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Original Article

The expression of actate dehydrogenase A (ADA) in NSCLC stem cells (SC) affects tumor progression and metastasis

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Abstract: Lactate dehydrogenase A (LDH-A) can catalyze the transition between lactate and pyruvate. LDH-A up-regulation can be found in various human malignant tumor cells, as it is possibly involved in tumor progression and invasion or metastasis. This study aimed to investigate the role of LDH-A expression in non-small cell lung cancer (NSCLC) tumor stem cells and tumor progression or metastasis. A total of 68 NSCLC tumor tissue samples were collected to test LDH activity. LDH-A inhibitor oxamate sodium was used for treatment at different concentrations. RNA interference (RNAi) approach inhibited LDH-A expression. Cell proliferation rate was measured by MTT assay. Transwell assay analyzed cell invasion potency. RNAi treated A549 cells were injected into mice for observing tumorigenesis. NSCLC tumor tissues had significantly higher LDH activity than normal tissues ($P<0.05$). In more advanced clinical stage, enzyme activity was even higher. After suppressing LDH-A activity by oxamate sodium or gene expression by RNAi, cell proliferation inhibitory rate was remarkably elevated ($P<0.05$). Transwell results showed significantly suppressed invasion potency ($P<0.05$), whilst tumorigenesis ability of A549 cells was inhibited by suppressing LDH-A expression ($P<0.05$). NSCLS tumor tissues have significantly higher LDH-A expression than normal tissues. In conclusion, suppression of LDH-A expression effectively suppresses lung cancer cell proliferation or tumorigenesis.

Keywords: LDH-A, non-small cell lung cancer, RNA interference, oxamate sodium, cell proliferation, cell invasion, tumorigenesis

Introduction

Lung cancer is the most prevalent and deadly malignant tumor worldwide, and about 85% of lung cancer cases belong to non-small-cell lung carcinoma (NSCLC) [1]. With the progression of medical science and development of targeted drugs, NSCLC treatment efficiency has been radically increased, as both survival rate and life quality of patients are significantly improved. However, there are still certain NSCLC patients manifesting rapid disease progression or aggravation by other reasons [2, 3]. Therefore, the study of NSCLC disease progression and cancer cell metastasis regulation, and manipulation of NSCLC progression via modulating critical factors in these processes, have become a major field for medical research.

Energy metabolism of lung cancer stem cells depends on cellular glycolysis. The production of lactate by glycolysis and derivative pathway in cancer cells can provide energy for cellular activity, which is correlated with cancer cell invasion potency [4, 5]. The acidic by-products from cancer cell metabolism can decrease pH value in the micro-environment, thus potentiating invasion and metastasis of cancer cells [5]. K-RAS gene mutation is frequently occurred in NSCLC patients. Such mutation can long-termly activate proliferation signal of cancer cells to facilitate cell proliferation, and increase glycolysis of cancer cells, thus potentiating lactate production and eventually facilitating cancer cell invasion and metastasis [6, 7].

Based on these evidences, scholars have proposed that pharmaceutical or molecular biological approach to inhibit glycolysis or lactate production in lung cancer stem cells could effectively inhibit disease progression [8]. During

Table 1. Artificially synthesized nucleic acid sequence for cell transfection

Name	Sequence
anti-LDH	CATTCATTCCACTCCATACAG AAGTAAGTAAGGTGAGGTATG
Scramble	CATCTTCAGCACCATATAC GAGTAGAAGTCGTGGTATA

lactate metabolism, lactate dehydrogenase A (LDH-A) plays a crucial role as it can catalyze transformation between lactate and pyruvate. LDH-A up-regulation can be found in various human malignant tumor cells, as it is possibly involved in tumor progression and invasion or metastasis via catalyzing lactate production [9, 10]. This study thus aimed to study the effect of LDH-A expression in NSCLC tumor stem cells and its correlation with tumor progression or metastasis.

Materials and methods

Research subjects

A total of 68 NSCLC patients who were admitted in the department of respiratory surgery in Qiqihar Medical College Affiliated First Hospital (Heilongjiang, China) from August 2014 to August 2016 were recruited, including 44 males and 24 females (age between 48 and 72 years, average age = 54.1 ± 6.7 years). All patients were first diagnosed as lung cancer, and did not receive any chemo- or radio-therapy or surgery. All patients were diagnosed based on clinical history and CT or MRI imaging, with reference to NSCLC guideline (2015, 1st edition). There were 8, 12, 20 and 28 patients diagnosed as stage I, II, III and IV NSCLS, respectively. Both cancer tissues and tumor adjacent tissues were collected during the surgery. Cancer tissues were divided into two parts, of which one half was immediately sent for LDH-A activity assay, and the other half was fixed in 10% formalin and prepared into 4 consecutive slices in paraffin embedding (5 μm thickness) for histopathology analysis. This study has been approved by the ethical committee of Qiqihar Medical College Affiliated First Hospital (Heilongjiang, China), and all research subjects signed informed consents.

LDH-A activity assay

Cancer tissues and adjacent tissues were cut into small pieces, rinsed twice in pre-cold PBS (pH 7.4), and re-suspended in fresh PBS (pH 7.4). Tissues were homogenized on ice and then centrifuged at 12000 g for 10 min to collect the supernatant for measuring LDH-A activity using test kit (GENMED). In brief, buffer A in test kit was aliquot into 96-well plate, followed by adding 10 μL tissue homogenate supernatant. After mixing with buffer B, the mixture was incubated at 25°C for 5 min. The microplate reader was adjusted to zero, and reaction buffer C was added for measuring absorbance (A) value at 340 nm wavelength. One single unit (U) of enzymatic activity was defined as the amount of enzyme to decrease A340 value by 1.0 at 25°C and pH 7.5 [11].

Western blot

Tissues collected from the surgery were homogenized and mixed with 100 μL cell lysis buffer. After centrifugation at 13000 g for 10 min, the supernatant was collected for protein electrophoresis and Western blot analysis. SDS-PAGE used 15% separating gel and 5% condensing gel for separation. Target protein was transferred to PVDF membrane, which was blocked in 5% defatted milk powder for 1 hour at 37°C, rinsed in TBST and incubated in primary antibody working solution (mouse anti-human LDH-A specific antibody and mouse anti-human β-actin antibody, 1:2000 dilution) for 4°C overnight incubation. TBST was used to rinse excess primary antibody, and horseradish peroxidase-labelled goat anti-mouse IgG secondary antibody (1:1000) was added for 1 hour incubation at room temperature. After TBST rinsing, the membrane was developed in freshly prepared DAB substrate for 10 min, and was quenched in distilled water. Western blot images were processed by gel imaging analysis system to detect integrity gray value of target bands. Relative expression of LDH-A in the samples was corrected using β-actin as the internal reference.

RNA interference

Based on mRNA sequence of LDH-A (Genebank access number: NM_003884), RNAi sequence was designed. Anti-LDH and negative control (NC) sequences were chemically synthesized. All base pairs of sequence were modified by methylation. Nucleotide sequences were synthesized by Sangon (China) as shown in **Table 1**. Liposome transfection kit INTERFERin™ (Polyplus transfection) was used for cell transfection. Lung cancer cell line A549 was purchased from Cell Bank, Chinese Academy of

Table 2. qRT-PCR for primer sequence

Primer		Sequence
β-tublin	F	5'-TGTCCCGATGGCGAGTGTTT-3'
	R	5'-CCTGTTGGCCATAGTACTGC-3'
LDH-A	F	5'-GAGAGTGCTTATGAGGTGAT-3'
	R	5'-ACAAGGTCTGAGATTCCATT-3'

Science. Cryopreserved cells were resuscitated and cultured in DMEM culture medium (Gibco) containing 10% fetal bovine serum (FBS) and gentamycin until reaching log-growth phase. Cells were then digested by trypsin, counted and diluted to 3×10^5 in fresh medium for inoculating in 96-well plate. After 24 hours incubation, transfection was performed following the manual instruction of test kit [12]. Cells were divided into three groups: anti-LDH group, negative group and empty control group. Empty control group used PBS for transfection. 1 μL Lipofectamine 2000 (Invitrogen, US) was diluted in 50 μL antibiotics-free, serum-free DMEM medium for 5 min incubation. All groups were added with miR-21 expression plasmid, negative control plasmid or PBS for preparing transfection reagent. 100 μL transfection reagent was added into each well. The 96-well plate was incubated at 37°C with 5% CO_2 for 6 hours. Antibiotic-/serum-free medium was switched for DMEM medium containing 10% FBS and gentamycin for 48~72 hours for further assays [10].

qRT-PCR

Those cells with successful transfection were tested for LDH-A expression level by qRT-PCR using primers shown in **Table 2**. One-step total RNA extraction reagent TRIZOL (Invitrogen) was used to extract total RNA, using RNA from prostate gland hyperplasia tissues as the control. qRT-PCR reagent (TianGen) was used for qRT-PCR. Reverse transcription PCR was firstly performed at 37°C for 2 hours. cDNA obtained from reverse transcription was used as the template in qRT-PCR, under the following conditions: 95°C for 5 min, followed by 40 cycles each containing 95°C for 1 min, 63.1°C for 30 s and 72°C 3 min. PCR products were tested in 1% agarose gel electrophoresis. Relative expression level of LDH-A was calculated by gel imaging analyzer.

Effects of LDH-A enzymatic activity on NSCLC

Specific inhibitor for LDH-A enzyme, oxamate sodium (Sigma), was used to inhibit LDH-A activity in lung cancer cell line A549 to observe its effect on cell proliferation and metastasis. A549 cells were inoculated into 96-well plate (100 μL each well), plus gradient concentrations of oxamate sodium solutions (0, 10, 20, 30, 40, 50 and 60 μmol/L) for 12 hours or 24 hours incubation. 10 μL MTT solution (5 mg/mL in PBS pH 7.5) was then added for 4 hours incubation. 150 μL DMSO was added into each well to completely solve MTT. ELISA apparatus was used to test A values of each well at 490 nm wavelength. Cell survival rate = (OD value of drug treatment group - OD value of empty group)/(OD value of control group - OD value of empty group) × 100%. Cell survival curve was plotted.

Transwell assay for cell invasion and migration potency

Following manual instruction of Transwell assay kit. 60 μL matrix gel (5 mg/mL, BD) was paved in the upper chamber of Transwell, which was air-dried at 4°C. HCT cells were incubated until log-growth phase, and were digested in trypsin. Cells were then washed in serum-free medium and re-suspended. Cell density was adjusted to 1×10^6/mL. 200 μL cell suspension was added into the upper chamber of Transwell with pavement. 600 μL culture medium containing 10% FBS was added into the lower Transwell chamber, which was incubated for 24 hours at 37°C with CO_2. Residual matrix gel and cells were gently removed by swab cotton. Cells in the lower chamber were stained by crystal violet for 30 min under room temperature, followed by rinsing in 10% acetic acid. OD_{570} was measured by a microplate reader. Each experiment was performed in triplicates.

Xenograft of human NSCLC cells

NSCLS xenograft assay was used to evaluate the effect of LDH-A activity on tumorigenesis potency of NSCLC. NSCLC cells were incubated at 37°C for 2 hours, and were re-suspended in PBS for courting. NOD/SCID mice (N = 12, both males and females, 6 weeks age, body weight = 32.6 ± 2.5 g) were equally divided into three groups, with each mouse receiving 10^5 cells by intra-peritoneal injection. All mice were fed with normal diet and were kept at 25°C with 60 ± 10% humidity. Animal protocols followed the guidance of Institutional Animal Care and Use Committee stipulated by NIH.

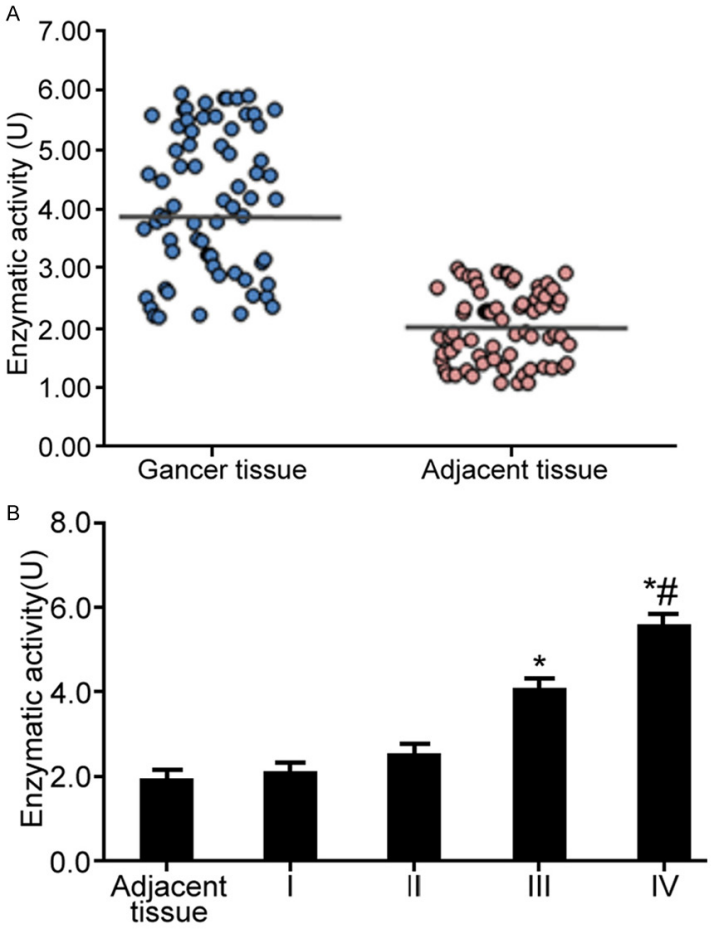

Figure 1. Differential LDH-A activity in NSCLC patient cancer tissues. A. LDH-A activity in cancer tissues and adjacent tissues; B. Differential LDH-A expression in NSCLC patients at different clinical stages. *, P<0.05 compared to tumor adjacent tissues; #, P<0.05 compared to stage III patients.

Statistical analysis

SPSS17.0 was used for data analysis. All results were presented as mean ± standard deviation (SD). Student t-test and analysis of variance (ANOVA) were used for comparison between two groups or among multiple groups, respectively. Q-test was used for between-group comparison. A statistical significance was defined when P<0.05. An extreme significance was defined when P<0.01.

Results

LDH activity in NSCLS tissues

LDH assay kit was used to test LDH-A activity in NSCLC cancer tissues. As shown in **Figure 1**, NSCLC tissues had average activity of LDH-A at 3.95U, whilst the activity in adjacent tissues was 1.92U, with significant difference (P<0.05). By further analyzing cancer tissues from different NSCLC patients, we found gradually increased LDH-A activity in NSCLC patients with advanced clinical grade (P<0.05).

Differential expression of LDH-A proteins

Western blot was used to test LDH-A expression at protein levels. Results were analyzed in gel imaging system. Using β-actin as the reference, relative expression level of LDH-A was calculated. As consistent with enzymatic activity, NSCLC patient cancer tissues showed significantly higher LDH-A expression level than adjacent tissues (**Figure 2**).

RNA interference to inhibit LDH-A expression

Oligonucleotide sequence used for RNA interference was designed based on mRNA sequence of LDH-A, and was used to transfect lung cancer cell line A549. Total RNA was extracted from cells for measuring relative mRNA expression level of LDH-A by qRT-PCR. As shown in **Figure 3**, after transfection, A549 cells showed significantly lowered mRNA expression of LDH-A (P<0.05 compared to empty control group), indicating successful RNA interference of cells.

Effects of LDH-A activity on cell proliferation

To analyze the effect of LDH-A activity on lung cancer cell proliferation potency, we used small molecule inhibitor of LDH-A, oxamate sodium, to suppress LDH-A activity in A549 cells, or used RNAi approach to inhibit LDH-A expression in cells. After 12 hours or 24 hours of incubation, MTT was employed to measure the effect of different treatment on cell proliferation rate. As shown in **Figure 4A**, oxamate sodium effectively inhibited A549 cell proliferation. With gradually increased oxamate sodium concentration, its inhibitory effects on A549

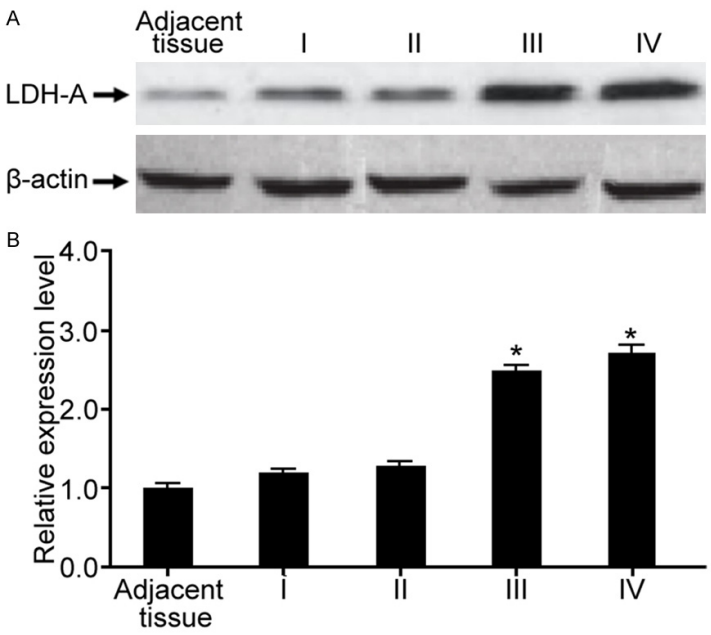

Figure 2. Differential LDH-A expression in NSCLC cancer tissues. A. Western blot for LDH-A expression; B. Differential LDH-A expression in NSCLS patients at different clinical stages. *, P<0.05 compared to cancer adjacent tissues.

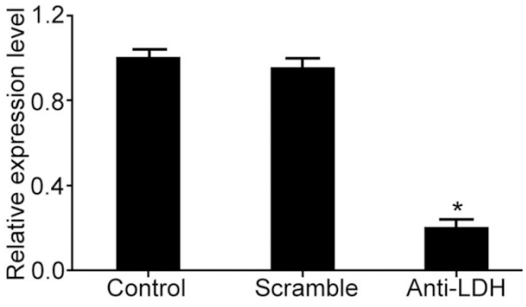

Figure 3. qRT-PCR for LDH-A mRNA expression in A549 cell line. *, P<0.05 compared to control group.

cell proliferation was potentiated. RNAi assay showed that decreased LDH-A expression level in lung cancer cells inhibited cell proliferation (P<0.05).

Invasion potency of A549 cell line

After 24 h of stable transfection, Transwell assay was used to measure the invasion potency of A549 cells with successful transfection. As shown in **Figure 5**, RNAi inhibited LDH-A expression and significantly decreased invasion potency of A549 cells (P<0.05). By measuring OD570, successful inhibition of LDH-A expression by RNAi decreased invasion potency of A549 cells by 65% (P<0.05).

Effects of LDH-A on tumorigenesis of lung cancer cells

Using lung cancer cell xenograft assay, we observed the effect of RNAi targeting LDH-A expression on tumorigenesis potency of A549 cells. As shown in **Figure 6**, we found significantly decreased tumorigenesis potency of A549 cells in experimental group (P<0.05 compared to control group), indicating that LDH-A expression could suppress tumorigenesis potency of lung cancer cells.

Discussion

Malignant tumor cells had different energy metabolism in contrast with normal cells. Abundant researches showed that, even under aerobic condition, malignant tumor cells adopt glycolysis pathway to acquire energy [4]. Further research showed that cancer cells can enhance their metastasis and invasion potency via acidic by-products from metabolism such as lactate, in addition to energy acquirement by glycolysis [13]. Therefore, the investigation of modulation on critical enzymes in glycolysis pathway of cancer cells, and the identification of effective inhibitor to suppress glycolysis of cancer cells, can effectively suppress cancer cell metastasis, cut-off energy source of cancer cells, and inhibit proliferation process [12], thus managing cancer disease progression.

In this study, we first measured LDH activity in cancer tissues from NSCLC patients at different stages, and found significantly higher LDH activity in cancer tissues compared to adjacent tissues, with even higher LDH activity in tumors from patients with advanced clinical stage. Zhang et al found significantly elevated LDH-A expression in gastric cancer, and its close correlation with tumorigenesis of cancer cells [14]. Previous studies showed significantly higher LDH-A activity in multiple human tumor cells. As abovementioned, cancer cell acquires energy via glycolysis pathway, which depends on catalyzing of pyruvic acid into lactate by LDH-A, thus supporting cell proliferation and metabolism without oxygen [14-16].

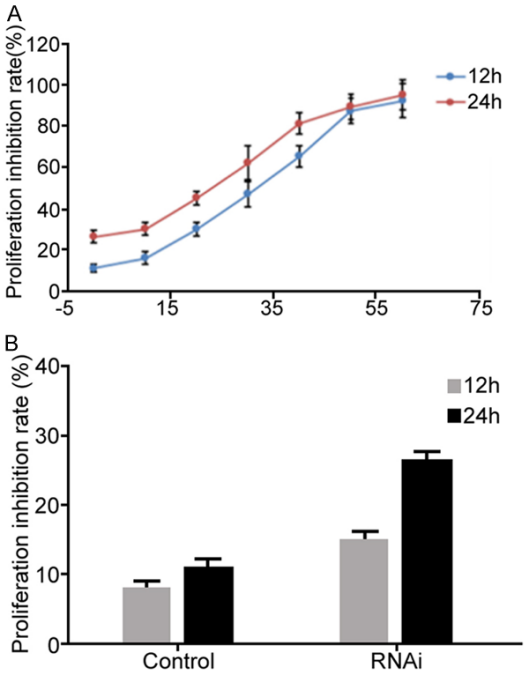

Figure 4. Effects of LDH-A on A549 cell proliferation. A. MTT assay for the effect of oxamate sodium on A549 cell proliferation after 12 h or 24 h incubation; B. MTT assay for the effect of RNAi targeting LDH-A expression on cell proliferation. *, P<0.05 compared to control group at the same time point.

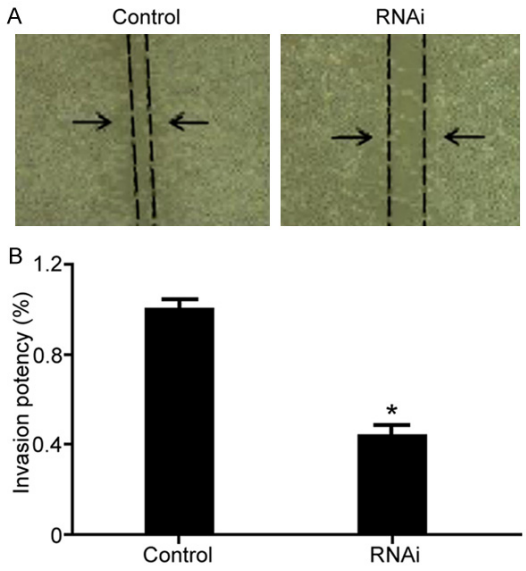

Figure 5. Transwell assay for invasion potency of A549 cells. A. Transwell assay showing A549 cell invasion; B. Differential invasion potency of A549 cells. *, P<0.05 compared to control group.

To further study the effect of LDH-A expression and activity on lung cancer cell proliferation, migration and tumorigenesis, we used lung cancer cell line A549 as the research target, on which LDH-A specific inhibitor oxamate, or RNAi approach was used to inhibit LDH-A activity or to suppress LDH-A expression. Results showed that either approach significantly suppressed cell proliferation potency, and remarkably down-regulated invasion or tumorigenesis potency of A549 cells. Yang et al found that LDH-A inhibitor oxamate sodium effectively suppressed NSCLC cell proliferation, as it can arrest H1395 cells at G2 phase for inducing cell apoptosis [16], consistent with our results. Therefore, it is concluded that up-regulation of LDH-A expression in NSCLC cells could potentiate cell proliferation or invasion/metastasis potency.

Previous study showed that human malignant tumor cells facilitate lactate biosynthesis via glycolysis pathway. Such lactate produced by abnormal metabolism can alter micro-environment in which cells survive, thus providing favorable conditions for tumor cell invasion and migration. It can also induce pathological process of other malignant tumors via inducing TGF-β2 expression, including enhancing MMP-2 expression for further acceleration of cancer cell proliferation or metastasis [17, 18]. The specific inhibitor of LDH-A, or suppression of LDH-A expression by molecular biology approach could probably block or decrease lactate biosynthesis inside cancer cells, thus inhibiting cell proliferation or spreading [19, 20].

Lung cancer is one common malignant tumor in humans, with high malignancy, rapid progression and prominent mortality rate, thus severely affecting public health. Therefore, the diagnosis and treatment of lung cancer has become a central question for both clinicians and patients [21]. This study demonstrated the role of LDH-A in cell proliferation or invasion of NSCLC cancer cells, and showed that inhibition of LDH-A expression effectively suppressed proliferation or spreading of NSCLC cancer cells. However, this study lacked the cytotoxicity study of LDH-A inhibitor on normal cells. In future, this question can be addressed by *in vitro* approach to discuss the effect of LDH-A on normal cells. Meanwhile, animal model can be generated to discuss the toxicity of LDH-A *in vivo*, thus providing more evidence for the promising value of LDH-A inhibitor to manage disease progression of NSCLC patients.

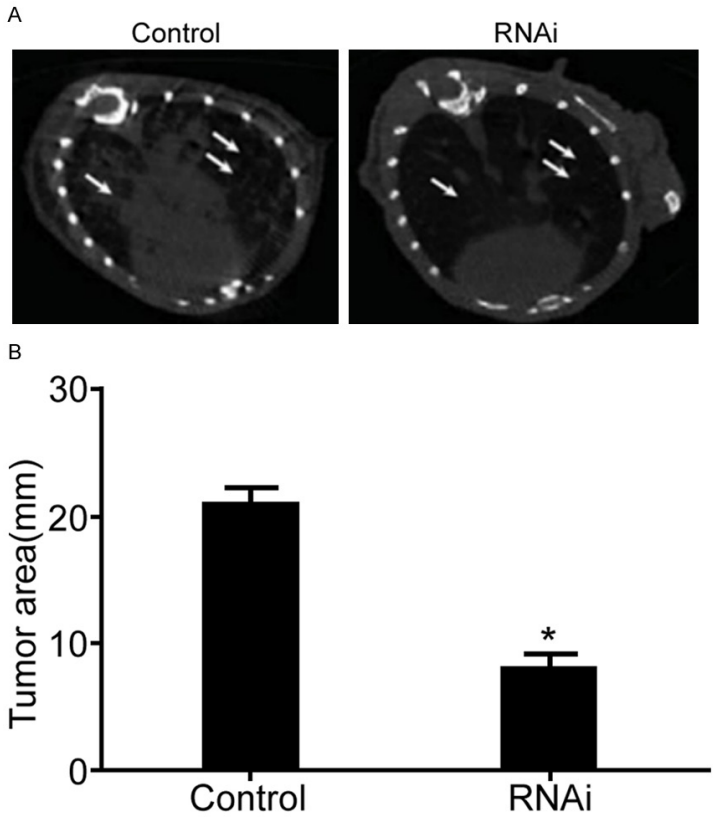

Figure 6. Effects of LDH-A expression on tumorigenesis of A549 cells. A. Ultrasound examination for tumorigenesis potency of A549 cells; B. Differential tumorigenesis of A549 cells. *, P<0.05 compared to control group.

Conclusion

NSCLC patients had significantly higher LDH-A expression level in cancer tissues compared to normal tissues. LDH-A inhibitor or RNAi approach to suppress LDH-A expression could effectively inhibit proliferation and tumorigenesis potency of lung cancer cells.

Disclosure of conflict of interest

None.

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