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# S100A4 is frequently overexpressed in lung cancer cells and promotes cell growth and cell motility

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ABSTRACT

S100A4, a small calcium-binding protein belonging to the S100 protein family, is commonly overexpressed in a variety of tumor types and is widely accepted to associate with metastasis by regulating the motility and invasiveness of cancer cells. However, its biological role in lung carcinogenesis is largely unknown. In this study, we found that S100A4 was frequently overexpressed in lung cancer cells, irrespective of histological subtype. Then we performed knockdown and forced expression of S100A4 in lung cancer cell lines and found that specific knockdown of S100A4 effectively suppressed cell proliferation only in lung cancer cells with S100A4-overexpression; forced expression of S100A4 accelerated cell motility only in S100A4 low-expressing lung cancer cells. *PRDM2* and *VASH1*, identified as novel upregulated genes by microarray after specific knockdown of S100A4 in pancreatic cancer, were also analyzed, and we found that *PRDM2* was significantly upregulated after S100A4-knockdown in one of two analyzed S100A4-overexpressing lung cancer cells. Our present results suggest that S100A4 plays an important role in lung carcinogenesis by means of cell proliferation and motility by a pathway similar to that in pancreatic cancer.



## 1. Introduction

Primary lung cancer is one of the most frequently occurring malignancies; it is the leading cause of cancer-related death in men and the second leading cause in women in Japan [1]. Lung cancers are divided into two major types, non-small cell lung carcinoma (NSCLC) and small cell lung carcinoma (SCLC); the former accounts for approximately 85% of all lung cancer cases, and the typical histopathological types are adenocarcinoma, squamous cell carcinoma, and large cell carcinoma [2]. Although the 5-year survival rate has significantly improved over the last 30 years, the overall survival rate is still 17% in the USA [3]. Therefore, there is an urgent need for improvement of clinical management of patients with lung cancer.

One potentially interesting molecular marker of lung cancer is S100A4, which belongs to the S100 family of calcium binding proteins and is characterized by the EF-hand motif and is emerging as a multifunctional player in cell proliferation, differentiation, metabolism, adhesion, motility, angiogenesis and signal transduction [4]. To date, 22 members have been assigned to the family, and most of them are mapped to human chromosome 1q21 [5].

S100A4 protein has been reported to be important in human carcinogeneses in various types of cancer, including those of urinary bladder [6], breast [7], colorectum [8], stomach [9], pancreas [10] and prostate [11]. We previously analyzed S100A4 in pancreatic cancer and reported that its overexpression is associated with cell growth, motility, and invasion, particularly to nerves [12–14].

Expression of S100A4 in lung cancer has been reported; 60% (81 of 135) in NSCLC [15] and 67.2% (137 of 204) in squamous cell carcinomas [16]. Furthermore, upregulation of S100A4 is significantly associated with poor prognosis in lung adenocarcinoma [17]. We previously reported that S100A4 is frequently upregulated in pancreatic cancer [14] and that siRNA-mediated knockdown of *S100A4* causes apoptosis and inhibition of cell motility only in cells with upregulated S100A4 [12]. In contrast, forced expression of S100A4 accelerated cell motility only in cells with downregulated S100A4 [13]. It is of great interest to analyze whether or not S100A4 has similar functions in lung cancer. In the present study, we report that the expression of S100A4 is frequently upregulated in lung cancer cell lines and that the siRNA-mediated knockdown suppresses cell growth in S100A4-upregulated lung cancers.

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## 2. Materials and methods

### 2.1. Lung cancer cell lines and cell culture

In this study, a total of 14 human lung cancer cell lines were used; seven adenocarcinomas (A549, PC9, LK87, 11–18, HLC-1, LCSC#2, HT-1), three squamous cell carcinomas (Sq-1, Sq-19, HS-24), two large cell carcinomas (Lu65, Lu99c), and two small cell carcinomas (SBC-3, LK79). These cells were also used in our previous studies [18,19]. An immortalized normal human lung epithelial cell, BEAS-2B, was also used as the control; this cell line was maintained in E-MEM medium supplemented with 10% FCS.

### 2.2. RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNAs were isolated using a RNeasy Midi Kit (QIAGEN, Valencia, CA). Then RNAs were run on a 1% agarose gel, and concentrations were spectrometrically measured at 260 and 280 nM using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific Inc., Pittsburgh, PA). An aliquot of 2 μg of each extracted RNA was used for cDNA synthesis by High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantitative RT-PCR (qRT-PCR) using the ABI PRISM 7000 (Applied Biosystems) was performed as described [20], and the data were normalized using the β2-microglobulin (*B2M*) mRNA as the internal control [21]. Primers and probes were previously designed [12,13].

### 2.3. siRNA transfection

Short interference RNA targeting against *S100A4* was used as described [12]; siRNA corresponding to nucleotides 215–233 relative to the initiating codon (5′-GGACAGAUGAAGCUGCUUUdTdT-3′ and 5′-AAAGCAGCUUCAUCUGUCCdTdT-3′) was used [22], and siRNA against luciferase (*GL2*) was used as the control [23]. These siRNAs were purchased from Integrated DNA Technologies MBL (Nagoya, Japan). A total of $2 \times 10^5$ cells were plated in 6-well plates, allowed to adhere for 24 h, and transfected using the Lipofectamine RNAi MAX reagent (Invitrogen, Carlsbad, CA) or Oligofectamine reagent (Invitrogen) according to the manufacturer's instructions.

### 2.4. Forced expression of S100A4 in lung cancer cell lines

The previously cloned S100A4-expressing plasmid, pcDNA6-S100A4 [13] was used. Transfection of the plasmid was performed using a total of $2 \times 10^5$ cells plated in 6-well plates. Cells were allowed to adhere for 24 h, and the transfection was done using Lipofectamine 2000 reagent (Invitrogen) according to the supplier's protocol. An empty plasmid pcDNA6/*Myc*-His was also transfected as the control. To assess transfection efficiency, green fluorescent protein (GFP) reporter plasmid pSUPER.retro.neo + GFP was co-transfected with either pcDNA6-S100A4 or pcDNA6/*Myc*-His in 6-well culture dishes. The molar ratio was as follows; pcDNA6 plasmid: reporter plasmid = 10: 1. Briefly, $3 \times 10^4$ cells/well were seeded on BD BioCoat Fibronectin 4-well CultureSlides (BD Biosciences, Bedford, MA) 24 h after the co-transfection. The fluorescent images were obtained 48 h after transfection with a Zeiss LSM5 PASCAL confocal microscope system (Carl Zeiss Inc., Thornwood, NY) as described previously [24]. Cells with successful transfection were used for analyses of cell growth and motility after induction of S100A4.

### 2.5. Western blotting

The proteins from harvested cells were extracted, and their concentrations were measured using the DC protein assay kit (Bio-Rad, Hercules, CA). A 40 μg aliquot of each protein was electrophoresed in a SuperSep Ace 5–20% polyacrylamide gradient gel (Wako, Japan) and subjected to Western blotting as described previously [25]. The antibodies used were rabbit anti-S100A4 polyclonal antibody (DakoCytomation, Denmark), mouse anti-β actin monoclonal antibody (Sigma, St. Louis, MO), and horseradish peroxidase conjugated anti-mouse or anti-rabbit immunoglobulin antibodies (Amersham Biosciences Corp., Piscataway, NJ); conditions followed by the manufacturer's recommendations. Signals were visualized by reaction with ECL Detection Reagent (Amersham Biosiences Corp.) and digitally processed using LAS 4000 Plus with ImageQuant TL software (GE Healthcare, Piscataway, NJ).

### 2.6. Cell proliferation assay

A total of $1 \times 10^4$ cells was plated in 24-well plates 24 h after transfection. Cells of each well were harvested and counted every 24 h up to 120 h using Countess Automated Cell Counter (Invitrogen) according to methods from the supplier. These cells were pelleted for further investigation of S100A4 expression. All experiments were performed in triplicate.

### 2.7. Scratch assay

Each $2 \times 10^5$ cell aliquot was seeded in a 6-well plate after the siRNA or plasmid transfection. Cells were grown for 24 h to create a confluent monolayer. Then the bottom of each well was scraped with a 200 μl-micro-pipette tip. This time point was set as time 0. Plates were washed twice with PBS to remove detached cells and incubated with the complete growth medium. Wounded empty spaces were measured and photographed over time until they were closed. Each scratch assay was performed in triplicate.

### 2.8. Statistical analysis

All experiments were performed in triplicate. A two-tailed Student's *t*-test was used for statistical analyses of comparative data using Microsoft Excel software (Microsoft Corporation, Tokyo, Japan). All significance tests were two-sided, and values of $P < 0.05$ were considered as significant.

## 3. Results and discussion

### 3.1. Expression analysis of S100A4

Because lung cancer is the number one killer in Japanese males, we thought it will be of interest to analyze S100A4 in this tumor type also. We first examined S100A4 expression by Western blotting. Representative results are shown in Fig. 1A; 14 lung cancer cell lines covering four major histological types (adenocarcinoma, squamous cell carcinoma, large cell carcinoma, and small cell lung carcinoma) and BEAS-2B, a cell line established from normal bronchial epithelium as the control, were analyzed. Distinct expression levels of S100A4 were observed among cell lines, but high levels of S100A4 expression were frequent when compared with that of BEAS-2B; they were detected in six of seven adenocarcinomas, two of three squamous cell carcinomas, one of two large cell carcinomas, and one of two small cell carcinomas. Two adenocarcinomas (11–18 and HLC-1) and two SCLCs (SBC-3 and LK79) were selected as representing the low- and high-level expressing cells,

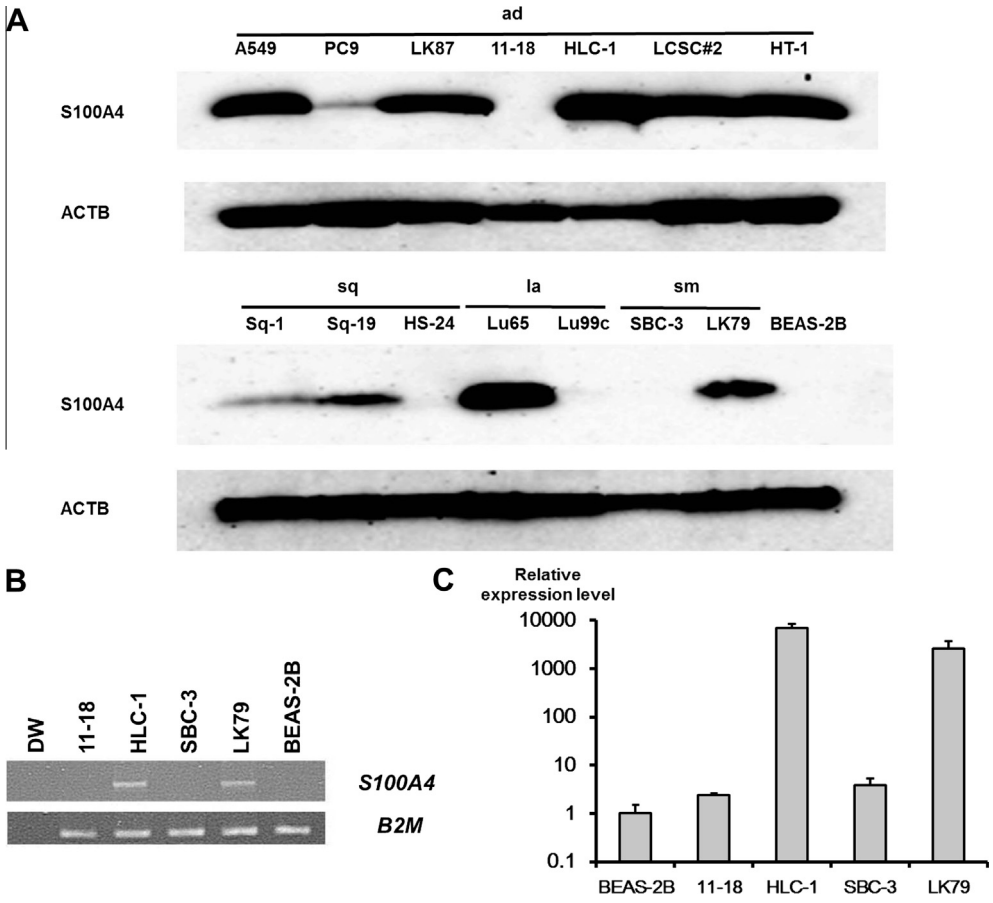


**Fig. 1.** Expression analyses of S100A4 in lung cancer cell lines. (A) Western blot analyses of S100A4 protein, which were determined by anti-S100A4 rabbit polyclonal antibody. Expression of β-actin (ACTB) was monitored as the internal control. The immortalized normal human lung epithelial cell BEAS-2B was used as the control. Ad, sq, la, and sm denote adenocarcinoma, squamous cell carcinoma, large cell carcinoma, and small cell lung carcinoma, respectively. (B, C) Expression of S100A4 in BEAS-2B and four selected lung cancer cell lines was determined by (B) RT-PCR, (C) qRT-PCR, respectively. Expression of the *B2M* gene was monitored as the internal control.

respectively, and were subjected to further characterization. Results of RT-PCR are also shown in Fig. 1B and C.

### 3.2. siRNA-mediated knockdown against S100A4 in lung cancer cell lines

To address the question whether S100A4 could serve as a therapeutic target for lung cancer, we employed siRNA-mediated knockdown in an attempt to deplete the expression of S100A4 in lung cancer cell lines. As shown in Supplementary Fig. 1, the specific suppression of expression of S100A4 by the siRNA was confirmed by Western blotting and qRT-PCR analyses. In Fig. 2A, suppression of proliferation was observed only in the S100A4-overexpressing cell lines HLC-1 and LK79. In contrast, no growth suppression was observed in the 11–18 and SBC-3 cell lines that did not express S100A4; it was highly likely that knockdown of S100A4 specifically suppressed the growth in S100A4-overexpressing cell lines. No morphological differences were observed after knockdown of *S100A4* in all the analyzed cells.

In order to investigate whether S100A4 knockdown affects cell motility, scratch assays were performed. As shown in Fig. 2B, silencing of S100A4 reduced the speed of refilling wounded empty spaces in HLC-1 and LK79, whereas no such differences were evident in 11–18 and SBC-3 (Supplementary Fig. 2). Speed to fill wounded empty spaces seemed to depend on the speed of cell growth, and it is likely that downregulation of S100A4 does not suppress cell motility of lung cancer cells with S100A4 overexpression (Fig. 2C).

### 3.3. Effects of forced expression of S100A4

We previously observed that forced expression of *S100A4* induced cell motility without stimulation of cell growth in human pancreatic cancer cell lines [13]. In this study, we analyzed both cell proliferation and motility in lung cancer cell lines using the same method as we did in pancreatic cancer. In order to monitor the efficiency of transfection, we performed co-transfection of pSUPER.retro.neo + GFP with pcDNA6-S100A4. Forced expression was successful in cell lines 11–18, HLC-1, SBC-3 and LK79, as shown in Supplementary Fig. 3A. Results of RT-PCR and Western blotting also supported successful transfection (Supplementary Fig. 3B, C). It is notable that no significant morphological differences were evident after the introduction of S100A4 (data not shown). In a pattern similar to that with pancreatic cancer, no proliferative accelerations were observed in lung cancer cells with either S100A4-low expression (Fig. 3A) or S100A4-overexpression (Supplementary Fig. 4A). It is notable that acceleration of cell motility was evident in S100A4-low expressing lung cancer cells after forced S100A4 expression (see Fig. 3B), but not in S100A4-overexpressing cells (Supplementary Fig. 4B). Statistical analysis indicated that this acceleration of cell motility is significant ($P < 0.01$ for 11–18 and $P < 0.001$ for SBC-3) (see Fig. 3B).

### 3.4. Possible molecular regulators were induced to express after knockdown of S100A4

To investigate the possible roles played by S100A4 in lung carcinogeneses, we analyzed the expression of the *PRDM2/RIZ*

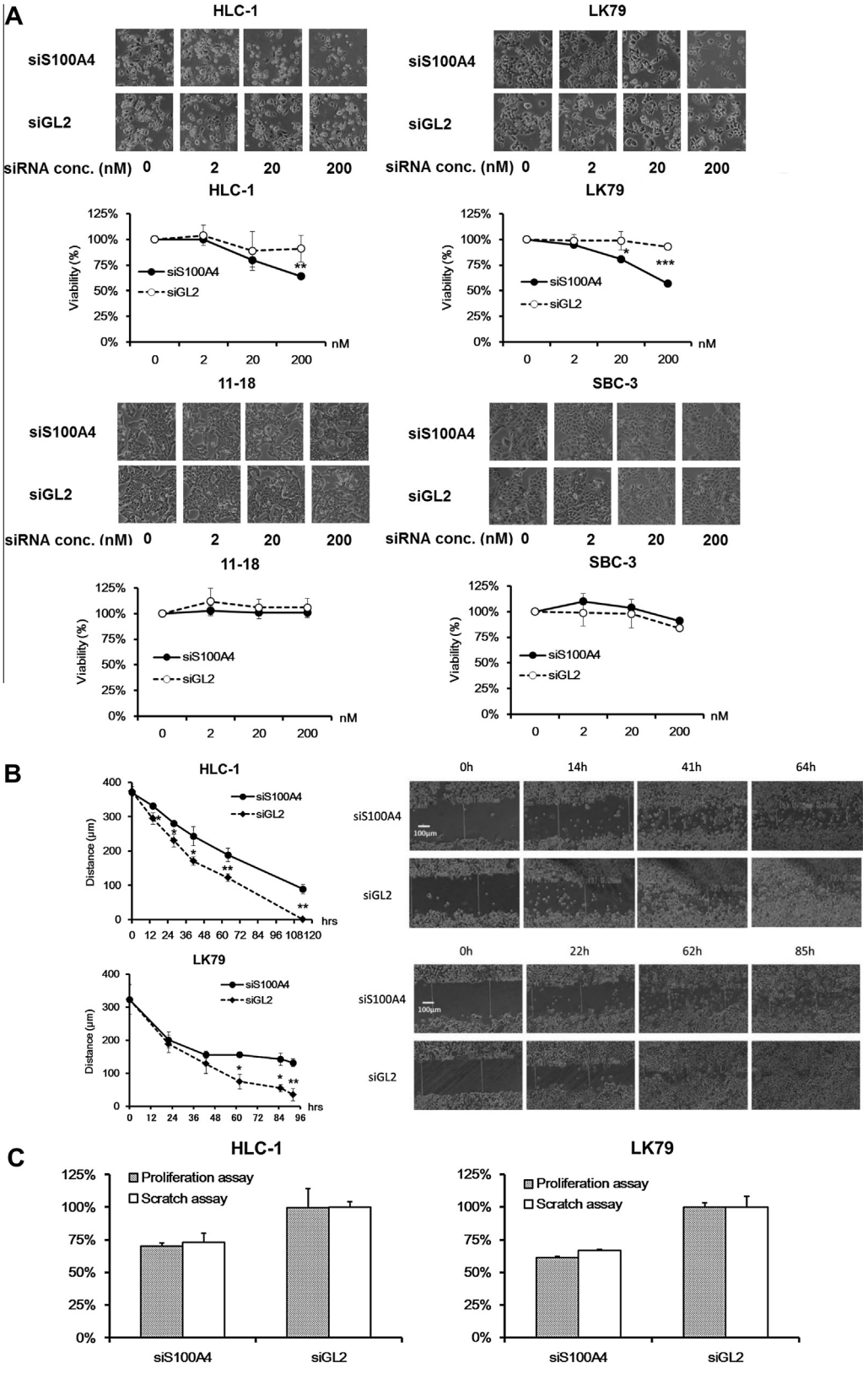


**Fig. 2.** Effects of specific S100A4-knockdown on cell growth and motility. (A) Results of morphological and cell proliferation analyses after siRNA-mediated knockdown of S100A4 at 2, 20, and 200 nM in HLC-1, 11–18, LK79, and SBC-3. Solid and dotted lines denote knockdown against *S100A4* and *GL2*, respectively. Significant differences in cell proliferation were observed only in HLC-1 and LK79; *, **, and *** denote $P < 0.05$, $P < 0.01$, and $P < 0.001$ respectively. (B) Results of the scratch assay after siRNA-mediated knockdown of S100A4 at 200 nM in HLC-1 and LK79, the S100A4-overexpressing cells. Cells were seeded at 24 h after the siRNA transfection, and 24 h after the cell-seeding, the bottom of each well was scraped with the fine end of a micro-pipette tip. This time point was set as time 0. Distances were measured at various time points and are graphically demonstrated at the right; then statistical analyses were done. Significant differences between cell migrations were observed in both HLC-1 and LK79; * and ** denote $P < 0.05$ and $P < 0.01$ respectively. Representative pictures are shown in the right columns. (C) Comparison of results of the cell proliferation assay and the scratch assay after siRNA-mediated knockdown of *S100A4* at the same concentration (200 nM). There were no statistically significant differences. *GL2* was used as the control.

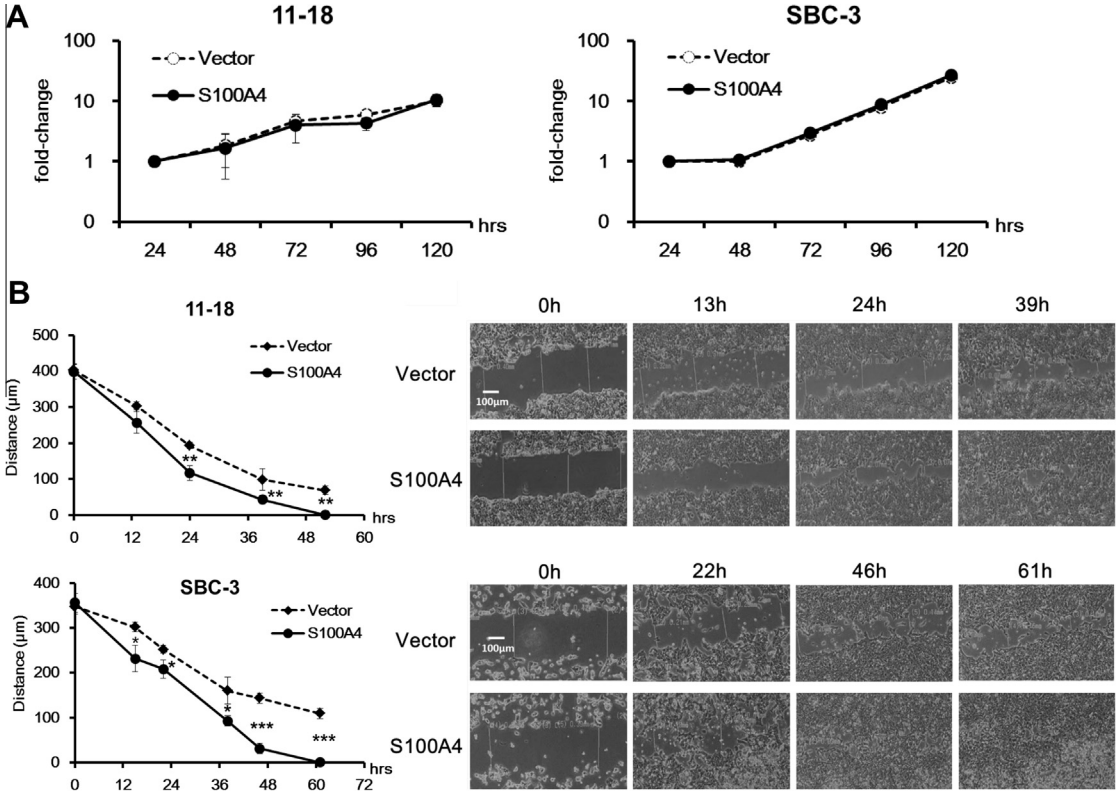


**Fig. 3.** Effects of forced expression of *S100A4* on cell growth and motility in S100A4-low expressing cells. (A) Cell proliferation assay demonstrated no differences after forced expression of S100A4 in 11–18 and SBC-3, the S100A4-low expressing cells. Dotted and solid bars denote empty vector (Vector) and S100A4-expression vector (S100A4) transfected cells, respectively. (B) Results of the scratch assay after forced expression of S100A4 in 11–18 and SBC-3. Cells were seeded at 24 h after the transfection, and, 24 h after the cell-seeding, the bottom of each well was scraped with the fine end of a micro-pipette tip. This time point was set as time 0. Distances were measured at various time points and are graphically demonstrated at the right; then, statistical analyses were done. Significant differences between cell migrations were observed in both 11–18 and SBC-3; *, **, and *** denote $P < 0.05$, $P < 0.01$, and $P < 0.001$ respectively. Representative pictures are shown in the right columns.

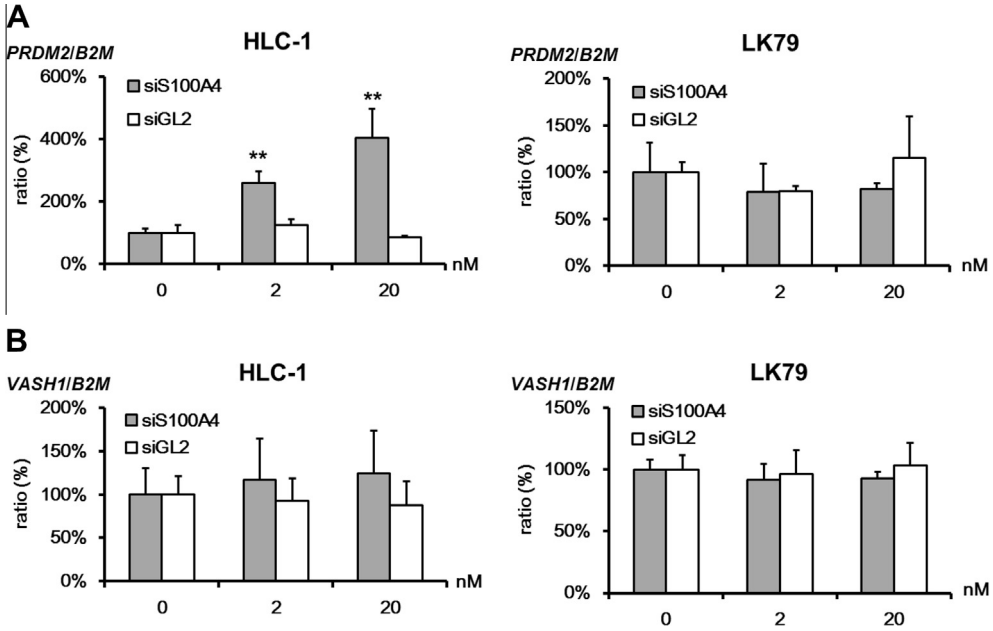


**Fig. 4.** Results of qRT-PCR of *PRDM2* and *VASH1* after siRNA-mediated knockdown of S100A4 (2, 20 nM). *B2M* was used as the internal control. Expression levels were normalized using the mock-transfected (labeled as 0 nM) cells. *PRDM2* was significantly upregulated at 72 h after the transfection only in HLC-1 ($P < 0.01$), and no other differences were observed.

and *VASH1/Vasohibin* genes, because these genes were upregulated after siRNA-mediated knockdown of *S100A4* in pancreatic cancer in our previous study [12]. After 72 h of S100A4-knockdown in HLC-1 and LK79 by qRT-PCR, upregulation of *PRDM2* was observed in one of the two analyzed cell line, HLC-1 (Fig. 4A). In contrast, no expression change was observed in *VASH1* (Fig. 4B).

*PRDM2* (PR domain containing 2, with ZNF domain), which is also named retinoblastoma protein-binding zinc finger protein (*RIZ*), was isolated as a candidate tumor suppressor gene by a functional screening for RB-binding proteins [26], and aberrations of this gene were observed in a number of tumors including pancreatic, gastric, and colon cancers [27,28]. Furthermore, methylation mediated suppression of this gene in cancers of the breast and lung as well as in neuroblastoma has been reported [29]. It is anticipated that suppression of *PRDM2* expression is one of the downstream events of S100A4 upregulation in lung adenocarcinoma. Further investigations are necessary for elucidation of the function of both S100A4 and PRDM2 in human carcinogenesis.

### 3.5. Future studies and conclusion

In this study, we investigated the expression of S100A4 and the biological responses in lung cancer cells after its downregulation and upregulation. We found that S100A4 is frequently overexpressed in lung cancer, irrespective of the histopathological subtype, that knockdown of S100A4 suppressed cell growth only in lung cancer cells with high levels of S100A4, and that forced upregulation of S100A4 promoted cell motility only in cells with S100A4-low level expression. These features are similar to those we reported in pancreatic cancer cells. Our present results suggest that S100A4 may be one of the biomarkers for patients with lung cancer and that suppression of S100A4 may benefit patients with S100A4-upregulated lung cancer. Further investigations, including the characterization of genes downstream from S100A4, are necessary for obtaining valuable clues to improving the clinical management of patients with lung cancer.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.04.025.

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