Biochemical and Biophysical Research Communications 454 (2014) 157–161

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Overexpression of SAMD9 suppresses tumorigenesis and progression during non small cell lung cancer

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ARTICLE INFO

Article history:
Received 7 October 2014
Available online 17 October 2014

Keywords:
Non small cell lung cancer
Carcinogenesis
Progression
SAMD9

ABSTRACT

The Sterile Alpha Motif Domain-containing 9 (SAMD9) gene has been recently emphasized during the discovery that it is expressed at a lower level in aggressive fibromatosis and some cases of breast and colon cancer, however, the underlying mechanisms are poorly understood. Here, we found that SAMD9 is down-regulated in human non-small cell lung cancer (NSCLC). Furthermore, knockdown of SAMD9 expression is increased the invasion, migration and proliferation in H1299 cells *in vitro* and overexpression of SAMD9 suppressed proliferation and invasion in A549 cells. Finally, depletion of SAMD9 increases tumor formation *in vivo*. Our results may provide a strategy for blocking NSCLC tumorigenesis and progression.

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1. Introduction

NSCLC remains the leading cause of cancer-related deaths in the world as well as in China and represents 75–80% of lung cancer cases and accounts for approximately 1.2 million new cases worldwide each year [1,2]. Although the incidence remains high, identification of molecular factors holds promise for further improvement of treatment in NSCLC. However, few of such prognostic factors have been clarified for predicting the tumorigenesis and progression of NSCLC.

In order to select means in effective controls of NSCLC, we must complete understanding of mechanisms responsible for NSCLC development and progression. The Sterile Alpha Motif Domain-containing 9 (SAMD9) gene is located in chromosome 7q21.2 of the human genome, and separated by approximately 12 kb. Tt plays important roles in normophosphatemic familiar tumoral calcinosis (NFTC) [3,4]. SAMD9 has been demonstrated to be associated with aggressive fibromatosis, breast, and colon cancers and it is expressed at a lower level [5]. However, very few studies focus on the expression and function of SAMD9 in human NSCLC. Human SAMD9 expression can be downregulated by tumor necrosis factor (TNF) [4] or by type I [6] and type II interferons (IFNs) [7], and it is classified as an interferon stimulated gene (ISG). Recently, the human SAMD9 is expressed in normal breast tissues and primary tumor tissues, it was shown to exhibit higher expression levels in normal tissues [5].

In our current study, knockdown of SAMD9 expression is increased the proliferation and invasion in H1299 cells *in vitro*. Furthermore, overexpression of SAMD9 increases proliferation and invasion in A549 cells *in vitro*. Depletion of SAMD9 increases tumor formation progression *in vivo*. Thus, these results indicate that SAMD9 is involved in NSCLC.

2. Material and methods

2.1. Cell culture and specimens

H358, PC9, H1299, and A549 and WI-38 cells were obtained from the American Type Culture Collection (Manassas, VA, USA), and maintained at 37 °C and 5% CO_2 in DMEM medium supplemented with 10% fetal bovine serum (Invitrogen).

Clinical samples were retrospectively collected from patients ($n = 40$) who underwent surgical resection between May 2005 and October 2008 at General Hospital of Tianjin Medical University. All of the lung cancer samples were pathologically validated as NSCLC. Normal tissue samples (the distance from tumor: >5 cm) from 40 patients included in the study were verified by pathologists. No patients received chemotherapy or radiotherapy prior to the operation. This study was approved by the Institutional Review Board of the General Hospital of Tianjin Medical University and written consent was obtained from all participants.

2.2. Plasmid, shRNA, and transfection

To construct the SAMD9 expression vector, the entire coding sequence of the SAMD9 was amplified by PCR using the Pfu DNA

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http://dx.doi.org/10.1016/j.bbrc.2014.10.054
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Polymerase (Thermo Scientific, Rockford, IL, USA). The PCR product was cloned into the pcDNA3 (Invitrogen). The construct was confirmed by sequencing. shRNAs of SAMD9 were purchased from RiboBio (Shanghai, China). For transient transfection, 2×10^5 cells were plated into 6-well plates and kept in antibiotic-free medium for 24 h before transfection. The cells were then transfected with and then transfected with the shRNA or plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. For generation of stable depleted SAMD9 expression, cells were transfected with SAMD9 shRNA. Two days after transfection, cells were trypsinized, transferred to 10 cm cell culture dishes and selected by complete medium plus 1 mg/ml of G418 (Sigma–Aldrich, St. Louis, MO, USA) for about 2 weeks. The mRNA and protein levels were determined by RT-qPCR and Western blotting.

2.3. Real-time quantitative reverse transcription PCR (RT-qPCR)

2 μg of the total RNAs extracted with Trizol reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. The RT-qPCR analysis was performed using the Fast SYBR Green Master Mix System (Invitrogen) according to the manufacturer's instructions. The targeted gene relative quantification was given by the CT values, and the CT value of Actin was subtracted to obtain ΔCT. The experiment was performed in triplicate. The relative SAMD9 mRNA expression.

2.4. Wound healing assay

Cells were seeded in 12-well plates and allowed to form confluent monolayers. The monolayers were then scratched horizontally using a sterile 100 ml pipette tip. Then cells were cultivated in medium containing 0.5% FBS overnight and exposed to different concentration of agents. Cells were washed with PBS and cultivated in DMEM medium. Bright field images of the randomly selected views along the scraped line were taken. Migration was quantified by a semi-automated computer-assisted procedure by a person blinded with respect to the experimental-treatment.

2.5. Western blot

Cells were lysed in lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 0.55 NP-40) with 0.1% protease inhibitor cocktail III (Calbiochem, San Diego, CA, USA). The membranes were blocked in 5% skimmed milk and incubated with primary antibodies overnight (SAMD9 1:3000, Origene, Rockville, MD, USA; β-actin 1:6000, Sigma) and then with horseradish peroxidase-conjugated anti-mouse antibodies (1:4000, Sigma–Aldrich). Specific proteins were visualized with the enhanced chemiluminescence detection reagent (Pierce Biotechnology, Rockford, IL, USA).

2.6. MTT, colony formation, and transwell assays

MTT and colony formation assays were used to evaluate the ability of cell proliferation. For MTT assay, 5×10^3 transfectioned cells were seeded in 96-well plates per well. Then 24, 48, 72 h after transfection, the cells were incubated with 10 μl MTT (0.5 mg/ml; Sigma–Aldrich) at 37 °C for 4 h. The medium was then removed, and precipitated Formosan was dissolved in 100 μl DMSO. The absorbance at 490 nm was detected using a micro-plate autoreader (Bio-Rad, Richmond, CA, USA). For the colony formation assay, the number of viable cell colonies was determined after inoculation of 150 cells/well in triplicate in 12-well plates. The cells were stained with crystal violet. The ability to form colonies was evaluated by determining the colony formation number.

Transwell assay was used to evaluate the ability of cell invasion. The invasion of cells *in vitro* was measured by the invasion of cells through Matrigel-coated transwell inserts (8 μm pore size, BD Biosciences, San Jose, CA, USA). 1×105 cells in 500 μl of serum-free medium were added to the upper chamber, Medium (DMEM/F12) containing 20% FBS were added into the lower chamber. After incubation for 16 h, cells that invaded the Matrigel were stained and counted under a microscope in six predetermined fields.

2.7. Bioluminescence imaging and analysis

1×10^6 shRNA-SAMD9 and control cells were washed and harvested in 0.1 ml PBS and injected into the mammary fat pad of thirty-nine. Bioluminescence images were obtained by using the Xenogen IVIS system 5 min after injection. Analysis was 6 performed with LIVINGMAGE software (Xenogen, Alameda, CA, USA).

2.8. Statistics

The hypothesis test for significance between two groups with complete randomized design or paired design utilized the Student's *t* test. The statistical significance was set to $P < 0.05$. All data quantification and statistical analysis were performed using SPSS 18.0 software (Chicago, IL, USA).

3. Results

3.1. SAMD9 is low-regulated in NSCLC

To determine whether SAMD9 has a role in NSCLC, we first examined SAMD9 expression levels in NSCLC cell lines (H358, PC9, H1299, and A549) and the normal lung cell line (WI-38). We observed that SAMD9 mRNA levels were low-regulated in NSCLC cell lines compared with the normal lung cell line by RT-qPCR (Fig. 1A). Furthermore, SAMD9 protein levels were also low-regulated in NSCLC cell lines by Western blot (Fig. 1B). Importantly, we examined SAMD9 mRNA expression levels in 40 cases of NSCLC tissues and the adjacent normal lung tissues. We observed that SAMD9 mRNA levels were down-regulated in NSCLC cancer tissues compared with normal lung tissues by RT-qPCR (Fig. 1C). Furthermore, SAMD9 protein levels were also down-regulated in NSCLC tissues by Western blot (Fig. 1D). These results indicate that SAMD9 is low-regulated in human NSCLC.

3.2. Downexpression of SAMD9 increases the invasion, migration and proliferation of human NSCLC cell lines in vitro

To examine whether SAMD9 affected cell invasion, migration and proliferation in NSCLC cell biology, we used shRNAs targeting SAMD9 to inhibit the SAMD9 expression in H1299 cells. The effective knockdown of SAMD9 was confirmed by RT-qPCR and Western blot assays. The SAMD9 expression levels were significantly lower in shSAMD9-transfected H1299 cells (Fig. 2A). Next, we used transwell assay to demonstrate the effects of SAMD9 on the invasive potential of H1299 cells. We found that H1299 cells transfected with SAMD9 shRNAs display higher transwell migratory rates than the control cells (Fig. 2B). Wound assay revealed shSAMD9-1 and shSAMD9-2 cells migrate much faster than the control cells (Fig. 2C). Furthermore, soft agar colony formation assay also revealed shSAMD9-1 and shSAMD9-2 cells formed more colonies than control cells (Fig. 2D). These data indicate that SAMD9 suppress the invasion, migration and proliferation of NSCLC cells *in vitro*.

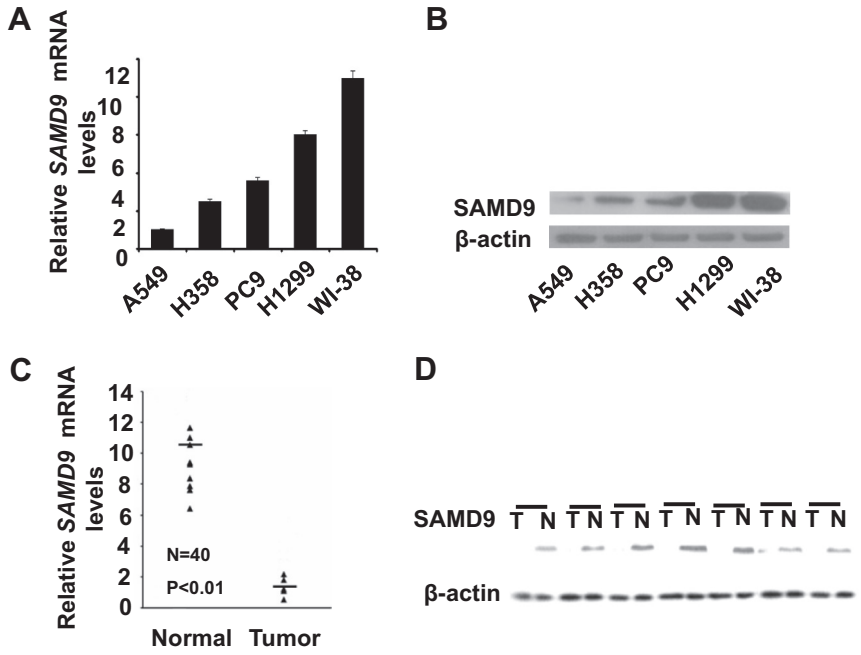

Fig. 1. SAMD9 expression in different NSCLC cell lines and normal lung cell line. (A) SAMD9 mRNA levels examined by RT-qPCR. (B) SAMD9 protein levels examined by Western blot. NSCLC cell lines: H358, PC9, H1299, A549. Normal lung cell line: WI-38. (C) The SAMD9 mRNA expression in NSCLC and the adjacent normal lung determined by RT-qPCR ($n = 40$). (D) The SAMD9 protein expression in NSCLC (T) and adjacent normal lung (N) determined by Western blot ($n = 7$).

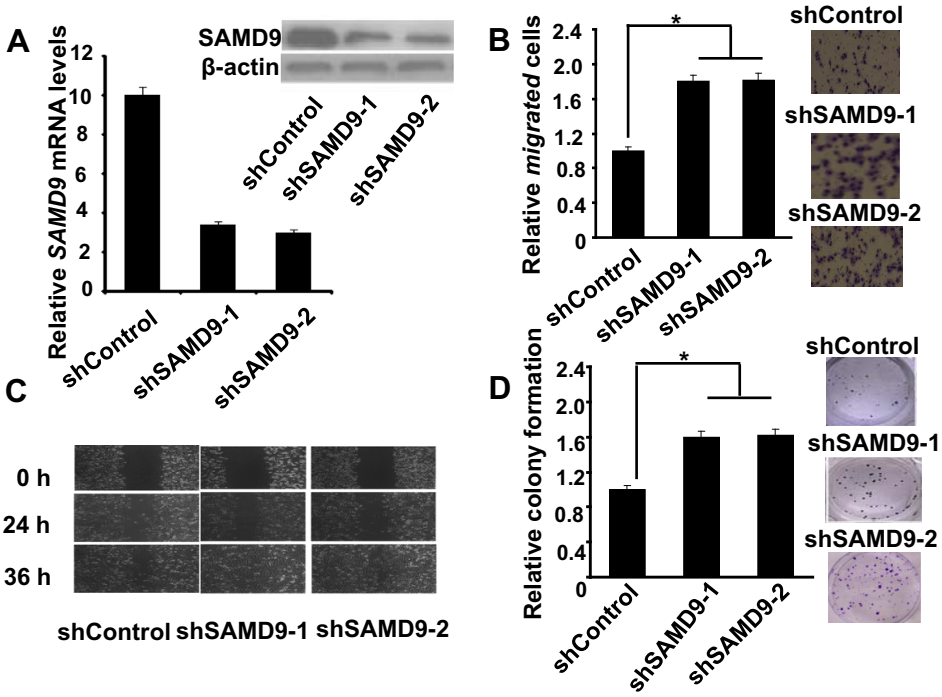

Fig. 2. Downexpression of SAMD9 increases NSCLC cell invasion, migration and proliferation. (A) RT-qPCR and Western blot analysis of SAMD9 expression in H1299 cells transfected with control and SAMD9 shRNAs. (B) Transwell analysis of SAMD9 shRNAs-transfected and control cells. (C) Scratch analysis of SAMD9 shRNAs-transfected and control cells. (D) Colony formation analysis of SAMD9 shRNAs-transfected and control cells. Scale bar = 150 μm. $^{*}P < 0.01$.

3.3. Overexpression of SAMD9 inhibits the proliferation and invasion of NSCLC cell lines in vitro

To investigate the role of SAMD9 in NSCLC cell proliferation and invasion, A549 cells were transfected with pcDNA3-SAMD9. The SAMD9 expression levels were significantly increased in SAMD9-transfected A549 cells by RT-PCR and Western blot (Fig. 3A and B). Next, MTT assay was used to evaluate proliferation. The result indicated that pcDNA3-SAMD9 significantly inhibited A549 proliferation at 48 h after transfection, compared with the parental and control cells (Fig. 3C). Furthermore, we performed transwell assay to demonstrate the effects of SAMD9 on the

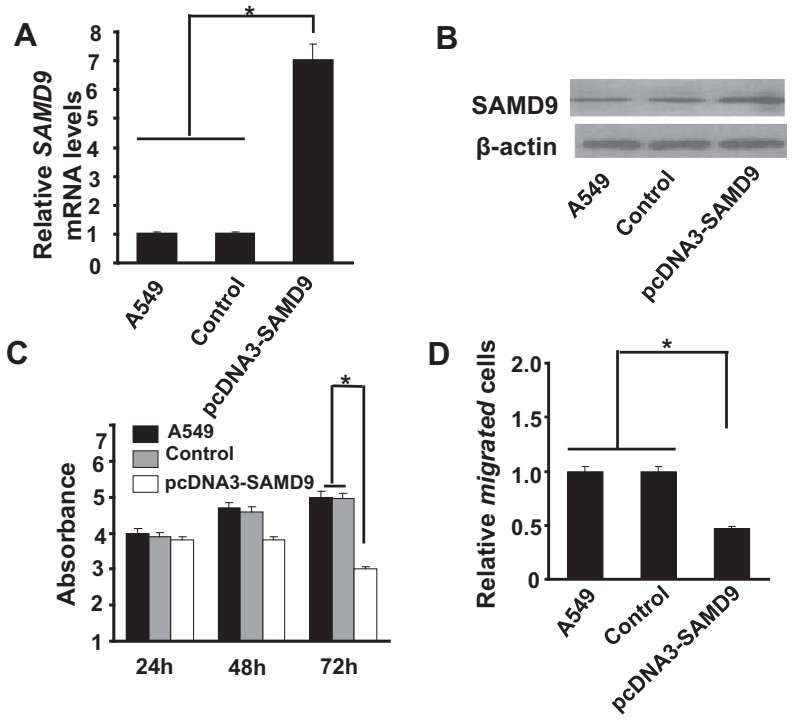

Fig. 3. Overexpression of SAMD9 inhibites the proliferation and invasion of NSCLC cell lines *in vitro*. (A) RT-qPCR analysis of SAMD9 expression in A549 cells were transfected with pcDNA3-SAMD9 and the control. (B) Western blot analysis of SAMD9 and β-actin expression in cells were transfected with pcDNA3-SAMD9 and the control. (C) MTT analysis of transfected pcDNA3-SAMD9 and the control cells. (D) Transwell analysis of transfected pcDNA3-SAMD9 and the control cells.*$P < 0.01$.

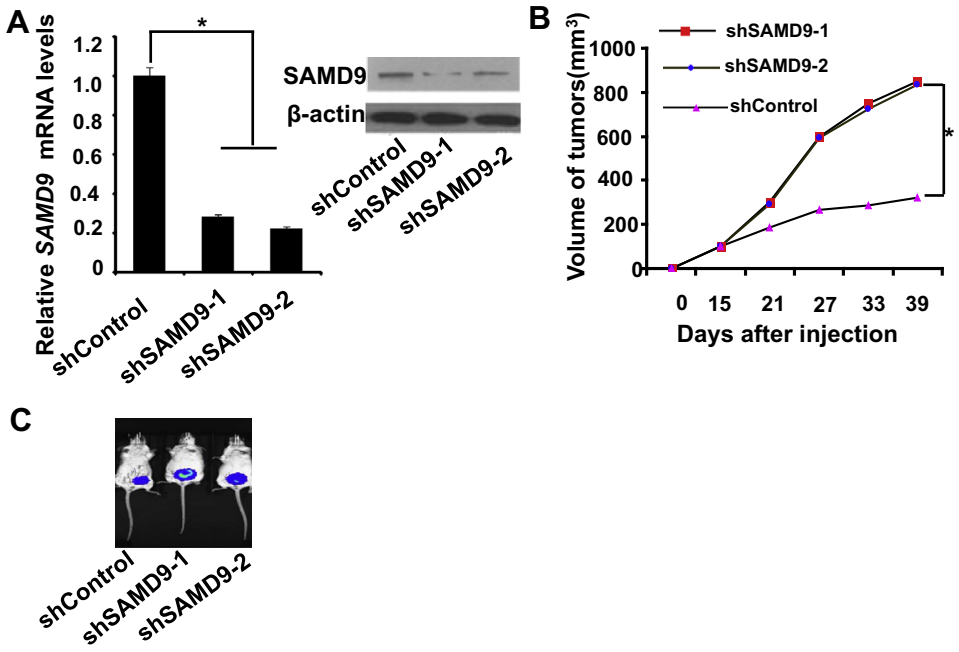

Fig. 4. Depletion of SAMD9 promotes NSCLC tumor formation and invasion *in vivo*. (A) H1299 cells stably expressing SAMD9 shRNA (shSAMD9-1 and shSAMD9-2) or (shControl) were lysed and SAMD9 expression was detected by RT-qPCR and Western blot. (B) 1 × 107 SAMD9 shRNA or shControl cells were inoculated into the mammary fat pad of SCID mice and tumor growth was recorded with a caliper-like instrument. Tumor volumes were calculated according to the formula volume = (width 2 × length)/2. (C) After thirty-nine days, bioluminescence imaging of luciferase activity were photographed. *$P < 0.01$.

invasive potential of A549 cells. The results show that SAMD9 overexpression inhibited invasion (Fig. 3D). These data indicate that SAMD9 inhibites the proliferation and invasion of NSCLC cell lines *in vitro*.

3.4. Depletion of SAMD9 promotes NSCLC tumor formation and invasion in vivo

To further determine the effect of altered SAMD9 expression on NSCLC cell growth, we established two stable SAMD9 shRNA-transfected H1299 cell lines. RT-qPCR and Western blot analyses showed decreased levels of SAMD9 expression in the SAMD9 shRNA-transfected H1299 cell lines (Fig. 4A). We then injected these cells into SCID mice (1×10^7 per mouse) to evaluate the effect of SAMD9 knock down on NSCLC tumor growth. Tumor formation and volume of the tumor in each mouse were examined, measured and recorded for 39 days and tumor growth curves were determined. As shown in (Fig. 4B), tumor formation and tumor growth of SAMD9 shRNA-transfected cells were significantly increased in nude mice when compared with those of control cells. Thirty-nine days after inoculation, bioluminescence imaging of luciferase activity were photographed, SAMD9 shRNA-transfected cells were much larger than those control cells (Fig. 4C). These results indicate that SAMD9 deficiency promotes the tumorigenicity and invasion of human NSCLC cells.

4. Discussion

Highly resistant non-small cell lung cancer is one of the major causes of cancer death across the world and angiogenesis has emerged as an integral process in promoting the growth and metastasis of NSCLC [1]. SAM domains, one of the most common protein domains found in eukaryotic cells, are protein–protein interaction modules that perform a large number of different functions [8]. Because of the great variety of known functions, the presence of a SAM domain does not necessarily involve a specific function or pathway, but an array of possible functions. SAMD9 the ability to form SAM polymers has been suggested that SAMD9 may have a similar function participating in protein complexes [9]. SAMD9 is expressed at a lower level in aggressive fibromatosis and some cases of breast and colon cancer. In the present study, we investigated the involvement of SAMD9 in the human NSCLC. We have identified that SAMD9 significantly down-regulated in NSCLC cancer tissues compared with normal lung tissues. In a subsequent study, we used shRNAs targeting SAMD9 to inhibit the SAMD9 expression in H1299 cells. We demonstrated that downexpression of SAMD9 increases the proliferation and invasion of human H1299 cell lines *in vitro*. It was reported that cell proliferation, invasion and migration of cancer cells at a primary site are important steps in the process of metastasis [10]. Our important finding supports this. Furthermore, A549 cells were transfected with pcDNA3-SAMD9. These data indicate that SAMD9 inhibits the proliferation and invasion of NSCLC cell lines *in vitro*. Finally, depletion of SAMD9 promotes NSCLC tumor formation and invasion *in vivo*.

In conclusion, we have demonstrated that SAMD9 expression is significantly down-regulated in NSCLC. Overexpression of SAMD9 inhibits the tumorigenicity *in vitro* and knockdown of SAMD9 promotes tumorigenicity *in vivo*. Functional analyses indicated that SAMD9 may be a potential biomarker in metastatic NSCLC, Whether the SAMD9 is a biomarker to evaluate patient prognosis is an issue that will be investigated in future studies.

Conflict of interest

The authors have no financial interests in or financial conflict with the subject matter discussed in this manuscript.

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