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Caspase Recruitment Domain-Containing Protein 9 Expression is a Novel Prognostic Factor for Lung Adenocarcinoma

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Purpose: Caspase recruitment domain-containing protein 9 (CARD9) is expressed at high levels in bone marrow cells and has a crucial role in innate immunity. Current studies indicate that CARD9 also plays a key role in tumor progression, but there are few reports on the role of CARD9 in lung cancer. The aim of this study was to clarify the role of CARD9 in lung adenocarcinoma.

Patients and Methods: Lung adenocarcinoma tumor samples from 74 patients who underwent complete resection at Kobe University Hospital from January 2014 to December 2014 were analyzed by immunohistochemistry. The role of CARD9 in cancer cells was analyzed using lung cancer cell lines treated with CARD9 siRNA.

Results: High expression of CARD9 was observed in 32.4% of tumors, and compared to low expression of CARD9, high expression was associated with poorer overall survival ($P = 0.0365$). Univariate and multivariate analyses showed that high expression of CARD9 was an independent prognostic factor. Knockdown of CARD9 in lung adenocarcinoma cells inhibited proliferation but did not increase apoptosis. In addition, CARD9 activated the NF- κ B pathway in a lung adenocarcinoma cell line.

Conclusion: CARD9 was shown to be an independent prognostic factor of poor outcome for lung cancer and may represent a molecular target for treatment.

Keywords: CARD9, lung adenocarcinoma, spiral array, immunohistochemistry

Introduction

To date, certain clinical factors and tumor stages have been used as prognostic tools for non-small-cell lung cancer (NSCLC). However, prognosis can vary widely even among patients with tumors of the same stage. Most reports show that 20% of patients with stage I NSCLC experience recurrence after surgery,¹ and the efficacy of adjuvant treatment for stage I patients is controversial.² Some patients with stage I NSCLC receive adjuvant chemotherapy, which is similar to what patients at stage II or higher receive.³ Therefore, it is important to identify the prognostic markers that predict patient prognosis in NSCLC.

Caspase recruitment domain-containing protein 9 (CARD9) is a central regulator of innate immunity.⁴ Myeloid cells, including macrophages and dendritic cells, express high levels of CARD9.⁴ Pathogens can activate CARD9 signaling, which regulates innate immune responses and affects adaptive immunity.^{5,6} Current research reports that CARD9 plays a key role in tumor progression,⁷ although

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there have been few reports on the role of CARD9 in lung cancer and even fewer on its potential as a prognostic biomarker for lung cancer.

Therefore, the aim of this study was to clarify the prevalence and clinical significance of CARD9 expression in NSCLC. In this study, we will introduce a spiral array containing 74 NSCLC postoperative specimens, which was used in our analysis.

Patients and Methods

Patients and Spiral Array Construction

A lung cancer spiral array was constructed with surgical specimens from 74 consecutive patients who underwent surgery for lung cancer and were diagnosed with adenocarcinoma at Kobe University Hospital from January 2014 to December 2014. The protocol for constructing the spiral array was described previously.⁸ The methods of data collection and analysis were approved by the institutional review board (permission number: 160,117), and written informed consent was obtained from all the patients.

Immunohistochemistry

Tissue sections from spiral array blocks were deparaffinized and rehydrated using standard procedures.⁹ Briefly, slides were deparaffinized and treated to a heat-induced

antigen retrieval procedure by incubating them for 20 min in pH 7.8 Tris-EDTA-citrate buffer in an autoclave at 121°C. A primary antibody against CARD9 (1:200) (polyclonal; Sigma-Aldrich, MO) was applied at 37°C for 60 min. We classified the stained specimens into two groups, low immunostaining and high immunostaining, for statistical analysis (Figure 1). CARD9 high was defined as a case in which there were 50% more positive cells than there were in normal tissue.

Cell Culture and siRNA Interference

Lung adenocarcinoma cell lines A549 and PC9, human large cell lung cancer cell line H460, human lung squamous cell carcinoma cell line H520, and human small cell lung cancer cell lines DMS53 and DMS114 were purchased from ATCC (Manassas, VA, USA). The lung adenocarcinoma cell line H118 was purchased from RIKEN Cell Bank (Riken, Tsukuba, Japan). These cells were cultured at 37°C with 5% CO₂ in RPMI-1640 medium (Wako, Japan) containing 10% fetal calf serum. The tumor cells were transfected with a CARD9 siRNA (Thermo Fisher Scientific, MA) or with Silencer® Select Negative Control No. 1 siRNA (Thermo Fisher Scientific, MA), which had no effect on any gene expression.

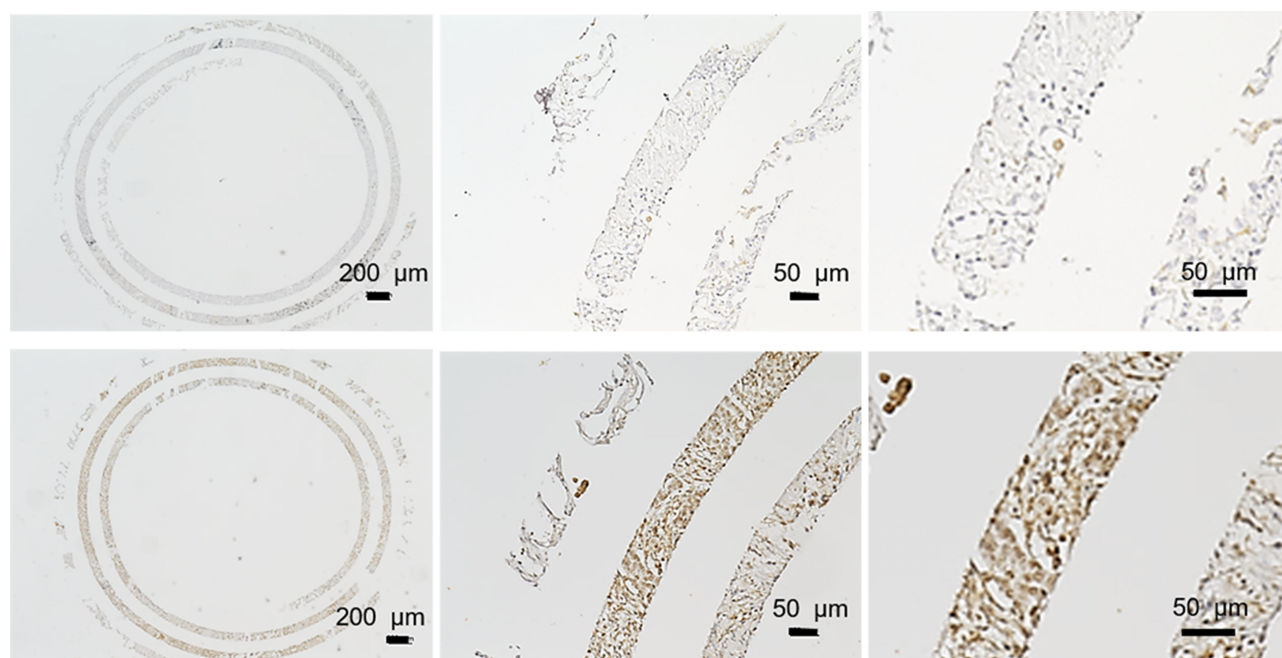


Figure 1 Immunohistochemical staining of CARD9 protein in lung adenocarcinoma.

Notes: Spiral array blocks of each sample were immunohistochemically stained. The upper column shows low CARD9 expression, and the lower column shows high CARD9 expression.

Real-Time PCR Analysis

RNA was isolated from various lung cancer cell lines (A549, PC9, II18, H460, H520, DMS53 and DMS114) according to the protocol of the manufacturer of Sepasol®-RNAI Super G (NACALAI TESQUE, INC. Japan). Extracted and purified RNA was reverse transcribed to generate cDNA using a PrimeScript RT Reagent Kit (Takara, Japan). Real-time PCR was performed using Thermal CyclerDice® Real Time System II (Takara, Japan). Relative mRNA levels were calculated with the $\Delta\Delta C_t$ method using *GAPDH* mRNA as an internal control. The primers used in this study were as follows: 5'-CCCTCACGCATCACACCTTAC-3' and 5'-GCACACCCACTTTCCGTTTG-3' for *CARD9* and 5'-CCACTCCTCCACCTTTGAC-3' and 5'-ACCCTGTTGCTGTAGCCA-3' for *GAPDH*.

Cell Proliferation Assay

A549, PC9 and II18 cells in the logarithmic growth phase were seeded at a density of 5×10^3 cells/well in a 96-well plate and were precultured for 24 h in a 5% CO₂ incubator. The cells were transfected with a *CARD9* siRNA to knockdown *CARD9* and then were cultured for 0, 24 and 48 h at 37°C with 5% CO₂. A total of 10 μ L of Cell Counting Kit-8 solution (CKK-8; Dojindo, Kumamoto, Japan) was added to each well, and the cells were incubated for another 30 min. Absorbance was measured at 450 nm using a spectrophotometer (Bio-Rad, iMark microplate reader, CA, USA).

Flow Cytometry

PC9 cells were transfected with a *CARD9* siRNA or Silencer® Select Negative Control siRNA and cultured at 37°C with 5% CO₂ for 48 h. Detection of apoptosis was performed by using Annexin V-FITC and propidium iodide (PI) and a BD FACSVerse System (BD Bioscience, Franklin Lakes, NJ).

Western Blot Analysis

A detailed protocol for Western blotting has been described previously.¹⁰ Antibodies against the following proteins were used in this study: β -actin (#4967), NF- κ B p65 (#8242) and phospho-NF- κ B p65 (#3033). These antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA).

Statistical Analysis

All statistical analyses were performed by using EZR version 1.37 (Saitama Medical Center Jichi Medical

University; <http://www.jichi.ac.jp/saitama-sct/SaitamaHP.files/statmed.html>; Kanda, 2018) and a graphical user interface for R version 3.4.1 (The R Foundation for Statistical Computing, Vienna, Austria).^{9,11} Differences between the two groups were analyzed by Pearson χ^2 tests or Fisher exact tests. For the univariate analysis, all cumulative survival was estimated by the Kaplan–Meier method, and differences were assessed by Log rank test. A multivariate regression analysis was conducted according to the Cox proportional hazard model. All P values reported are 2-sided, and a $P < 0.05$ was considered significant.

Results

Patient Characteristics

The median age of patients was 71.5 years old (range 49–85 years old). Forty-one of the included patients were men, and 33 of the patients were women. Regarding the pathological data, 26 tumors were T1, 36 were T2 and 1 was T3. Although 59 patients were negative for lymph node metastasis, 8 patients had tumors that were categorized as N1 and 7 were N2 (Table 1).

Relationship Between *CARD9* Expression and Clinicopathologic Factors

CARD9 was expressed in the cytoplasm of adenocarcinoma cells, as detected by immunohistochemistry. Figure 1 shows a typical *CARD9* immunostaining result. In adenocarcinoma, high expression of *CARD9* was seen in 32.4% of tumors, and low expression was seen in 67.6% of analyzable tumor samples. Compared to samples with low expression of *CARD9*, those with high levels correlated with the median survival ($P = 0.0365$; Figure 2). The results of univariate and multivariate analyses are shown in Tables 2 and 3, respectively. High expression of *CARD9* was revealed to be an independent prognostic factor of poor outcome. Due to the relatively small number of cases in this study, lymph node metastasis was not found to be significantly different between the groups following univariate analysis.

Effect of *CARD9* on Lung Cancer Cells

To confirm the effect of *CARD9* on lung cancer cell lines, *CARD9* mRNA levels in various lung cancer cell lines (A549, PC9, II18, H460, H520, DMS53 and DMS114) were analyzed by real-time PCR (Figure 3). The results

Table 1 Association Between CARD9 Expression and Clinicopathological Variables in Lung Adenocarcinoma

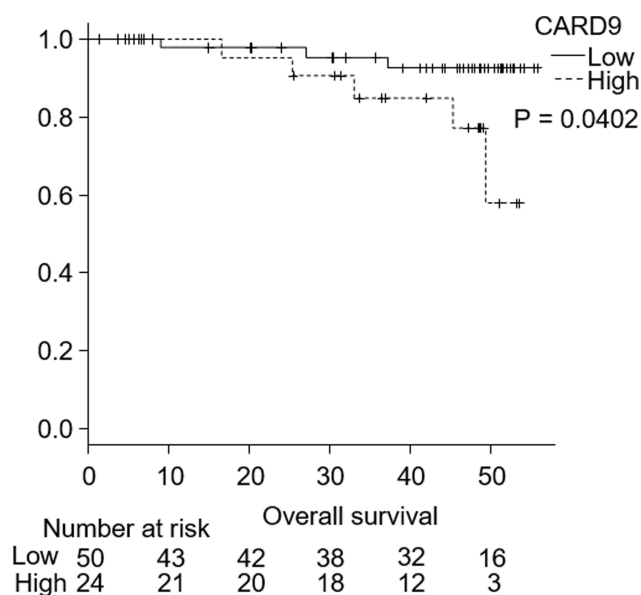
| | Patients (n) | CARD9 Expression Level | | p |
|-----------------------|--------------|------------------------|------------|--------|
| | | High | Low | |
| Number of cases | 74 | 24 (32.4%) | 50 (67.6%) | |
| Age [median(range)] | | 72 (55–83) | 71 (49–85) | |
| Male | 41 | 16 | 25 | 0.217 |
| Smoking history | 44 | 19 | 25 | 0.0228 |
| Lobectomy | 56 | 19 | 37 | 0.775 |
| Partial resection | 18 | 5 | 13 | |
| pT1 | 36 | 14 | 22 | 0.124 |
| pT2 | 37 | 9 | 28 | |
| pT3 | 1 | 1 | 0 | |
| pN0 | 59 | 20 | 39 | 0.437 |
| pN1 | 8 | 1 | 7 | |
| pN2 | 7 | 3 | 4 | |
| pM0 | 74 | 24 | 50 | |
| Pleural infiltration | 27 | 10 | 17 | 0.609 |
| Vascular invasion | 29 | 10 | 19 | 0.803 |
| Lymphatic invasion | 22 | 6 | 16 | 0.597 |
| pStage IA | 36 | 14 | 22 | 0.314 |
| pStage IB | 18 | 5 | 13 | |
| pStage IIA | 11 | 1 | 10 | |
| pStage IIB | 2 | 1 | 1 | |
| pStage IIIA | 7 | 3 | 4 | |
| Adjuvant chemotherapy | 23 | 6 | 17 | 0.593 |
| EGFR mutation | 16 | 6 | 10 | 0.739 |

Abbreviations: EGFR, epidermal growth factor receptor; CARD9, caspase recruitment domain-containing protein 9.

suggested that CARD9 has a possible role in various types of lung cancer cell lines.

Proliferation of Lung Adenocarcinoma Cells

A549, PC9 and H18 cells were transfected with CARD9 siRNA, and mRNA expression was analyzed 24 hours later. In all these cell lines, the mRNA expression of CARD9 was significantly reduced compared to that in the control cells (Figure 4A). Then, the proliferation of these cell lines was analyzed by the

**Figure 2** Kaplan-Meier curves of the overall survival of lung adenocarcinoma patients.

Notes: The Kaplan-Meier method was conducted by Log rank test. High CARD9 immunostaining was associated with a worse prognosis in lung adenocarcinoma patients.

Table 2 Univariate Analysis of CARD9 Expression and Clinical Features of Lung Adenocarcinoma Cases

| | Cut Off | Median Survival Time | 95% Confidence Interval | p |
|----------------------|---------------------------|----------------------|-------------------------|--------|
| CARD9 | Low High | NA NA | NA-NA 1502-NA | 0.0365 |
| pT factor | <2 ≥2 | NA NA | NA-NA NA-NA | 0.286 |
| pN factor | 0 ≥1 | NA NA | NA-NA 1131-NA | 0.0826 |
| Pleural infiltration | 0 ≥1 | NA NA | NA-NA NA-NA | 0.237 |
| Vascular invasion | 0 1 | NA NA | NA-NA NA-NA | 0.0874 |
| Lymphatic invasion | 0 1 | NA NA | NA 1502-NA | 0.585 |
| Smoking | Never vs. Ever or Current | NA NA | NA-NA NA-NA | 0.233 |

Abbreviations: CARD9, caspase recruitment domain-containing protein 9; NA, not available.

Table 3 Multivariate Analysis of CARD9 Expression and Clinical Features of Lung Adenocarcinoma Cases

| | Cut Off | Hazard Ratio | Lower 95% Confidence Interval | Upper 95% Confidence Limit | P |
|----------------------|--------------------------|--------------|-------------------------------|----------------------------|---------|
| CARD9 | Low or High | 18.02 | 1.438 | 225.8 | 0.02498 |
| pT1 or 0 | <2 vs ≥2 | 3.74 | 0.2382 | 58.74 | 0.3478 |
| pN1 or 0 | 0 vs ≥1 | 6.714 | 0.5492 | 82.07 | 0.136 |
| Pleural infiltration | 0 vs ≥1 | 0.6383 | 0.0395 | 10.31 | 0.7518 |
| Vascular invasion | 0 vs 1 | 2.342 | 0.2199 | 24.94 | 0.4808 |
| Lymphatic invasion | 0 vs 1 | 0.352 | 0.04674 | 2.652 | 0.31 |
| Smoking | Never vs Ever or Current | 1.81 | 0.15 | 21.85 | 0.6405 |

Abbreviation: CARD9, caspase recruitment domain-containing protein 9.

CCK8 method. Growth inhibition was observed in CARD9 knockdown PC9 cells. No clear growth inhibition was observed in A549 and H18 cells. (Figure 4B). This result suggested that CARD9 may promote cell proliferation in PC9 cells.

Effect of CARD9 on Apoptosis in Lung Adenocarcinoma Cells

PC9 cells were transfected with CARD9 siRNA, and apoptosis was analyzed by flow cytometry. The proportion of apoptotic cells was almost the same between the CARD9 knockdown PC9 cells and control cells (Figure 5A). This suggests that CARD9 does not have a role in suppressing apoptosis in lung adenocarcinoma.

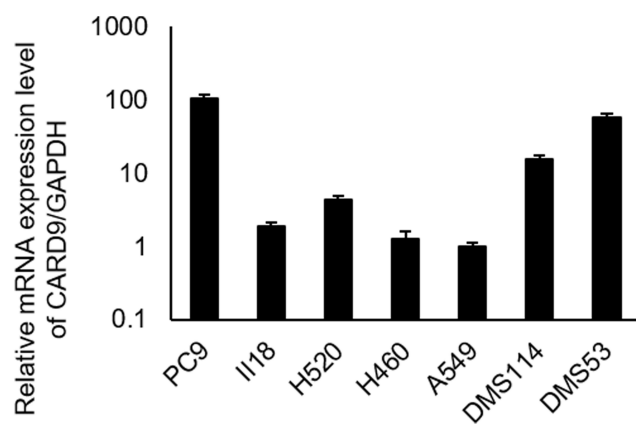


Figure 3 mRNA expression levels of CARD9 in various lung cancer cells.

Notes: These were analyzed by using real-time PCR. The mRNA expression values of CARD9 were normalized to those of GAPDH in the indicated lung cancer cells are shown.

Effect of CARD9 on the NF-κB Pathway in Lung Adenocarcinoma Cells

Western blot assays were performed to determine the activity of the NF-κB pathway. In PC9 cells transfected with CARD9 siRNA, p-NF-κB levels were lower relative to total NF-κB than they were in cells treated with the negative control siRNA (Figure 5B). This demonstrated that CARD9 promotes activation off the NF-κB pathway in a lung adenocarcinoma cell line.

Discussion

In this paper, we show for the first time that CARD9 is expressed in lung cancer cells and is a prognostic indicator of poor outcome.

To date, there have been many reports of prognostic biomarkers in NSCLC, but few have been used in clinical practice. It is a well-known fact that DNA microarray methods have the advantage of enabling assessment of a large amount of gene expression,^{12,13} but the disadvantage is that information about proteins cannot be obtained. We applied spiral array immunostaining as a simpler and faster method of predicting prognosis.

The human CARD9 locus has long been known to be an inflammatory bowel disease susceptibility factor, and the basic mechanism of CARD9 signaling activity has been elucidated.^{7,14} In addition, in recent years, CARD9 has been implicated in tumorigenesis, as CARD9 promotes anti-tumor T cell responses through various mechanisms, thereby activating proinflammatory carcinogenic STAT3 or anti-tumor suppressor functions in malignant cells.¹⁵ It has also been found to play a facilitating role in tumorigenesis.^{16,17}

According to immunohistochemical staining results in a previous study, compared to that in normal tissues,

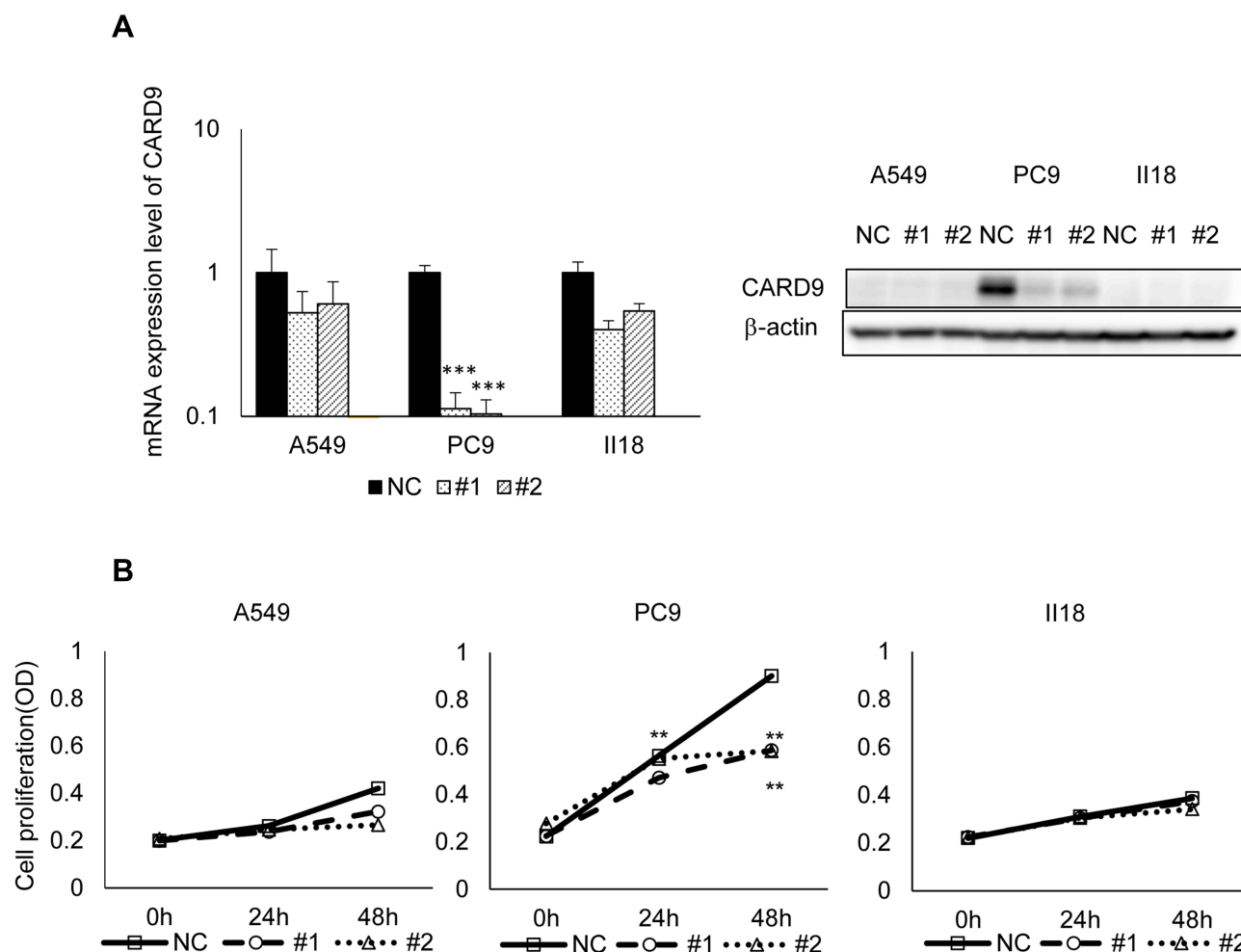


Figure 4 Knockdown efficiency of CARD9 siRNA and tumor growth inhibition test.

Notes: (A), Twenty-four hours after transfection of CARD9 siRNA, CARD9 mRNA expression levels in A549, PC9 and II18 cells were analyzed by using real-time PCR and Western blotting. *** $P < 0.001$. (B), The growth inhibition effect of CARD9 siRNA in A549, PC9 and II18 cells is shown. The proliferation of PC9 cells treated with CARD9 siRNA was suppressed compared to that of control cells. ** $P < 0.01$.

CARD9 expression is increased mainly in tumor-infiltrating leukocytes in colon cancer tissues but not in cancer cells.¹⁸ Furthermore, CARD9 immunoreactivity was histologically observed in macrophages, and CARD9 was reported in patients with colon cancer. CARD9 expression is associated with tumor progression as well as liver metastasis of colon carcinoma cells.¹⁸

Regarding the distribution of CARD9 in cells, a previous study revealed that CARD9 is expressed in the cytoplasm of oral squamous cell carcinoma cells (OSCC) and OSCC cell lines.¹⁹ This previous study also showed that, with respect to the role of CARD9, down-regulation of CARD9 is associated with suppression of OSCC growth and migration and that this is regulated through the NF- κ B pathway. The arguments in this previous study seem to support our conclusions.

However, another study found that CARD9 suppressed MDSC expansion and IDO production in mice and prevented lung cancer by suppressing the noncanonical NF- κ B pathway in CARD9 knockdown mice and inflammatory cells, and the mechanism was reported. There are two known signaling pathways that lead to inactivation of NF- κ B: canonical and noncanonical.^{20–23} A common regulatory mechanism in these two pathways is the activation of the I κ B kinase (IKK) complex, which phosphorylates I κ B. The signals that stimulate the classical pathway are the binding of ligands (such as LPS) to the Toll-like receptor (TLR) superfamily and the binding of a ligand (TNF- α) to the TNF receptor.²⁴ These proteins recruit adapter proteins, such as TRAFs, to the intracellular domain of the receptor. This adapter also recruits the IKK complex. In the classical pathway the IKK complex is composed of a homodimer

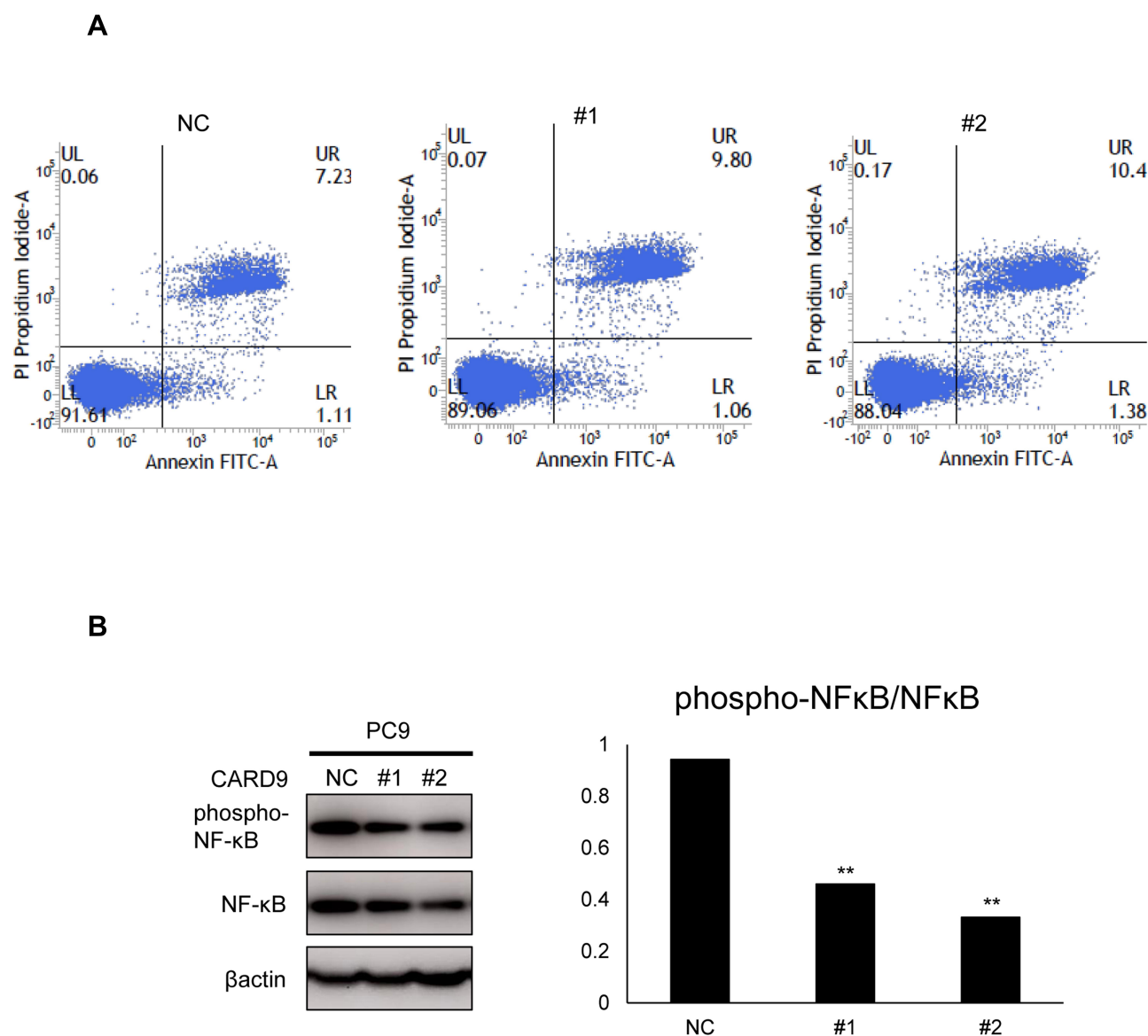


Figure 5 Apoptosis assay.

Notes: (A) Apoptosis of PC9 cells treated with CARD9 siRNA was evaluated by flow cytometry. Annexin V-positive and PI-negative populations (right lower column) represent cells in early apoptosis. No difference in apoptosis was detected between CARD9 knockdown PC9 cells and control cells. (B) NF-κB signaling in PC9 cells was analyzed by Western blotting. The right panel shows the relative expression values of phospho-NF-κB compared to total NF-κB. ** $P < 0.01$.

or heterodimer consisting of IKK α and IKK β and a scaffold protein NEMO (NF-κB essential modulator).²⁵

IκB that is bound to the NF-κB dimer is degraded by the proteasome system upon phosphorylation by the IKK complex. After the degradation of IκB, the activated NF-κB dimer exposes its nuclear localization signal sequence (NLS), which allows its translocation into the nucleus.²⁶ After it translocates into the nucleus NF-κB, induces the expression of various target genes.

Nonclassical pathways function in the development of lymphoid organs to produce B and T cells. Known factors that stimulate these pathways are lymphotoxin β

and BAFF (B cell activating factor).²⁷ When a ligand binds to a cell surface receptor, NIK (NF-κB inducing kinase) is activated and phosphorylates the IKK complex. The IKK complex in this pathway is a homodimer of IKK α , which does not include NEMO. The phosphorylated and activated IKK complex phosphorylates p100 (IκB domain) of the p100/RelB complex, which triggers the p100/RelB complex to undergo limited degradation to generate the active p52/RelB complex. This complex translocates to the nucleus and induces the expression of various target genes.²⁵

In the current study, we demonstrated that CARD9 enhances the canonical NF- κ B pathway to promote cell proliferation, as observed in the lung cancer cell line PC9. Therefore, inconsistencies with previous reports may be because the mechanism of NF- κ B activation in the non-canonical pathway seen in mice is defined by the balance with the mechanism of NF- κ B activation in the canonical pathway that is seen in human cell lines.

The limitation of the present study is the relatively small sample size. In addition, relatively few deaths occurred. However, we were convinced that the clinical data were supported by in vitro data.

Conclusion

CARD9 was shown to be an independent prognostic factor for poor outcome in lung adenocarcinoma. CARD9 may be a promising molecular target for lung cancer, and adjuvant therapy may be considered for CARD9-positive lung cancer.

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Disclosure

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