Received: 23 November 2018 | Revised: 15 March 2019 | Accepted: 19 March 2019

DOI: 10.1002/jcla.22897

RESEARCH ARTICLE

Long non-coding RNA KDM5B anti-sense RNA 1 enhances tumor progression in non-small cell lung cancer

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Background: The long non-coding RNAs (lncRNAs) have been shown as a novel class of transcripts with no protein coding functions. LncRNAs can play diverse roles in cancer cell proliferation, differentiation, metastasis, and apoptosis. However, the exact contributions of lncRNA KDM5B anti-sense RNA 1 (KDM5BAS1) to non-small cell lung cancer (NSCLC) remain poorly understood.

Methods: In current study, we have unraveled a novel function of KDM5BAS1 in NSCLC.

Results: We found that KDM5BAS1 was significantly overexpressed in tumor specimens and selected cancerous cell lines. Meanwhile, higher KDM5BAS1 expression predicted poor overall survival. Increased KDM5BAS1 expression can promote proliferation or migration and inhibit apoptosis in H1838 and H1299 cells. Furthermore, knocking down of KDM5BAS1 levels can also reduce tumor growth in in vivo implantation experiments. Overexpression of KDM5BAS1 also decreased the caspase-3 immunostaining but enhanced Ki-67 staining.

Conclusion: Taken together, our findings indicated that KDM5BAS1 might play an oncogenic role in NSCLC and provided clues into pharmacological intervention targeting KDM5BAS1.

KEYWORDS

KDM5BAS1, lncRNA, NSCLC, oncogenesis

1 | INTRODUCTION

The lung cancer has been reported to be one of the most deadly cancers world-wide with relatively high mortality.[1,2] Among all lung cancer cases, over eighty percent have non-small cell lung cancer (NSCLC) characteristics with poor prognosis.[1,2] More than half million patients die from lung cancer, and the mortality remains high for years.[3] The occurrence of NSCLC has been ascribed to multiple origins.[4] Therefore, due to the complex nature of lung cancer progression, novel biomarkers for identifying NSCLC patients require further investigation.

The long non-coding RNAs (lncRNAs) represent a class of RNAs with at least 200 nucleotides in length.[5,6] The lncRNAs play critical roles in various physiological and biological processes.[7,8] Previously, lncRNAs have been regarded as mock transcripts with no significant functions primarily due to the fact that they do not reside in coding sequences.[9] However, recent studies have argued that lncRNAs could behave as oncogenes or tumor suppressors with great contributions to the cancer development. For example, lncRNA RGMB-AS1 has been reported to be significantly upregulated in NSCLC specimens and negatively correlates with repulsive guidance molecule b (RGMB) expression, suggesting that RGMB-AS1 may play an oncogenic role in NSCLC.[10] Recently, Deng et al showed that lncRNA AFAP1-AS1 displayed higher expression in NSCLC tissues and predicted poor prognosis and survival.[11] Tang et al[12] employed a lncRNA microarray to confirm that combinatorial use of three lncRNAs (RP11-397D12.4, AC007403.1, and ERICH1-AS1) can act as biomarkers to predict the

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tumorigenesis of NSCLC. Inversely, Wang et al found that downregulation of lncRNA TUSC7 in NSCLC cell lines and tissues could promote the NSCLC cell proliferation and dictate poor survival.[13] Similarly, the lncRNA GAS5 also showed a tumor suppressive role in NSCLC by inhibiting miR-23 as exemplified by Mei et al's work.[14] Therefore, lncRNAs can possibly be applied for NSCLC diagnosis and act as putative targets for pharmacological intervention.

The lncRNA KDM5B anti-sense RNA 1 (KDM5BAS1), also named as non-coding RNA activating 2 (ncRNA-A2) or ENSG00000228288, is universally expressed in various tissues such as lung and liver.[15] KDM5BAS1 can be expressed bi-directionally with KDM5B and has multiple splice variants, 550-750 nt in length.[15] It has been shown that KDM5BAS1 expression appears to stimulate Kelch-like protein 12 (KLHL12) gene expression in HeLa cells.[15] However, the exact function of KDM5BAS1 in NSCLC remains poorly understood. Therefore, in the current study, the effect of KDM5BAS1 on the progression of NSCLC was investigated.

2 | MATERIALS AND METHODS

2.1 | Cell culture and human specimens

Six NSCLC cell lines (H1299, H2228, H522, H1838, A549, and H358) and MRC-5 normal lung cell line all were purchased from American Type Culture Collection (Manassas, VA, USA) and cultured in RMPI-1640 medium with 5% fetal bovine serum (FBS, Sigma, Shanghai, China) plus streptomycin (50 μg/mL; Sigma, Shanghai, China) in humidified 5% CO_2 at 20°C. The 293T cell line was purchased from the Shanghai Institute of Cell Biology (Shanghai, China). The NSCLC specimens were surgical archives at the First Hospital of Qiqihaer City from June 2011 to May 2013. Signed formal consent forms were obtained from all patients. The research for human specimens was reviewed and formally approved by the Ethics Committee of the First Hospital of Qiqihaer City.

2.2 | KDM5BAS1 knockdown and transfection

The cDNA for lnc-KDM5BAS1 was amplified by PCR and cloned into the pCDNA3.1 vector (Sigma, Shanghai, China). The lnc-KDM5BAS1 small interfering RNAs (si-KDM5BAS1) were synthesized by Sigma (Shanghai, China). All transfections were implemented by Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc, Waltham, MA, USA) according to the manufacturer's protocols. Following transfection for 36 hours, the culture was refreshed with fresh medium. All plasmids were verified by sequencing experimentally.

2.3 | Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNAs were isolated from NSCLC cell lines (H1838 and H1299) and human samples with Trizol reagent (Invitrogen, Carlsbad, CA, USA). Totally, 2 ng total RNA in a volume of 20 μL containing 1 mmol/L dNTP Mix (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was used to obtain complementary DNA. The mixture was maintained at 70°C for 5 minutes, and then, a mixture composed of 5× RT buffer, 10 U/μL reverse transcriptase, and 100 U/μL RNase inhibitor was added (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as the control. Reactions were carried out by the ABI PRISM® 7000 Sequence Detection System (Applied Biosystem, Foster City, USA) according to the manufacturer's protocols. The expression of KDM5BAS1 was quantified by the $2^{-\Delta\Delta Ct}$ method.[16] The experiments were performed triplicates. The primer sequences were as follows: *KDM5BAS1*: sense: 5′-GTGCCTAATAGGTATC-3′; anti-sense: 5′-TGTTAGCTTAGCATGCGTT-3′; *GAPDH*: sense: 5′-TCATTGACCTCAACTA CATGGTTT-3′; anti-sense: 5′-GAAGATGGTGATGG GATTTC-3′. The experiments were performed with at least triplicates.

2.4 | Migration and invasion assay

A 12-well transwell plate (TIANGEN, Shanghai, China) with 8-μm-pore membranes was used to measure the migration. About 1×10^5 transfected cells were suspended in serum-free medium and then plated into upper chambers. H1838 and H1299 cells were resuspended 36 hours following transfection and loaded into the upper chamber (10^4 cells per well) in RPMI-1640 medium (Sigma, Shanghai, China). The lower chambers were supplemented with RPMI-1640 plus 5% FBS. Following 24 hours incubation, the upper chambers were removed and cells migrating into the lower chambers were fixed with 5% polytetrafluoroethylene (PFA) and stained with 0.5% crystal violet. For migration assay, a similar protocol was performed as stated for the invasion assay, in the absence of Matrigel. The final concentration of cells was ~10^5 cells in each chamber. Following incubation for 24 hours, the cells were loaded into 5% PFA and stained with crystal violet. Results were evaluated under a Leica fluorescent microscope (DM-IRB; Leica Microsystems GmbH, Wetzlar, Germany).

2.5 | Proliferation assay

We used the Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Kumamoto, Japan) to evaluate the proliferation. Following transfection for 48 hours, H1838 and H1299 cells were resuspended and seeded into a 12-well plate (10^4 cells per well) for 5 days. Twenty microliter MTT solutions (Sigma, Shanghai, China) were added into the culture with final concentration of 20 mg/mL for 2.5 hours. The solution was shaken for 5 minutes, leading to complete solubilization. The proliferation was evaluated each day for 5 days. Crystalline formazan was dissolved in 300 μL 5% sodium dodecyl sulfate solution for 24 hours, and the optical density at 490 nm was evaluated using the Spectramax M5 microplate monitor (Molecular Devices, CA, USA) following the manufacturer's instructions.

2.6 | Cell cycle assay

After transfection for 48 hours, H1838 and H1299 cells were harvested and washed with cold PBS. Then, cells were fixed with 70% ethanol at 4°C overnight. The fixed cells were then stained with propidium iodide (PI, Sigma, Shanghai, China) at 4°C for 60 minutes in the dark. The fraction of cells in G0/G1, S, and G2/M phases was measured using fluorescence-activated cell sorting (FACS; BD Bioscience, Mansfield, MA, USA). The whole experiments were carried out with at least triplicates. Data analysis was implemented with FACS (BD Bioscience).

2.7 | In vivo implantation and immunohistochemistry

About 2 × 10^6 H1299 cells expressing pcDNA-KDM5BAS1 or si-KDM5BAS1 were injected subcutaneously into the nude mice. The animal research was approved by the Ethics Committee of the First Hospital of Qiqihaer City. Totally, 15 mice (age, 4-5 weeks; average weight, 14.9 g) were used in this study. Mice were housed at 20°C, 50%-55% humidity, light-dark cycle of 12 hours. Ad libitum access to food and water was provided. Thirty days later, mice were sacrificed by sodium amobarbital overdose (250 mg/kg, Sigma, Shanghai, China) by intraperitoneal injection and tumor weights were evaluated. The nude mice were purchased from the Model Animal Research Center (Nanjing, China). Tumor samples were fixed in formalin-fixed and paraffin-embedded and cut into 5 μm sections with microtome. After deparaffinization and rehydration, antigens were retrieved with 1× Cytomation target retrieval solution (DakoCytomation, Hamburg, Germany) in a decloaker chamber. Slides were then incubated with hydrogen peroxide for 2 minutes. After rinsing twice with TBS-0.2% Tween 20, slides were analyzed using Ki-67 ELISA kit (Sigma, Shanghai, China). For caspase-3 staining, slides were incubated with primary antibodies directed against caspase-3 (Sigma, Shanghai, China) for 30 minutes at room temperature. After washing with TBST twice, slides were incubated with anti-goat horseradish peroxidase-conjugated secondary antibodies (Sigma, Shanghai, China). Images were shown with 200× magnification.

2.8 | Statistical analysis

Data were expressed with mean ± SD. Statistical significance was determined by Student's *t* test (SPSS, version 15.0, Inc, Chicago, IL, USA), and the significance was identified if $P < 0.05$. Kaplan-Meier survival curve was tested using log-rank test. Fisher exact test was used to evaluate the correlation between lncRNA and different clinicopathological characteristics.

3 | RESULTS

3.1 | The KDM5BAS1 is upregulated in NSCLC as well as selected cell lines

Since KDM5BAS1 was identified recently but no functional roles have been identified for KDM5BAS1 in NSCLC,[15] we investigated the role of KDM5BAS1 in NSCLC. We quantified the level of KDM5BAS1 in NSCLC and paired normal tissues. It was found that KDM5BAS1 expression was significantly upregulated in NSCLC tissues in a total of 82 samples (Figure 1). Correlation analysis for different clinicopathological features revealed that KDM5BAS1 was significantly correlated with tumor size, metastasis, and TNM stages (Table 1). However, we did not find significant association between

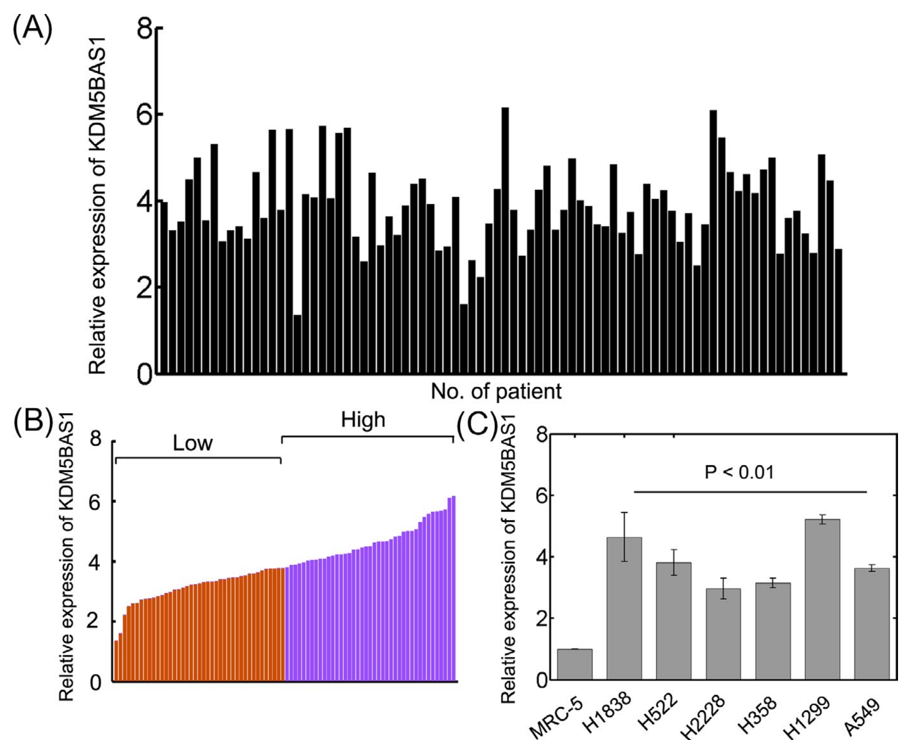

FIGURE 1 KDM5B anti-sense RNA 1 (KDM5BAS1) was upregulated in non-small cell lung cancer (NSCLC). (A) The relative expression of lnc-KDM5BAS1 in human specimens (n = 82), $P < 0.05$. (B) The median value of the relative expression was used as the cutoff. The expression of lnc-KDM5BAS1 is sorted in ascending order. Dark orange: low expression (n = 41); light purple: high expression (n = 41). (C) Relative expression of lnc-KDM5BAS1 in selected NSCLC cell lines compared with normal lung cell line MRC-5, $P < 0.01$. Data were represented as mean ± SD. Negative bars were shown

TABLE 1 Correlation between the lnc-KDM5B anti-sense RNA 1 (KDM5BAS1) and clinicopathological factors

Factors	No.	lnc-KDM5BAS1 expression		
		Low (n %)	High (n %)	*P*-value
Age				
<55	39	21 (53.8)	18 (46.2)	0.637
≥55	43	20 (60.6)	23 (39.4)	
Gender				
Male	45	20 (44.4)	25 (55.6)	0.375
Female	37	21 (56.8)	16 (43.2)	
Tumor size				
≤2	32	22 (68.8)	10 (31.2)	0.012*
>2	50	19 (38.0)	31 (62.0)	
Metastasis				
Absent	35	24 (68.6)	11 (31.4)	0.007**
Present	47	17 (36.2)	30 (63.8)	
Histological type				
Adenocarcinoma	38	16 (42.1)	22 (57.9)	0.268
Squamous cell carcinoma	44	25 (56.8)	19 (43.2)	
TNM stage				
0-I	31	24 (77.4)	7 (22.6)	<0.0001*
II-IV	51	17 (33.3)	34 (66.7)	

*$P < 0.05$.
**$P < 0.01$.

KDM5BAS1 and other factors such as age, histological type, and gender (Table 1). We used the median level of KDM5BAS1 expression as the cutoff, and the samples were divided into two groups with either low or high KDM5BAS1 expression (Figure 1B). Similarly, we also found that in selected NSCLC cell lines, KDM5BAS1 expression was also increased compared with a normal lung cell line MRC-5 (Figure 1C). The expression of KDM5BAS1 in A549 cells was also lower compared with that in H1838 and H1299 cells (Figure 1C). These results indicated that KDM5BAS1 expression was significantly upregulated in NSCLC samples as well as cell lines to possibly promote cancer progression. Since H1838 and H1299 cells had relatively higher KDM5BAS1 expression, these cell lines were used for further analysis.

3.2 | High KDM5BAS1 level correlates with poor survival

We further explored whether KDM5BAS1 was associated with NSCLC patient survival. We obtained the Kaplan-Meier plot, and the results showed that higher KDM5BAS1 expression led to poor overall survival (Figure 2, dashed line). The lower branch may drop to around 20% by the end of the follow-up (Figure 2). These results suggested that high KDM5BAS1 expression was correlated with decreased overall survival.

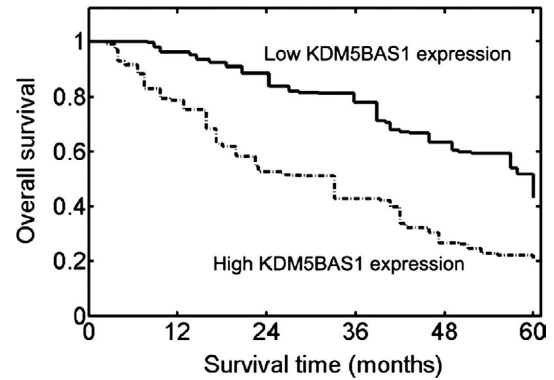

FIGURE 2 Kaplan-Meier survival curves for patients with non-small cell lung cancer. Log-rank test was used. Patients with higher lnc-KDM5B anti-sense RNA 1 expression correlated with a significantly poor survival ($P = 0.003$). A 5-y follow-up was implemented

3.3 | KDM5BAS1 promotes NSCLC proliferation, migration, and inhibits apoptosis

The data described above indicated that KDM5BAS1 might advance NSCLC oncogenesis. To deeply investigate the function of KDM5BAS1 in further details, we either knocked down or overexpressed KDM5BAS1 levels in H1838 and H1299 cells. The knockdown and overexpression efficiency was verified by PCR (Figure 3,B). It seemed that the efficiency in KDM5BAS1 knockdown and overexpression was more evident in H1299 cells compared with that in H1838 cells (Figure 3,B). By evaluating the proliferation of H1838 and H1299 cells with altered KDM5BAS1 expression, we found that KDM5BAS1 overexpression could significantly increase the proliferation of both cell lines (Figure 3,D, green curves). On the contrary, lowering KDM5BAS1 levels markedly decreased the proliferation of H1838 and H1299 cells (Figure 3,D, blue curves). To further clarify the effect of KDM5BAS1 in NSCLC cell lines, we performed the migration assay. It was found that pcDNA-KDM5BAS1 transfection substantially increased the migration of H1838 and H1299 cells compared with untreated control (Figure 3). The invasion of H1838 and H1299 cells was also elevated by pcDNA-KDM5BAS1 transfection (Figure 3). Meanwhile, we obtained qualitatively consistent results by knocking down KDM5BAS1 as reduced KDM5BAS1 expression inhibited the migration and invasion in both cell lines (Figure 3,F). Cell cycle analysis further showed that KDM5BAS1 overexpression could lead to decreased sub-G1 fraction which indicated that KDM5BAS1 may also inhibit apoptosis (Figure 3). These results suggested that KDM5BAS1 could promote migration, invasion, and proliferation in NSCLC cells possibly by reducing apoptosis.

3.4 | The KDM5BAS1 can enhance tumor growth in H1299-inoculated nude mice

We also performed in vivo implantation experiments to identify the effect of KDM5BAS1. H1299 cells were either untreated or transfected with pcDNA-KDM5BAS1 or si-KDM5BAS1 for 36 hours.

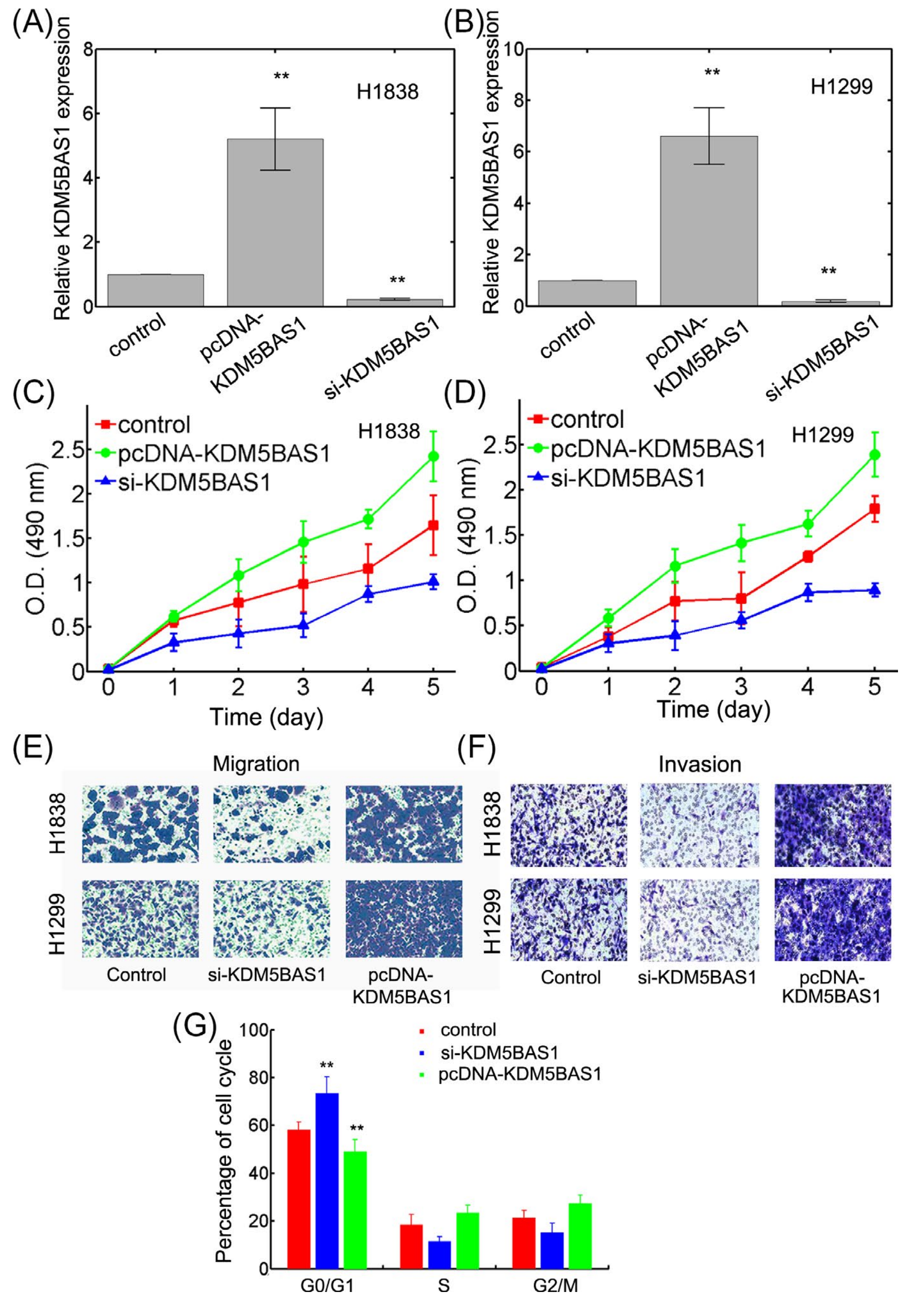

FIGURE 3 lnc-KDM5B anti-sense RNA 1 (KDM5BAS1) increases the oncogenesis of non-small cell lung cancer in vitro. Transfection efficiency of si-KDM5BAS1 or pcDNA-KDM5BAS1 in (A) H1838 and (B) H1299 cell lines was confirmed. $^{**}P < 0.01$. Negative bars were shown. (C) The proliferation assay for H1838 cells. Red: control; green: pcDNA-KDM5BAS1; blue: si-KDM5BAS1. The statistical significance can be detected between either pcDNA-KDM5BAS1 or si-KDM5BAS1 group and the control group, $P < 0.01$. (D) Proliferation assay for H1299 cells. Qualitatively similar results can be observed. (E) Transwell migration assays for H1838 (top) and H1299 (bottom) cells either left untreated (control) or transfected with si-KDM5BAS1 or pcDNA-KDM5BAS1 plasmids. (F) Transwell invasion assays for H1838 (top) and H1299 (bottom) cells either untreated (control) or transfected with either si-KDM5BAS1 or pcDNA-KDM5BAS1 plasmids. (G) The cell cycle analysis was performed for H1299 cells either left untreated (control) or transfected with si-KDM5BAS1 or pcDNA-KDM5BAS1 using flow cytometry. $^{**}P < 0.01$

Then, H1299 cells were resuspended and subcutaneously injected into the rear flank of nude mice (age, 4-5 weeks; average weight, 14.9 g). We found that compared with the control condition, KDM5BAS1 overexpression could significantly increase the tumor growth (Figure 4) as indicated by increased tumor weight and volume (Figure 4B,C). Meanwhile, KDM5BAS1 knockdown can result in attenuated tumor growth as evident by dramatically decreased tumor weight and volume (Figure 4,C). Furthermore, KDM5BAS1 knockdown resulted in enhanced caspase-3 staining, suggesting that reducing KDM5BAS1 might induce apoptosis (Figure 4D). Overexpressing KDM5BAS1 inhibited apoptosis of xenograft tumors (Figure 4D). Immunostaining also showed that elevation in KDM5BAS1 levels led to enhanced Ki-67 staining, indicating that KDM5BAS1 could exacerbate tumor growth (Figure 4E). These results suggested that KDM5BAS1 could promote tumor growth in H1299-inoculated nude mice.

4 | DISCUSSION

Recent advances in biological technology have substantially promoted our understanding on the roles of lncRNAs. Numerous lncRNAs have been shown to play important and diverse roles in malignant tumors. Despite considerable progress in cancer research, the exact molecular mechanisms of NSCLC occurrence and development remain poorly understood.

Tremendous efforts have been devoted to identifying the correlation between lncRNA and tumor development especially in

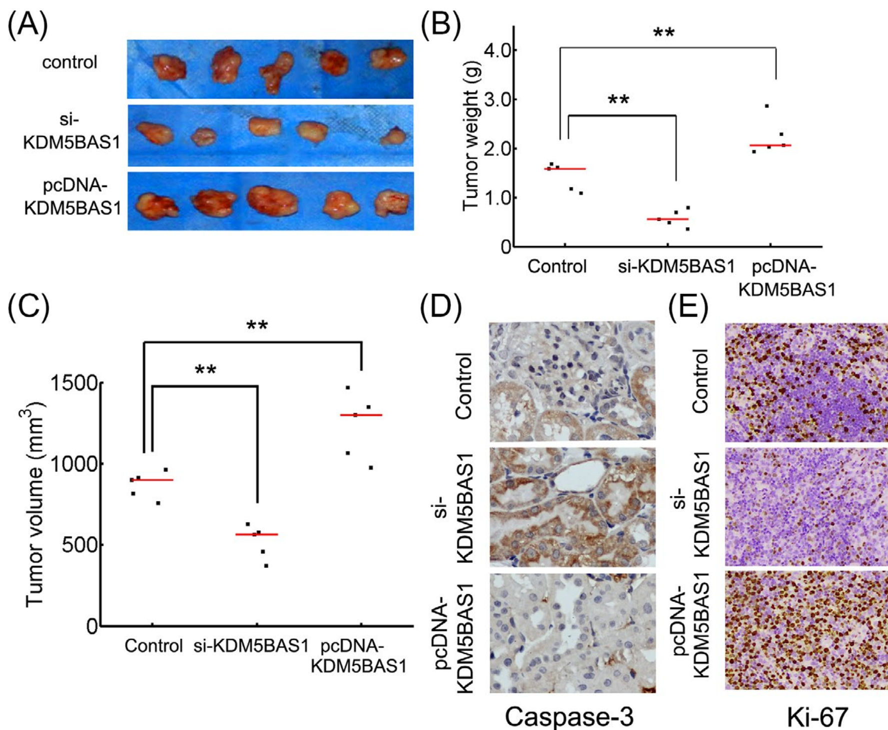

FIGURE 4 The lnc-KDM5B anti-sense RNA 1 (KDM5BAS1) can promote tumor growth. (A) The growth of solid tumors in nude mice injected with H1299 cells either left untreated or transfected with pcDNA-KDM5BAS1 or si-KDM5BAS1. After 30 d, the tumors were sectioned. (B) The tumor weight and (C) tumor volume were quantified. Red bars denote the median values. n = 5 for each group. (D) Caspase-3 and (E) Ki-67 immunostaining for in vivo implantation with H1299 cells. The H1299 cells were either left untreated or transfected with pcDNA-KDM5BAS1 or si-KDM5BAS1. ** $P < .01$

recent years. It is known that over 90% mammalian genomes are non-coding sequences which do not lead to the production of proteins. The microRNA and lncRNA are important constituents.[17] Owing to the complex nature in tumor microenvironment, the lncRNA can play various roles in oncogenesis possibly in a tumor type-specific manner. Furthermore, since lncRNAs may also act as biological or prognostic markers even at very early stage, probing into the potential linkage between lncRNAs and various tumors has attracted much attention in recent years.[18-21] In current study, we identified an oncogenic role for KDM5BAS1 in NSCLC. We noticed that KDM5BAS1 expression was significantly upregulated in NSCLC specimens as well as cell lines (Figure 1). By drawing the Kaplan-Meier survival curves, we found that patients with higher KDM5BAS1 expression were correlated with poor overall survival (Figure 2). Furthermore, KDM5BAS1 overexpression can markedly promote proliferation and migration in H1838 and H1299 cells (Figure 3). Implantation experiments showed that increasing endogenous KDM5BAS1 levels can accelerate xenograft tumor growth (Figure 4). The xenograft tumor sections exhibited elevated Ki-67 as well as reduced caspase-3 staining with pcDNA-KDM5BAS1 transfection (Figure 4). These results together suggested that KDM5BAS1 possibly functions as an oncogenic lncRNA in NSCLC.

The lncRNA KDM5BAS1 is universally expressed in various tissues such as lung and liver.[15] However, whether the lncRNA KDM5BAS1 potentially affects tumor development especially in NSCLC has never been reported. Noticeably, it has been shown that KDM5BAS1 expression appears to stimulate KLHL12 gene expression in HeLa cells possibly as an enhancer.[15] The KLHL12 gene product has been shown to mediate Wnt/β-catenin signaling via its ubiquitin ligase activities.[22] Aberrant Wnt/β-catenin signaling activation is usually associated with tumor progression such as loss of tumor suppressor Merlin.[23] Therefore, it is possible that KDM5BAS1 can exert its tumorigenic function through its indirect effect on Wnt/β-catenin signaling. Whether there exist other mechanisms where KDM5BAS1 can mediate its oncogenic effects remains to be investigated in further details.

There are also some limitations in current research. For example, whether KDM5BAS1 mediates broad oncogenic effect in other types of tumors remains elusive and demands further investigation. Meanwhile, whether KDM5BAS1 plays consistent or different roles in various tumors should also be explored. Yang et al recently analyzed the lncRNA profiles in NSCLC and identified many lncRNAs which play functional roles in NSCLC progression.[24] Besides KDM5BAS1, more attention should be paid to unravel the intrinsic mechanisms about how these lncRNAs are coordinated in NSCLC development.

Previous studies have implied that NCI-H358 and NCI-H358M cell lines might be identical,[25-27] and this misidentification may complicate the conclusion if this cell line was used. However, since the expression of KDM5BAS1 in H358 cells was relatively lower compared with that in H1838 and H1299 cell lines (Figure 1C), the selection of specific NSCLC cell lines was not affected in our study. In addition, our main studies are based on H1838/H1299 cell lines and human samples, and we argued that these results may imply an oncogenic role for KDM5BAS1 at least in well-characterized NSCLC cell lines and human specimens.

In conclusion, we have for the first time showed that KDM5BAS1 could promote NSCLC progression. The oncogenic role of KDM5BAS1 has been extensively investigated in current research through multiple strategies. Due to the effect on NSCLC

progression, our results collectively implied that KDM5BAS1 might act as a putative and novel diagnostic marker at least in NSCLC. Meanwhile, effective therapeutic intervention targeting KDM5BAS1 may serve as a plausible strategy for treatment in NSCLC. This potentially novel application might be experimentally verified in future.

ACKNOWLEDGMENT

None.

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How to cite this article: Lou B, Wei D, Zhou X, Chen H. Long non-coding RNA KDM5B anti-sense RNA 1 enhances tumor progression in non-small cell lung cancer. *J Clin Lab Anal*. 2020;34:e22897. https://doi.org/10.1002/jcla.22897