Short hairpin RNA-directed LDHA silencing using liposomal nanovectors for the effective treatment of human hepatocellular carcinoma

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Abstract

Background

Lactate dehydrogenase A (LDHA), which converts pyruvate to lactate, is over-expressed in many malignant tumors. We will analyze the correlation between LDHA expression and clinicopathological features of HCC. We further applied biodegradable polyethyleneimine containing multiple disulfide bonds (SS-PEI) as an effective non-viral vector for the safe intracellular delivery of plasmid LDHA-shRNA-EGFP in vitro and in vivo for the treatment of liver cancer.

Methods

We first retrospectively analyzed the correlation between the expression of LDHA in liver cancer and the clinical case characteristics of patients and the prognosis of anti-PD1/PD-L1 therapy. Further, I synthesized a nanomaterial SS-PEI/pLDHA-shRNA-EGFP that targets and inhibits the expression of LDHA. Through cytological and animal experiments, it is confirmed that the effectiveness and safety of inhibiting tumor growth.

Results

Our studies showed that patients with hepatocellular carcinoma (HCC) with high LDHA expression have poorer overall survival and disease-free survival. The expression of PD-L1 in HCC was positively correlated with the expression of Ki-67, PD-L1 and the infiltration of Regulatory T cells, CAF cells in the tumor microenvironment (p < 0.05). The objective response rate of anti-PD1/PD-L1 immunotherapy in HCC patients with low LDHA expression was significantly higher than patients with high LDHA expression (p < 0.05). Transfection experiments showed that the SS-PEI/pLDHA-shRNA-EGFP complexes could be used to transfect various types of HCC cell lines in vitro, inducing reduced expression of LDHA and cell growth inhibition. In addition, treatment of mice with SS-PEI/pLDHA-shRNA-EGFP by tail vein injection significantly inhibited the growth of subcutaneous xenograft tumors. Meanwhile, the complexes revealed relatively low cytotoxicity in vitro and no toxicity was observed in vivo.

Conclusions

The expression of LDHA in HCC is closely related to tumor proliferation and immune escape. Patients with low LDHA expression of HCC are more sensitive to anti-PD1/PD-L1 immunotherapy. Targeting LDHA in vivo with a specific short hairpin RNA delivered via SS-PEI is a promising therapy for patients with HCC.

Background
Hepatocellular carcinoma (HCC) is one of the most prevalent and lethal malignant tumors worldwide [1], and it is treated commonly using surgery and chemotherapy [2]. However, its rapid progression, high metastasis rate, and profound chemoresistance make this fatal disease very difficult to treat [3, 4]. As a result, the long-term survival rate of patients with liver cancer is very low [5, 6], highlighting the urgent need for novel effective therapeutic approaches to HCC.

The preference of cancer cells to produce energy by a high rate of glycolysis followed by lactic acid fermentation, even under adequate oxygen, has been termed the Warburg effect or aerobic glycolysis [7–9]. This phenomenon stems from discoveries that the activity or levels of a number of glycolytic enzymes have changed, causing them to act as human tumor oncogenes or suppressors in cancer cells [10–12]. Thus glycolytic enzymes have become a promising target for tumor therapy [13, 14]. Lactate dehydrogenase A (LDHA), which converts pyruvate to lactate, can enhance the proliferation, invasion, and metastasis of malignant tumor cells [15–17]. More importantly, our previous study confirmed that stable knockdown of LDHA in HCC cells inhibited tumor growth in vivo [18], indicating that LDHA is a potential therapeutic target in the majority of cases of HCC.

However, in vivo gene therapy has proven difficult because of lack of non-toxic and effective systemic delivery methods. To the best of our knowledge, no specific LDHA inhibitors have been developed. In our previous study, we developed biodegradable polyethyleneimine-containing multiple disulfide bonds (SS-PEI) as a carrier system that displayed low cytotoxicity, and strong small interfering RNA (siRNA) binding ability and intracellular siRNA release, leading to significant and robust target gene silencing in liver cancer animal models [19]. This strategy not only enhances targeting of siRNAs to tumor tissues, but also protects against degradation by nucleases, thus enhancing the efficacy of the encapsulated siRNAs. However, in that study we did not evaluate whether the SS-PEI could be used to deliver plasmids and to deliver agents via systemic intravenous delivery. In addition, we only detected the transfection efficiency via the decreased amount of mRNA indirectly.

In the present study, we will further analyze the correlation between LDHA expression and clinicopathological features of HCC. And aimed to deliver construct pLDHA-shRNA-EGFP (a plasmid expressing the enhanced green fluorescent protein (EGFP) and a short hairpin RNA (shRNA) targeting LDHA) via mice tail veins using SS-PEI. We also aimed to evaluate the transfection efficiency on pEGFP protein expression directly using fluorescence microscopy and fluorescence activated cell sorting (FACS) and assessing the safety of nanomaterials and effectiveness in inhibiting tumor growth.

Materials And Methods

Patient characteristics

119 cases of HCC, which were diagnosed at the Department of Pathology, Ren Ji Hospital of Shanghai Jiaotong University between 2019 and 2021, were obtained. All patients were followed after surgery with the collection of detailed and complete clinical data. The follow-up time ranged from 1.5 to 108 months,
with a median duration of 72 months. 20 out of the 119 patients with HCC received PD1/PD-L1 immune checkpoint treatment (Atezolizumab) at least 3 cycles. For evaluation of the efficacy of cancer treatment, we referred to the Response Evaluation Criteria in Solid Tumors (RECIST 1.1). The efficacy of anti-PD1/PD-L1 treatment is classified as complete remission (CR), partial remission (PR), stable disease (SD), and progressive disease (PD) according to the RECIST criteria. The effective rate was defined as: (CR cases + PR cases)/total cases × 100%

The study protocol was approved by the Ethics Committee of Shanghai Jiao Tong Medical University affiliated Ren Ji Hospital and all experimental methods were carried out in accordance with approved guidelines of Shanghai Jiao Tong Medical University affiliated Ren Ji Hospital. Written informed consent was obtained from the patients for publication of this study.

**Synthesis and characterizations of bioreducible polyethyleneimine (SS-PEI)**

SS-PEI was synthesized by chemical coupling of 3′-dithiobispropanoic acid (DTPA) with the low molecular weight PEI (LWPEI; 800 Da) at an equal molar ratio via an 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)/ N-hydroxysuccinimide (NHS) activation reaction, as described previously. The molecular weight and polydispersity of SS-PEI were determined using gel permeation chromatography (GPC) relative to polyethylene oxide (PEO) standards, as described previously [19].

**Cloning of LDHA-shRNA-EGFP gene and expression construct**

A short hairpin RNA (shRNA) sequence was cloned for LDHA knockdown (LDHA-shRNA target sequence: AAAGTCTTCTGATGTCATA, scrambled control (NC)-shRNA control sequence: TTCTCCGAACGTGTCACGT). The hairpin sequences were cloned into vector pcDNA3.1/EGFP separately.

**Particle size and zeta-potential measurements**

SS-PEI/shRNA complexes were prepared by adding an SS-PEI solution in HEPES buffer (20 mM, pH 7.4) to the shRNA, followed by vortexing for 5 s and incubating at room temperature for 30 min. The particle size and surface charge of the formed complexes were measured at 25°C using a Nanosizer NS90 (Malvern Instruments Ltd., Malvern, UK).

**Cell culture and transfection**

Human HCC cell lines HepG2, HCCLM3, Hep3B, and SMMC-7721 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), and maintained in Dulbecco’s modified Eagle’s medium (DMEM) (GIBCO) supplemented with 10% fetal bovine serum (FBS) (GIBCO), 1% penicillin–streptomycin (GIBCO) at 37°C and 5% CO₂. In a typical shRNA plasmid transfection experiment with SS-PEI, 15 × 10³, 6 × 10⁴, and 0.3 × 10⁶ cells were plated in 96, 24, and 6-well plates respectively. Cells were grown for 24 h before starting the transfection experiments. pNC-shRNA-EGFP (0.1 µg, 0.5 µg, and 2 µg) or pLDHA-
shRNA-EGFP (0.1 µg, 0.5 µg, 2 µg) and SS-PEI (nanoparticle to plasmid ration (N/P) = 15) in sterilized HEPES buffer (10 µL, 20 mM, pH 7.4) were gently mixed and incubated at an ambient temperature for 30 min. The complexes were incubated for 5 h with transfected cells, which were then grown for 72 h post transfection, after which different assays were performed. Lipofectamine 2000 (Lipo2000) was used as a positive control for all the transfection experiments, which were performed according to manufacturers’ protocols. Phosphate-buffered saline (PBS) or pNC-shRNA-EGFP was used as the negative controls. Each transfection experiment was followed by FACS to show the variations in EGFP expression.

**Fluorescence activated cell sorting analysis activated**

The EGFP expression from pNC-shRNA-EGFP and pLDHA-shRNA-EGFP transfection was quantified using a FACS assay, as described previously[20]. In brief, the percentage of transfected cells was obtained by analyzing approximately 10,000 cells harvested in 1% FBS containing Dulbecco's phosphate-buffered saline (DPBS). Duplicate cultures from two independent cultures were pooled and analyzed immediately using flow cytometry in a Becton and Dickinson flow cytometer equipped with a fixed laser source at 488 nm.

**Quantitative real-time PCR analysis**

Cells were collected at 72 hours after shRNA plasmid transfection. Total RNA was isolated using a TRIZOL kit (Omega), and cDNA was synthesized using a cDNA synthesis kit (Takara). Quantitative real-time PCR (qPCR) was performed using a SYBR Green PCR Master Mix (Takara) on the Roche 480 system (Roche). The primers used in the qPCR reaction were the $LDHA$ forward primer 5′-TATGGAGTGGAATGAATGTTGC-3′ and reverse primer 5′-CCCTTAATCATGGTGGAAACTC-3′, and the ACTB (encoding $\beta$-actin; control) forward primer 5′-AAAGACCTGTACGCCAACAC-3′ and reverse primer 5′-GTCATACTCCTGCTTGCTGAT-3′).

**Western blotting analysis**

Forty-eight hours after transfection, cells were collected and washed with PBS. Total proteins were extracted from the lysate and fractionated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred onto a nitrocellulose membrane for western blotting analysis. $\beta$-actin was used as the reference.

**Cell viability studies using the cell counting kit 8 (CCK8) assay**

Cells (15×10^3 cells) were seeded in a 96-well plates and transfection was performed as described above. At 72 h post-transfection, cell viability was evaluated using the CCK8 assay. Each experiment was carried out in triplicate. The CCK8 assay on the cytotoxicity of the nanovectors against HepG2 cells was performed using the same steps as mentioned above, but only SS-PEI at different concentrations was incubated with HepG2 cells. Lipo2000 alone was used as positive control and PBS alone was used as negative control.
In vivo treatment of HepG2 xenograft models

All animal experimental procedures were performed in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals and were approved by Shanghai Jiao Tong Medical University affiliated Ren Ji Hospital. Four to six-week-old male nude mice were purchased from SLAC Laboratory Animals (Shanghai, China). Then, \(5 \times 10^6\) HepG2 cells were injected subcutaneously into the flank of the mice to initiate tumor growth. Tumor size was measured every three days using a caliper in two dimensions and calculated using the formula: (length) \(\times\) (width)\(^2\)/2. When the tumor volume reached approximately 150 mm\(^3\), the animals were divided into two groups: SS-PEI with pNC-shRNA-EGFP as a control, or SS-PEI with pLDHA-shRNA-EGFP, using eight mice per in group. SS-PEI/ pNC-shRNA-EGFP and SS-PEI/ pLDHA-ShRNA-EGFP were injected into the tail vein of mice every other day five times. The amount of pNC-shRNA-EGFP or pLDHA-ShRNA-EGFP complexes was 4 µg at an optimal N/P ratio of 15/1. Three days after the final injection, three mice from each group were euthanized. Some of the tumors were digested using trypsin to obtain parenchyma cells and some were stored at −80°C for subsequent study. Two weeks after the final injection, the remaining five mice from each group were euthanized and their tumors were excised and weighed. Part of the tumor was digested using the trypsin method for western blotting and another portion of the tumor was fixed and paraffin-embedded for immunostaining and apoptosis analysis according to the procedures mentioned below. Organs, including the liver, heart, spleen, lung, and kidney, were obtained from each mouse and weighted.

Apoptosis analysis using the terminal deoxynulceotidyl transferase nick-end-labeling (TUNEL) assay

For the TUNEL assay, an in situ apoptosis detection kit (Roche Applied Science, Penzberg, Germany) was used to detect apoptotic cells in the tumor tissue. Briefly, the tumor sections (5-µm) were treated with 20 µg/mL of proteinase K in distilled water for 10 minutes at room temperature. To block endogenous peroxidase, the slides were incubated in methanol containing 3% hydrogen peroxide for 20 minutes and sections were then incubated with equilibration buffer and terminal deoxynucleotidyl transferase. Finally, the sections were incubated with an anti-digoxigenin-peroxidase conjugate. The peroxidase activity in each tissue section was shown by the application of diaminobenzidine. Sections were counterstained with hematoxylin.

Immunohistochemistry (IHC) staining and assessment

After Paraffin embedded samples and microtome sectioning (5-µm slices), the slides were processed for staining. Primary antibodies recognizing LDHA, Ki-67, PD-L1, CD8, FOXP3, CD206, and FAP were purchased from Abcam. Sections were assessed by two independent observers. The slides were scored for the intensity of staining (0 to 3) and the percentage of stained cells, with scores of 0 (0%), 1 (1–9%), 2 (10–49%), and 3 (50–100%). Immunohistochemistry (IHC) score (0 to 9) was defined as the product of the staining intensity and percentage of stained cells. Protein expression was judged as positive when the IHC score was greater than or equal to four.
CD8, FOXP3, CD206, and FAP are specific markers of the cytotoxic T cell, regulatory T cells, M2 macrophage, and FAP+ CAF, respectively [21, 22]. After IHC staining, the numbers of immune cells and CAF cells infiltrated in tumor tissue were analyzed and counted by Visiopharm software (VISIPPHARM, Denmark). All IHC results were evaluated by two experienced observers who were blinded to the condition of the patients. Where discrepancies occurred between the two readers, the two readers reached a consensus.

Liver and kidney function test

Four-week-old BALB/c male mice were ordered from SLAC and divided into two groups of five mice. SS-PEI/pLDHA-shRNA-EGFP (N/P ratio = 15/1) or PBS as control were injected via the tail vein of the nude mice. Two weeks after injection, the mice were sacrificed, and their blood was collected for liver and kidney function detection. The detected biological markers included alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), blood urea nitrogen (BUN), creatinine clearance (CCR), and uric acid (UA).

Statistical analysis

Data were presented as mean ± standard deviation. Statistical differences between the groups were compared using one-way analysis of variance and t tests. A P value < 0.05 was considered statistically significant. To explore the association between recurrence-free survival and LDHA expression, a Kaplan-Meier survival analysis was performed. Two-sided p values of less than 0.05 were considered to be statistically significant. All statistical analyses were performed using SPSS version 16.0 (IBM Corp., Armonk, NY, USA).

Results

Human HCC expresses high levels of LDHA, which correlates with poor overall survival

Cancers with a high rate of glycolysis have been noted to have a worse clinical outcome. To determine whether the expression of LDHA in HCC correlated with clinical prognosis in patients with HCC, LDHA protein levels were analyzed using immunohistochemistry in a cohort of 119 HCC cases. LDHA was expressed at moderate or high levels (an IHC score of 2 or 3) in 60.5% of HCC (n = 72 tumors), with weak or no expression (having an IHC score of 0 or 1) being detected in normal liver samples. Consistent with our clinical data in HCC, LDHA protein expression was significantly upregulated in HCC cell lines compared with that in normal liver cells (Fig. 1A). We segregated patients with HCC into two categories: Those with LDHA expression above the mean were considered high expression and those below the mean were considered low expression (Fig. 1B). Table 1 shows the patients’ clinicopathological characteristics and the correlations between LDHA expression and clinical factors. Among the two LDHA expression scores, we found no significant differences in the distribution according to age, gender, hepatic cirrhosis, tumor number, or lymph node metastasis. However, we did observe significant
correlations between LDHA and tumor size, histological grade, vascular invasion, and T stage. We also found that high expression of LDHA was associated with poor overall survival (P = 0.023) and disease-free survival (P = 0.014; Fig. 1C, D).
Table 1
Immunohistochemical expression of LDHA and its association with clinic pathological variables

<table>
<thead>
<tr>
<th>Character</th>
<th>n</th>
<th>LDHA (n = 119)</th>
<th>$\chi^2$</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>negative</td>
<td>positive</td>
<td></td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 60</td>
<td>90</td>
<td>38</td>
<td>52</td>
<td>1.149</td>
</tr>
<tr>
<td>&gt; 60</td>
<td>29</td>
<td>29</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>97</td>
<td>38</td>
<td>59</td>
<td>0.023</td>
</tr>
<tr>
<td>Female</td>
<td>22</td>
<td>9</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td><strong>Tumor size(cm)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 5</td>
<td>48</td>
<td>9</td>
<td>39</td>
<td>14.49</td>
</tr>
<tr>
<td>&gt; 5</td>
<td>71</td>
<td>38</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td><strong>Tumor differentiation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>well</td>
<td>5</td>
<td>4</td>
<td>1</td>
<td>7.759</td>
</tr>
<tr>
<td>moderate</td>
<td>103</td>
<td>42</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>poor</td>
<td>11</td>
<td>1</td>
<td>10</td>
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<tr>
<td><strong>Vascular invasion</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>yes</td>
<td>79</td>
<td>24</td>
<td>55</td>
<td>8.173</td>
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<tr>
<td>no</td>
<td>40</td>
<td>23</td>
<td>17</td>
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<tr>
<td><strong>Hepatic cirrhosis</strong></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>yes</td>
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<td>41</td>
<td>66</td>
<td>0.616</td>
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<tr>
<td>no</td>
<td>12</td>
<td>6</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td><strong>Number of tumor</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥ 2</td>
<td>23</td>
<td>6</td>
<td>17</td>
<td>2.145</td>
</tr>
<tr>
<td>1</td>
<td>96</td>
<td>41</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td><strong>T stage</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>40</td>
<td>25</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>T2</td>
<td>58</td>
<td>20</td>
<td>38</td>
<td>17.362</td>
</tr>
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</table>
LDHA can catalyze pyruvic acid to lactic acid. High expression of LDHA can lead to increased lactate in the tumor microenvironment and changes the immune status of the tumor microenvironment[23]. Therefore, we were very interested in the correlation between LDHA expression and tumor proliferation, immune status for ICH (Fig. 1E). Our study showed that the expression of LDHA correlated with Ki-67 and PD-L1 (p < 0.05). The expression of LDHA was not correlated with the infiltration of cytotoxic T cells and M2 macrophages in the tumor microenvironment (p > 0.05). Interestingly, LDHA was positively correlated with FAP^+ CAF and regulatory T cell infiltration in the tumor microenvironment (p < 0.05) (Table 2).

<table>
<thead>
<tr>
<th>Character</th>
<th>n</th>
<th>LDHA (n = 119)</th>
<th>χ²</th>
<th>p value</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>negative</td>
<td>positive</td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>21</td>
<td>2</td>
<td>19</td>
<td></td>
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<tr>
<td>Pathological N stage</td>
<td></td>
<td>1.378</td>
<td>0.24</td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>113</td>
<td>46</td>
<td>67</td>
<td></td>
</tr>
<tr>
<td>N1</td>
<td>6</td>
<td>1</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

Table 2
Pearson correlation coefficients between LDHA expression with expression of PD-L1 and tumor environment-related cells counts

<table>
<thead>
<tr>
<th>Factor</th>
<th>LDHA expression</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>correlation coefficients</td>
</tr>
<tr>
<td>Ki-67</td>
<td>0.311</td>
</tr>
<tr>
<td>PD-L1</td>
<td>0.347</td>
</tr>
<tr>
<td>Cytotoxic T cell</td>
<td>-0.117</td>
</tr>
<tr>
<td>Regulatory T cells</td>
<td>0.245</td>
</tr>
<tr>
<td>M2 macrophage</td>
<td>0.216</td>
</tr>
<tr>
<td>FAP^+ CAF</td>
<td>0.270</td>
</tr>
</tbody>
</table>

Because LDHA expression of HCC is related to the immune status of the tumor microenvironment, we further analyzed the correlation between the expression of LDHA and the anti-PD1/PD-L1 immune therapy. We found that anti-PD1/PD-L1 treatment was effective in 33.3% of patients in the group with low expression of LDHA, whereas it was only 9.09% effective in the group with high expression of LDHA (Table 3).
## Table 3
Comparison of clinical efficacy between two groups of patients

<table>
<thead>
<tr>
<th>group</th>
<th>CR + PR</th>
<th>SD + PD</th>
<th>Efficacy</th>
<th>p</th>
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</thead>
<tbody>
<tr>
<td>LDHA low expression</td>
<td>3</td>
<td>6</td>
<td>33.33%</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>LDHA high expression</td>
<td>1</td>
<td>10</td>
<td>9.09%</td>
<td></td>
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</table>

### Components and formulation of SS-PEI and Characterization of SS-PEI/shRNA complexes

The SS-PEI polymer was readily prepared by chemical coupling of the 3-dithiobispropanoic acid (DTPA) and LWPEI via an EDC/NHS activation reaction, as described previously (Fig. 2A). Gel retardation and dynamic light scattering (DLS) experiments were carried out to detect the formation of SS-PEI/shRNA complexes. Figure 2B shows the capability of SS-PEI to bind shRNA as a function of the N/P ratio. As the N/P ratio increased from 1/8 to 12/1, the mobility of shRNA was completely retarded at and above an N/P ratio of 8/1, indicating complexation between SS-PEI and the shRNA. To further confirm complexation, DLS was used to measure the particle sizes and surface charges of the SS-PEI/shRNA complexes. SS-PEI could efficiently condense shRNA into nanoscale particles with an average diameter of approximately 80 to 170 nm (Fig. 2C). Moreover, as the N/P ratio increased from 3/1 to 24/1 and the surface charge of the complexes increased from approximately −17 to +23 mV (Fig. 2D).

### Transfection efficiency and cytotoxicity evaluation of SS-PEI on cancer cells

To ascertain whether SS-PEI could mediate plasmid LDHA-shRNA-EGFP (pLDHA-shRNA-EGFP) delivery to silence LDHA expression, the transfection of SS-PEI was performed in vitro using HepG2 cells and pLDHA-shRNA-EGFP. The transfection efficiency was optimized as a function of N/P ratios, ranging from 0 to 30/1 (Fig. 3A). The transfection efficiency was evaluated by FACS analysis of EGFP expression. At an optimal N/P ratio of 15/1, the complexes of SS-PEI/plasmid LDHA-shRNA-EGFP could efficiently transfect approximately 90% of HepG2 cells (Fig. 3A).

Experiments were performed to determine the transfection efficiency of SS-PEI at the N/P ratio of 15/1 in four HCC cell lines (i.e. HepG2, HCCLM3, Hep3B, and SMMC-7721). SS-PEI transfected approximately 90% of HepG2, approximately 50% of HCCLM3, approximately 20% of Hep3B, and approximately 40% of SMMC-7721 cells, as evaluated by observing the green fluorescence using a fluorescence microscope (Fig. 3B) and FACS analysis of the EGFP expression (Fig. 3C). There was no significant difference in transfection efficiency between SS-PEI and Lipo2000 in HCCLM3, Hep3B, and SMMC-7721 cells, while SS-PEI exhibited a higher transfection efficiency in HepG2 cells (approximately 90%) compared to Lipo2000 (approximately 75%).

To examine whether SS-PEI has an effect on cell viability, HepG2 cells were exposed to SS-PEI at varying polymer concentrations in the range of 1–60 µg/mL, Lipo2000 as positive control, and PBS as negative
control, and then subjected to a CCK8 assay. SS-PEI showed lower cytotoxicity than Lipo2000 at a concentration of 60 µg/mL (Fig. 3D).

SS-PEI mediated LDHA-shRNA-EGFP plasmid delivery into hepG2 cells to inhibit cell growth in vitro

The functional activity of the *LDHA*-shRNA was evaluated by calculating cell viability after transfecting pLDHA-shRNA-EGFP with SS-PEI or Lipo2000 as a control. Cell viability was measured using the CCK8 assay. At the optimized N/P ratio of 15/1, the SS-PEI/pLDHA-shRNA-EGFP complexes could kill approximately 50% HepG2 cells whereas Lipo2000/pLDHA-shRNA-EGFP could kill only approximately 40% of the cells (Fig. 4A). By contrast, SS-PEI with pNC-shRNA-EGFP showed no significant cytotoxicity compared with approximately 10% dead cells in case of Lipo2000 with pNC-shRNA-EGFP. Thus, the LDHA-shRNA-EGFP plasmid was functional in this experiment and probably killed the cells via inhibiting LDHA protein expression.

RT-PCR analysis showed that the mRNA expression of *LDHA* in the PBS group, Lipo2000/pNC-shRNA-EGFP group, and SS-PEI/pNC-shRNA-EGFP group was not significantly different (Fig. 4B). The Lipo2000/pLDHA-shRNA-EGFP significantly decreased the mRNA expression of *LDHA* (by approximately 80%), while the SS-PEI/pLDHA-shRNA-EGFP lowered the expression of mRNA of LDHA even further (by approximately 90%), and was significantly different to that in the Lipo2000/pLDHA-shRNA-EGFP group.

LDHA shRNA-mediated tumor regression in nude mice

We identified the transduction of tumor cells *in vivo* by determining the protein level of LDHA using western blotting. Successful *LDHA* knockdown in the tumors was confirmed by western blotting at day 3 and day 14 after the final injection (Fig. 5A).

All reports to date indicate that humans lacking LDHA develop normally but display exertional myopathy [24]; therefore, we exercised the mice twice a week to examine their exertional tolerance and there was no significant difference between the SS-PEI/pLDHA-shRNA-EGFP group and SS-PEI/pNC-shRNA-EGFP control group. We also note no inability to eat and drink, or lethargy. In fact, mice treated with pLDHA-ShRNA-EGFP did not lose weight compared with those treated with pNC-ShRNA-EGFP (Fig. 5B). There was also no significant difference in liver, heart, spleen, lung, and kidney weights between these two groups (Fig. 5C).

Our previous study showed that the SS-PEI/pLDHA-shRNA-EGFP was mainly accumulated in the liver and kidney; therefore, we next evaluated the effect of the SS-PEI/pLDHA-shRNA-EGFP on liver and kidney functions. The mice were treated with SS-PEI/pLDHA-shRNA-EGFP or PBS as a control and blood was collected to detect liver and kidney function. In contrast to the PBS control, the levels of liver and kidney biomarkers did not increase significantly in mice treated with the complexes (Table 4). These results suggested that the SS-PEI/pLDHA-shRNA-EGFP has no adverse effect on liver and kidney functions *in vivo.*
To determine the effects of nanoplex treatment on tumor growth *in vivo*, we used SS-PEI to deliver pLDHA-shRNA-EGFP in a HepG2 model. Once the tumor volume reached to 150mm$^3$, SS-PEI/pLDHA-shRNA-EGFP, or SS-PEI/pNC-shRNA-EGFP as a control, were injected into the tail vein of mice every other day five times. Mice were weighed and tumor volumes was measured every three days. Two weeks after the final injection, mice were euthanized and their tumors were excised, weighed, measured, and snap frozen for molecular studies or submitted for histopathology. Nanoplex treatment in formed HepG2 tumors led to profound tumor volume inhibition at day 6, 9, 12,15, and 18 compared with that in the control group (P < 0.001)(Fig. 6A,B). Significant tumor weight decreases were also observed in the mice treated with the nanoplexes (1.466 ± 0.095 g) compared with that in the control group (0.806 ± 0.122 g) (P < 0.001)(Fig. 6C).

We next assessed the changes in LDHA protein levels in tumors using immunohistochemistry. Loss of LDHA protein was noted in HepG2 tumor cells treated with SS-PEI/pLDHA-shRNA-EGFP compared with that in cells transfected with SS-PEI/pNC-shRNA-EGFP as a control. An example of this potent knockdown is shown in Fig. 6D. Then, a TUNEL assay was performed to investigate whether the treatments induced apoptosis in HepG2 tumor cells. TUNEL staining showed that the number of apoptotic cells increased dramatically in tumors treated with SS-PEI/pLDHA-shRNA-EGFP compared with those in the tumors treated with SS-PEI/pNC-shRNA-EGFP (Fig. 6D). Thus, the decreased tumor growth could be attributed to increased cell apoptosis. We further sought to determine the relationship between the expression of LDHA and final tumor weight. There was a highly significant correlation between the mRNA level of LDHA and final tumor weight (Fig. 6E).

**Discussion**

LDHA has attracted significant research attention in tumor biology because it is broadly upregulated in many tumors[25, 26]. Consistent with other malignant tumors, we demonstrated aberrant expression of LDHA in a large percentage of HCC tissues, with relatively low expression in normal liver tissues. Furthermore, LDHA expression correlated positively with tumor size, histological grade, vascular invasion, and T stage. Patients with high LDHA expression have poorer overall and disease-free survival. This suggested that LDHA might provide a survival and proliferative advantage *in vivo* and is a potential therapeutic target.
Our data also showed that LDHA plays an important role in hepatic tumor growth and inducing immune escape. By IHC analysis, we found that the LDHA expression in HCC was positively correlated with Ki-67 ($r = 0.311, p = 0.05$). Ki-67 is an important indicator of tumor proliferation [27]. This suggests that the high expression of LDHA is closely related to the rapid proliferation of HCC. LDHA expression of tumors can produce more lactate. Many recent studies have confirmed that lactate is an important factor in inducing tumor immune escape[28]. Therefore, we compared the correlation between LDHA expression and tumor immune escape-related indicators. First, it was interesting that LDHA was positively correlated with PD-L1 expression ($r = 0.347, p = 0.011$). PD-L1 is an important immune checkpoint for inducing tumor immune escape [29]. The high expression of LDHA producing more lactic acid is likely to induce an increase in PD-L1 expression, resulting in immune escape. We further compared the correlation between tumor LDHA expression and tumor microenvironment immune cells and CAF cell infiltration. The results showed that the expression level of LDHA was positively correlated with the number of regulatory T cells and CAF cells infiltrated in the tumor microenvironment. Regulatory T cells are important immune cells that induce tumor immune escape[30], and CAF cells are not only the most major stromal cells in the tumor microenvironment but also can induce tumor immune escape[31]. This indicates that the expression of LDHA in HCC may also change the immune status of tumors by regulating the infiltration of Regulatory T cells and CAF cells.

In view of the above findings in the IHC analysis, LDHA may play an important role in tumor immune escape, so we retrospectively analyzed the effect of LDHA expression level in HCC on the sensitivity of anti-PD1/PD-L1 immunotherapy. Our clinical research results show that the objective response rate of anti-PD1/PD-L1 immunotherapy in patients with liver cancer with low LDHA expression is significantly higher than that in patients with liver cancer with high LDHA expression. These findings of my study suggest that LDHA plays an important role in inducing tumor proliferation and immune evasion and immunotherapy resistance in HCC. Targeted inhibition of LDHA may become an important means to inhibit liver cancer and restore the sensitivity of immunotherapy. Hence, how to inhibit LDHA expression of HCC has become an urgent technical problem to be solved.

There are several inhibitors of LDHA, however, most of the inhibitors are either inactive in cell-based assays or are not specific[32]. Thus, the LDHA-shRNA-EGFP plasmid was designed to target LDHA mRNA. The development of low-toxic and potent siRNA delivery systems is a crucial step for the success of siRNA-based cancer therapy. In this study, the SS-PEI polymer[19] to serve as a pLDHA-shRNA carrier is that this polymer is able to condense pLDHA-shRNA into nanoscaled and positively-charged complexes. The SS-PEI complexes could be cleaved inside the cells to facilitate intracellular pLDHA-shRNA release, which mediate efficient intracellular pLDHA-shRNA delivery, leading to significant silencing of LDHA mRNA expression in vitro.

Although the results of the present and previous studies showed that many human cancers have higher LDHA levels compared with that in normal tissues, LDHA is also expressed in liver, spleen, heart, lung, kidney, and red blood cells of normal tissues[33, 34]. This raises the concern that inhibition of LDHA might have deleterious consequences. Previous reports noted that humans lacking LDHA might develop
normally but display exertional myopathy [24]. This suggests that inhibiting LDHA might have minimal off-target affects in normal tissue. In fact, no apparent toxicity was detected after LDHA shRNA treatment of mice in this study. Thus, delivery of LDHA shRNA via SS-PEI is a potentially efficacious treatment with low toxicity for patients with HCC. In our study, the SS-PEI/pLDHA-shRNA-EGFP complexes significantly inhibited the expression of LDHA and the proliferation of HepG2 cells both in vitro and in vivo. Meanwhile, the SS-PEI/pLDHA-shRNA-EGFP complexes exhibited lower cytotoxicity in vitro and had no adverse effects on mice. These results indicate that SS-PEI/pLDHA-shRNA-EGFP complexes not only has low cytotoxicity, but also can effectively inhibit LDHA expression and tumor growth by intravenous injection.

However, several problems remain to be resolved. First, further research is needed to decrease the clearance of SS-PEI particles by the reticuloendothelial system and prolong their circulation time to potentially increase delivery to tumors. Second, since SS-PEI is mainly accumulated in the liver, SS-PEI should be formulated for long-term circulation in vivo to minimize its undesirable hepatic accumulation. In future experiments, we will verify the effect of using small interference targeting to increase the sensitivity to anti-PD1/PD-L1 immunotherapy in animal experiments.

**Conclusion**

In summary, the expression of LDHA in HCC is closely related to tumor proliferation and immune escape. Patients with low LDHA expression in HCC are more sensitive to anti-PD1/PD-L1 immunotherapy. shRNA targeting LDHA delivered via SS-PEI represents a novel and potent therapy that could inhibit tumor growth with low toxicity. Ultimately, our goal is to develop the most effective method for delivering targeted shRNAs to tumors in vivo to improve the outcomes for patients with HCC.

**Declarations**

**Ethics approval and consent to participate:** All studies related to IHC staining of hepatocellular carcinoma samples and the evaluation of treatment efficacy were approved by the Ethics Committee of Shanghai Jiao Tong Medical University affiliated Ren Ji Hospital and all experimental methods were carried out in accordance with approved guidelines of Shanghai Jiao Tong Medical University affiliated Ren Ji Hospital.

**Consent for publication:** Not applicable

**Availability of data and materials:** The datasets used and/or analyzed during the current study are available from the corresponding authors Jianjun Liu on reasonable requests.

**Competing interests:** The authors declare that they have no competing interests

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Authors’ contributions: Xiang Zhou, Chao Lin and Jianjun Liu designed the experiments. Xiang Zhou, Ruohua Chen and Chao Lin performed the experiments. Xiang Zhou, Ruohua Chen and Jianjun Liu analyzed the data. Xiang Zhou and Ruohua Chen wrote this manuscript. Gang Huang, Jianjun Liu reviewed and edited the manuscript.

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References


**Figures**
Figure 1

Patients with HCC expressing high LDHA protein levels have a poor clinical outcome. (A) Protein levels of LDHA in one normal liver cell line (L02) and four cancer cell lines (HepG2, Hep3B, SMMC-7721, and HCCLM3) were examined using western blotting. LDHA protein expression was significantly upregulated in HCC cell lines compared with that in normal liver cells. (B) Representative photomicrographs showing LDHA levels in normal liver tissues and HCC (200×). (C) Kaplan–Meier survival data for patients with...
HCC, separated by their LDHA IHC scores. Patients with high LDHA expression (n = 72; IHC score 2 or 3) had a worse overall survival compared with patients with low LDHA expression (n = 49; IHC score 0 or 1). (D) Kaplan–Meier survival data for patients with HCC, separated by their LDHA IHC score. Patients with high LDHA expression (n = 72; IHC score 2 or 3) had worse disease-free survival compared with patients with low LDHA expression (n = 49; IHC score 0 or 1). (E) Representative ICH staining of Ki-67, PD-L1 expression and tumor environment-related cells (200×).

Figure 2

Components and formulation of SS-PEI and characterization of SS-PEI/shRNA complexes. (A) Structure of bioreducible polyethylenimine (SS-PEI).

(B) Gel retardation assay of SS-PEI/shRNA complexes formed at N/P ratios of 1/8, 1/4, 1/2, 4, 8, and 12. Control: DNA (naked shRNA). As the N/P ratio increased from 1/8 to 12/1, the mobility of shRNA was
completely retarded at and above an N/P ratio of 8/1, indicating complexation between SS-PEI and the shRNA. (C) Particle size of SS-PEI/shRNA complexes formed at N/P ratios of 3, 6, 12, and 24 detected by dynamic light scattering (DLS). SS-PEI could efficiently condense shRNA into nanoscale particles with an average diameter of approximately 80 to 170 nm. (D) Zeta potential of SS-PEI/shRNA complexes formed at N/P ratios of 3, 6, 12, and 24 detected by dynamic light scattering (DLS).
Transfection efficiency and cytotoxicity evaluation of SS-PEI on cancer cells. (A) Transfection efficiency of the complexes of SS-PEI/plasmid LDHA-shRNA-EGFP at different N/P ratios in HepG2 cells. (B) Fluorescence microscopy images of HepG2, Hep3B, SMMC-7721, and HCCLM3 cells. All the left side panels show bright field images of the corresponding fluorescence images (right side) of cells treated with the complexes of SS-PEI/plasmid LDHA-shRNA-EGFP. The complexes of SS-PEI/plasmid could efficiently transfect approximately 90% of HepG2 cells. (C) FACS analysis of EGFP expression in HepG2, Hep3B, SMMC-7721, and HCCLM3 cells transfected by complexes of SS-PEI/plasmid LDHA-shRNA-EGFP or Lipo2000/plasmid LDHA-shRNA-EGFP. (D) Cell viability of SS-PEI at varying polymer concentrations and Lipo2000 towards HepG2 cells. The percentage of cell viability was determined relative to control cells (treated with PBS). SS-PEI showed lower cytotoxicity than Lipo2000.

Figure 4

Effect of SS-PEI-mediated LDHA-shRNA-EGFP plasmid delivery on cell growth in HepG2 cells. (A) Effect of SS-PEI/shRNA-mediated LDHAgene silencing on the growth of HepG2 cells. The number of active cells was assessed using a CCK8 assay at 72 h after HepG2 cells were transfected with SS-PEI/plasmid LDHA-shRNA-EGFP, SS-PEI/plasmid NC-shRNA-EGFP, Lipo2000/plasmid LDHA-shRNA-EGFP, or Lipo2000/plasmid NC-shRNA-EGFP. (B) Effect of SS-PEI/shRNA-mediated LDHAgene silencing on the mRNA expression of LDHA. The mRNA expression of LDHAwas assessed by RT-PCR at 72 h after HepG2 cells were transfected with SS-PEI/plasmid LDHA-shRNA-EGFP, SS-PEI/plasmid NC-shRNA-EGFP, Lipo2000/plasmid LDHA-shRNA-EGFP, or Lipo2000/plasmid NC-shRNA-EGFP. The Lipo2000/pLDHA-shRNA-EGFP significantly decreased the mRNA expression of LDHA (by approximately 80%), while the SS-PEI/pLDHA-shRNA-EGFP lowered the expression of mRNA of LDHA even further (by approximately 90%).
Figure 5

The toxicity of SS-PEI-mediated LDHA-shRNA-EGFP plasmid delivery in vivo. (A) Successful LDHA knockdown in the tumors was confirmed by western blotting at day 3 and day 14 after the final injection. (B) mice treated with pLDHA-ShRNA-EGFP did not lose weight compared with those treated with pNC-ShRNA-EGFP. (C) There was also no significant difference in liver, heart, spleen, lung, and kidney weights between these two groups.
Figure 6

growth could be attributed to increased cell apoptosis. (E) There was a highly significant correlation between the mRNA level of \textit{LDHA} in tumors and the final tumor weight. Results were normalized to the \textit{LDHA} expression of a control tumor.

**Supplementary Files**

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