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Elevated expression of JAM-A promotes neoplastic properties of lung adenocarcinoma

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Key words

Adenocarcinoma, JAM-A, lung, Neoplasia, tight junction

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A cell–cell adhesion protein, junctional adhesion molecule-A (JAM-A), has been shown to be involved in neoplasia of various organs. However, the fundamental role of JAM-A in tumorigenesis is still under debate because dysregulated expression of this protein has distinct effects, playing opposite roles in carcinogenesis depending on the target tissues. In the present study, we found elevated levels of JAM-A expression in lung adenocarcinoma and its preinvasive lesions, including atypical adenomatous hyperplasia and adenocarcinoma *in situ* by immunohistochemistry. We also showed that suppression of constitutive JAM-A expression conferred target cells with increased susceptibility to apoptosis in lung adenocarcinoma cells. Consequently, inhibition of JAM-A activity decreased colony-forming capability *in vitro* and tumorigenicity *in vivo*. The transformed phenotype following suppression of JAM-A expression was sufficient to reduce motile and invasive capacities. Importantly, knockout of *JAM-A* had striking effects on cells. Our observations suggest that increased expression of JAM-A promotes neoplasia of lung adenocarcinoma. In addition, an anti-JAM-A antibody efficiently reduced cell proliferation and provoked apoptosis, indicating the potential feasibility of JAM-A-inhibitory cancer therapy.

Tight junctions (TJ) are the apical-most intercellular adhesion structures in epithelial and endothelial cells, forming the closest contacts between adjacent cells.[1–5] TJ are not simple static constituents but are dynamic structures having a complex molecular composition, including claudin family members, occludin, and scaffolding proteins. Junctional adhesion molecule proteins (JAM) are transmembrane glycoproteins that belong to the immunoglobulin superfamily, the most well-characterized members of which are JAM-A, JAM-B, and JAM-C.[6] JAM-A is expressed predominantly at TJ of epithelial and endothelial cells. JAM-A has been shown to have a unique expression profile in a wide variety of tissues, including the lung, heart, central nervous system, liver, kidney, and lymph nodes.[7,8] Previous studies have shown that JAM-A is involved in various cellular physiologies such as cell–cell adhesion, platelet activation, leukocyte migration, angiogenesis, and formation of cell morphology.[6,9,10]

Accumulated evidence has revealed that compromised TJ are responsible for tumor pathologies in carcinogenesis and that phenotypic changes caused by deregulated expression of TJ proteins are involved in the tumorigenicity and malignant behaviors of carcinoma cells.[1–5] Consistently, JAM-A has been shown to be a contributing factor for the development and progression of various types of cancer.[11–15] For example, JAM-A is overexpressed in cancers such as those arising in the breast and lung, whereas its expression is downregulated in other cancers such as pancreatic and endometrial cancers. However, the functional importance of JAM-A in tumorigenesis is still under debate because dysregulated JAM-A expression has distinct effects on target tissues and cells, playing opposite roles (i.e. oncogenic-stimulatory and, conversely, tumor-suppressive effects in a carcinogenic scenario).[11,16–18]

In the present study, we examined the expression of JAM-A in lung adenocarcinoma tissues and its precursor lesions by immunohistochemistry. We also investigated the functional relationship between JAM-A expression and malignant behaviors of adenocarcinoma cells of the lung. Our observations suggested that elevated expression of JAM-A promotes neoplasia of lung adenocarcinoma, indicating that JAM-A is a potential therapeutic target for this malignancy.

Materials and Methods

Patient samples. To examine JAM-A expression in neoplasia of lung adenocarcinoma, we studied 72 cases of archived formalin-fixed, paraffin-embedded tissue samples prepared from surgically resected materials (Table S1). Experienced surgical pathologists who have been certified by the Japan Society of Pathology independently evaluated H&E-stained slides of all specimens and immunohistochemical results of JAM-A expression. JAM-A expression was examined in normal lung tissue (non-neoplastic alveolar epithelium), preinvasive precursor

lesions of lung adenocarcinoma, including atypical adenomatous hyperplasia and adenocarcinoma *in situ*, and adenocarcinoma. Informed consent was obtained from all patients for pathological assessment of their specimens, and the ethics committee of Sapporo Medical University School of Medicine approved the present study (approval number: #282-242). Lung neoplasias were classified according to histological types using the World Health Organization guidelines.[19]

Immunohistochemistry. Tissue sections were deparaffinized in xylene, rehydrated through a graded series of ethanol and PBS, and incubated in 3% H_2O_2 for 10 min to block endogenous peroxidase activity. After antigen retrieval by microwave heating (95°C for 30 min) in 10 mM Tris/1 mM EDTA buffer, sections were incubated overnight at 4°C with a primary polyclonal antibody against JAM-A (ab52647; Abcam, Cambridge, UK). The sections were then incubated with EnVision (Dako, Glostrup, Denmark) for 30 min at room temperature, and color was developed using 3,3'-diaminobenzidine tetrachloride (Dako) as the chromogen. The slides were subsequently counterstained with hematoxylin. Appropriate positive and negative controls were used in each experiment.

Immunohistochemical staining positivity was semiquantitatively analyzed by considering the percentage of positive cells and staining intensity. A score was assigned on the basis of the percentage of positively stained tumor cells (proportion score) as follows: 10, staining in 91%–100% of the cells; 9, staining in 81%–90% of the cells; 8, staining in 71%–80% of the cells; 7, staining in 61%–70% of the cells; 6, staining in 51%–60% of the cells; 5, staining in 41%–50% of the cells; 4, staining in 31%–40% of the cells; 3, staining in 21%–30% of the cells; 2, staining in 11%–20% of the cells; 1, staining in 1%–10% of the cells; 0, staining in 0% of the cells. Another score was determined on the basis of immunoreactivity intensity (intensity score) as follows: 3 + , strong; 2 + , moderate; 1 + , weak, and 0, negative. The final score was obtained by multiplication of the proportion and intensity scores. In cases of invasive adenocarcinoma, we analyzed JAM-A immunoreactivity in invasive parts excluding the non-invasive component because JAM-A expression was also observed in non-invasive adenocarcinoma.

Cell culture and transfection. We obtained a lung adenocarcinoma cell line, LHK2, that was established by our colleagues (Drs Y. Hirohashi and T. Torigoe, Department of Pathology, Sapporo Medical University School of Medicine).[20] LHK2 is a poorly differentiated lung adenocarcinoma cell line that was established from pleural effusion of a 68-year-old Japanese male patient with advanced lung cancer who died of the disease. Analysis of LHK2 cells by next-generation sequencing (Thermo Fisher Scientific, Waltham, MA, USA) revealed that LHK2 cells harbor wild-type *TP53*, *KRAS*, *ALK*, and *EGFR*, but have mutations in *KIT* (Met 541 Leu) and *STK11* (Met335 Ile), as well as deletion of *PDGFRA* (our unpublished observation). The cells were maintained in DMEM (Sigma, St Louis, MO, USA), supplemented with 10% FBS (Thermo Fisher Scientific), 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma).

LHK2 cells were transfected with a JAM-A-specific or control small-interfering RNA (siRNA) using Lipofectamine™ RNAiMAX (Thermo Fisher Scientific). Sequences of siRNA are 5′-GCCUUAUUUGUCUUCUACATT-3′ for JAM-A siRNA #1 and 5′-CUGUUGUGCCUCUUCAUAUTT-3′ for JAM-A siRNA #2.

We also generated an LHK2 cell line in which the *JAM-A* gene was permanently knocked out by transfection of a CRISPR/Cas9 plasmid. Briefly, we searched for guide RNA target sites using the free software CRISPR direct.[21] The sequence of the target site for the human *JAM-A* gene is 5′-GGACAAAGGCGCAAGTCGAG-3′. A vector expressing non-coding guide RNA, orange fluorescent protein (OFP), and Cas9 nuclease was constructed by using a GeneArt CRISPR Nuclease Vector Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Cells were transfected with a vector by using Lipofectamine™ 3000 (Thermo Fisher Scientific). Two days after transfection, OFP-positive cells were sorted by FACS Aria (BD Biosciences, Franklin Lakes, NJ, USA) and collected into a 96-well plate at the concentration of 1 cell/well. Loss of JAM-A protein expression was screened by western blotting. After screening, genomic DNA was extracted from candidate cell clones by using a DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) and analyzed by direct sequencing after PCR-based amplification around the guide RNA target site. Primer sequences are as follows: 5′-AGAGCAGCGGTTCCTTACAC-3′ (forward) and 5′-GCC TTCCTCCAACCTCTGAC-3′ (reverse).

Western blotting. Aliquots of whole cell lysates (20 μg) were separated on 12% SDS-PAGE and electroblotted onto nitrocellulose membranes. The membranes were then immunoblotted with antibodies against JAM-A (#36-1700; Thermo Fisher Scientific), cleaved caspase 3 (#9664; Cell Signaling Technology, Danvers, MA, USA), and β-actin (Sigma). The membranes were incubated with appropriate peroxidase-labeled secondary antibodies (Dako), and bands were visualized using enhanced chemiluminescence (GE Healthcare, Buckinghamshire, UK).

Evaluation of apoptosis and cell proliferation. We prepared cell blocks by using the sodium alginate method as described elsewhere.[22] Briefly, collected cells were washed with PBS, and the cells were fixed in 10% formalin solution for 24 h at 4°C. After centrifugation, aggregated cells were gently suspended in 1% sodium alginate solution (Wako Pure Chemical Industries, Osaka, Japan). Then, 1 M calcium chloride was added to form a gel and the fixed cell-containing gel was embedded in paraffin.

Cell blocks were cut and prepared for H&E specimens to evaluate their morphology. Then, apoptosis and cell proliferation were assessed in histological sections made from cell blocks by carrying out immunohistochemistry using antibodies against cleaved caspase 3 (#9664; Cell Signaling Technology) and Ki-67 (MIB-1 clone; BioGenex, Fremont, CA, USA), respectively. Number of positive cells was counted using a microscope (Olympus, Tokyo, Japan) by counting at least 100 cells in each representative field of interest.

Invasion assay. A cell suspension (1×10^5 LHK2 cells in DMEM without FBS) was added to a Corning BioCoat Matrigel™ Invasion Chamber (pore size, 8 μm; Thermo Fisher Scientific); DMEM containing 10% FBS was added to the lower chamber to create a chemotactic gradient. Number of invasive cells was then estimated after 24 h of incubation. The lower surface of the upper chamber was wiped with a cotton swab, and cells that passed through the filters onto the lower surface of cell culture inserts were quantitated by counting the transmigrated cells. Invading cells were fixed and visualized by 0.04% crystal violet in 70% ethanol for 5 min. Cells of interest were then counted using a microscope (Olympus).

Cell proliferation assay. Cell proliferation was assessed by incorporation of BrdU into cell DNA. Cells were grown on 35-mm glass-base dishes coated with rat tail collagen. Cells were incubated for 2 h after treatment with 20 μM BrdU and

were then fixed in cold absolute ethanol. After the samples had been further incubated with 2 N HCl at room temperature for 20 min, they were incubated with a monoclonal anti-BrdU antibody (#5292; Cell Signaling Technology) at room temperature for 1 h and then with Alexa 488 (green)-conjugated anti-mouse IgG antibody (Thermo Fisher Scientific) at room temperature for 1 h. DAPI (Sigma) was also used for counterstaining of nuclei in the cells. Number of cells with BrdU-labeled nuclei was counted using a microscope (Olympus), by counting at least 100 cells per dish.

Colony-forming assay in 2D culture. LHK2 cells (2.5×10^3 cells/well) were plated in six-well plates. The plates were incubated for 10–14 days, and cells were stained with 0.04% crystal violet for 15 min at room temperature followed by fixing with methanol for 15 min. Cell clusters of at least 50 cells were defined as positive colonies, and visible colonies were counted.

***In vivo* xenograft model.** We injected cells of parental LHK2 and its transfectants (2×10^5 cells in 25 μL/mouse) into the back skin of 5-week-old female athymic nude mice (BALB/cAJcl-*nu/nu*; CLEA Japan, Tokyo, Japan). Animals were killed when skin ulceration of the primary tumor occurred. Tumor volumes were calculated in two dimensions in a time-dependent method after inoculation of cells. Volume (V) of the primary tumor was calculated as follows: $V = (\pi/6) \times (L \times W^2)$, where L is the length representing the largest tumor diameter and W is the perpendicular width of the tumor. Primary tumors were examined macroscopically and then the tumors were subjected to histological evaluations. Maintenance and handling of animals were conducted using protocols approved by the Animal Care and Use Committee of Sapporo Medical University School of Medicine (approval number: #12-040).

Statistical analysis. All data are expressed as means ± standard deviations. Data analysis was carried out using EZR software version 1.32.[23] Data were analyzed using a two-tailed unpaired Student's t-test. P-value <0.05 was considered statistically significant.

Results

JAM-A expression in neoplasia of lung adenocarcinoma. A number of previous studies have shown dysregulation of JAM-A expression in various types of cancer.[11–15] Therefore, we first evaluated the expression of JAM-A in lung adenocarcinoma and its preinvasive lesions, including atypical

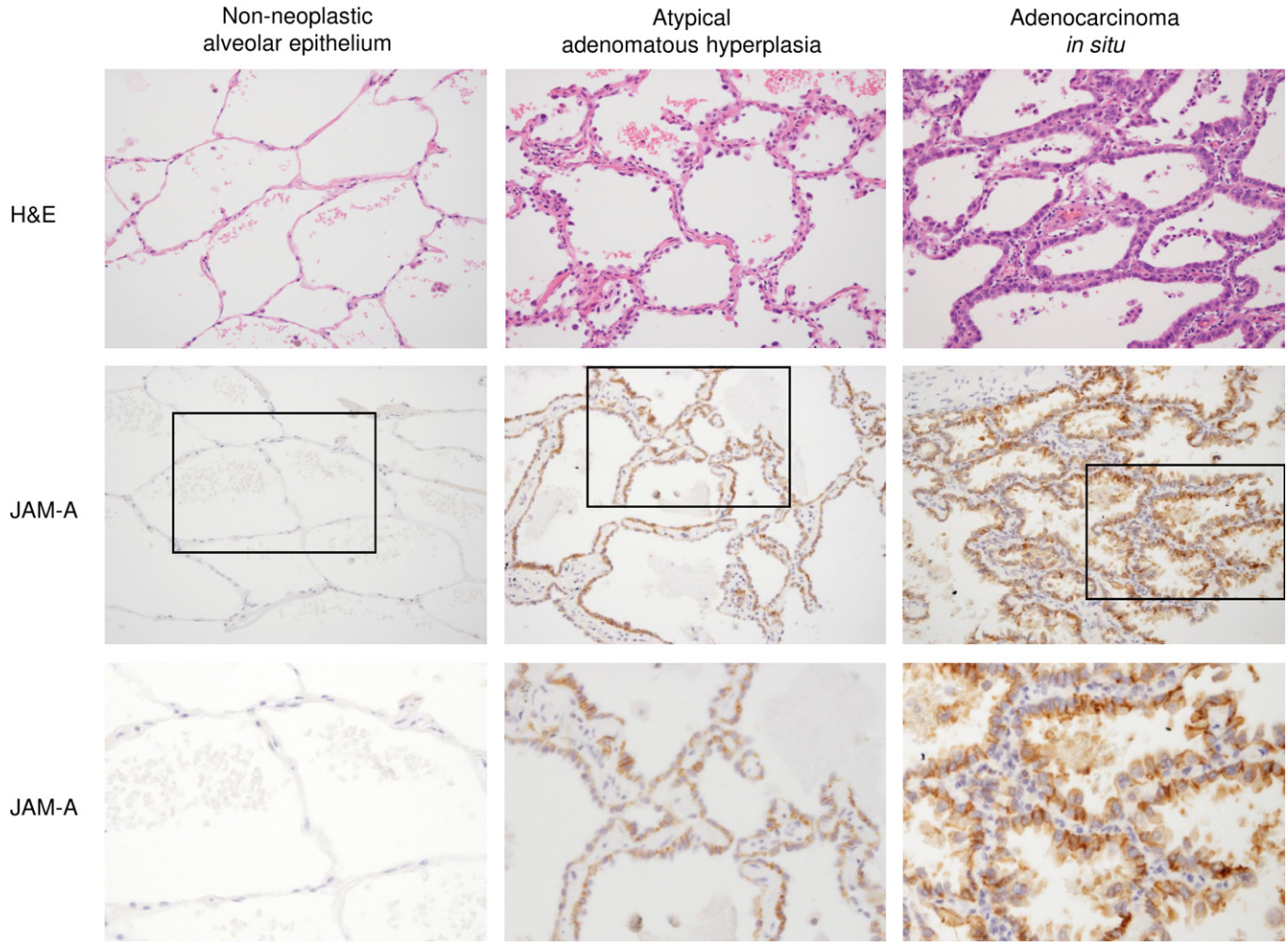

Fig. 1. Junctional adhesion molecule-A (JAM-A) expression in preinvasive neoplasia of lung adenocarcinoma, including atypical adenomatous hyperplasia and adenocarcinoma *in situ*. Upper panel, H&E staining; middle and lower panels, immunohistochemistry of JAM-A. Lower panel (×200) is a magnified view of the rectangular area in the middle panel image (×100) for each histology.

adenomatous hyperplasia and adenocarcinoma *in situ* (Figs 1,2; Table S2). Immunohistochemical analyses showed elevated levels of JAM-A expression in adenocarcinomas, including acinar, papillary, solid, and mucinous types. We also observed increased levels of JAM-A expression in atypical adenomatous hyperplasia and adenocarcinoma *in situ*. In particular, JAM-A expression abruptly, not gradually, appeared at the interface of atypical adenomatous hyperplasia and normal (non-neoplastic) alveolar tissue (Fig. S1).

We carried out semiquantitative analysis of JAM-A expression by immunohistochemistry, considering both the proportional and intensity scores (Table 1). JAM-A-positive signals were observed in atypical adenomatous hyperplasia, adenocarcinoma *in situ*, and invasive adenocarcinoma ($P < 0.001$ *vs* normal tissue). Importantly, cases of adenocarcinoma *in situ* and invasive adenocarcinoma showed higher scores than those of atypical adenomatous hyperplasia ($P = 0.015$ and $P = 0.013$, respectively). These observations suggested the involvement of enhanced JAM-A expression that can potentially lead to neoplastic transformation of pneumocytes in an early phase during lung carcinogenesis and that can promote neoplasia of lung adenocarcinoma.

In contrast, while strong immunopositivity of JAM-A was observed even in cases of non-neoplastic alveolar epithelium (intensity scores of 2 + in 17 cases and 3 + in 7 cases; Table S2), there were significantly lower scores of JAM-A expression in normal tissue (Table 1). Since JAM-A has been reported to be expressed in endothelial cells in the lung and brain,[24] JAM-A expression was also observed in endothelial cells of the capillary in the alveolar wall, although its expression was not present in all of the endothelial cells of the lung vasculature (data not shown).

There was no significant difference between expression levels of JAM-A in histological subtypes of adenocarcinoma, including acinar, papillary, solid, and mucinous types ($P = 0.466$). In addition, a correlation was not found between subcellular localization of JAM-A protein and various lesions in neoplasia of lung adenocarcinoma. Strong expression of JAM-A was an independent indicator of several clinicopathological variables, including age ($P = 0.183$), gender ($P = 0.147$), tumor size ($P = 0.082$), primary tumor stage ($P = 0.073$), lymph node involvement ($P = 0.141$), and tumor stage ($P = 0.175$). Nonetheless, Kaplan–Meier analysis could not demonstrate a positive relationship between increased

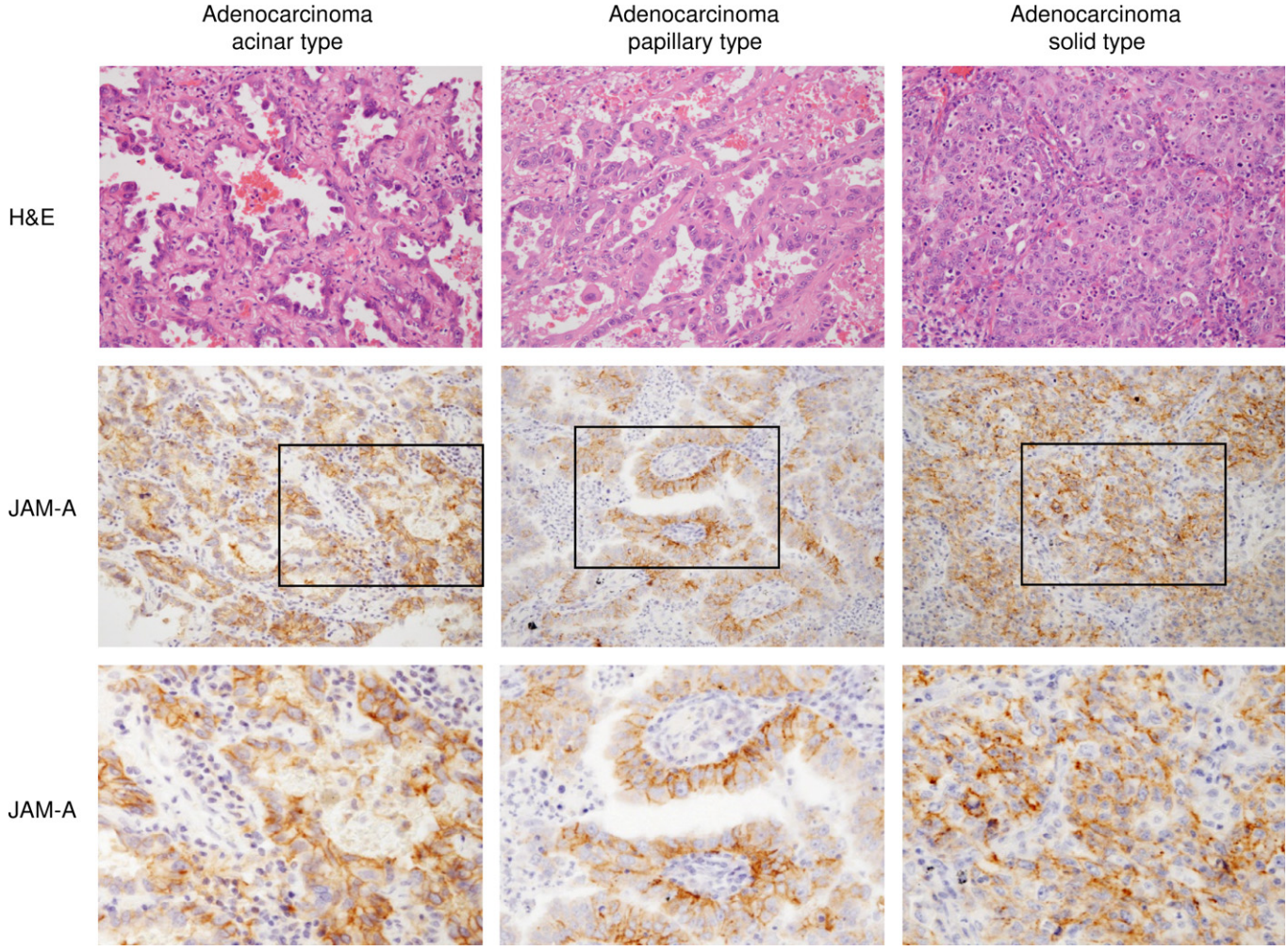

Fig. 2. Junctional adhesion molecule-A (JAM-A) expression in lung adenocarcinoma, including its histological subtypes. Upper panel, H&E staining; middle and lower panels, immunohistochemistry of JAM-A. Lower panel (×200) is a magnified view of the rectangular area in the middle panel image (×100) for each histology.

Table 1. Junctional adhesion molecule-A expression in neoplasia of lung adenocarcinoma

	No.	Proportional score	Intensity score	Total score	*P*-value *vs* AE	*P*-value *vs* AAH	*P*-value *vs* AIS	*P*-value within subtypes
AE	82	1.8 ± 2.1	1.0 ± 1.0	3.4 ± 5.3				
AAH	29	6.9 ± 3.1	2.4 ± 0.8	18.6 ± 11.0	<0.001			
AIS	29	8.3 ± 1.6	3.0 ± 0.2	24.9 ± 5.2	<0.001	0.015		
Adenocarcinoma	53	8.5 ± 1.7	3.0 ± 0.2	25.5 ± 5.4	<0.001	0.013	0.744	
Acinar type	29	8.7 ± 1.7	3.0 ± 0.2	26.2 ± 5.5	<0.001	0.009	0.532	0.466
Papillary type	16	7.9 ± 1.9	2.9 ± 0.3	24.5 ± 5.7	<0.001	0.017	0.871	
Solid type	7	8.6 ± 1.7	3.0 ± 0.4	24.7 ± 7.5	<0.001	0.015	0.913	
Mucinous type	1	8	3	24	NA	NA	NA	

JAM-A expression was semiquantitatively analyzed by immunohistochemistry, considering both the proportional and intensity scores. Data are expressed as means ± standard deviations. We carried out statistical analysis providing *P*-values. *P*-value <0.05 was considered statistically significant by the Mann–Whitney *U*-test, compared to JAM-A expression in tissue types as indicated. AAH, atypical adenomatous hyperplasia; AE, non-neoplastic alveolar epithelium; AIS, adenocarcinoma *in situ*; NA, not applicable.

expression of JAM-A and poor overall survival (data not shown).

Suppression of JAM-A expression induces apoptosis and inhibits cell proliferation. To examine the effects of JAM-A expression on tumor cells, we established both JAM-A-silenced cells and cells with stably knocked out JAM-A (Fig. 3). In these experiments, we used LHK2 lung adenocarcinoma cells, which were characterized by various differentiation phenotypes of the lung.[20] Consistently, this cell line showed epithelial cell morphology having spontaneous differentiation capability of gland-like structures in a normal 2D culture condition (Fig. 3a).

We transfected LHK2 cells with JAM-A-specific siRNAs to establish several different transfectants. We finally obtained transfectants, designated as JAM-A siRNA #1 and JAM-A siRNA #2, showing that JAM-A expressions were efficiently suppressed but were similar in these siRNA transfectants (Fig. 3b, left panel). We also transfected a CRISPR/Cas-9 plasmid targeting the *JAM-A* gene into LHK2 cells to generate cells with permanently knocked out JAM-A, designated as JAM-A KO cells. A clonal transfectant was isolated by subsequent screening for confirming the absence of constitutive JAM-A expression, showing that JAM-A protein expression was not detectable in JAM-KO cells (Fig. 3b, right panel).

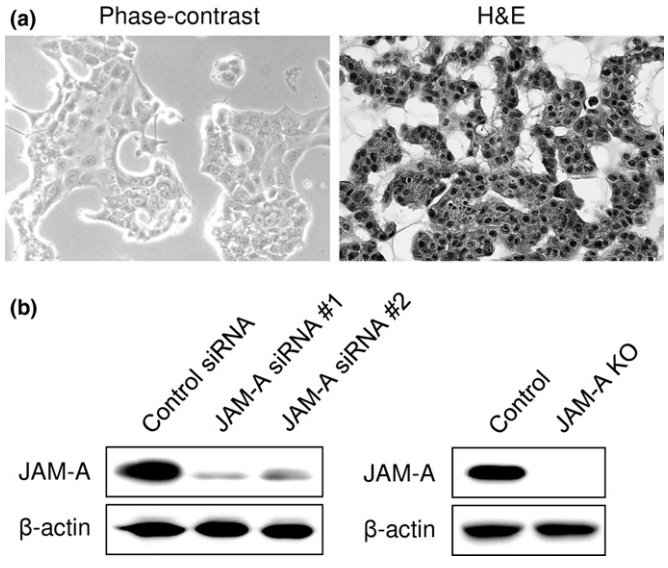

Fig. 3. Generation of junctional adhesion molecule-A (JAM-A)-silenced and knocked out LHK2 cell lines. (a) Representative images of phase-contrast microscopy of cultured cells and H&E slide of a cell block sample. (b) Western blotting of JAM-A in cells as indicated.

To investigate the effects of altered JAM-A signaling on apoptosis and cell proliferation, cell blocks were made from cultured cells and they were subjected to immunohistochemistry by incubation with antibodies against the cleaved form of caspase 3 and Ki-67, respectively (Fig. 4). Suppression of JAM-A expression significantly induced apoptosis, which was characterized by scattered apoptotic bodies in H&E specimens, a morphological feature of apoptosis showing scattered pyknotic nuclear fragments. Consistent with these observations, cells positive for cleaved caspase 3 were significantly increased in JAM-A-silenced LHK2 cells. Cell growth was also decreased when JAM-A expression was suppressed. More striking effects on the expression of cleaved caspase 3 and cell growth were observed in JAM-A KO cells. Although JAM-A KO cells should have higher sensitivity to apoptosis, apoptosis was not notably increased in JAM-A KO cells compared with that in JAM-A-silenced cells. Expression levels of JAM-A inversely correlated with the apoptosis-sensitizing and cell proliferation-inhibitory effects, suggesting that JAM-A expression is important for determining the sensitivity of cells to apoptosis and the regulation of cell growth.

Suppression of JAM-A expression decreases cellular motility and invasiveness. To determine whether JAM-A modulates cellular motility and invasiveness, we carried out assays to examine these malignant behaviors with or without Matrigel™ using LHK2 cells (Fig. 5). When JAM-A expression was suppressed by siRNA transfection, cellular motility was significantly reduced as assessed by transmigrating activity between biomembranes. In addition, suppression of JAM-A expression significantly decreased the invasiveness of JAM-A-expressing cells. These effects were more evident in the absence of constitutive JAM-A expression following knockdown of the *JAM-A* gene. Our observations suggested that the expression level of JAM-A modulates motile and invasive properties of lung adenocarcinoma cells.

Suppression of JAM-A expression inhibits tumorigenicity *in vitro* and *in vivo*. Being consistent with the apoptosis-sensitizing effect of JAM-A inhibition, JAM-A downregulation significantly suppressed colony-formation capability, as evidenced by a decreased number of colonies *in vitro* (Fig. 6a,b). To determine whether JAM-A affects tumorigenicity *in vivo*, we used an LHK2 xenograft tumor mouse model. Suppression of JAM-A expression inhibited tumorigenicity *in vivo*, showing a

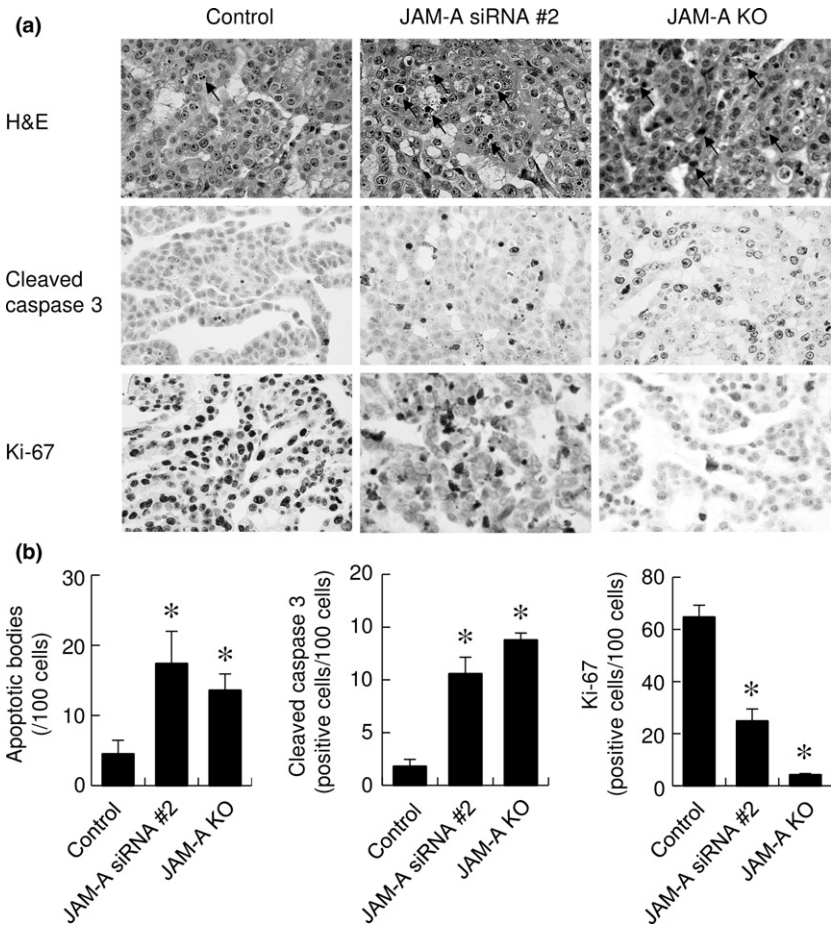

Fig. 4. Suppression of junctional adhesion molecule-A (JAM-A) expression induces apoptosis and inhibits cell proliferation. (a) H&E staining and immunohistochemistry of cleaved caspase 3 and Ki-67 in cell block samples that were made from LHK2 cells as indicated. Arrows, apoptotic bodies. (b) Quantitative analyses of apoptotic bodies and cells positive for cleaved caspase 3 and Ki-67 by immunohistochemistry. *$P < 0.05$ *vs* control cells.

markedly decelerated rate of tumor growth in mice (Fig. 6c). Our observations indicated a mechanistic link between expression level of JAM-A and tumorigenicity of lung adenocarcinoma cells in *in vitro* and *in vivo* settings.

Anti-JAM-A antibody inhibits cell growth and induces apoptosis. To examine the pathological significance of elevated levels of JAM-A protein in carcinogenesis, we next used an anti-JAM-A polyclonal antibody that recognizes the extracellular domain (N-terminal) of this protein (Fig. 7). *In vitro* experiments demonstrated that the anti-JAM-A antibody significantly reduced proliferation of LHK2 cells in a titer-dependent method of the antibody (Fig. 7a). Further analyses using cell blocks that were made from cultured LHK2 cells showed that the JAM-A antibody inhibits cell growth, as assessed by a BrdU incorporation assay (Fig. 7b,c; left panels). Treatment with this antibody also increased positive cells labeled with the cleaved form of caspase-3 (Fig. 7b,c; right panels). These results supported our observations that inhibition of JAM-A activity interferes with tumor proliferation and induces apoptosis, being consistent with results of previous studies.[18,25] Our findings indicate that JAM-A protein is a potential therapeutic target for lung adenocarcinoma.

Discussion

In the present study, we demonstrated that suppression of JAM-A expression limits the oncogenic potential of lung adenocarcinoma cells, suggesting that elevated expression of JAM-A promotes neoplasia of lung adenocarcinoma. As JAM-A has been shown to exert oncogenic effects on cultured cells,[11–15] it is plausible for JAM-A expression to be potentially involved in the development and/or progression of these malignancies. Accumulated data have revealed that certain types of TJ proteins are signaling molecules that are directly involved in the regulation of cellular functions, including proliferation, differentiation, and apoptosis.[1–5] Therefore, it is not surprising that dysregulated expression of JAM-A leads to several tumor pathologies of the lung during carcinogenesis.

Consistent with our observations, a previous study showed that JAM-A overexpression correlates with tumor progression in non-small-cell lung cancers.[13] That study revealed that JAM-A was highly expressed in 37% of lung cancers and that JAM-A expression was significantly associated with clinicopathological variables, including cancer stage, lymph node involvement, and overall survival. In contrast, we did not obtain positive results for a relationship between JAM-A overexpression and any clinicopathological parameters, as well as patient outcome. We cannot provide a definite explanation for our results. However, we clearly demonstrated that expression levels of JAM-A are increased not only in invasive adenocarcinomas but also in their preinvasive lesions. Our observation supports the possibility that JAM-A is not required for late-stage tumor development and progression once JAM-A overexpression potentially leads to neoplastic transformation of pneumocytes in an early phase of lung carcinogenesis. Alternatively, the failure to verify the clinical significance of JAM-A overexpression might be mainly because of limited numbers of well-characterized patients with long-term follow up.

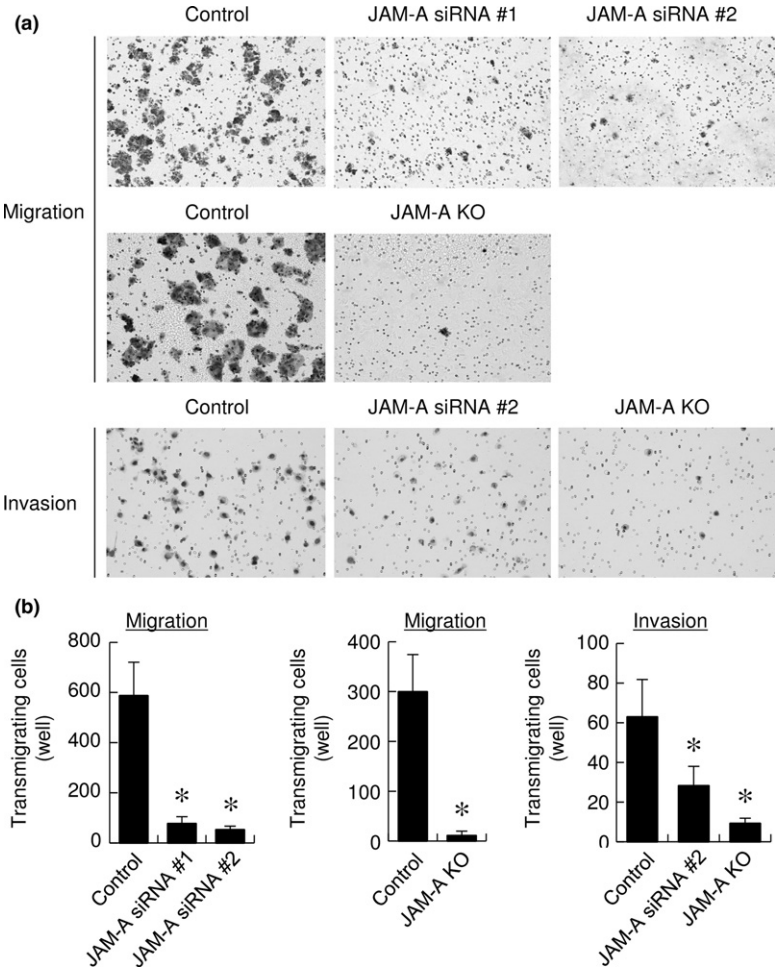

Fig. 5. Suppression of junctional adhesion molecule-A (JAM-A) expression inhibits cellular motility and invasiveness. (a) Boyden chamber assays with or without Matrigel™ to show cell migration and invasiveness in LHK2 cells as indicated. (b) Quantitative analyses of transmigration and invasion properties. $*P < 0.05$ *vs* control cells.

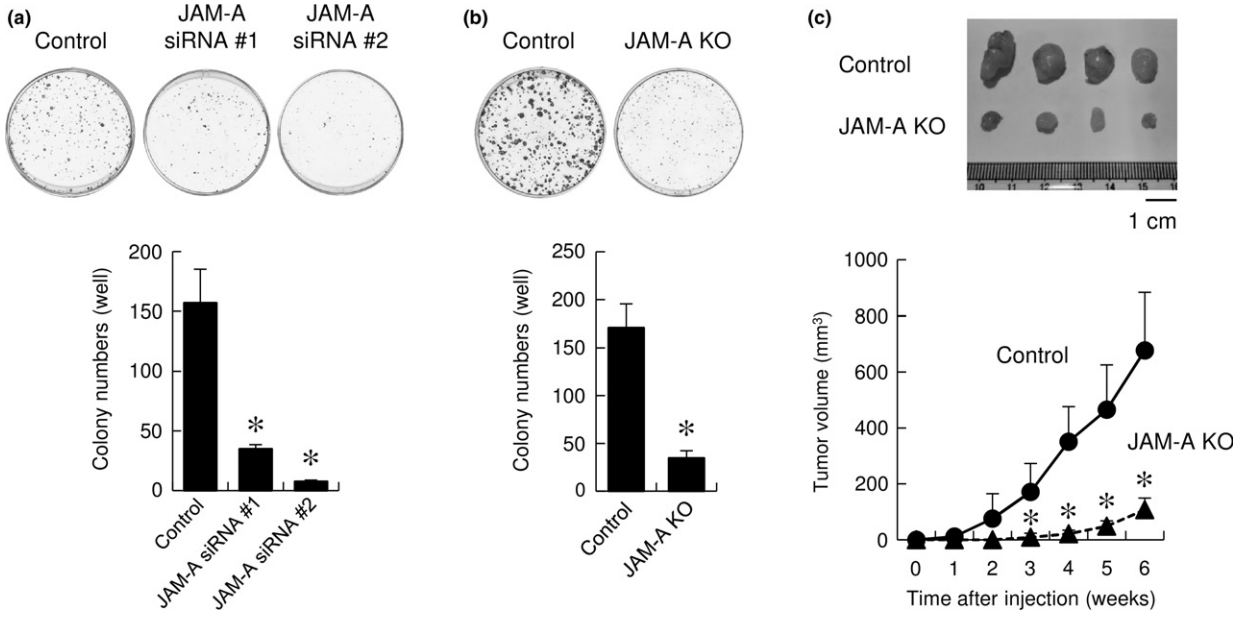

Fig. 6. Suppression of junctional adhesion molecule-A (JAM-A) expression inhibits tumorigenicity *in vitro* and *in vivo*. (a,b) Colony formation assays in LHK2 cells with JAM-A knocked down by JAM-A-specific siRNAs and LHK2 cells with JAM-A knocked out. Representative images of tissue culture plates (upper panels) and quantification of colony numbers (lower panels). (c) Xenograft transplantation mouse model. JAM-A knocked-out LHK2 cells grew significantly slower than did control cells. Representative tumors that developed in mice 6 weeks post-injection (upper panel) and cell growth in a time-dependent method (lower panel). $*P < 0.05$ *vs* control cells.

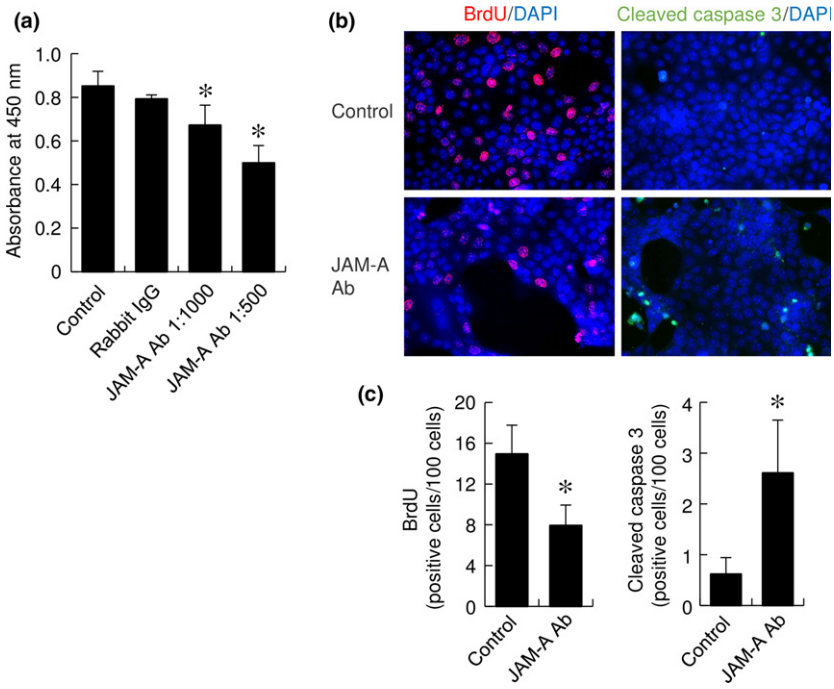

Fig. 7. Anti-junctional adhesion molecule-A (JAM-A) antibody reduces cell growth and provokes apoptosis. (a) Cell growth inhibition 24 h after treatment with different titers of anti-JAM-A antibody, as assessed by the WST1 assay in LHK2 cells. Rabbit IgG was used as a control. (b,c) Anti-JAM-A antibody-mediated activity for inhibition of cell proliferation and activation of apoptosis. (b) Cell blocks made from cultured LHK2 cells after 24 h treatment with anti-JAM-A antibody (1:500) were subjected to immunohistochemistry as indicated. (c) Quantification analyses of positive cells labeled with BrdU and cleaved caspase 3. $^*P < 0.05$ *vs* control cells.

Published data also showed that silencing of JAM-A expression inhibits cell proliferation and leads to decreased colony-formation capability in various types of lung cancer cells.[13] We obtained similar results, but this report is the first study showing elevated expression of JAM-A in precursor lesions of lung adenocarcinoma, and showing that JAM-A expression affects various malignant properties of cancer cells, including cell proliferation, apoptotic sensitivity, and colony-forming capability, *in vitro* and *in vivo*. Finally, we proposed the potential feasibility of JAM-A-inhibitory cancer therapy. We exclude the possibility that modulation of JAM-A expression had specific effects on LHK2 cells because the effects of JAM-A expression were not cell type-specific (data not shown). Consistently, accumulated data have shown that JAM-A has multidisciplinary roles in carcinogenesis.[11–15]

Although our results showed increased expression of JAM-A in neoplasia of lung adenocarcinoma, the underlying molecular mechanism remains to be clarified. Because of the significance of signaling complexity in the regulatory mechanism of JAM-A, dysregulated signaling resulting from a multifactorial process involving various genetic alterations in carcinogenesis of the lung might offer a possible explanation for the molecular mechanism. The signaling pathways involved may modulate the activity of different types of signaling factors that have yet to be identified as JAM-A regulators. Another possibility is that deregulated expression of JAM-A might be directly associated with cumulative alterations of aberrant signalings such as MAPK, PI3K, and Akt/β-catenin pathways. This explanation is based on evidence that these signalings are closely associated with various tumor pathologies that are caused by JAM-A overexpression.[25–27] It is thus reasonable to assume that dysregulation of JAM-A leads to the promotion of various malignant behaviors of tumor cells such as cell proliferation, migration, epithelial–mesenchymal transition, and dedifferentiation accompanied by the gain of stem-cell features.[17,25–27]

Results of the present study indicate that JAM-A is not only a possible biomarker for lung adenocarcinoma but also a plausible therapeutic target for this malignancy. Based on the suppressive effect of an antibody against JAM-A on malignant behaviors of lung adenocarcinoma cells, we believe that specific inactivation of deregulated JAM-A protein would have a notable impact on malignant potential of human tumors with overexpressed JAM-A, suggesting the potential feasibility of JAM-A-inhibitory cancer therapy. However, the exact role of deregulated JAM-A expression and its effects on carcinogenesis as well as the expression pattern and the underlying regulatory mechanism of increased JAM-A expression in human cancers remain to be clarified. Therefore, future studies are needed for a better understanding of the regulatory mechanism of JAM-A overexpression and its molecular impacts on neoplasia of lung adenocarcinoma.

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Disclosure Statement

Authors declare no conflicts of interest for this article.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. JAM-A expression in atypical adenomatous hyperplasia of the lung. H&E staining (upper left panel) and immunohistochemistry of JAM-A. Right panels (dotted line and arrows) represent the boundary of atypical adenomatous hyperplasia (right) and normal (non-neoplastic) alveolar tissue (left). Lower panels are magnified views of the rectangular areas in the upper right image.

Table S1. Clinicopathological features of human tissue samples.

Table S2. JAM-A expression in neoplasia of lung adenocarcinoma.