Received: 10 April 2021 | Revised: 11 August 2021 | Accepted: 16 August 2021

DOI: 10.1111/cas.15116

Cancer Science WILEY

ORIGINAL ARTICLE

LncRNA AC020978 facilitates non–small cell lung cancer progression by interacting with malate dehydrogenase 2 and activating the AKT pathway

Fei Xu[1] | Qian Hua[2] | Aimi Zhang[1] | Zhang Di[1] | Yining Wang[1] | Li Zhao[1] | Hao Yang[3] | Jianjun Liu[1] | Gang Huang[1,3]

[1]Department of Nuclear Medicine, Renji Hospital, School of Medicine, Shanghai Jiaotong University, Shanghai, China

[2]Department of Nuclear Medicine, The Second Affiliated Hospital of Soochow University, Suzhou, China

[3]Shanghai Key Laboratory of Molecular Imaging, Shanghai University of Medicine and Health Sciences, Shanghai, China

Correspondence

Gang Huang and Jianjun Liu, Department of Nuclear Medicine, Renji Hospital, School of Medicine, Shanghai Jiaotong University, Shanghai 200127, China.
Emails: huanggang@sumhs.edu.cn (GH); RJnuclear@126.com (JL)

Hao Yang, Shanghai Key Laboratory of Molecular Imaging, Shanghai University of Medicine and Health Sciences, Shanghai 201318, China.
Email: yangh@sumhs.edu.cn

Funding information

Shanghai Municipal Education Commission (Class II Plateau Disciplinary ConstructionProgram of Medical Technology of SUMHS, 2018-2020); National Natural Science Foundation of China, Grant/Award Number: 81830052, 81530053 and 81903065; Shanghai Key Laboratory of Molecular Imaging, Grant/Award Number: 18DZ2260400

Abstract

Long non–coding RNA AC020978 (lncRNA AC020978) is an oncogenic regulator of non–small cell lung cancer (NSCLC). However, the function of AC020978 in regulating NSCLC metastasis and the potential molecular mechanism remains largely unknown. In this study, we evaluated the expression levels of AC020978 in a series of NSCLC tissues using FISH assays and found that higher AC020978 expression levels were closely associated with metastasis and unfavorable prognosis. Functional studies showed that AC020978 promoted NSCLC migration and invasion both in vitro and in vivo. Further investigation demonstrated that AC020978 interacted with malate dehydrogenase 2 (MDH2) and maintained MDH2 stability. Knockdown of MDH2 weakened the facilitating effect on cell metastasis and 2-hydroxyglutarate (2-HG) metabolism in AC020978-overexpressed NSCLC cells. RNA sequencing, bioinformatic analysis, and western blotting revealed that AC020978 was associated with the AKT signaling pathway. Taken together, our findings revealed that AC020978 might serve as a prognostic biomarker and activate the AKT pathway by stabilizing MDH2, leading to metastasis and progression of NSCLC.

KEYWORDS

AKT, lncRNA, MDH2, metastasis, NSCLC

1 | INTRODUCTION

Lung cancer is the most common cancer, accounting for approximately 1.6 million deaths or 28% of all deaths from cancer globally each year.[1] Clinical research has shown that 81% of patients with clinically detected lung cancer live for less than 5 years. In addition, most cases are diagnosed at a distant stage because of the typically asymptomatic nature of the early stages of the disease.[2]

Fei Xu and Qian Hua contributed equally to this article.

[Correction added on 21 Sept 2021, after first online publication: Author contribution has been added in this version.]

Non–small cell lung cancer (NSCLC) is the most common subtype and represents 83% of lung cancer cases with molecularly heterogeneous tumors.[3] Patients with advanced stage NSCLC may die within 18 months after diagnosis due to a greater propensity for metastasis during tumor development.[4] Infiltrative growing malignant tumors grow persistently and spread locally in situ. They disseminate to other tissues through the circulatory and lymphatic systems. Thus, improved understanding the biological subtypes and the molecular mechanism of NSCLC metastasis will have tremendous clinical significance for early diagnosis and biomarker-directed therapies.[5]

Long non–coding RNA (lncRNA) are defined as transcripts longer than 200 nucleotides with little to no protein coding potential[6] that participate in the transcription, translation, and regulation of protein function at multiple levels.[7] Many lncRNA are exhibited aberrantly in cancers compared with para-carcinoma tissue of the same origin and play key roles in promoting tumorigenesis.[8] Following the development of high-throughput sequencing and bioinformatics analysis, the prominent role of lncRNA in the regulation of tumor progression has gained widespread attention. LncRNA have been found to serve as critical regulatory factors in carcinogenesis and metastasis.[9] Many hallmarks of cancer, especially in the metastasis-associated phenotype, are related to lncRNA.

In an RNA-seq analysis in our previous study, AC020978 was identified as an upregulated oncogenic lncRNA.[10] However, our previous study did not investigate the function of AC020978 in regulating NSCLC metastasis, and the potential molecular mechanism needs to be further elucidated. In this study, we collected clinical samples of metastatic lung cancer and identified an AC020978-involved regulatory mechanism in the metastasis process of NSCLC.

2 | MATERIALS AND METHODS

2.1 | Cell culture and treatment

Human NSCLC cell lines were purchased from the Cell Bank of the Chinese Academy of Science. Cells were cultured in a humidified incubator with 5% CO_2 at 37°C in DMEM (Gibco) containing 10% FBS (Gibco), 100 units/mL penicillin, and 100 μg/mL streptomycin (Gibco). Each cell line was confirmed to be mycoplasma-free.

2.2 | Transwell assay

Transfected A549 and H1299 cells (1.0×10^4/well) cultured in 200 μL medium without FBS were seeded into the upper chamber (8-μm pore size chamber inserts; Corning). The lower chamber was filled with 500 μL medium with 10% FBS. After 24 hours, the cells that had migrated through the membrane were fixed in 4% paraformaldehyde for 20 minutes, stained with 0.1% crystal violet for 10 minutes, and counted through an inverted microscope.

2.3 | Wound-healing assay

Transfected A549 and H1299 cells were plated into six-well plates and cultured for 24 hours. Artificial wounds were scratched with a 200-μL sterile pipette tip across the monolayers and images were captured at 0, 24, and 48 hours.

2.4 | In vivo tumorigenesis assay

A metastatic model was constructed by injecting the tail veins of 4-week male BALB/c nude mice with 1.0×10^6 A549 cells with either sh-NC or sh-0978. Six nude mice in each group were killed 8 weeks after injection, and the lung tissues were obtained. For subcutaneous tumors, 4-week male BALB/c nude mice were inoculated with 1×10^7 A549 cells with sh-NC in the left flank and sh-0978 in the right flank. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Renji Hospital.

2.5 | RNA-pulldown assay and mass spectrometry analysis

Full-length sense and antisense of AC020978 were transcribed with a MEGAscript T7 Transcription Kit (Thermo Fisher Scientific) in vitro. Then the transcripts were labeled with biotin using a Pierce RNA 3′ End Desthiobiotinylation Kit (Thermo Fisher Scientific). We performed a pulldown assay following the manufacturer's protocol for the Pierce Magnetic RNA-Protein Pull-Down Kit (Thermo Fisher Scientific) for the whole lysates of A549 cells. The pulldown complexes were analyzed by mass spectrometry and western blot.

2.6 | RNA immunoprecipitation assay

An RNA immunoprecipitation (RIP) assay was performed as described previously.[11,12] Briefly, 2.0×10^7 A549 or H1299 cells were used with a Magna RNA-Binding Protein Immunoprecipitation Kit (Millipore) according to the manufacturer's instructions. Cell lysates were incubated with RIP buffer. Then, 4 μg of MDH2 antibody or control anti–mouse IgG was incubated with protein A/G magnetic beads in RIP Wash Buffer for 30 minutes at room temperature. After washing with RIP Wash Buffer three times, beads were added to the acquired RIP lysates and incubated at 4°C overnight. On the second day, after being washed three times, the beads were resuspended in Proteinase K Buffer and incubated at 55°C for 30 minutes. Finally, RNA was acquired using RNA extraction buffer and analyzed by quantitative real-time PCR.

2.7 | FISH

Seventy-eight paired NSCLC tissues samples, including tumors and adjacent noncancerous tissues, were obtained from Renji Hospital

and approved for use by the institutional clinical research ethics committee. None of the patients had received radiotherapy or chemotherapy before surgery. Paraffin-embedded tumor tissue slides were deparaffined, rehydrated, and pretreated with 3% H_2O_2 for 15 minutes. Then slides were preincubated with hybridization solution at 37°C for 1 hour, followed by hybridization with a digoxigenin (DIG)-labeled probe at 42°C for 24 hours. After hybridization, each sample was washed in 5×, 1×, and 0.2× sodium saline citrate for 5 minutes, incubated with anti–DIG-HRP at 4°C overnight, and then incubated with a tyramide signal amplification fluorescent signal reaction solution for 30 minutes.[13] Finally, each sample was sealed with tablets containing DAPI. The sequences of probes are listed in Table S1. AC020978 expression was quantified using a visual grading system based on the extent of staining (1 = 0%-25%, 2 = 26%-50%, 3 = 51%-75%, and 4 = 76%-100%) and the intensity of staining (0 = no staining, 1 = weak staining, 2 = moderate staining, and 3 = strong staining). Final scores were computed by multiplying the percentage score and the intensity score of positive cells.[14]

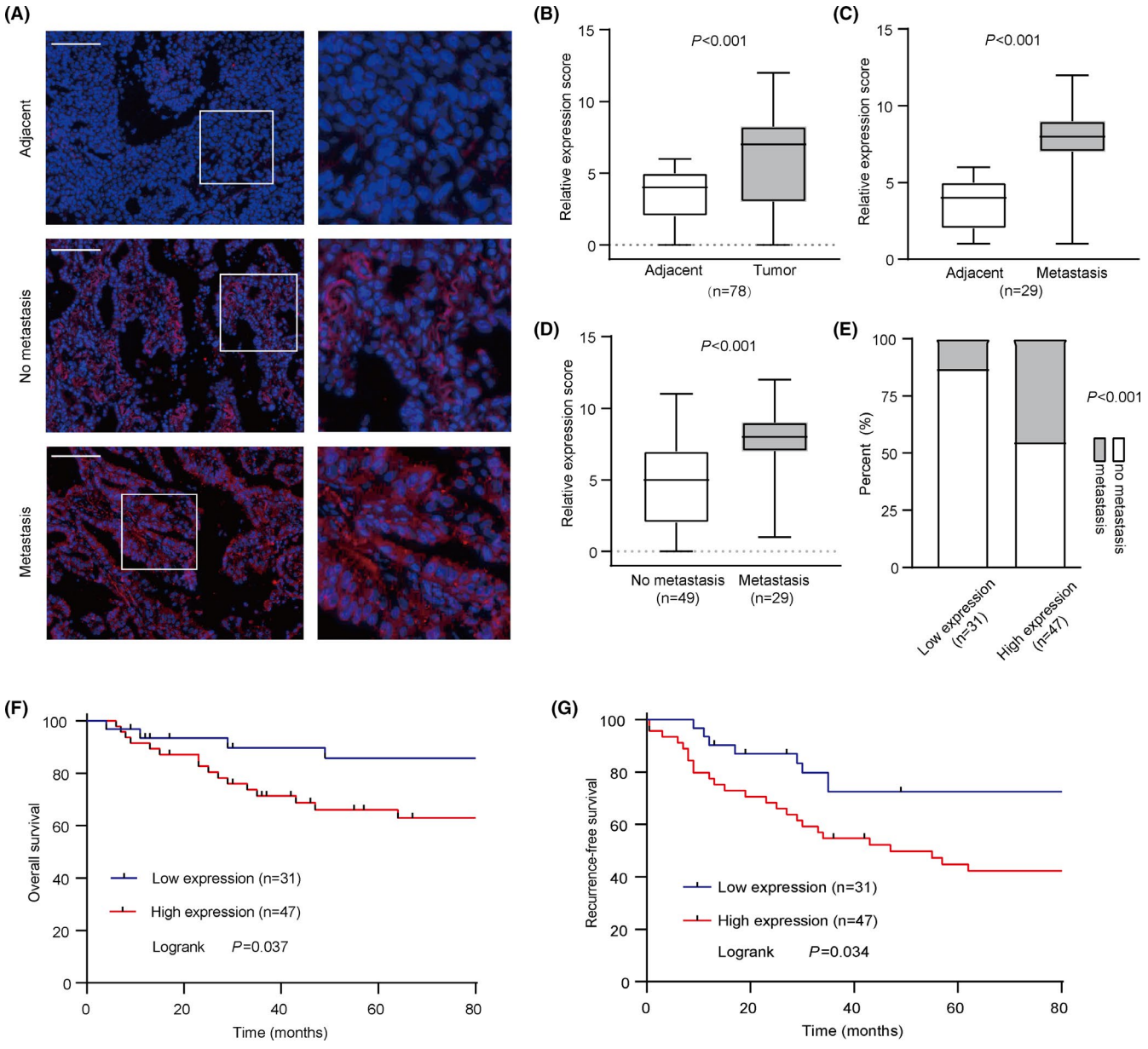

FIGURE 1 Long non–coding RNA (lncRNA) AC020978 is clinically relevant to metastatic non–small cell lung cancer (NSCLC). A, Representative FISH images of AC020978 expression in metastatic tumor tissue, non–metastatic tumor tissue, and adjacent normal tissue (blue, DAPI; red spot, positive staining; scale bar = 100 μm). B, Relative AC020978's FISH expression levels in 78 pairs of NSCLC tissues and adjacent normal tissues. C, Relative AC020978's FISH expression levels in 29 pairs of metastatic NSCLC tissues and adjacent normal tissues. D, Relative AC020978's FISH expression levels in metastatic (n = 29) and non–metastatic (n = 49) NSCLC tissues. E, The association between metastasis and AC020978 expression. F and G, Kaplan-Meier analysis of overall and recurrence-free survival in NSCLC patients with low (n = 31) and high (n = 47) AC020978 expression; logrank test (high expression: score 7-12; low expression: score 0-6)

2.8 | Immunofluorescence

Immunofluorescence was performed as described previously.[15] Expression and localization of the proteins were observed under a confocal microscope system (Olympus BX61). The antibodies used are listed in Table S2.

2.9 | Immunohistochemistry

Immunohistochemistry was performed following a previous report.[16] Tumor tissues were fixed and embedded in paraffin and stained with H&E. Immunostaining for MDH2, p-AKT (Ser473), and AKT were performed in transplanted subcutaneous tumors. The antibodies used are listed in Table S2.

2.10 | Statistical analysis

Statistical analysis was performed using SPSS 23.0 software and GraphPad Prism 8.0. All data were presented as the mean ± SD and reproduced in triplicate experiments. Student's *t* test was used to compare continuous variables and the χ^2-test or Fisher's exact test were used to compare categorical variables. The survival curves were calculated using the Kaplan-Meier method, and differences were assessed by logrank tests. Cox multivariate regression analysis was performed to detect the independent factors that influenced survival and recurrence. All results were reproduced in triplicate experiments. A *P*-value < .05 was considered significant.

TABLE 1 Correlation of the expression of AC020978 in non–small cell lung cancer with clinicopathologic features

		AC020978		
Parameter	Number	High[a] (n)	Low (n)	*P*-value
Age (y)				
≥60	40	23	17	.65
<60	38	24	14	
Gender				
Female	22	17	5	.07
Male	56	30	26	
T stage				
T1+T2	73	43	30	.64
T3+T4	5	4	1	
N stage				
N0	49	22	27	<.01
N ≥ 1	29	25	4	
M stage				
M0	63	33	30	<.01
M1	15	14	1	
Stage				
I + II	46	22	24	<.01
III + IV	32	25	7	
Mortality				
Survive	42	20	22	.02
Die	36	27	9	

Note: χ^2; $P < .05$ was considered statistically significant; n = 78 patients.
[a]AC020978 high expression: score 7-12; low expression: score 0-6.

3 | RESULTS

3.1 | Long non–coding RNA AC020978 is clinically relevant to metastatic non–small cell lung cancer

FISH assays were performed in 78 paraffin-embedded tissues to investigate the clinical relevance of AC020978 in metastatic NSCLC. Based on the AC020978 expression score, we divided all the tissues into a high expression group and a low expression group. The results showed that there was a notable positive correlation between AC020978 expression level and NSCLC metastasis (Figure 1A). AC020978 was remarkably overexpressed in lung cancer tissues (n = 78), especially in metastatic NSCLC tissues (n = 29), compared with adjacent normal tissues (Figure 1B,C). The results of quantitative real-time PCR (qRT-PCR) demonstrated that lung cancer tissues in the high expression score group had a higher RNA level of AC020978 (Figure S1A). Moreover, AC020978 was significantly overexpressed in metastatic (n = 29) versus nonmetastatic (n = 49) NSCLC tissues (Figure 1D). A high expression level of AC020978 in the primary tumor indicated an increased risk for lymphatic metastasis or distant metastasis (Figure 1E). Kaplan-Meier survival analysis of subgroups revealed that high AC020978 expression was negatively correlated with overall survival (OS) as well as recurrence-free survival (RFS) in NSCLC patients (Figure 1F,G). Detailed information on AC020978 expression and clinicopathologic parameters is exhibited in Table 1. High levels of AC020978 were significantly associated with lymphatic metastasis ($P < .01$), distant metastasis ($P < .01$), and higher TNM stage ($P < .01$) in NSCLC patients. Moreover, multivariate Cox regression analyses illustrated that the AC020978 expression level was an independent prognostic indicator for NSCLC patients (hazard ratios [HR] = 1.763; 95% confidence interval [CI], 1.028-3.097; $P = .048$; [Table S3]). These observations suggested that AC020978 might be a useful marker for NSCLC metastasis and prognosis.

3.2 | Long non–coding RNA AC020978 depletion inhibit metastasis of non–small cell lung cancer both in vitro and in vivo

In our previous study, using RNA sequencing, we found that AC020978 was a 428-nt transcript without protein-coding potential as well as one of the top-scoring upregulated lncRNA in NSCLC.[10,14] Furthermore, the expression level of AC020978 was higher in NSCLC tissues as well as NSCLC cell lines.[14] To

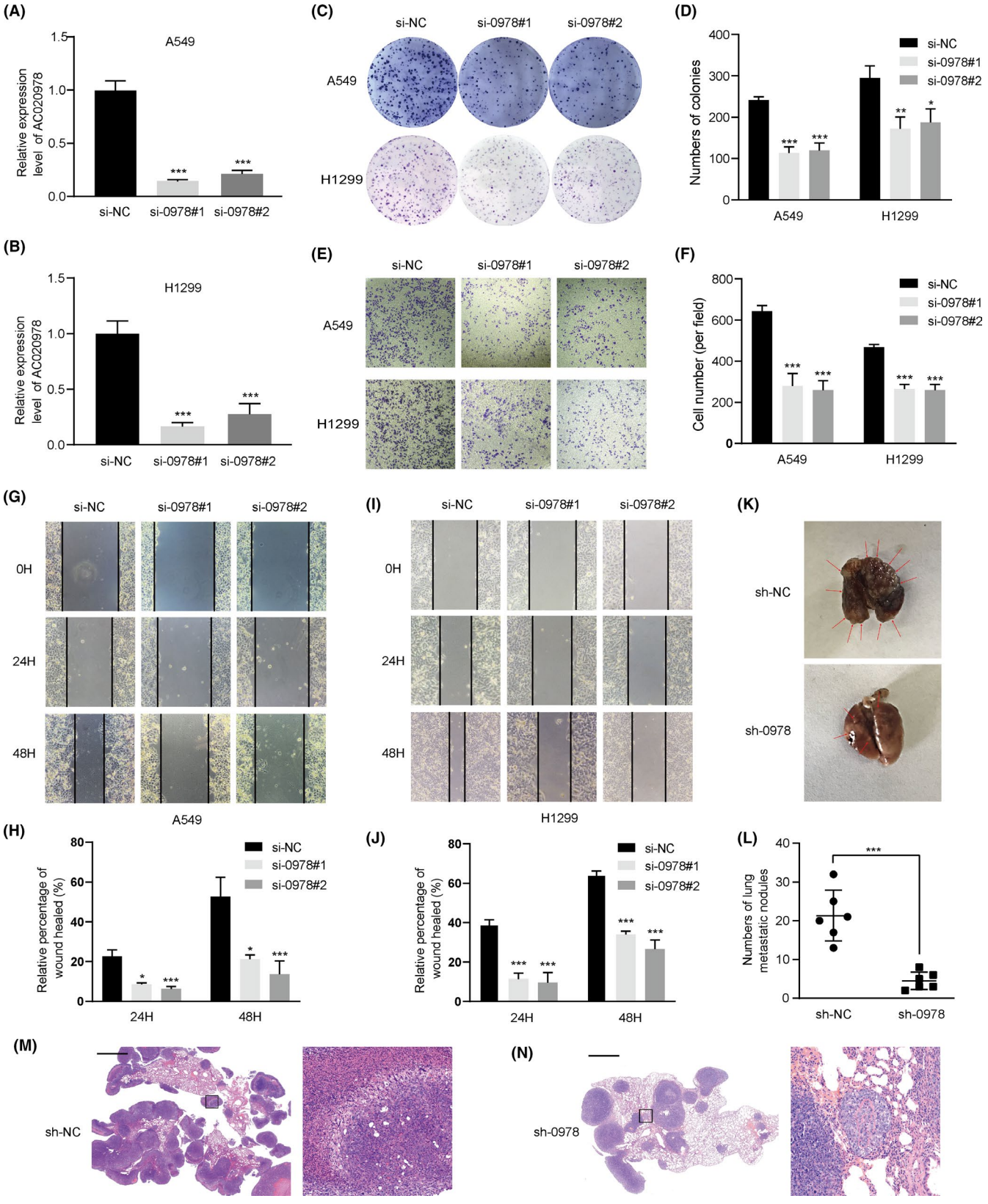

FIGURE 2 Long non–coding RNA (lncRNA) AC020978 depletion inhibits metastasis of non–small cell lung cancer (NSCLC) both in vitro and in vivo. A and B, Real-time PCR was used to analyze the knockdown efficiency of si-0978 in A549 and H1299 cell lines. C and D, Colony formation assays of NSCLC cells transfected with si-NC or si-0978. Bars on the right panel represent the number of formed clones. E and F, Transwell assays in indicated NSCLC cells. Bars on the right panel represent the number of migrated cells. G-J, Representative and quantitative results of the wound healing assays in indicated cells. Bars represent the relative percentage of wound healed. K, Metastatic tumor cells were injected into nude mice; the representative photographs of gross lungs with red arrows point to lung surface tumor nodules. L, Quantification of macroscopic metastatic nodules on the lung surfaces (n = 6). M and N, Magnification areas revealed microscopic metastatic nodes in the lung (scale bar = 2 mm). $^{*}P < .05$, $^{**}P < .01$ and $^{***}P < .001$

investigate the function of AC020978 on metastasis in NSCLC cells, we designed two different siRNA (si-0978#1 and si-0978#2), and qRT-PCR analysis showed that both of efficiently silenced AC020978 in A549 and H1299 cell lines (Figure 2A,B). Colony formation assays showed that knockdown of AC020978 could significantly inhibit the colony formation ability of A549 and H1299 cells (Figure 2C,D). Transwell assays showed that knockdown of AC020978 remarkably suppressed the migration ability

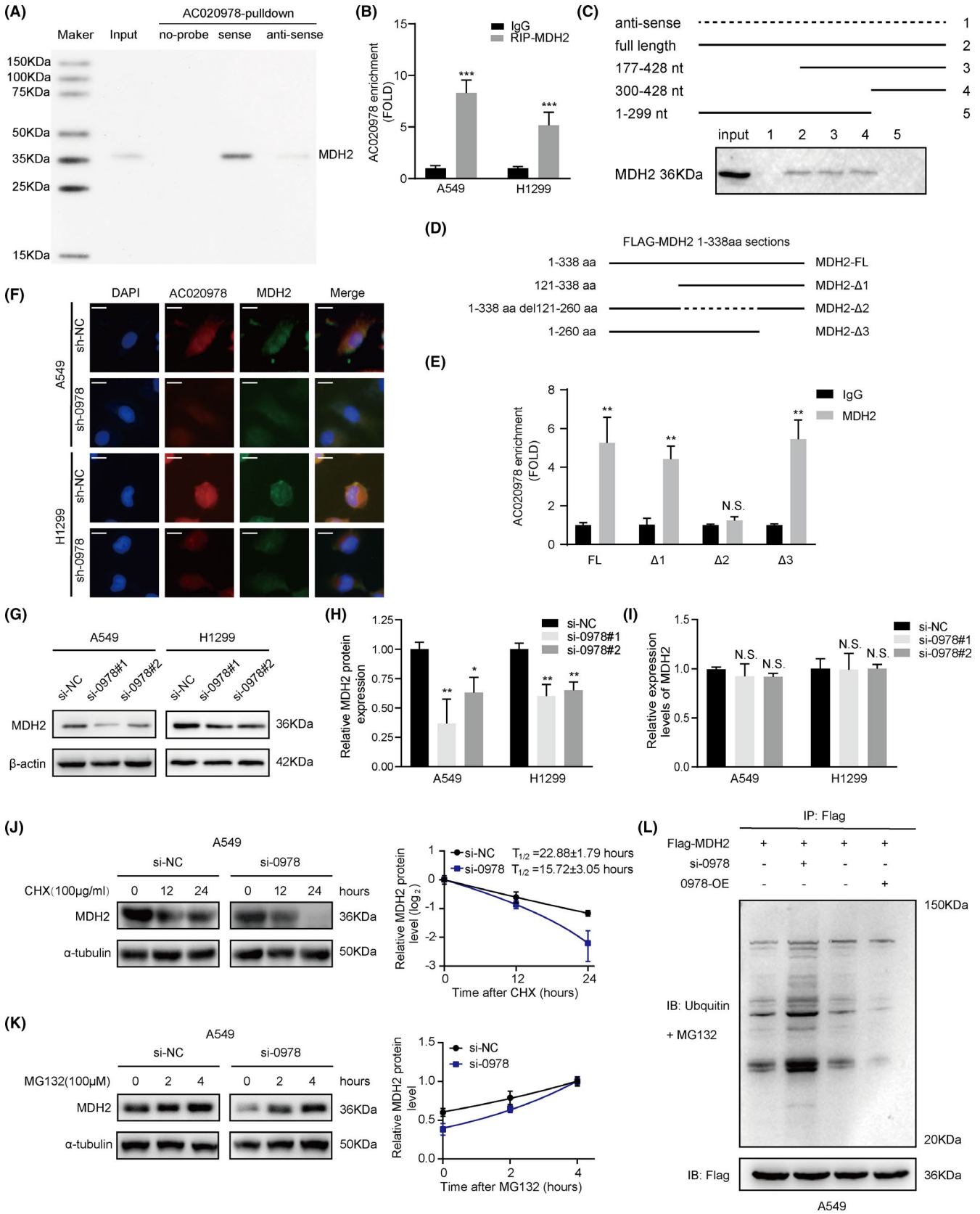

FIGURE 3 Long non–coding RNA (lncRNA) AC020978 binds to malate dehydrogenase 2 (MDH2) and stabilizes MDH2. A, Western blot analysis of the specific combination of MDH2 with AC020978 from RNA-pulldown assay. B, RIP assay using anti–MDH2 antibody showed that MDH2 interacted with AC020978. C, Schematic representation of AC020978 truncates and RNA-pulldown assay is used to examine the interaction between MDH2 and the different mutants of AC020978. D, Schematic representation of the Flag-tagged MDH2 truncates for RIP assay. E, The relative quantification of AC020978 expression in RNA-protein complexes immunoprecipitated with anti–Flag. F, Representative immunofluorescence staining displayed the expression level of MDH2 and AC020978 with different treatment in A549 and H1299 cells (scale bar = 10 μm). G and H, Western blotting was used to evaluate the protein levels of MDH2 in AC020978-KD A549 and H1299 cells. I, Quantitative real-time PCR (qRT-PCR) was used to measure the mRNA expression level of MDH2 in AC020978-KD cell lines. J, Cells were treated with cycloheximide (CHX, 100 ng/mL) and simultaneously transfected with si-0978 or si-NC, and MDH2 protein levels were measured. K, Cells were treated with MG132 (100 μmol/L) and simultaneously transfected with si-0978 or si-NC, and MDH2 protein levels were measured. L, A549 cells co–expressing Flag-MDH2 and AC020978-KD (or AC020978-OE) were immunoprecipitated with Flag antibody to detect the binding of ubiquitin. NS, not significant; $^{*}P < .05$, $^{**}P < .01$ and $^{***}P < .001$

of A549 and H1299 cells (Figure 2E,F). Moreover, the result of wound healing assays verified that migration and invasion abilities were suppressed in A549 and H1299 cells transfected with si-0978#1 and si-0978#2 (Figure 2G-J). The detection of markers for epithelial-mesenchymal transition also showed that AC020978 facilitated the invasion of A549 and H1299 cells (Figure S1B,C). To further validate the effect of AC020978 on tumor migration and invasion in vivo, we constructed a nude mouse metastasis model by tail vein injection of transfected tumor cells. The results showed that the AC020978 depletion group had a less aggressive phenotype with migration and invasion (Figure 2K). The number of metastatic nodules on the lung surface in the sh-0978 group was remarkably less than in the control group (Figure 2L). H&E staining showed that the sh-0978 group had smaller and fewer microscopic metastatic tumor nodules compared with the control group (Figure 2M,N). Taken together, these findings suggested that AC020978 plays an important part in the metastasis of NCSLC in vitro and in vivo.

3.3 | Long non–coding RNA AC020978 binds to malate dehydrogenase 2 and stabilizes malate dehydrogenase 2

Many studies have revealed that lncRNA may exert their functions by interacting with specific proteins.[17] Gene set enrichment analysis (GSEA) revealed that the function of AC020978 was highly associated with the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (Figure S2A). Our previous study conducted a biotin RNA-pulldown assay and mass spectrometry.[14] We focused on mitochondrial protein MDH2, one of the top five enriched AC020978-binding partners, which plays a crucial role in the TCA cycle. Western blotting results demonstrated that total MDH2 (Figure 3A) and mitochondrial MDH2 (Figure S2B) were abundant in AC020978-pulldown lysates. Furthermore, the results of RIP assays showed that AC020978 was characteristically enriched using MDH2 antibody compared with IgG (Figure 3B). To further explore how AC020978 binds to MDH2, a series of AC020978 truncated mutants were constructed for RNA-pulldown assays to explore the binding sites of AC020978 interacting with MDH2. The results verified that the MDH2-specific binding sequence was located within 300 and 428-nt long region (Figure 3C). Meanwhile, Flag-tagged full-length (FL) or truncated MDH2 were transfected into A549 cells, and the potential interactions were detected by RIP assay. The results showed that the 121-260 aa segment of MDH2 was sufficient to bind with AC020978 (Figure 3D,E).

To explore the molecular function of the interaction between AC020978 and MDH2, we analyzed the effects of AC020978 depletion on MDH2 expression. RNA FISH assays of AC020978 combined with immunofluorescence detection of MDH2 in A549 and H1299 cells showed a significant decline of MDH2 expression in sh-0978 cells (Figure 3F). In addition, the results of western blotting showed that knockdown of AC020978 significantly decreased the expression of MDH2 in protein level (Figure 3G,H and Figure S2C), while the relative expression level of MDH2 mRNA remained fairly constant compared with the control group (Figure 3I). Therefore, we proposed a hypothesis that AC020978 might regulate the protein stability of MDH2. To confirm the above assumption, we used protein synthesis inhibitor cycloheximide to observe the degradation of MDH2. Notably, we found that overexpression of AC020978 enhanced the stability of MDH2 (Figure S3A), and knockdown of AC020978 led to a faster speed of MDH2 degradation compared with the control group (Figure 3J and Figure S3B). Simultaneously, the effect of protein degradation caused by AC020978 depletion was obviously recovered by MG-132 (Figure 3K and Figure S3C). We performed an in vitro ubiquitination assay of A549 and H1299 cells transfected with si-0978 or pcDNA-0978. The results revealed that knockdown of AC020978 increased the level of ubiquitination of MDH2 protein, while the ubiquitin-mediated degradation effects of MDH2 were abrogated in AC020978 overexpressed A549 and H1299 cells (Figure 3L and Figure S3D). In conclusion, AC020978 directly binds to MDH2 and contributes to maintaining MDH2 protein stability through the ubiquitin-proteasome degradation pathway.

3.4 | Long non–coding RNA AC020978 promotes metastasis of non–small cell lung cancer via stabilizing malate dehydrogenase 2

Based on the results described above, we further explored the functional relevance of the interaction between AC020978 and MDH2. First, we transfected plasmid, which carries AC020978

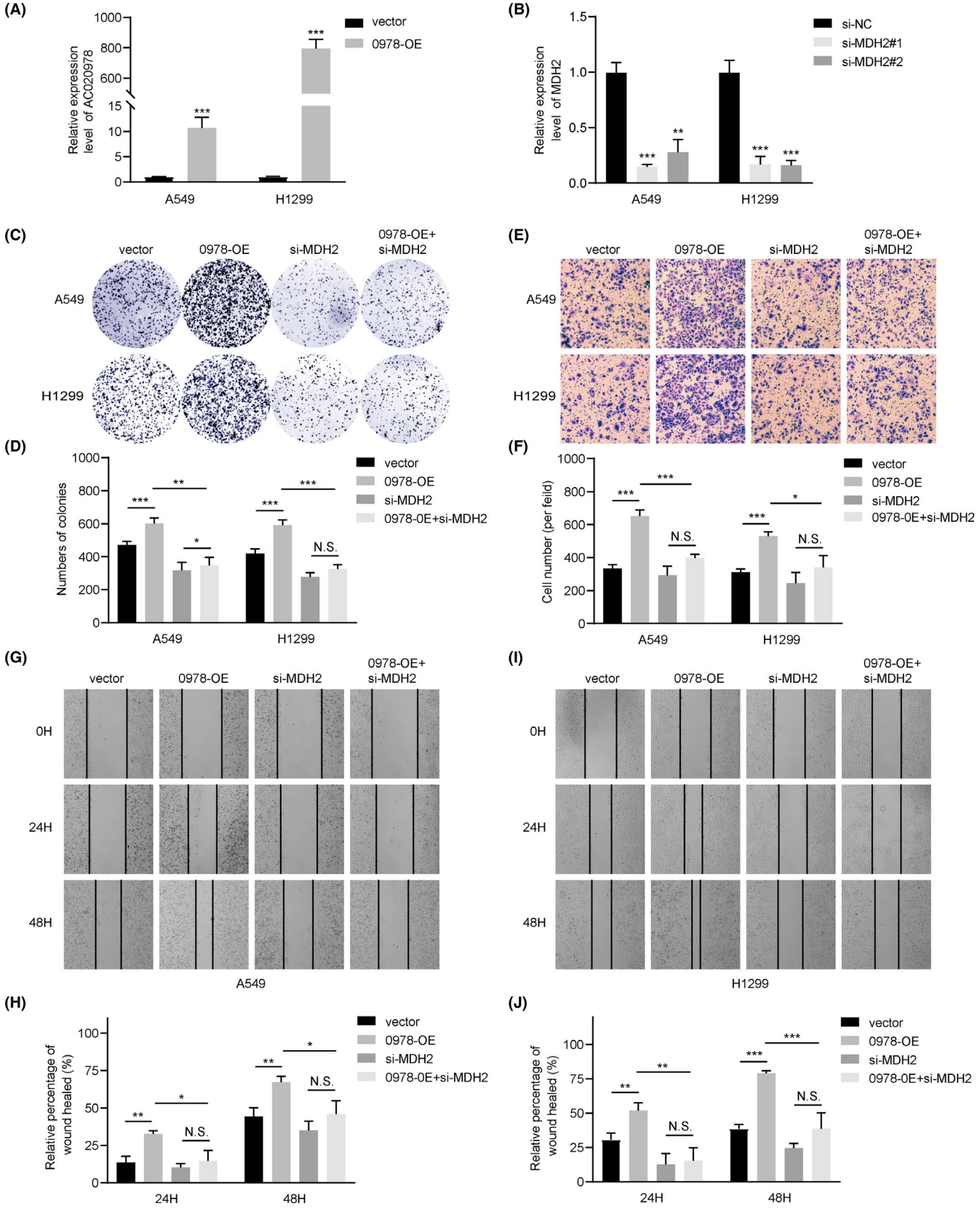

FIGURE 4 Long non–coding RNA (lncRNA) AC020978 promotes metastasis of non–small cell lung cancer (NSCLC) through stabilizing malate dehydrogenase 2 (MDH2). A, The expression of AC020978 mRNA was assessed after transfection with 0978-OE and vector in A549 and H1299 cells. B, The expression of MDH2 mRNA was assessed after downregulation of MDH2 in A549 and H1299 cells. C and D, Representative and quantitative results of the colony formation assay in vector, 0978-OE, si-MDH2, and 0978-OE+si-MDH2 transfected cells. E and F, Representative and quantitative results of the transwell assays in vector, 0978-OE, si-MDH2, and 0978-OE+si-MDH2 transfected cells. G-J, Representative and quantitative results of the wound-healing assays in vector, 0978-OE, si-MDH2, and 0978-OE+si-MDH2 transfected cells. NS, not significant, $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$

(0978-OE), compared with vector pcDNA-4/TO (Invitrogen). The results of qRT-PCR analysis showed that the expression of AC020978 was significantly elevated in the 0978-OE group (Figure 4A). Meanwhile, two different siRNA targeting MDH2 (si-MDH2#1, si-MDH2#2) were designed and both could efficiently silence MDH2 in A549 and H1299 cells (Figure 4B). Colony formation assays showed that silencing of MDH2 significantly inhibited the colony formation ability of A549 and H1299 cells and counteracted the cancer-promoting effects of AC020978 (Figure 4C,D). Meanwhile, the result of transwell and wound healing assays indicated that si-MDH2 co–transfection remarkably abolished the promoting effects of AC020978 on NSCLC migration and invasion (Figure 4E-J). These data implied that depletion of MDH2 remarkably inhibited cell migration and invasion of NSCLC and knockdown of MDH2 could reverse the facilitating effects of AC020978 in cell migration and invasion. In other words, AC020978-mediated MDH2 stabilization contributes to the migration and invasion of NSCLC.

3.5 | Long non–coding RNA AC020978 promotes the accumulation of 2-hydroxyglutarate via stabilizing malate dehydrogenase 2

High expression levels of AC020978 in lung cancer tissues guaranteed the abnormally high protein levels of MDH2, which exhibited enzyme promiscuity in catalyzing the reduction of α-ketoglutarate (α-KG) to the oncometabolite 2-hydroxyglutarate (2-HG) using

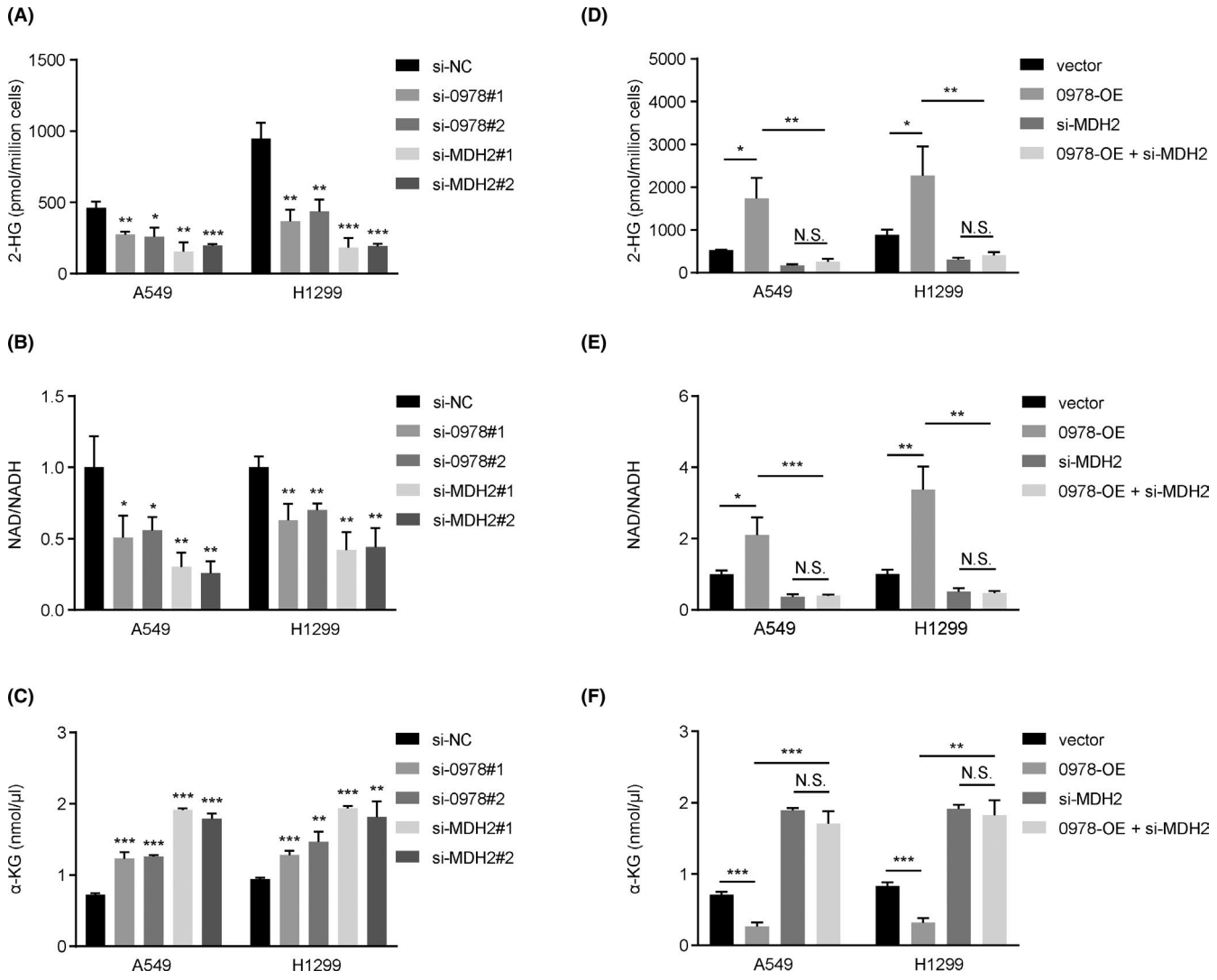

FIGURE 5 Long non–coding RNA (lncRNA) AC020978 promotes the accumulation of 2-HG through stabilizing malate dehydrogenase 2 (MDH2). A and D, Intracellular accumulation of 2-hydroxyglutarate (2-HG) was determined in A549 and H1299 cells transfected with si-NC, si-0978, si-MDH2, vector, 0978-OE, si-MDH2, and 0978-OE+si-MDH2. B and E, Relative NAD/NADH ratio in A549 and H1299 cells transfected with si-NC, si-0978, si-MDH2, vector, 0978-OE, si-MDH2, and 0978-OE+si-MDH2. C and F, Intracellular α-KG level in A549 and H1299 cells transfected with si-NC, si-0978, si-MDH2, vector, 0978-OE, si-MDH2, and 0978-OE+si-MDH2. NS, not significant; $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$

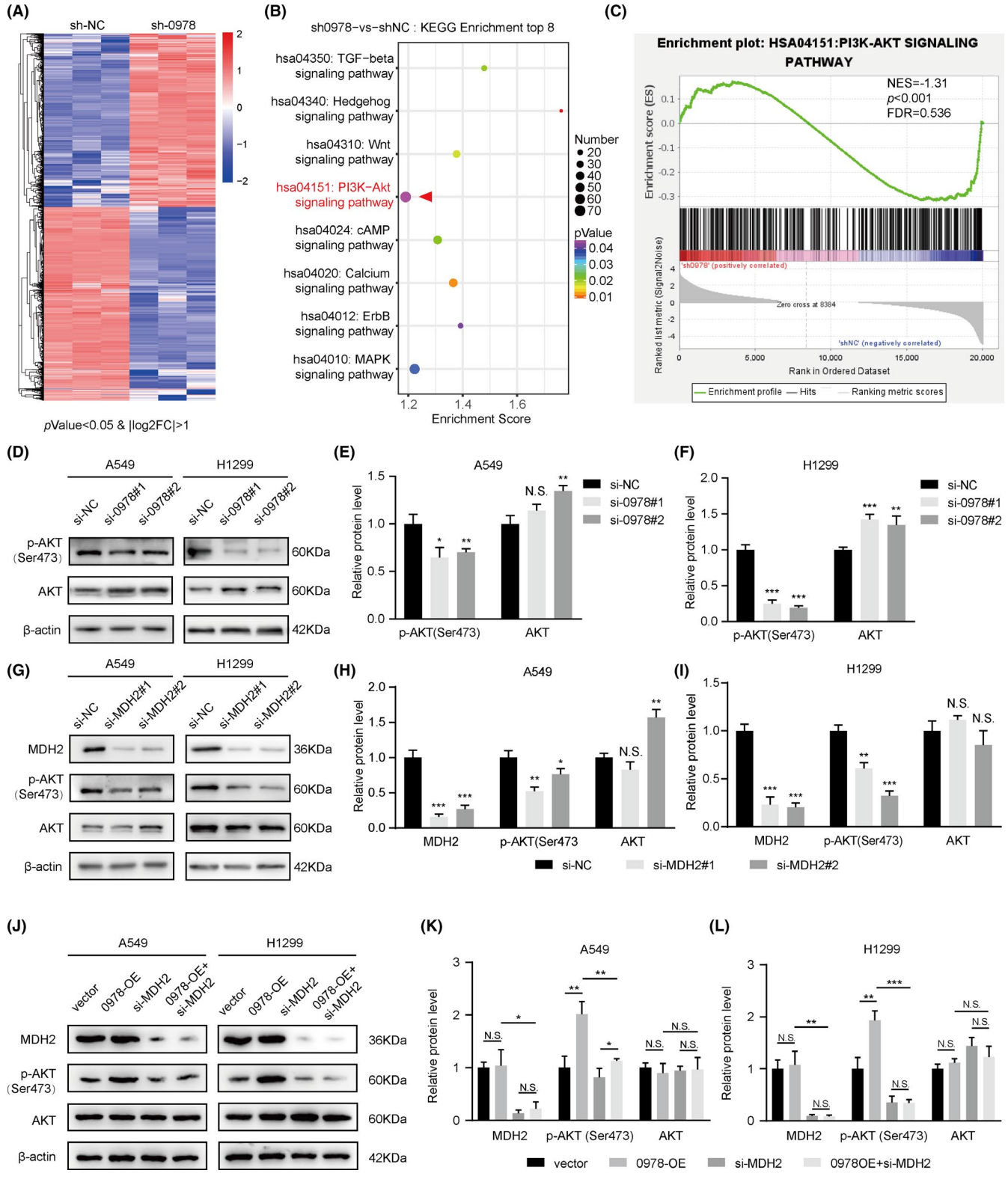

FIGURE 6 Long non−coding RNA (lncRNA) AC020978 activates the PI3K-AKT signaling pathway via malate dehydrogenase 2 (MDH2). A, Heatmap of differentially expressed downstream genes in AC020978-knockdown and corresponding control cells. B, Top eight enriched signaling pathways of the differentially expressed genes in AC020978-knockdown and control groups. C, Gene set enrichment analysis assays for the correlation of the PI3K-AKT signaling pathway and AC020978 according to the RNA-seq. D-F, Western blotting was used to evaluate the expression levels of AKT and p-AKT (Ser473) in si-0978-transfected non−small cell lung cancer (NSCLC) cells. G-I, Western blotting was used to evaluate the expression levels of MDH2, p-AKT (Ser473), and AKT in si-MDH2-transfected non−small cell lung cancer (NSCLC) cells. J-L, Western blotting evaluated the expression levels of MDH2, p-AKT (Ser473), and AKT in vector, 0978-OE,si-MDH2, and 0978-OE+si-MDH2-transfected NSCLC cells. NS, not significant; *$P < .05$, **$P < .01$ and ***$P < .001$

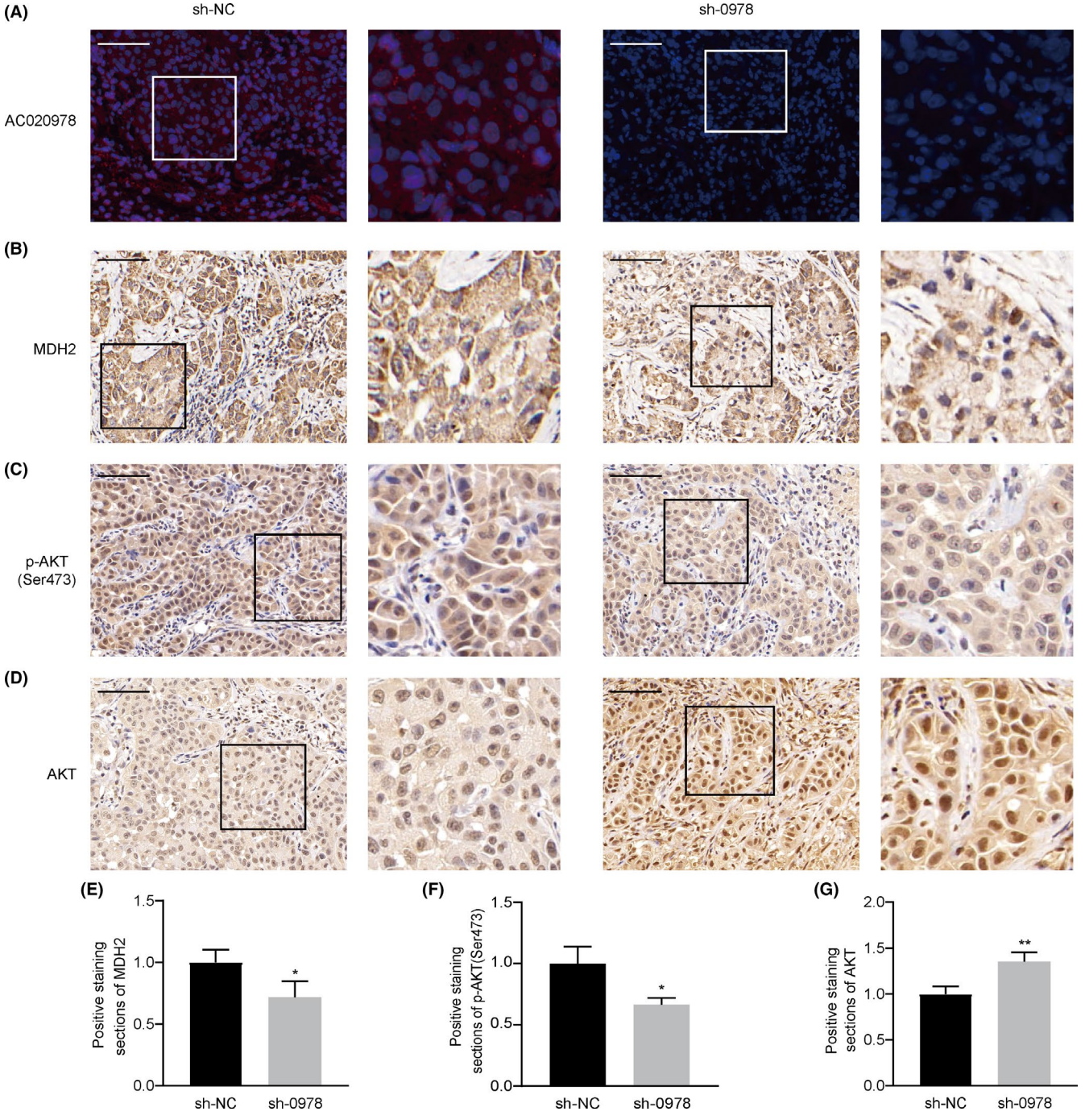

FIGURE 7 Long non–coding RNA (lncRNA) AC020978 facilitates non–small cell lung cancer (NSCLC) progression by stabilizing malate dehydrogenase 2 (MDH2) and activating the AKT pathway in vivo. A, Representative FISH images revealed the expression levels of AC020978 in sh-NC and sh-0978 xenograft tissues (scale bar = 100 μm). B-D, Representative immunohistochemistry staining images MDH2, p-AKT (Ser473), and AKT from sh-NC and sh-0978 tumors (scale bar = 100 μm). E-G, Quantification of the positive staining sections of MDH2, p-AKT (Ser473), and AKT levels in sh-NC and sh-0978 tumors. $^{*}P < .05$, $^{**}P < .01$

NADH as a cofactor.[18] To explore the effect of the 0978-MDH2 axis on metabolism, we further detected upstream and downstream metabolites of MDH2. The result showed that depletion of AC020978 or MDH2 significantly reduced 2-HG levels as well as the NAD/NADH ratio (Figure 5A,B) and remarkably increased the α-KG level (Figure 5C). Overexpression of AC020978 could upregulate 2-HG levels and the NAD/NADH ratio, as well as reduce the α-KG level. However, in the absence of MDH2, AC020978 had little effect on the regulation of these metabolites (Figure 5D-F). Our data confirmed the effect of AC020978 on MDH2-based metabolic regulation.

3.6 | Long non–coding RNA AC020978 activates the PI3K-AKT signaling pathway via malate dehydrogenase 2

To explore the key downstream signaling pathways regulated by AC020978, we used sh-AC020978 and sh-NC stably transfected A549 cells to compare their gene expression discrepancy through whole transcriptome sequencing. The result showed that 1551 genes were upregulated and 1715 genes were downregulated ($P < .05$) in sh-0978 A549 cells (Figure 6A and Figure S4A). Gene ontology enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis revealed that the differentially expressed genes were enriched for cell signaling transduction (Figure S4B,C). We further conducted the top eight enriched pathways and observed that the PI3K-AKT signaling pathway contained the maximum number of differentially expressed genes (Figure 6B). The results of GSEA revealed that a substantial subset of genes involved in the PI3K-AKT signaling pathway was downregulated after knockdown of AC020978 (Figure 6C). Subsequently, the result of western blotting demonstrated a remarkable reduction of p-AKT (Ser473) in A549 and H1299 cells with AC020978 or MDH2 depletion (Figure 6D-I). Therefore, we proposed a hypothesis that AC020978 might activate the PI3K-AKT signaling pathway through stabilizing MDH2. Indeed, overexpression of AC020978 had little effect on activating AKT in the absence of MDH2. (Figure 6J-L). Overall, our findings demonstrated that AC020978 activates the PI3K-AKT signaling pathway by stabilizing MDH2.

3.7 | Long non–coding RNA AC020978 facilitates non–small cell lung cancer progression by stabilizing malate dehydrogenase 2 and activating the AKT pathway in vivo

To further verify the above results, we used sh-AC020978 and sh-NC-stably transfected A549 cell lines and transplanted them into nude mice to generate xenograft models. FISH assays of xenograft tumors showed that the expression levels of AC020978 were noticeably reduced in sh-0978 xenograft tissues (Figure 7A). Immunohistochemical staining with MDH2, p-AKT (Ser473) and AKT of xenograft tissues validated the important role of AC020978 in vivo (Figure 7B-D). Depletion of AC020978 significantly decreased the expression of MDH2 and p-AKT (Ser473) in protein level but increased the protein level of AKT in vivo (Figure 7E-G). Combined with our previous findings, we concluded that AC020978 promoted NSCLC migration and invasion through the AC020978/MDH2/AKT axis both in vitro and in vivo.

4 | DISCUSSION

Many studies have been carried out in past that highlight the role of lncRNA in the progression and metastasis in cancer. In the present study, we demonstrated that knockdown of AC020978 significantly inhibits the migration and invasion of NSCLC in vitro and in vivo.

Incremental evidence has demonstrated that lncRNA take part in cellular behaviors by binding with specific proteins.[19] In this research, we found that MDH2 was bound with AC020978 and functionally involved in AC020978-mediated NSCLC progression. MDH2 is the final enzyme in the mitochondrial TCA cycle, which catalyzes the inter-conversion of oxaloacetate and L-malate using NAD as a cofactor to generate reducing equivalents.[20] Recent studies have shown that MDH2 knockdown has a pronounced negative effect on cell proliferation and migration and increases the expression of PTEN.[21,22] In this study, we demonstrated that AC020978 interacts with MDH2 and maintains the stability of MDH2 through ubiquitin-mediated proteasome degradation. Rescued experiments confirmed that knockdown of MDH2 attenuated the cancer-promoting effect of AC020978 in migration and invasion. These results suggested that MDH2 is indispensable for AC020978-related metastatic progression of NSCLC. Our research went a step further than previous studies and revealed a new mechanism for MDH2 to promote lung cancer metastasis. Inhibition of MDH2 could be a prospective tumor-suppressing agent in the future. Moreover, AC020978 or MDH2 knockdown significantly reduced oncometabolite 2-HG levels as well

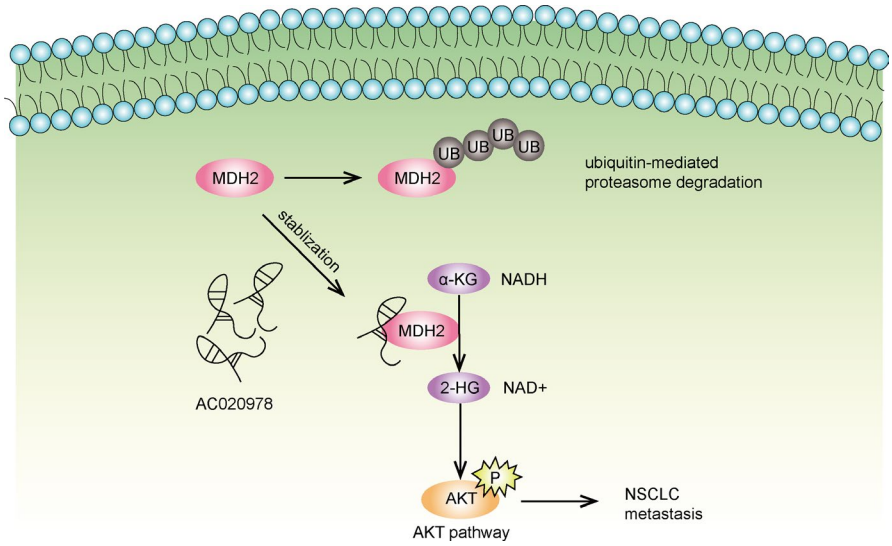

FIGURE 8 Proposed model illustrating the protective function of AC020978-mediated metastasis in non–small cell lung cancer (NSCLC)

as the NAD/NADH ratio. These results indicated that the AC020978-MDH2 axis facilitates metabolic dysfunction.

Long non–coding RNA have been declared to have multiple and diverse molecular mechanisms in the process of tumor development. Recent discoveries have revealed that lncRNA serve as key signal transduction mediators in cancer signaling pathways.[23] For instance, cancer pathway microarray analysis showed that lncRNA AK023391 is involved in the regulation of the PI3K/AKT signaling pathway.[24] To further explore downstream signaling pathways regulated by AC020978, whole transcriptome sequencing was performed in sh-0978 and sh-NC A549 cells. The KEGG network analysis and GSEA of these differentially expressed genes indicated that AC020978 may regulate the PI3K/AKT pathway, which is involved in cell proliferation, differentiation, migration, invasion, and survival.[23] The PI3K/AKT/mTOR signaling pathway is frequently overstimulated and influences the progression and metastasis in assorted types of cancers.[25,26] Our data confirmed that the phosphorylated levels of AKT were remarkably reduced after AC020978 knockdown and elevated after AC020978 overexpression.

Many studies have reported that 2-HG could stimulate the PI3K/AKT/mTOR pathway and further promote cancerogenesis.[27] The accumulation of 2-HG in tumors could inhibit chromatin-modifying enzymes as well as activate the mechanistic target of the PI3K/AKT/mTOR signaling pathway, thus dysregulating gene expression and promoting tumorigenesis.[27] We suspected that AC020978 activates the PI3K/AKT/mTOR pathway by enhancing the stability of MDH2 protein. As expected, subsequent rescued experiments confirmed that depletion of MDH2 reversed the activating effect of AC020978 in the AKT signaling pathway. These results demonstrated that the AC020978-MDH2 axis causes an increase in 2-HG levels and activates PI3K/AKT/mTOR signaling.

5 | CONCLUSIONS

In summary, we identified the metastasis-related function of lncRNA AC020978 in NSCLC. Furthermore, we demonstrated that AC020978 promotes migration and invasion of lung cancer through the AC020978/MDH2/AKT axis (Figure 8). These findings might provide a theoretical and experimental basis for revealing the mechanism of NSCLC metastasis. The newly identified AC020978/MDH2/AKT axis is a promising potential therapeutic target for NSCLC.

ACKNOWLEDGMENTS

This work was supported by research grants from the National Natural Science Foundation of China (Grant No. 81830052, 81530053, and 81903065), the Construction Program of Shanghai Key Laboratory of Molecular Imaging (18DZ2260400), and the Shanghai Municipal Education Commission (Class II Plateau Disciplinary Construction Program of Medical Technology of SUMHS, 2018-2020).

DISCLOSURE

The authors have no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

Data are available from the corresponding author upon reasonable request. The RNA-seq data that support the findings of this study are openly available in the NCBI SRA database (http://www.ncbi.nlm.nih.gov/bioproject/702551; BioProject accession number: PRJNA702551).

ORCID

Fei Xu https://orcid.org/0000-0003-3071-3186

REFERENCES

1. Herbst RS, Morgensztern D, Boshoff C. The biology and management of non–small cell lung cancer. *Nature*. 2018;553:446-454.
2. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2019. *CA Cancer J Clin*. 2019;69:7-34.
3. Miller KD, Nogueira L, Mariotto AB, et al. Cancer treatment and survivorship statistics, 2019. *CA Cancer J Clin*. 2019;69:363-385.
4. Ahmad S, Khan MY, Rafi Z, et al. Oxidation, glycation and glycoxidation-the vicious cycle and lung cancer. *Semin Cancer Biol*. 2018;49:29-36.
5. Arbour KC, Riely GJ. Systemic therapy for locally advanced and metastatic non–small cell lung cancer: a review. *JAMA*. 2019;322:764-774.
6. Sun Q, Hao Q, Prasanth KV. Nuclear long noncoding RNAs: key regulators of gene expression. *Trends Genet*. 2018;34:142-157.
7. Wang J, Cai H, Dai Z, Wang G. Down-regulation of lncRNA XIST inhibits cell proliferation via regulating miR-744/RING1 axis in non–small cell lung cancer. *Clin Sci*. 2019;133:1567-1579.
8. Beermann J, Piccoli MT, Viereck J, Thum T. Non–coding RNAs in development and disease: background, mechanisms, and therapeutic approaches. *Physiol Rev*. 2016;96:1297-1325.
9. Jiang C, Li X, Zhao H, Liu H. Long non–coding RNAs: potential new biomarkers for predicting tumor invasion and metastasis. *Mol Cancer*. 2016;15:62.
10. Hua Q, Jin M, Mi B, et al. LINC01123, a c-Myc-activated long non–coding RNA, promotes proliferation and aerobic glycolysis of non–small cell lung cancer through miR-199a-5p/c-Myc axis. *J Hematol Oncol*. 2019;12:91.
11. Zhang C, Ge S, Gong W, et al. LncRNA ANRIL acts as a modular scaffold of WDR5 and HDAC3 complexes and promotes alteration of the vascular smooth muscle cell phenotype. *Cell Death Dis*. 2020;11:435.
12. Chen J, Jin J, Zhang XI, et al. Microglial lnc-U90926 facilitates neutrophil infiltration in ischemic stroke via MDH2/CXCL2 axis. *Mol Ther*. 2021 Sep 1. 29:2873–2885.
13. Zhou M, Hu H, Han Y, et al. Long non–coding RNA 01126 promotes periodontitis pathogenesis of human periodontal ligament cells via miR-518a-5p/HIF-1alpha/MAPK pathway. *Cell Prolif*. 2021;54:e12957.
14. Hua Q, Mi B, Xu F, et al. Hypoxia-induced lncRNA-AC020978 promotes proliferation and glycolytic metabolism of non–small cell lung cancer by regulating PKM2/HIF-1alpha axis. *Theranostics*. 2020;10:4762-4778.
15. Ottaviani S, Stebbing J, Frampton AE, et al. TGF-β induces miR-100 and miR-125b but blocks let-7a through LIN28B controlling PDAC progression. *Nat Commun*. 2018;9:1845.
16. Wen X, Liu X, Mao YP, et al. Long non–coding RNA DANCR stabilizes HIF-1alpha and promotes metastasis by interacting with NF90/NF45 complex in nasopharyngeal carcinoma. *Theranostics*. 2018;8:5676-5689.
17. Zhang W, Chen LI, Wu J, et al. Long noncoding RNA TUG1 inhibits osteogenesis of bone marrow mesenchymal stem cells via Smad5 after irradiation. *Theranostics*. 2019;9:2198-2208.

18. Rzem R, Vincent MF, Van Schaftingen E, Veiga-da-Cunha M. L-2-hydroxyglutaric aciduria, a defect of metabolite repair. *J Inherit Metab Dis*. 2007;30:681-689.
19. Hua Q, Mi B, Huang G. The emerging co–regulatory role of long noncoding RNAs in epithelial-mesenchymal transition and the Warburg effect in aggressive tumors. *Crit Rev Oncol Hematol*. 2018;126:112-120.
20. Shi Q, Gibson GE. Up-regulation of the mitochondrial malate dehydrogenase by oxidative stress is mediated by miR-743a. *J Neurochem*. 2011;118:440-448.
21. Shelar S, Shim E-H, Brinkley GJ, et al. Biochemical and epigenetic insights into L-2-hydroxyglutarate, a potential therapeutic target in renal cancer. *Clin Cancer Res*. 2018;24:6433-6446.
22. Zhuang Y, Xiang J, Bao W, et al. MDH2 stimulated by estrogen-GPR30 pathway down-regulated PTEN expression promoting the proliferation and invasion of cells in endometrial cancer. *Transl Oncol*. 2017;10:203-210.
23. Lin C, Yang L. Long noncoding RNA in cancer: wiring signaling circuitry. *Trends Cell Biol*. 2018;20:287-301.
24. Huang Y, Zhang J, Hou L, et al. LncRNA AK023391 promotes tumorigenesis and invasion of gastric cancer through activation of the PI3K/Akt signaling pathway. *J Exp Clin Cancer Res*. 2017;36:194.
25. Wu Y, Zhang Y, Qin X, Geng H, Zuo D, Zhao Q. PI3K/AKT/mTOR pathway-related long non–coding RNAs: roles and mechanisms in hepatocellular carcinoma. *Pharmacol Res*. 2020;160:105195.
26. Liu Z, Mo H, Sun L, et al. Long noncoding RNA PICSAR/miR-588/EIF6 axis regulates tumorigenesis of hepatocellular carcinoma by activating PI3K/AKT/mTOR signaling pathway. *Cancer Sci*. 2020;111:4118-4128.
27. Jezek P. 2-Hydroxyglutarate in cancer cells. *Antioxid Redox Signal*. 2020;33:903-926.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Xu F, Hua Q, Zhang A, et al. LncRNA AC020978 facilitates non–small cell lung cancer progression by interacting with malate dehydrogenase 2 and activating the AKT pathway. *Cancer Sci*. 2021;112:4501–4514. https://doi.org/10.1111/cas.15116