silencing of APOC1 may be a valuable strategy for cancer treatment.

Several other studies have reported high serum levels of other apolipoproteins in patients with several types of cancers.[20,31,32] Contrarily, some studies indicated that serum levels of apolipoproteins A-1 or A-2 were decreased in pan-

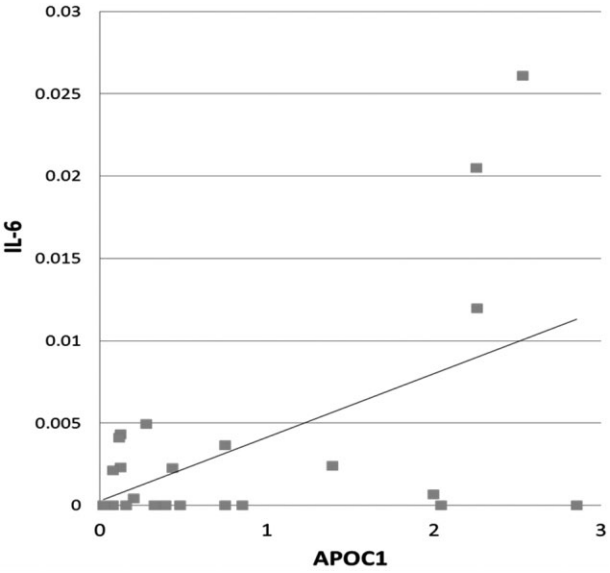

Figure 4 Correlation between interleukin (IL)-6 and apolipoprotein C-1 (APOC1) expression in tumor tissue from lung cancer patients. The plot of the expression of IL-6 as a function of APOC1 expression in lung cancer patients showed a moderately positive relationship. The value of Pearson's correlation was 0.537, $P < 0.05$. The total patient number was 30.

creatic cancer patients compared with those in healthy volunteers.[33] These facts suggest that different apolipoproteins may have different roles in tumorigenesis or in different tumors. Therefore, further analyses of alternative proteins that are differentially expressed in pre- or postoperative tumors, rather than sera, of lung cancer patients may provide novel and valuable information.

The most relevant end point for assessing the prognostic value of a marker is its association with OS. APOC1 seems to have a strong biological effect on prognosis. As age, stage, gender, and smoking history all influence the prognosis of lung cancer, only multivariant analysis that adjusts for these factors is more likely to make a conclusion. A multi-phase approach for the tumor marker clinical trials has been proposed.[34] Our study with a small sample size can only serve a marker phase I trial, to demonstrate a marker may be of interest for further exploration.

Conclusion

In conclusion, we found that the level of tumor APOC1 was highly expressed in late stage lung cancer. Further research to determine the molecular mechanisms underlying APOC1 and IL-6 interrelationships in cancer cells is warranted, and will likely lead to the discovery of new therapies for lung cancer by targeting APOC1.

Acknowledgments

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Disclosure

No authors report any conflict of interest.

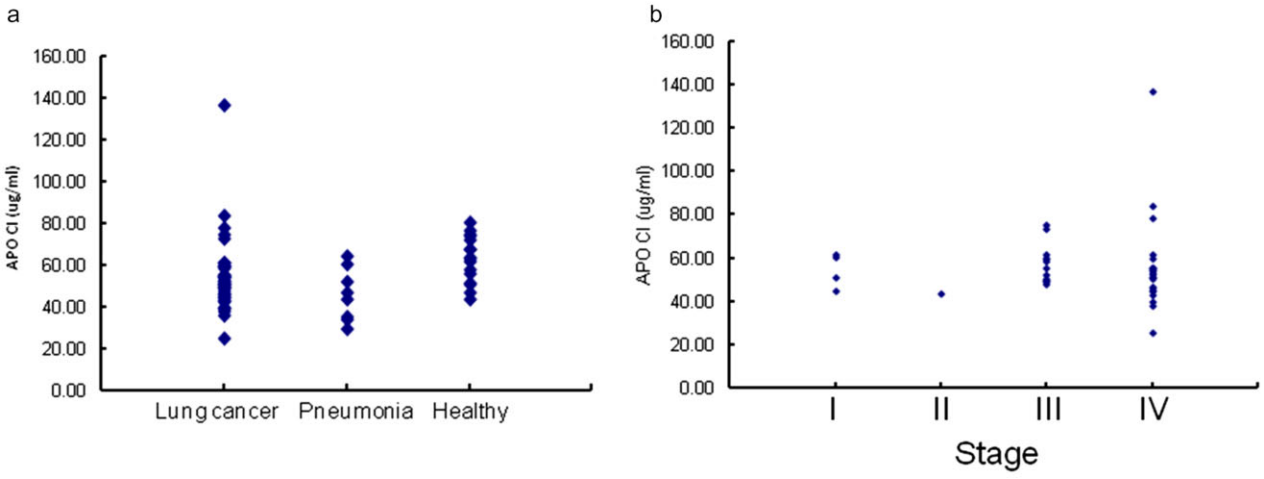

Figure 5 Serum apolipoprotein C-1 (APOC1) levels cannot be used as prognostic marker of lung cancer patients. (**a**) The protein level of APOC1 determined by enzyme-linked immunosorbent assay, including those with lung cancer and inflammatory disease, and healthy controls. (**b**) Serum APOC1 levels was determinate at various stage of lung cancer patients. Total number of serum examined was 72 (lung cancer patients, $n = 48$; pneumonia patients, $n = 16$; healthy volunteers, $n = 8$).

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