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Long noncoding RNA FAM201A mediates the metastasis of lung squamous cell cancer *via* regulating ABCE1 expression

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Abstract. – OBJECTIVE: Long noncoding RNA (lncRNA) family with sequence similarity 201-member A (FAM201A) is a novel lncRNA promoting the development of various cancers. However, the biological function of FAM201A on the metastasis of lung squamous cell carcinoma (LSCC) remains unknown. The aim of this study was to explore the molecular mechanism of FAM201A and its target protein in advanced LSCC.

PATIENTS AND METHODS: Quantitative Polymerase Chain Reaction (qPCR) was applied to evaluate FAM201A expression in lung cancer tissues. The impact of high FAM201A expression on the overall survival in patients with lung cancer was tested using the log-rank test. The relevance between aberrant FAM201A and clinicopathological characteristics in patients with lung cancer was analyzed using the Chi-square tests. Cell proliferation was assayed using the Cell Counting Kit-8 (CCK-8) and a transwell assay, and the mice xenograft models were applied to determine the promoting effects of FAM201A on LSCC *in vitro* and *in vivo*. The underlying regulatory mechanism was explored through RNA transfection, qPCR, and Western blotting. The correlation between ATP-binding cassette transporter E1 (ABCE1) and FAM201A expression was verified using Spearman's correlation coefficient.

RESULTS: FAM201A is aberrantly elevated in tissues from patients with non-small cell lung cancer. High levels of FAM201A expression were more likely to present in patients with squamous type, M1 stage, and inferior overall survival. Differential expression was found between non-metastatic and metastatic squamous carcinoma, but not in adenocarcinoma. FAM201A knockdown inhibits cell proliferation, migration, and invasion of LSCC cells *in vitro*, and represses tumor growth *in vivo*. Furthermore, ABCE1 in LSCC cells was downregulated by silencing FAM201A. The tissue level of ABCE1 was positively correlated with FAM201A expression in patients with LSCC.

CONCLUSIONS: FAM201A may markedly induce migration and invasion of LSCC, resulting in the M1 stage and poor survival. These findings suggest the FAM201A-ABCE1 axis as a novel therapeutic target in LSCC.

Key Words:

Lung squamous cell carcinoma, Long noncoding RNA family with sequence similarity 201-member A, ATP-binding cassette transporter E1, Metastasis.

Introduction

Lung cancer ranks first in both cancer incidence and the leading cause of cancer-related mortality globally[1]. Two histological subtypes, adenocarcinoma (51%) and squamous cell carcinoma (30%), mainly constitute non-small cell lung cancer (NSCLC), representing 85% of all lung cancers[2]. Each subtype manifests distinctive genomic mutation profiles, with corresponding targeted drugs[3]. Increasingly targetable mutations that initiate tumorigenesis have recently resulted in significant improvement in adenocarcinoma therapeutic options and efficacy[3]. A significant survival benefit from targeted treatments is more frequently observed among non-smokers and younger patients[4,5]. Over 40% of adenocarcinomas, which possess different genomic mutations (EGFR, BRAF, ALK, and ROS1 rearrangements)[6] demonstrate various degrees of response to targeted treatments[7-9]. However, fewer targetable mutations are currently treatment options in squamous cell carcinoma. Genetic studies of the aberrant expression underlying lung squamous cell carcinoma (LSCC) development will help us fully understand the tumorigenesis of LSCC, furthering the identification of the promising therapeutic targets that may ameliorate the inferior survival of patients.

Long noncoding RNAs (lncRNAs) are a kind of long RNA (containing more than 200 nucleotides) with no protein-coding capacity[10,11]. Various lncRNAs have been found to be involved in both health maintenance and diseases[12], including cancers[13]. Using transcription factors-miRNA-lncRNA network motifs, lncRNA LINC00319, IQCH-AS1,

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and MARKAPK5 have been predicted to play an important role in LSCC pathogenesis[14]. However, a validation based on cellular biology is still needed.

LncRNA family with sequence similarity 201-member A (FAM201A) is a long RNA transcribed from chromosome 9p13.1 with no open reading frame[15]. Originally, FAM201A was reported as the location for the common single-nucleotide polymorphisms both in the obsessive-compulsive disorder and Tourette's syndrome[16]. Recently, a study of radiosensitivity in esophageal squamous carcinoma investigated the relationship between FAM201A deregulation and radiotherapy tolerance[17]. However, the regulatory mechanism and downstream factor of FAM201A in LSCC remain undefined. In our study, we found that FAM201A was overrepresented in LSCC tissues and cancer cells. Furthermore, clinical feature analysis identified FAM201A as an oncogenic lncRNA associated with clinical progression and a helpful predictor for metastasis of LSCC. FAM201A function was explored to evaluate the biological influence of FAM201A on LSCC cell proliferation, migration, and invasion.

Patients and Methods

Patients, Corresponding Tissues and Follow-Up

Totally 107 lung cancer patients, who received no radiotherapy or chemotherapy before hospitalization, were enrolled at the General Hospital of the Ningxia Medical University (Yinchuan, Ningxia, China) from March 2014 to September 2014. All patients were diagnosed by pathology, divided into squamous carcinoma (n=56, LSCC) and adenocarcinoma (n=51). All corresponding tissues were acquired by biopsy (bronchofiberscope and fine needle aspiration) or surgery. Finally, subsequent tissues were all rapidly frozen at -80°C. Five-year-follow-up was implemented to attain overall survival. The study was approved by the Ethics Committee of the General Hospital of the Ningxia Medical University. Informed consent was received from all patients before this study.

RNA Extraction and Quantitative-PCR

Based on the manufacturer's protocol, total RNA was extracted from tissues and cultured cells using TRIzol solution (Thermo Fisher Scientific, Waltham, MA, USA). The total RNA was transcribed to cDNA using SuperScript IV Reverse Transcript (Thermo Fisher Scientific, Waltham, MA, USA). The prepared cDNA was measured by a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Then, in accordance with the protocol of kit, quantitative PCR for target RNA in tissues and cells was performed using PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) in a 96-well PCR plate on the Applied Biosystems 7500 Sequence Detection system. All expression of target RNA was calculated using the $2^{-\Delta\Delta Ct}$ method with glyceraldehyde-phosphate dehydrogenase (GAPDH) as the endogenous control. All primer sequences used in this study are presented in **Supplementary Table SI**.

Cell Culture

Roswell Park Memorial Institute-1640 (RPMI-1640) Medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, Waltham, MA, USA) was used to culture SK-MES-1 and NCI-H520. All of them were in an atmosphere with 5% CO^2 at 37°C, purchased from the Chinese Academy of Sciences (Shanghai Institute of Cell Biology, Shanghai, China).

Gene Knockdown and Cells Transfection

Short hairpin RNA (shRNA) and pGFP271-puro-RNAi expression vector were constructed to knockdown FAM201A expression (Invitrogen, Waltham, MA, USA). After LSCC cells (1×10^6 per well) were seeded in six-well plates overnight, each well was transfected with the expression vector or vector control, by Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) based on the protocol. PCR was applied to evaluate the efficacy of transfection.

Proliferation Ability of Tumor Cells

Cell viability (5 days) of LSCC cells was measured every 24 h utilizing Cell Counting Kit-8 (CCK-8; MedChemBio, Lexington, MA, USA) according to the manufacturer's protocol. The proliferation curves representing the proliferation ability were shaped by the absorbance at each time point.

Migration Ability and Invasion Ability in LSCC Cells

Transwell inserts with a polycarbonate membrane (8.0 μm pores; Corning, New York, NY, USA) were applied for migration assays. Membranes were additionally coated with Matrigel matrix (BD Biosciences, Franklin Lakes, NJ, USA) for invasion assays. After LSCC was transfected

with the expression vector or the vector control 24 hours later, 200 µl medium with tumor cells (5×10^5) was seeded into the upper chamber, while 600 µl medium supplemented 10 % fetal bovine serum was added in the lower chamber. Following incubation for 24 h (37°C in a 5 % CO_2), the cells on the upper surface of the polycarbonate membrane were removed. The cells attached to the bottom of the membrane were defined as migrated or invaded. All of those cells were fixed, stained, and being counted by the microscope (Leica DM20, Leica Microsystems Inc., Buffalo Grove, IL, USA).

Animal Experiment and Tumor Growth In Vivo

Four-week-old BALB/c-nude mice were purchased from the Laboratory Animal Center of Ningxia Medical University and fed in an atmosphere with a 12 h light/dark cycle under specific pathogen-free conditions. LSCC cells (2×10^5 cell in 100 ul) transfected with the expression vector (sh-FAM201A) or vector control (sh-vec) were respectively incubated subcutaneously into mice (each group n=5). The tumor sizes of every mouse were collected every week. In the fifth week, tumor weight was measured after the mice were sacrificed. All the procedures of these experiments obtained the approval from the Institutional Animal Care and Use Committee of General Hospital of Ningxia Medical University.

Western Blotting Assay

LSCC cells were resolved by RIPA buffer with 1 mmol/L protease inhibitors. A Bicinchoninic acid (BCA) Protein Assay Kit (Beyotime, Shanghai, China) was applied to measure the concentration of the protein samples according to the manufacturer's guidance. The proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transfected to a nitrocellulose polyvinylidene difluoride (PVDF) membrane (Bioss Antibodies, Beijing, China). PVDF membrane with primary antibodies (anti-ABCE1, 1:2000; Abcam, San Francisco, CA, USA) was incubated at 4°C overnight, using GAPDH (1:4000; Abcam, San Francisco, CA, USA) as the endogenous reference. Then, the target blot on the membrane was incubated with the secondary antibody with horseradish peroxidase (HRP, 1:8000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The Western blot band was photographed by an enhanced chemiluminescence detection system (ECL™, Pierce, Waltham, MA, USA) and quantified by Image J software.

Statistical Analysis

All data were analyzed using GraphPad Prism 6 software (San Diego, CA, USA), and presented as the mean ± standard deviation. The comparison between the two groups was carried out by Student's *t*-test. The correlation between ABCE1 and FAM201A expression was evaluated *via* Spearman's correlation coefficient. Each experiment was done independently three times. $p<0.05$ was defined as significant difference.

Results

Elevated FAM201A Expression is Highly Related to Metastatic LSCC

Firstly, lncRNA FAM201A expression in 107 patients' tissues (tumor and adjacent normal ones) was quantified using quantitative Reverse Transcription-Polymerase Chain Reaction. FAM201A expression in NSCLC tissues was higher than that in adjacent normal tissues (Figure 1A, $p<0.01$). Secondly, patients with NSCLC were divided into two groups based on the median level of lncRNA FAM201A expression (high group, n=54; low group, n=53). Clinicopathological analysis (Table I) revealed that elevated FAM201A expression was significantly related to the subtype of squamous carcinoma ($p=0.042$) and M1 stage ($p=0.013$). Based on the Kaplan-Meier analysis, a higher level of FAM201A expression was associated with inferior overall survival (Figure 1B, $p<0.05$). Thirdly, after further stratification of patients with NSCLC according to pathological subtype, we verified the relationship between FAM201A expression and tumor metastasis. Patients with M1-stage LSCC exhibited increased FAM201A expression than that exhibited by patients with M0-stage LSCC (Figure 1C, $p<0.01$), but this difference was not observed in adenocarcinoma (Figure 1D, $p>0.05$). Altogether, our results indicated that lncRNA FAM201A is highly expressed in NSCLC tissues, and it may be a helpful biomarker for predicting metastasis in patients with LSCC.

FAM201A Promotes LSCC Cell Proliferation, Migration, and Invasion In Vitro

The level of FAM201A expression in two LSCC cell lines (SK-MES-1 and NCI-H520) was decreased by synthesized short hairpin RNA to confirm the influence of lncRNA FAM201A on the biological behavior of tumor cells.

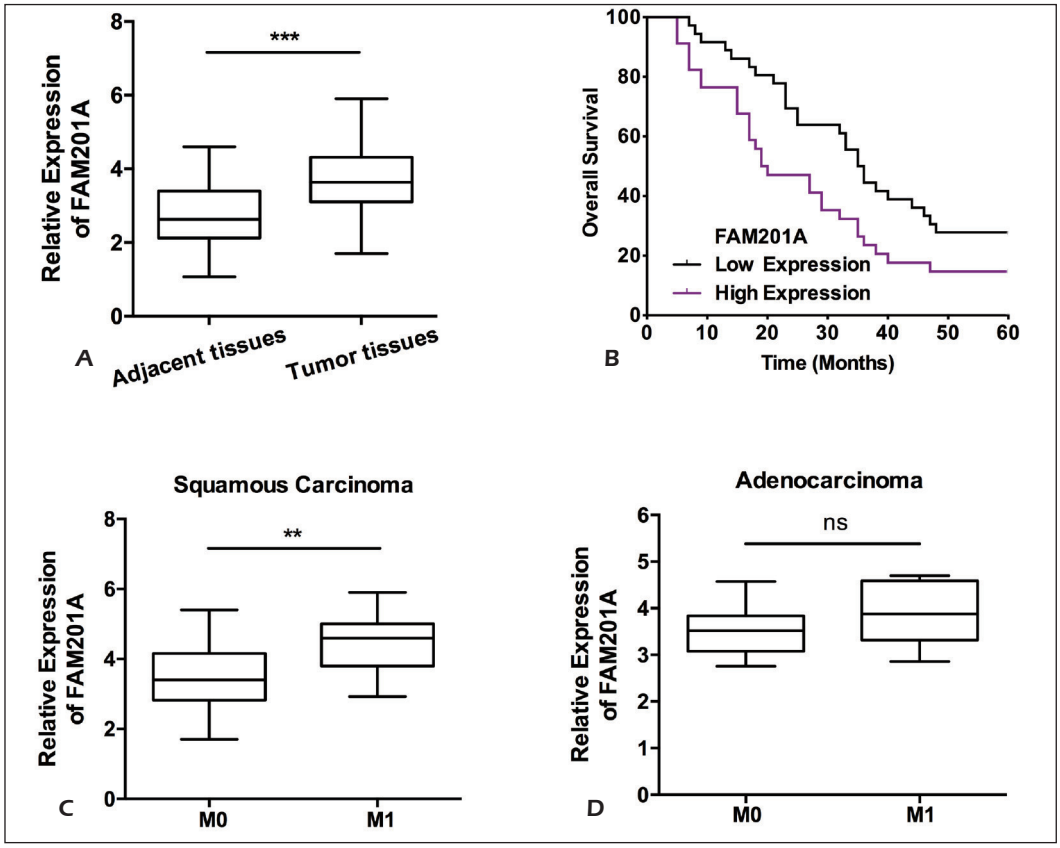

Figure 1. Elevated FAM201A expression is highly related to metastatic LSCC (**A**) Expression of lncRNA FAM201A in NSCLC tissues and adjacent normal tissues shown by qPCR. **B**, Overall survival of LSCC patients with high and low lncRNA FAM201A expression. **C**, Expression of lncRNA FAM201A in LSCC tissues with different M stage (M0, n=35; M1 n=21). **D**, Expression of lncRNA FAM201A in tissues of adenocarcinoma with different M stage (M0, n=34; M1 n=17). $^{**}p<0.01$, $^{*}p<0.05$ compared to the control group.

Table I. Relation between FAM201A expression and clinicopathological features in NSCLC (n = 107).

	Total n	FAM201A expression		*p*-value
		Low n = 54	High n = 53	
Gender				0.727
Male	73	37	36	
Female	34	16	18	
Age				0.205
≤60	53	23	30	
>60	52	29	23	
Pathology				0.042*
Squamous carcinoma	56	22	34	
Adenocarcinoma	51	32	19	
T stage				0.203
T1-2	67	30	37	
T3-4	40	23	17	
N stage				0.090
N0-1	43	26	17	
N2-3	64	28	36	
M stage				0.013*
0	69	41	28	
1	38	13	25	

Notes: *p < 0.05 represents statistical difference

Quantitative PCR showed the knockdown efficiency 48 h after transfection (Figures 2A and 2B, $p<0.01$). Cell proliferation in LSCC was limited, resulting from FAM201A silencing (Figures 2C and 2D, $p<0.05$). In addition, not only migration, but invasion of LSCC cells were weakened after the FAM201A level was restrained (Figures 3A and 3B, $p<0.05$). Therefore, these data suggest that lncRNA FAM201A can mediate the malignant abilities of LSCC cells *in vitro,* including proliferation, migration, and invasion.

FAM201A Silencing Inhibits LSCC Tumor Growth In Vivo

SK-MES-1 and NCI-H520 cells transfected with sh-FAM201A or sh-Vec were incubated in immune-deficient mice to induce xenograft tumor models. The experiment revealed that decreased FAM201A restrained the growth (tumor size, Figures 4A and 4B, $p<0.05$; tumor weight, Figures 4C and 4D, $p<0.05$) of LSCC tumor nodes, compared to that in the vector control mice. The macroscopic observation of tumor nodes is shown in Figures 4E and 4F. Thus, these results suggest that FAM201A dysregulation is involved in LSCC tumor formation *in vivo.*

FAM201A Modulates LSCC Development by Upregulating ABCE1

Based on bioinformatics analysis using TargetScan, we predicted that ATP-binding cassette transporter E1 (ABCE1), which exhibits increased expression in lung cancer, may be a downstream target for lncRNA FAM201A. This regulatory relation was verified based on a Western blot assay (Figures 5A and 5B, $p<0.01$). ABCE1 expression was declined in cancer cells transfected with sh-FAM201A, compared to that in the vector controls (Figure 5B). Moreover, ABCE1 expression in NSCLC tissues was higher than that in adjacent normal tissues (Figure 5C, $p<0.01$).

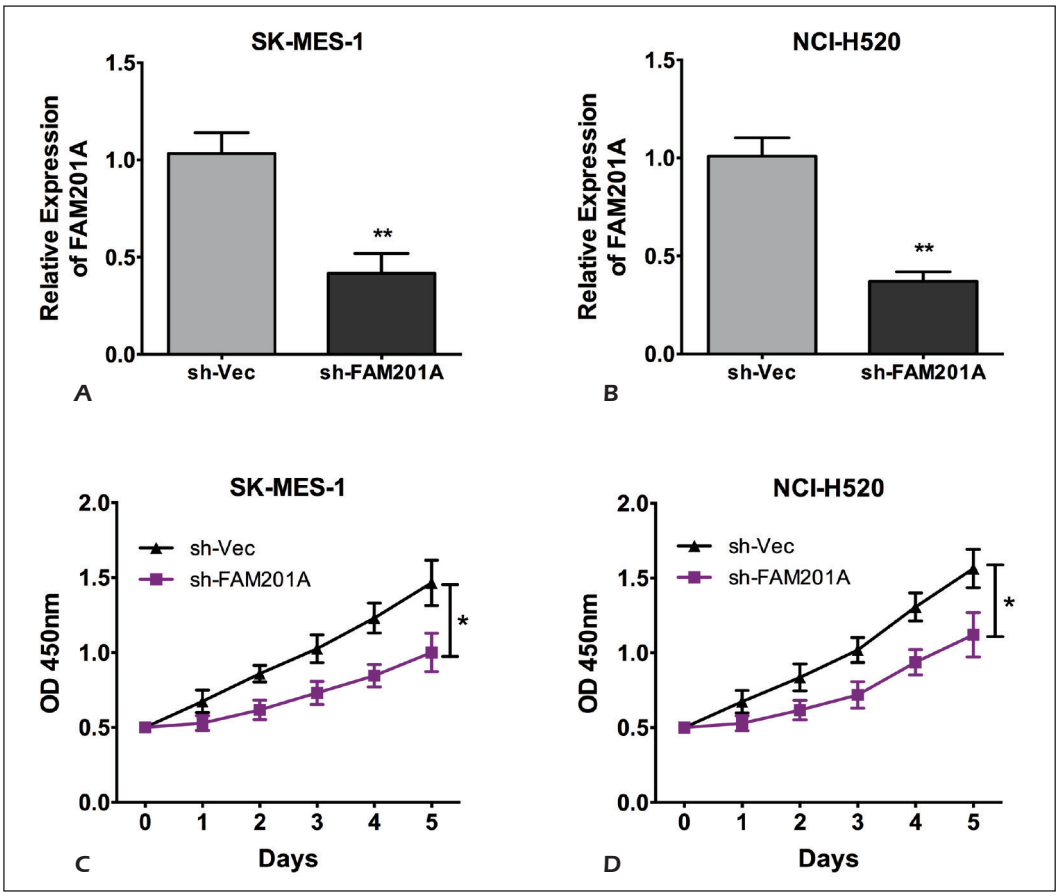

Figure 2. FAM201A promotes LSCC cells proliferation *in vitro*. **A**-**B**, FAM201A expression was silenced by shRNA targeting FAM201A in LSCC cells. **C**-**D**, Proliferative ability of LSCC cells transfected with shRNA or vector control measured by CCK-8 test. $^{**}p<0.01$, $^{*}p<0.05$ compared to the control group.

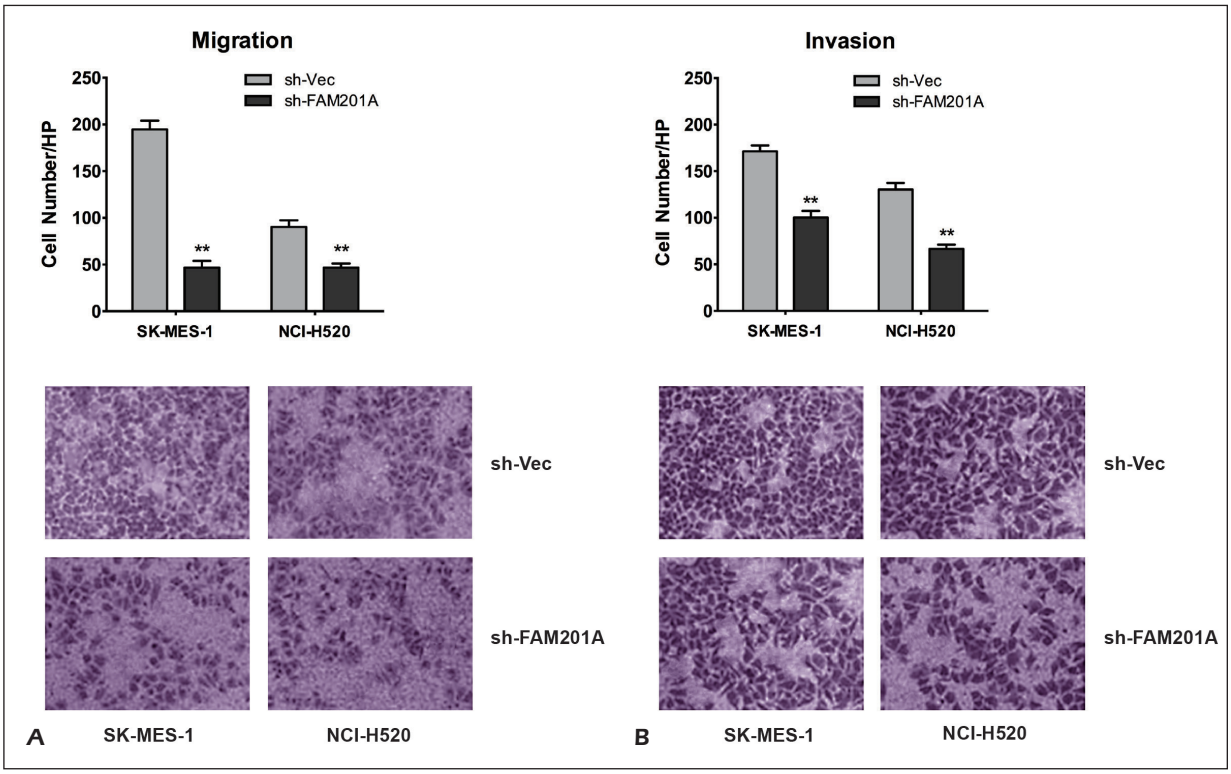

Figure 3. FAM201A promotes LSCC cells migration and invasion in vitro. **A**, Migration ability of LSCC cells after FAM201A knockdown (400×). **B**, Invasion ability of LSCC cells transfected with shRNA or controls. $^{**}p<0.01$, $^{*}p<0.05$ compared to the control group (400×).

A positive correlation between ABCE1 and FAM201A expression in human cancer tissues was verified (Figure 5D; r=0.4250, $p<0.0001$). Altogether, our data suggest that ABCE1 is an effector protein targeted by FAM201A in LSCC.

Discussion

Our study showed that the upregulation of lncRNA FAM201A is associated with tumor progression in patients with advanced LSCC. Although FAM201A will not function through a coding protein, it acts as a transcription mediator in the pathogenesis of several diseases. Bioinformatics and PCR validation revealed that downregulated FAM201A had an impact on the development of osteonecrosis of the femoral head[18]. Originally, FAM201A, named G-quadruplex-containing lncRNA, was found to modulate colon cancer cell migration by Matsumura et al[19]. Moreover, Chen et al[17] showed that the influence of FAM201A on radiotherapy efficacy was significant in esophageal squamous cell cancer by increasing ataxia telangiectasia mutated and mammalian target of rapamycin expression. However, some studies have investigated the influence of lncRNA FAM201A on the migration and invasion of cancer cells. In accordance with the results presented in previous reports, our study also revealed that ectopic-inclined FAM201A significantly induced the proliferation and growth of LSCC cells both *in vitro* and *in vivo* (Figures 2 and 4). In addition, we also found that FAM201A seems to be a significant indicator for the metastasis of squamous cell carcinoma, rather than adenocarcinoma (Figures 1C and 1D). Next, a migration and invasion assay in tumor cells verified the facilitation effect of FAM201A (Figure 3). Collectively, our results revealed that FAM201A is a promising predictor for distant metastasis and poor survival in patients with LSCC.

ABCE1, a highly conserved protein[20], can change ribosomal morphology, decrease the number of large ribosomal subunits, and increase the number of 80S polyribosomes, thus inhibiting the intracellular protein translation process[21,22]. It has been observed that ABCE1 is involved in

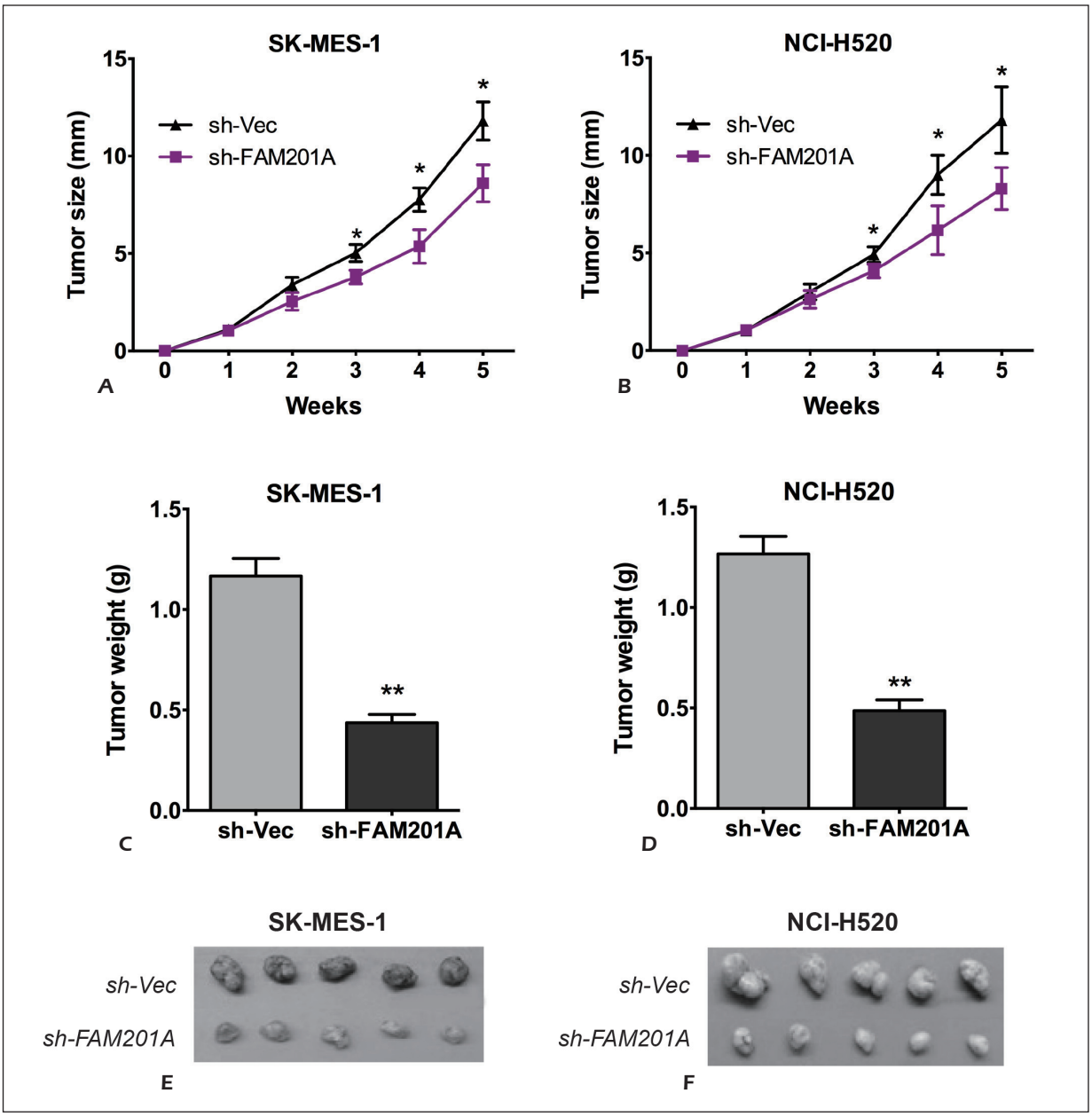

Figure 4. FAM201A silencing inhibits LSCC tumor growth in vivo. **A-B**, Tumor size between FAM201A knockdown mice and the control mice. **C-D**, Tumor weight of tumor nudes after stable knockdown of FAM201A. **E-F**, The macroscopic observation of tumor nudes. $^{**}p<0.01$, $^{*}p<0.05$ compared to the control group.

the development of several cancers, including breast cancer[23], small cell lung cancer[24], and NSCLC[25]. ABCE1 not only reduces the expression of tumor-suppressor gene growth arrest and DNA damage-inducible 45α[26], but also interacts with the cytoskeleton protein actin and increases cell motility, resulting in promoting the growth and metastasis of tumor cells[27]. The downregulation of ABCE1 increases the sensitivity of lung cancer to chemotherapeutics, including 5-fluorouracil[28], irinotecan[28], and doxorubicin[29]. Investigators are beginning to focus on the upstream regulatory signals of ABCE1. Tat interactive protein 60 kDa (Tip60) expression accelerates ABCE1 acetylation, affecting the biological functions of ABCE1 in A549 lung carcinoma cells[30]. In addition, the malfunction of iron-sulfur domains in ABCE1 intervenes in the progression of advanced lung can-

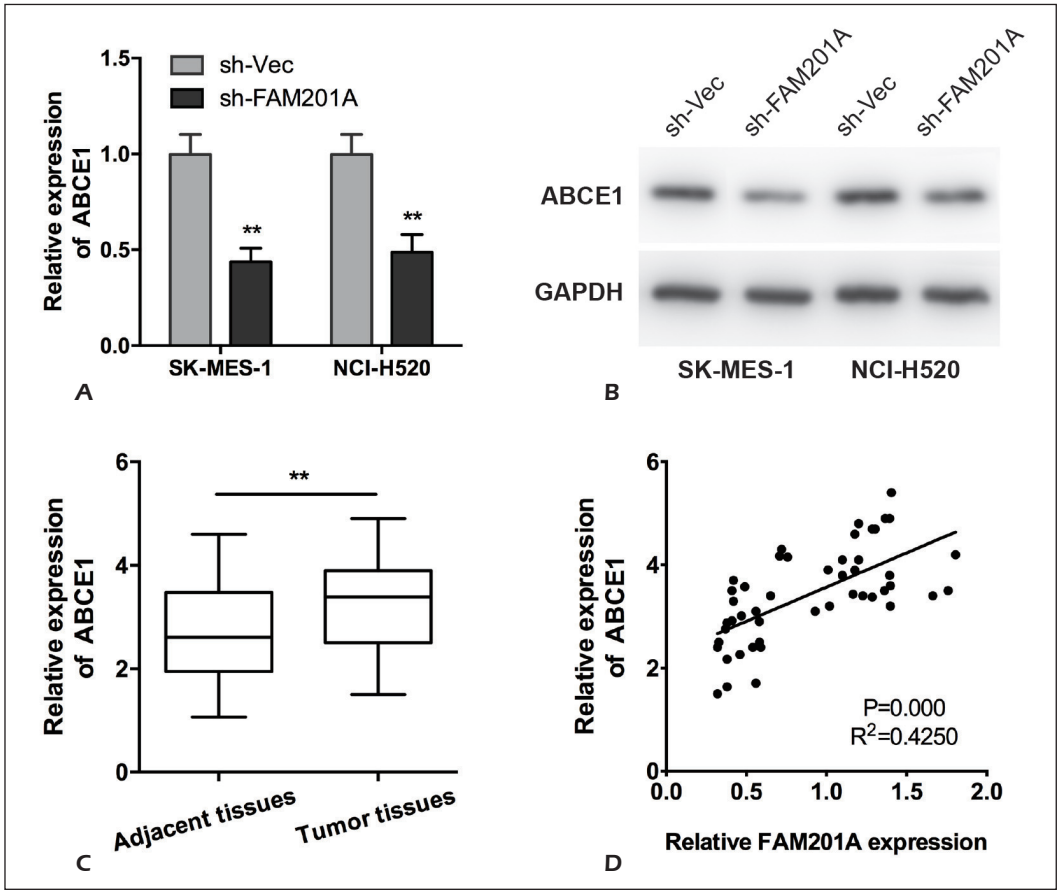

Figure 5. FAM201A modulates LSCC development by upregulating ABCE1. **A-B**, Western blot assay showed the ABCE1 protein expression in LSCC cells with or without FAM201A silencing. **C**, qPCR showed the ABCE1 expression in NSCLC tissues (n=107) and adjacent normal tissues (n=107). **D**, A positive correlation between the expression of ABCE1 and FAM201A in tumor tissues. $^{**}p<0.01$, $^{*}p<0.05$ compared to the control group.

cer. Furthermore, aberrant expression of monocyte-chemotactic protein 3 in tumor cells may mediate the upregulation of ABCE1[31].

Although ABCE1 is involved in the formation, metastasis, and even chemosensitivity of lung cancer, the molecular mechanism of ABCE1 modulation *via* lncRNA in the metastasis of lung cancer remains unknown. In the present study, ABCE1 expression could be repressed after FAM201A was silenced (Figure 5A and 5B). In addition, the association between ABCE1 and FAM201A was confirmed in human tissues. ABCE1 increased in cancerous tissues, showed a quantitative dependence on FAM201A levels. However, the specific molecular mechanism of ABCE1 regulation mediated by FAM201A has not been investigated in this work. This has become the limit of this research. Interfering with the expression of the downstream target proteins *via* binding microRNA as mRNA sponges is the molecular way in which lncRNAs mainly function[32,33]. It is found that ABCE1 is regulated by miR-96 to promote metastasis in breast cancer[34,35]. ABCE1 controls cell viability and growth of tumor cells in retinoblastoma, being inhibited by miR-145[36]. MicroRNA-299-3p increases the sensitivity of lung cancer to doxorubicin by inhibiting ABCE1[29]. All these indicate that FAM201A can mediate the expression of ABCE1 by modulating a certain miRNA in LSCC. This requires further exploration in the further investigations.

Conclusions

We have demonstrated that high levels of FAM201A expression are associated with metastatic LSCC with inferior overall survival. FAM201A may markedly induce the migration and invasion of LSCC. Furthermore, ABCE1 in LSCC is downregulated by silencing FAM201A. These data suggest that the lncRNA FAM201A-ABCE1 axis may be a novel therapeutic target in LSCC.

Data Availability Statement

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Disclosure of Financial Arrangements

The research and manuscript preparation are funded by Wei He.

Conflict of Interests

All authors of this article declare that they have no conflict of interest.

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