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REVIEW

Cholesterol-conjugated *let-7a* miRNA mimics: promising tools for HCC systemic therapy

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***Let-7* microRNA (miRNA) family members have been demonstrated to have potential therapeutic value in vitro and in vivo as tumor suppressors that act by regulating Ras at a posttranscriptional level. Previous studies on these miRNAs have primarily focused on certain cancers in which mutation and abnormal activation of *k-ras*/K-Ras occur, such as lung cancer and pancreatic cancers. However, the antitumor potential of *let-7* in the case of hepatocellular carcinoma (HCC) has remained untested. Moreover, a major hurdle that limits the clinical use of miRNAs through systemic delivery, including the delivery of *let-7* for HCC therapy, is the lack of an effective carrier for targeting tumors. Recently, we confirmed the antitumor efficacy of cholesterol-conjugated *let-7a* mimics ("*Chol-let-7a*") in vitro and in vivo and verified-for the first time-that *Chol-let-7a* can effectively carry *let-7a* to orthotopic tumors in the liver and successfully inhibit tumor growth in a preclinical model when delivered systemically. We also evaluated for the first time the potential damages that *Chol-let-7a* could cause to the liver and kidney at histological and ultrastructural levels after long-term systemic delivery, in which *Chol-let-7a* mainly reached and remained at these organs. Lastly, we showed that *Chol-let-7a* downregulated all 3 human *ras*/Ras at transcriptional and translational levels and primarily functioned in the cytoplasm. Overall, our data suggest that the use of cholesterol-conjugated miRNAs is a promising tool for HCC systemic therapy.**

Keywords: Hepatocellular carcinoma; let-7; systemic therapy; n-ras; off-target effects; liver; kidney

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Abnormal activation of the Ras signaling pathway is a crucial factor in carcinogenesis. Activating mutations in Ras proteins result in constitutive Ras signaling, which stimulates cell proliferation and inhibits apoptosis, and thereby leads to tumor development. Thus, blocking the Ras signaling pathway is a key strategy used in human cancer therapy [1].

Recently, the *let-7* microRNA (miRNA) family was demonstrated to hold potential for use in miRNA therapeutics based on the ability of these miRNAs to regulate Ras at a posttranscriptional level in vitro and in vivo [2]. Human cells contain at least 3 *ras* proto-oncogenes that encode closely related but distinct proteins that function abnormally in different human tumors [1]. Oncogenic mutations in *ras* are related to approximately 30% of all human cancers [1]. However, previous studies have reported that *ras* proto-oncogenes generated through mutations in common codons do not contribute to hepatocellular carcinogenesis [3-6]. Thus, studies on *let-7*-mediated blocking of Ras signaling have to date focused primarily on cancers in which mutations cause abnormal activation of K-Ras, such as lung cancer and pancreatic cancers [7-11]. Most studies related to this topic have mainly reported the miRNA effects in K-Ras- and H-Ras-related cancers, and the antitumor potential of *let-7* in the case of hepatocellular carcinoma(HCC) remains unknown.

Recent studies conducted by Calvisi and colleagues have suggested that wild-type Ras activity in human liver cancer can be promoted by a pathway different from that which activates mutated ras [12,13], and that activated (GTP-bound) pan-Ras, H-Ras, K-Ras, and N-Ras are markedly upregulated in human hepatocarcinogenesis and influence cancer progression and prognosis of HCC [12, 13]. Moreover, studies conducted in *Caenorhabditis elegans* [14,15] and computational screening (Targetscan,Pictar)have predicted that the 3′ untranslated regions(UTRs) of human *n-ras*, *k-ras*, and *h-ras* mRNA sequences all contain multiple *let-7* complementary sites (LCSs), which are essential for the regulation of Ras expression by *let-7*. These results suggest that *let-7* miRNAs are potential regulators of all 3 human Ras. Another encouraging finding was our confirmation that *let-7a* inhibited the growth and migration of HCC cells in vitro by downregulating all 3 human *ras* mRNAs and Ras proteins [16,17]. These results suggest that *let-7* can exert potential antitumor effects by downregulating all human Ras in HCC.

A major hurdle encountered in the clinical use of miRNAs—as in the case of any new drug or molecule used for systemic therapy—is the lack of an effective, non-toxic vehicle. Although miRNAs can potentially be used in cancer therapies [18], the safe and effective delivery of miRNAs into solid tumors remains a challenge. The reported studies have verified that certain carriers can be used for targeting miRNAs to tumors, including through systemic delivery. For treating cancer, viral vector-directed methods [19] have been used in order to reintroduce and restore tumor-suppressor miRNAs that are otherwise lost in cancer. Kota and colleagues [20] reported that adeno-associated virus-wrapped miR-26 inhibits cancer-cell proliferation in a mouse model of HCC. The use of self-complementary vectors might enhance tumor-cell transduction and therapeutic miRNA expression. Certain chemical modifications such as cholesterol labeling [21] and nanoparticle medicine delivery [22] have been used to enhance stability and thus avoid deregulation. Trang and colleagues [23] successfully delivered, systemically, synthetic miR-34a and *let-7* mimics targeting lung tissues by using a neutral lipid emulsion and caused a reduction in lung tumor in mice. Zhang *et al.* [24] designed a hepatocyte-targeting ligand to increase the efficiency of anti-miR-155 targeted delivery. These previous methods resulted in the production of strong antitumor effects of miRNAs. However, off-target uptake and effects were not the major focus of previous studies, and, in particular, the uptake and effects after long-term therapy remain unknown.

Recently, we showed that cholesterol-conjugated *let-7a* miRNA mimics ("*Chol-let-7a*") exhibited a high transfection rate when used with Lipofectamine®2000 and produced satisfactory antitumor effects on HCC cells in vitro [16,17]. Subsequently, we performed whole-animal imaging and verified that Cy5-labeled *Chol-let-7a* was mostly targeted to liver tissue and excreted from the kidney in nude mice [17]. This supported our hypothesis that *Chol-let-7a* might serve as a useful carrier for systemic HCC therapy.

Next, we confirmed that *Chol-let-7a* exerted antitumor effects on HCC growth when delivered by means of local injection in a subcutaneous xenograft model [25,26]. Subsequently, *Chol-let-7a* was shown to effectively carry *let-7a* to the target tumors and produce satisfactory antitumor effects in an orthotopic xenograft model when administered systemically [16]. These results also suggested that *Chol-let-7a* produces stronger inhibitory effects when administered systemically than when delivered through local injection. Moreover, the results of histopathological and ultrapathological studies suggested that *Chol-let-7a* induced only mild off-target effects, which were non-specific reaction changes, in the liver and kidney in nude mice (data not shown)[27]. Our studies also suggested that autophagy plays a key role in tumor inhibition, but not in the off-target effects of miRNAs [16]. Furthermore, we confirmed that *Chol-let-7a* downregulated all 3 human Ras at the transcriptional and translational levels and mainly functioned in the cytoplasm [16,23].

In published studies and reviews, the discussion has commonly been limited to the mutation- and frequency-dependent effects of the expression of a single Ras on tissues and tumor type [1,28]. However, the aforementioned results supported the hypothesis that *let-7a* exerts antitumor effects in HCC by regulating all 3 human Ras. The antitumor

effects of miRNAs attributed previously to the targeting of single K-Ras or H-Ras probably reflect cross-effects produced by changes in all 3 human Ras.

Evidence gathered over the past few years has indicated that strategies based on the modulation of miRNA activity could serve as a novel approach for treating cancer. MiRNAs play numerous crucial roles; they can act as both oncogenes ("oncomiRs," such as miR-155 and miR-221) or tumorsuppressors (such as miR-122, miR-34, and members of the *let-7* family) [29,30]. Certain miRNAs have been shown to function as potential miRNA therapeutics for liver cancer [18,31-33]. The therapeutic strategy applied involves the use of antagomirs for oncomiR inhibition or the restoration of tumor-suppressor miRNAs. Our results showed that *Chol-let-7a* and negative-control miRNA mimics ("*Chol-miR-Ctrl*"), which are both cholesterol-conjugated, modified forms of miRNA mimics, could effectively target miRNAs to liver tissues in vivo. Thus, cholesterol conjugation might be a useful modification for developing carriers for the systemic delivery of these potential miRNA therapeutic molecules for HCC treatment, particularly in the case of the tumor-suppressor miRNAs that are downregulated in HCC tumors.

Overall, our data suggest that *Chol-let-7a* is a promising therapeutic drug candidate for systemic treatment of HCC**.** The use of cholesterol-conjugated miRNAs might also serve as an effective tool for systemic HCC therapy.

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Authors' contributions

JG initiated the project. JG and ML analyzed data and wrote the manuscript. ML and JG performed most of the experiments, YX performed ultrasonic analysis, XL, WD and YH performed electronic microscopic analysis, JC, XZ, YL, TY, XL, LZ and JC performed molecular imaging, western blotting, immunohistochemical staining, H&E staining, and analysis. All authors read and approved the final manuscript.

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