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Longitudinal assessment of ultrasound-guided complementary microRNA therapy of hepatocellular carcinoma

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ABSTRACT

Hepatocellular carcinoma (HCC) is the second-leading cause of cancer related deaths worldwide and new strategies to efficiently treat HCC are critically needed. The aim of this study was to assess the longitudinal treatment effects of two complementary miRNAs (miRNA-122 and antimiR-21) encapsulated in biodegradable poly lactic-*co*-glycolic acid (PLGA) - poly ethylene glycol (PEG) nanoparticles (PLGA-PEG-NPs), administered by an ultrasound-guided and microbubble-mediated delivery approach in doxorubicin-resistant and non-resistant human HCC xenografts. Using *in vitro* assays, we show that repeated miRNA treatments resulted in gradual reduction of HCC cell proliferation and reversal of doxorubicin resistance. Optimized US parameters resulted in a 9–16 fold increase ($p = 0.03$) in miRNA delivery *in vivo* in HCC tumors after two US treatments compared to tumors without US treatment. Furthermore, when combined with doxorubicin (10 mg/kg), longitudinal miRNA delivery showed a significant inhibition of tumor growth in both resistant and non-resistant tumors compared to non-treated, and doxorubicin treated controls. We also found that ultrasound-guided miRNA therapy was not only effective in inhibiting HCC tumor growth but also allowed lowering the dose of doxorubicin needed to induce apoptosis. In conclusion, the results of this study suggest that ultrasound-guided and MB-mediated delivery of miRNA-122 and antimiR-21, when combined with doxorubicin, is a highly effective approach to treat resistant HCC while reducing doxorubicin doses needed for treating non-resistant HCC in longitudinal treatment experiments. Further refinement of this strategy could potentially lead to better treatment outcomes for patients with HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is the most common primary liver cancer and the second-leading cause of cancer-related deaths worldwide [1–5]. Currently treatment options are limited. Liver transplantation and surgical resection are currently the best available curative treatment options for early stages of the disease. However, shortage of transplant-organs, high risk of relapse [6–9], and the fact that the disease is often detected at advanced stages have restricted these options to only a fraction of the patient population [9–12]. In patients with intermediate stage HCC, trans-arterial chemoembolization (TACE) that allows delivering chemotherapeutic drugs locally to the tumor site is the most commonly offered therapy [13,14]. However, TACE is often contraindicated in patients with cirrhotic liver as the ischemic damage involved in embolization as well as drug related non-specific toxicities may lead to further compromised liver function in an already diseased liver [15,16]. Furthermore, HCC is prone to developing drug resistance (for example resistance to doxorubicin), hindering the efficacy of this procedure. At advanced stages of HCC, which involve 40% of the patients [17–19], very limited therapeutic options are available such as sorafenib, which offers limited survival benefit of only 2–3 months compared to untreated HCC patients [20,21]. Hence, new therapeutic strategies targeting the molecular basis of HCC are critically needed to treat patients with liver cancer of all stages.

Recently, it has been shown that targeted delivery of miRNAs can be an efficient strategy for blocking key cellular processes involved in HCC development and progression [22]. Specifically, targeting two complementary miRNAs, miRNA-122 and miRNA-21, that play critical roles in regulating the progression and drug resistance associated with HCC [23–30], were shown to act synergistically to result in significant

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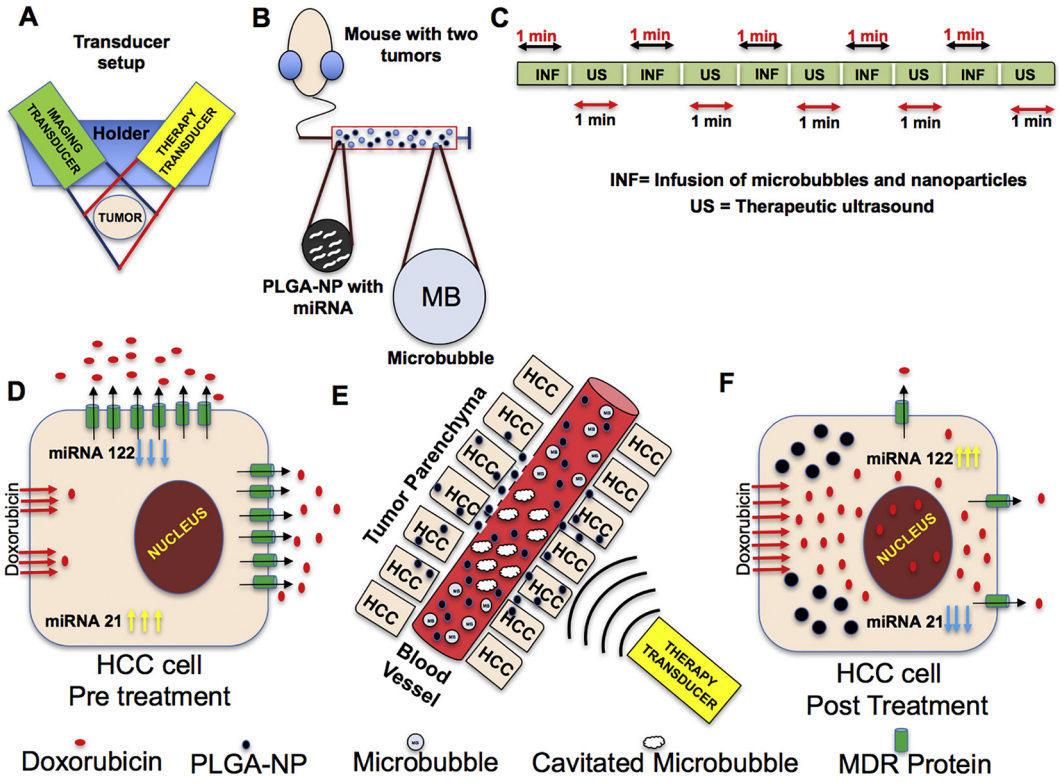

Fig. 1. Schematic drawing of experimental setup and delivery methods.
(A) Pictorial illustration of the alignment of two ultrasound transducers (imaging and treatment) for both localizing and treating tumors. (B) MiRNA-loaded PLGA-PEG-NPs were co-delivered intravenously with clinical grade microbubbles (MB) in mice bearing two HCC tumors, one of which served as intra-animal non-ultrasound treated control. (C) Summary of ultrasound treatment protocol of one 10-minute treatment cycle. (D) Schematic representation of doxorubicin-resistant HCC cell (pre-treatment) showing low levels of miRNA-122 and high levels of miRNA-21 as well as high expression levels of multi drug resistance (MDR) proteins which efflux doxorubicin from the cell. (E) Summary of principles of microbubble-enhanced sonoporation allowing PLGA-PEG-NP to penetrate across vascular endothelial barrier into the extravascular compartment and enter into the cytosol of HCC cells. (F) Schematic of doxorubicin-resistant HCC cells after treatment with therapeutic restoration of miRNA-122 while silencing miRNA-21 function, and down-regulation of MDR protein expression, thereby re-sensitizing resistant HCC to doxorubicin.

therapeutic effects after a single treatment [22]. To ensure that therapeutically-relevant doses of the miRNAs in intact functional forms are delivered to the tumors, miRNAs were loaded into biodegradable and FDA-approved poly lactic-*co*-glycolic acid (PLGA) nanoparticles and delivered into HCC using ultrasound guidance and microbubble-mediated sonoporation [22,31] (Fig. 1). In sonoporation, small, transient holes are generated in the tumor vasculature under the mechanical effects of ultrasound in the presence of microbubble, thereby enhancing therapeutic delivery of nanoparticles into the tumor stroma [22,31] (Fig. 1). Our group has previously shown that PLGA nanoparticles used in this study are non-toxic to cancerous and non-cancerous cells even at very high concentrations [32]. Also, several other groups have used PLGA nanoparticles for the treatment of liver disorders without observing any significant toxic effects [32–34].

In the current study, we hypothesized that repeated delivery of complementary microRNAs (miR-122 and antimiR-21) targeting miR-122 and miR-21 function by ultrasound-guided treatments can significantly enhance therapeutic effects of doxorubicin in both doxorubicin-resistant and non-resistant HCC (Fig. 1). We used HepG2 and Hep3B human HCC cell lines to investigate the cellular and molecular effects of repeated miR-122/antimiR-21 loaded PLGA-PEG-NP treatments in resistant and non-resistant HCC cells *in vitro* and tumor xenografts *in vivo*.

2. Materials and methods

2.1. Synthesis and characterization of miRNA-loaded PLGA-PEG-NPs

The synthesis and characterization of miRNA-122 and antimiR-21 loaded PLGA-PEG-NPs were performed as described previously [22] with minor modifications to increase miRNA encapsulation efficiency. Please see details in Supplementary methods section of the manuscript.

2.2. Cell culture

Human HepG2 and Hep3B HCC cells (ATCC, Manassas, VA) were grown in high glucose (4.5 g/L) Dulbecco's Modified Eagle's Medium (DMEM) and Modified Eagle's Medium (MEM), respectively (Invitrogen, Carlsbad, CA) supplemented with fetal bovine serum (10%), penicillin (100 U/mL) and streptomycin (100 μg/mL). The cells were cultured by incubating at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

2.3. Resistant cell line development

A HepG2 sub-line with resistance to doxorubicin was developed by continuous exposure of parental HepG2 cells to increasing concentrations of doxorubicin over several passages for selection of cells with resistance to 5 μM of doxorubicin by following the protocol described previously [35] (see Supplementary methods section for details). The drug resistance of the created cell line was further confirmed by demonstrating overexpression of multi drug resistant protein (MDR) of the