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Overexpression of *SALL4* in lung cancer and its importance in cell proliferation

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Abstract. Few target molecules have been identified that enable the diagnosis of lung cancer with high sensitivity and specificity, especially in the early clinical stages. Herein, we present the first evidence for mRNA overexpression of SALL4, a transcription factor essential for embryonic development and the self-renewal of embryonic stem cells, in lung cancer. Analysis using cancerous and noncancerous tissues revealed that the sensitivity and specificity of SALL4 mRNA were 85.1 and 92.9%, respectively, estimated using the cutoff value obtained from analyzing the receiver operating characteristic curve. Furthermore, comparison of paired tissues from the same patient revealed elevated SALL4 mRNA levels that were greater than two-fold in 93% of the specimens. *SALL4* mRNA was highly expressed even in the early clinical stages and there was no difference in the positivity rate between stage IA and other stages. An siRNA approach to determine the significance of *SALL4* expression revealed catastrophic growth inhibition of SBC-1 lung cancer cells that was induced by cell cycle arrest at the G1/early S phase. Therefore, SALL4 mRNA may be a candidate for use as support in the diagnosis of lung cancer, and may also represent a therapeutic target.

Introduction

To increase the efficacy of lung cancer therapy, it is important to identify candidate molecules that play crucial roles in cancer cell proliferation and would facilitate tumor detection with high sensitivity and specificity, even in early stages of the disease. Measurement of the gene expression of such molecules would support disease diagnosis, especially when very few cells are available and malignancy cannot be definitively diagnosed by histological or cytological methods. Previous studies have shown that some molecules are useful in distinguishing cancerous from noncancerous samples by measuring the expression of single or multiple genes in tissue, peripheral blood cells, or serum (1-4). However, in actual use as a marker to support the diagnosis, there has been a limit in the specificity and sensitivity because of non-negligible level of expression in noncancerous samples or low detection rate in cancerous samples even with high specificity.

Cancer cells have an ability of self-renewal through uncontrolled proliferation and dedifferentiation, similar to embryonic stem (ES) cells (5). Detection of the expression of molecules that are crucial to ES cell self-renewal might reveal essential aspects of carcinogenesis. Thus, if the expression of these molecules is up-regulated in cancers, these molecules represent ideal candidates to support cancer diagnosis. Some molecules such as Nanog, Klf4, Oct3/4, and Sall4, which are expressed during early embryonic development, are important in the maintenance of mouse ES cell self-renewal (6-9). These molecules also generate and maintain the ability of induced pluripotent stem (iPS) cells to self-renew in mice and humans (10-12). Nanog and Sall4 are key factors in maintaining the undifferentiated state and cell proliferation, respectively (13,14). Knockdown of Sall4 expression leads to catastrophic ES cell proliferation, and *Sall4* knockout mice do not survive to embryonic day 7 (14).

Sall4 is the mouse homolog of the *Drosophila* homeotic gene *spalt (sal)* and is required for the early development of the posterior head and anterior tail of *Drosophila* (15). *Sal* also regulates pattern formation and cell fate decisions in the wing disc, trachea, and sensory organs. Mutations in the human homolog *SALL4* are known to cause Okihiro syndrome (Duane-radial ray syndrome), characterized by limb deformities and loss of eye movement (16,17). In some cases, anomalies of the rectum, ear, heart, and kidney are observed.

The *SALL4*/*Sall4* gene is constitutively expressed in human and mice CD34-positive hematopoietic stem cells (18). Of note, the overexpression of the *Sall4* leads to leukemogenesis by increasing the number of leukemic cells with markers for stem cells in 50% of transgenic mice (18). In fact, *SALL4* is overexpressed in various types of human hematopoietic malignancies such as acute myelocytic and lymphocytic leukaemia (19,20). Moreover, SALL4 up-regulates the expression of the oncogene *Bmi-1* in human hematopoietic stem cells and leukemic cells (21). Bmi-1 activates telomerase reverse transcriptase, thereby inducing telomerase activity and leading to the transformation of human noncancerous epithelial cells (22).

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Bmi-1 also inhibits the function of INK4a/ARF, usually by disturbing cyclin-dependent kinases 2, 4, and 6 (23) indicating that Bmi-1 expression leads to the progression of the cell cycle from G1 to the S phase. These observations strongly suggest oncogenic potential for SALL4, allowing us to speculate that SALL4 might be overexpressed and potentially used as a diagnostic marker in human malignancies. As for this point, Cao *et al* reported the overexpression of SALL4 protein as a novel diagnostic marker for germ cell tumors of the testis and ovary and for metastatic germ cell tumors (24-26). However, there have been no studies that evaluated the sensitivity and specificity of SALL4 expression in the diagnosis of non-germ cell tumors. As for clinical use, especially in cases where a definite cytological (or histological) diagnosis cannot be obtained using limited numbers of cells, a gene amplification technique is ideal for the sensitive detection of the marker to support a diagnosis. Therefore, before the clinical study to determine diagnostic performance, we first examined whether SALL4 mRNA could actually be overexpressed in cancerous tissues from lung cancer patients. By transducing siRNA against *SALL4*, we also determined the significance of SALL4 expression in lung cancer cells.

Materials and methods

Patients and frozen tissue samples. Cancerous (44 non-small cell lung cancer; 2 small cell lung cancer, and 1 unknown tissue type) and noncancerous (14) tissue specimens were obtained from lung cancer patients after surgery. After informed consent was obtained, samples were dissected from these tissue specimens and immediately frozen in liquid nitrogen in a screw-capped cryotube. The tissue samples were stained with haematoxylin/eosin and reviewed by experienced pathologists. Clinicopathological factors and clinical stages were evaluated according to the criteria of the Japanese Society of Lung Cancer, which are based on the tumor-node-metastasis staging system.

Cell culture. The human lung cancer cell line SBC-1 (LC817) was cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich Japan K.K., Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen, Carlsbad, CA) and grown at 37˚C in a humidified atmosphere of 5% CO_2. SBC-1 cells show aggressive proliferative activity (doubling time: ~12 h in a logarithmic growth phase) compared to well-known cancer cell lines.

Quantification of SALL4 mRNA expression. The expression of *SALL4* mRNA was determined using quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) on an ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA). Total RNA was isolated using the ISOGEN reagent (Nippon Gene, Toyama, Japan), according to the manufacturer's instructions. The RNA concentration was determined using the GeneQuant DNA/RNA Calculator (Amersham Pharmacia Biotech, Uppsala, Sweden). The intron-spanning, gene-specific primers and fluorescent hybridization probes used in the qRT-PCR were: *SALL4* forward primer, 5'-AAG TGT AAG GGT CGG AGC AGT CT-3'; reverse primer, 5'-AAT GTC GAG GGT CCC ACA AA-3'; and probe, 5'-(FAM) CCA CGT TTA TCC GAG CCC CGC (TAMRA)-3'. qRT-PCR was performed using the TaqMan One-Step RT-PCR Master Mix reagent kit (Applied Biosystems). To compare *SALL4* mRNA expression in different samples, its levels were normalized to the levels of 18S ribosomal RNA (rRNA). The expression of 18S rRNA was determined using the TaqMan Ribosomal RNA control reagent kit (Applied Biosystems) according to the manufacturer's instructions. For each experiment, a calibration curve using control RNA from MKN45 cells was prepared. Briefly, using a computer algorithm, reporter and quenching dye emissions were analyzed during PCR amplification, and the intensity of fluorescent signals during each PCR cycle was detected. Amplification curves obtained from the serial dilutions of the control RNA were prepared, and the optimal signal intensity (threshold) was manually selected in the exponential phase of the curves. Finally, each PCR cycle number (threshold cycle, Ct) at each initial RNA concentration was determined to generate a calibration curve. The calibration curve was established as an *xy* plot (the log of the input amount [log ng of initial total RNA] as *x* and Ct as *y*). The expression level of the target mRNA in the unknown samples was determined from the Ct value. A control mix that lacked a template was included in each experiment as a negative control.

Transduction of small inhibitory RNA (siRNA) against SALL4. HP GenomeWide siRNA, designed to target the coding region (exon 2) of *SALL4* (GenBank accession no. NM-020436), was obtained from Qiagen (Tokyo, Japan). Single-strand RNAs were annealed by incubating each strand in the siRNA suspension buffer at 90˚C for 1 min and then at 37˚C for 1 h. Non-silencing control RNA (NSC, Qiagen) was used as a transduction control. The transduction of siRNA was performed using the Nucleofector II device and the Cell Line nucleofector kit V (Amaxa Inc. Gaithersburg, MD), according to the manufacturer's instructions.

In brief, ~2-4x10^6 cells were cultured under normal conditions to subconfluency, and 1x10^6 cells were transduced with siRNA or NSC in a cuvette. Next, 5x10^4 of these transduced cells were plated on 3 ml of medium supplemented with 10% FBS in a 6-well plate (Costar, Tokyo, Japan). After 48 h, the expression of the silenced mRNA was quantified by TaqMan RT-PCR. The cells were collected at different periods (24, 48, and 72 h) and subjected to cell number counts and cell cycle analysis. Prior to the assay, the cells in the 6-well culture plate were observed using the BZ-8100 fluorescent microscope (Keyence Co. Ltd, Osaka, Japan), immediately after removal from the incubator.

Cell cycle analysis. Cells plated onto 6-well culture dishes (Costar) were trypsinized and washed with FBS-free media and PBS. The cells were treated with 1 ml of hypotonic fluorochrome solution (50 μg/ml propidium iodide, 0.1% sodium citrate, 0.1% Triton X-100) at room temperature for 30 min. The cells were then kept on ice, and 20,000 cells/sample were analyzed on the EPICS XL flow cytometer (Beckman Coulter Inc., Fullerton, CA).

Statistical analysis. Statistical analysis of the expression level differences of *SALL4* mRNA was analyzed using the Mann-Whitney rank-sum test. Statistical significance was defined as

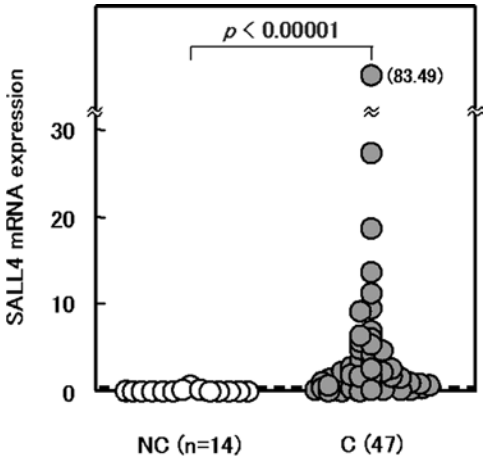

Figure 1. *SALL4* mRNA expression in lung cancer and noncancerous tissues. *SALL4* mRNA expression level was measured by quantitative RT-PCR and is reported relative to the amount of 18S rRNA. The dotted line shows the mean + 2 SDs of *SALL4* mRNA expression levels in noncancerous tissues. NC, noncancerous tissues; C, cancerous tissues. Statistical analysis was performed using the Mann-Whitney rank-sum test.

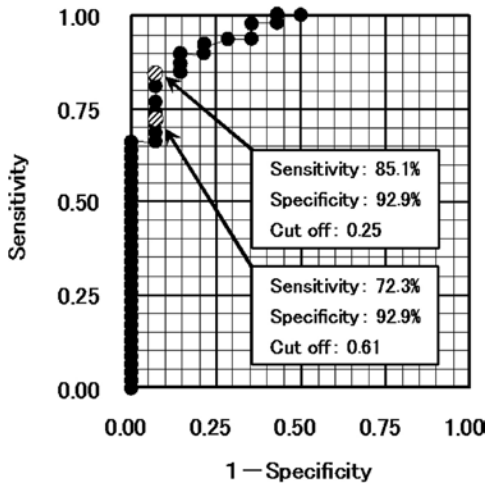

Figure 2. ROC curve for the detection of lung cancer. The curve was automatically generated from multiple points representing various cutoff values determined by StatFlex, version 5.0.

p<0.05. The receiver operating characteristics (ROC) curve was generated, and the sensitivity/specificity at various cutoff values was calculated using StatFlex software version 5.0 (Artech, Osaka, Japan). The software automatically arranges manually inputted data for both groups (noncancerous and cancerous tissues) according to the magnitude of the values. It then calculates the sensitivity and specificity at each cutoff value that is determined for each data set and automatically generates the ROC curve.

Results

Expression of SALL4 mRNA in lung cancers. RT-PCR was performed on noncancerous and cancerous tissue samples obtained from patients with lung cancer (Fig. 1). The mean *SALL4* mRNA expression level in cancerous tissues (5.46±12.77) was remarkably higher than that in noncancerous tissues (0.11±0.24; p<0.00001). The cutoff value for *SALL4* mRNA expression was set as the mean + 2 standard deviations (SDs; 0.58) of its expression in noncancerous lung tissue. *SALL4* mRNA expression was positive in 80.9% of the cancerous tissues (38 of 47; Fig. 1). In ROC analysis, however, an allowable sensitivity (72.3%) and high specificity (92.9%) was obtained at a cutoff value (0.61) close to the mean + 2 SDs of the expression level in noncancerous tissues (Fig. 2). Further, at a cutoff value (0.25) for which the difference between sensitivity and specificity was minimal, the sensitivity and the specificity using the ROC curve was 85.1 and 92.9%, respectively. In addition, *SALL4* mRNA expression was elevated more than two-fold in 92.9% (13/14) of cancer specimens that were obtained along with the adjacent noncancerous tissue samples. The remaining single cancerous specimen showed almost the same *SALL4* mRNA expression level as compared to the noncancerous tissues.

We then analyzed the relationships between the clinicopathological factors and *SALL4* mRNA expression in cancerous tissues. No statistically significant correlations of *SALL4* mRNA expression were found with gender, age, or

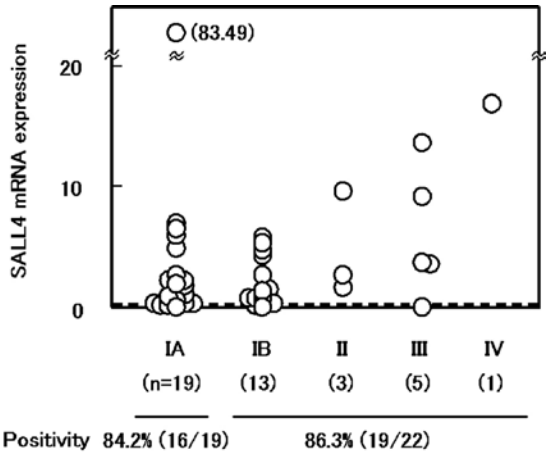

Figure 3. *SALL4* mRNA expression in each clinical stage of lung cancer. *SALL4* mRNA level was measured by quantitative RT-PCR and expressed relative to the amount of 18S rRNA. The dotted line shows the cutoff value (0.25) obtained from ROC analysis.

tissue type. Analysis of the clinical stages of lung cancer, as determined by tumor size, invasion, and metastasis to lymph nodes and distant organs, showed no significant difference in the positivity rate between the 2 groups when they were evaluated by the cutoff value (0.25) from ROC analysis [stage IA, 84.2% (16/19) vs. stage IB-IV, 86.3% (19/22), Fig. 3]. Overall, high *SALL4* expression levels were observed even in the very early stages of lung cancer, although the expression profile at advanced stages cannot be precisely evaluated because of the small number of samples.

Effect of SALL4 siRNA transduction on cell proliferation and cell cycle. To determine the role of *SALL4* expression in cancer cells, we transduced *SALL4* siRNA into lung cancer SBC-1 cells. Transduction of siRNA decreased the *SALL4* mRNA expression level to 43% of the baseline levels after 48 h (Fig. 4). Thereafter, we determined how the transduction of *SALL4* siRNA affects cell numbers. We observed a very slight increase in growth at 24 h after transduction of NSC

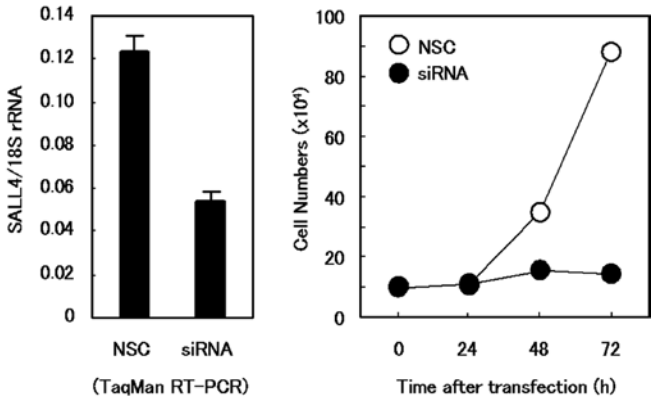

Figure 4. Silencing effect on *SALL4* mRNA expression and on the proliferation by siRNA transduction of SBC-1 cells. Cells were transduced with non-silencing control RNA or siRNA, and total RNA was extracted after a 48-h culture. Twenty nanograms of total RNA was used for quantitative measurement of *SALL4* mRNA expression by TaqMan RT-PCR. The expression level of *SALL4* mRNA in the transduced cells is presented relative to that of 18S rRNA. The data represent the mean value for triplicate measurements. For the proliferation curve, viable cell numbers were counted at 24, 48, and 72 h after the transduction. Open circles, control cells transduced with NSC RNA; closed circles, cells transduced with 2.0 μg *SALL4* siRNA. NSC, non-silencing control RNA; siRNA, small inhibitory RNA.

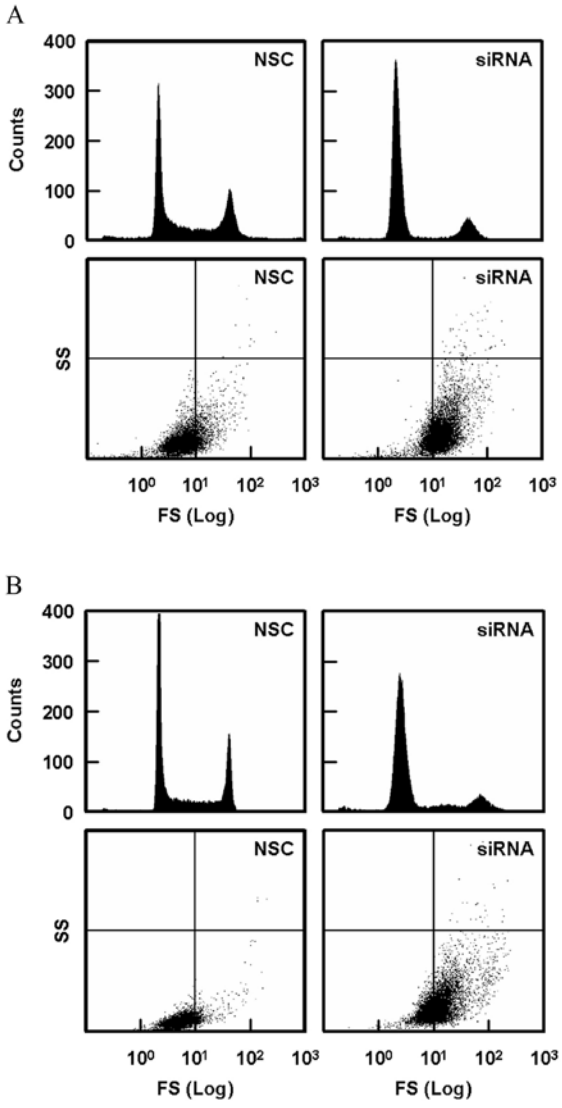

Figure 5. Flow cytometric analysis at 48 h (A) and 72 h (B) after transduction of *SALL4* siRNA into SBC-1 cells. Cells were stained with propidium iodide (PI) and subjected to FACS analysis. Cells identical to those stained with PI were analyzed for cytogram distribution determined by forward scatter (FS) and side scatter (SS). The data in the cytogram were obtained using the same gate as that used in the cell cycle analysis.

RNA and siRNA. Obvious inhibitory effects on cell numbers were observed in siRNA-transduced cells at 48 and 72 h after transduction (Fig. 4). The growth inhibition rate was 73.5 and 93.5% at 48 and 72 h after transduction, respectively. During the same periods, the cells transduced with NSC RNA dramatically increased in number without an obvious loss of viability, as determined by microscopic observation. After 72 h of transduction, there were few viable and normally dividing cells, and only relatively larger cell bodies and cell debris were observed.

We then examined how the cell cycle populations altered during the growth inhibition induced by the transduction of *SALL4* siRNA. The population in the sub-G1 phase did not increase at any time point after transduction. The siRNA-transduced cells showed increased numbers in the G1 phase (38.8-67.8%) and decreased numbers in the S phase (27.6-9.4%) after 48 h, demonstrating typical cell cycle arrest at the G1 and early S phases (Fig. 5A); no alteration was seen in the NSC siRNA-transduced cells. In addition to this phase profile, the overall cell distribution, as determined by 2 parameters (forward scatter, a cell size indicator; and side scatter, an intracellular content indicator), was observed. At 48 h, the cells transduced with siRNA showed an obvious shift to the upper right (Fig. 5A). At 72 h after transduction of NSC, the cells aggressively proliferated and a keen G2/M peak was observed. Additionally, the overall cell distribution altered with a smaller cell volume accompanied by the decreased distance between cells. At this period, in the cells transduced with *SALL4* siRNA, we observed an increase in the number of cells in the G1 phase (48.5-66.3%) and a decrease in the number of cells in the S phase (25.2-14.5%), with a slight increase in the number of cells entering the early S phase. During these periods, the cells did not enter into the full range of the S phase, suggesting that the cell cycle was arrested at the G1 phase accompanied by an arrest at the early S phase. At 72 h after transduction, the directional shift of the cell distribution to the upper right was more evident compared to that at 48 h, consistent with the inhibition of cell division shown by an apparently decreased G2/M population.

Discussion

This study investigated whether *SALL4* mRNA could be used as a marker for the diagnosis of lung cancer. In the present study, we have demonstrated for the first time that *SALL4* mRNA expression can be detected with high sensitivity and specificity in lung cancer tissues, although examined population is rather small. Many studies have analyzed the expression levels of certain candidate genetic markers for cancer diagnosis, but the clinical use of these genes was limited because of either low specificity due to non-negligible expression in noncancerous tissues or low detection rates even with high specificity. This study clarified that essential molecules in ES cell proliferation

are ideal genetic markers for the highly sensitive and specific detection of cancer.

Previous studies have not compared the expression profiles of other ES-related molecules (i.e., Nanog, Oct3/4, and Sox2) with that of SALL4 in human cancers. Interestingly, we recently observed increased Nanog expression in malignant cells in lung cancer patients (27), although the diagnostic performance of Nanog was not superior to that of SALL4 shown in this study. Further, preliminary studies identified a positive correlation between SALL4 and Nanog, although statistical significance was not reliable because of small sample sizes (data not shown). These findings suggest that the reciprocal regulation of gene expression between *SALL4* and Nanog, which has been observed in mouse ES cells (28), may occur in human cells. Thus, there is a possibility that several ES-related molecules co-operatively play pivotal roles in the behaviour of human cancer cells. However, the regulatory mechanisms of both gene expressions are not fully clarified and future analysis using a larger sample size is recommended to elucidate the correlation of these two genes.

In the ROC analysis, the 0.25 cutoff value was associated with a minimum difference between sensitivity and specificity. An important finding of our study is that *SALL4* mRNA was overexpressed even in the majority of the cancer patients at early clinical stages. Thus, we speculate that the quantitative determination of *SALL4* mRNA expression is of clinical value in the diagnosis of small and suspected neoplastic lesions in the lungs. In such cases, qRT-PCR might be useful when there are only a small number of cells per sample, as obtained by punch biopsy, needle/aspiration biopsies, sputum collection, or bronchoalveolar lavage. Therefore, to avoid inappropriate therapy in patients without cancer, the cutoff value should ideally be selected so that a high specificity is obtained. In most of the previous studies, the cutoff value in the analysis of the positivity has been customarily set as the mean + 2 SDs of the expression levels observed in the noncancerous tissues. In this study, the cutoff value close to the mean + 2 SDs of the expression level in noncancerous tissues was 0.61, and resulted in the same high level of specificity (92.9%) but lower sensitivity (72.3%). Thus, for clinical application, a cutoff value at least around the mean + 2 SDs of the expression level in noncancerous tissues or lower is recommended.

The detection of mRNA or protein expression is theoretically applicable for the clinical use of SALL4. Recently, one study demonstrated the SALL4 protein expression profiles in cancerous and noncancerous breast tissues using immunohistochemistry by their original antibody (29). In their study only 5 of 20 breast cancer specimens showed detectable SALL4 expression, resulting in low sensitivity, although the aims were not to examine the usefulness of SALL4 as a diagnostic marker. Moreover, using another antibody, Cao *et al* recently demonstrated that most of germ cell tumors showed high SALL4 protein expression but only 10 (esophageal, gastric, and colonic cancers) of 170 metastatic nongerm cell tumors including 8 breast and 12 lung cancers samples showed weak expression (25). From these facts and our results in this study, at least for lung cancer, the measurement of gene expression, which can be detected with highly sensitive RT-PCR, might be ideal for the diagnostic use of SALL4.

In this study, we additionally examined the significance of SALL4 expression in cancer cells, and siRNA experiments clarified that the constitutive expression of *SALL4* in cancer cells is indispensable for their proliferation. Although the speculated effects of siRNA on the differentiation state were not observed because of rapid and potent growth inhibition, our data are not inconsistent with previous observations that the overexpression of the *Sall4* promotes tumorigenesis in mice (18). However, it is still unclear whether the high SALL4 mRNA expression causes carcinogenesis or whether carcinogenesis leads to elevated SALL4 mRNA levels in human. Regarding this aspect, a recent study demonstrated interesting data that SALL4 up-regulates the expression of an oncogene Bmi-1 via its binding to *Bmi-1* promoter (21). In breast cancer, from the evidence of *Bmi-1* overexpression in cancer tissues (30) and the transformation of mammary epithelial cells by *Bmi-1* overexpression (22), SALL4 may drive the carcinogenesis via Bmi-1. In lung cancer, studies have not shown the possibility of carcinogenesis by Bmi-1. However, at least in this study, *SALL4* siRNA transduction in lung cancer cells dramatically inhibited cell growth via cell cycle arrest at the G1 and early S phases in this study, indicating that SALL4 drives the G1 phase transition of the cell cycle. These data can connect two facts that SALL4 induces *Bmi-1* expression (21) and that Bmi-1 down-regulates the tumor suppressor genes *INK4a/ARF* inhibiting the G1 to S phase cell cycle transition (23). Thus, *SALL4* expression could exert oncogenic potential in the development of human malignancies. Taken together with these findings and our data, it is suggested that the *SALL4* expression could be one of ideal targets to support the diagnosis, at least for lung cancer. However, clinical performance of this marker is still undetermined. Further study using numbers of clinical samples should be performed to know whether *SALL4* expression is detected from small numbers of cells and appropriate cutoff value can distinguish majority of cancers and other diseases.

Acknowledgements

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