Cholesterol-conjugated let–7a mimics: Antitumor efficacy and toxicity in preclinical xenograft models of human hepatocellular carcinoma

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Introduction

- Hepatocellular carcinoma (HCC) is the fifth (2008)/the sixth (2002) most common cancer worldwide; and the third (2008, 2002) most common cause of cancer mortality, and has high recurrence rates after surgery.
- Survival rates are 3% to 5% in cancer registries for the United States and developing countries.
- Chemotherapy and radiotherapy for HCC show limited efficacy and serious toxicity.
- New therapeutic strategies are urgently needed, particularly for the treatment of advanced tumours.

Figure 11. Estimated age-standardized incidence and mortality rates for liver cancer.

Figure FIGURE 10. Age-standardized Incidence Rates for Liver Cancer. Data shown per 100,000 by sex.
MiRNAs, Potential therapeutic molecules for HCC.

• *let*-7 miRNAs family, potential Therapeutic effects?

• A major challenge for the clinical utility of the miRNAs is the lack of an effective, non-toxic carrier.

Oncogenic mutations in *ras* are related to approximately 30% of all human cancers. However, previous studies have reported that ras proto-oncogenes generated through mutations in common codons do not contribute to hepatocellular carcinogenesis. Thus, studies on *let*-7-mediated blocking of Ras signaling have to date focused primarily on cancers with abnormal activation of K-Ras, such as lung cancer and pancreatic cancers. 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 HCC remains unknown.

Let-7, potential therapeutic molecule for HCC?

- Recent studies have suggested that wild-type Ras activity in human liver cancer can be promoted by a pathway different from that which activates mutated ras, 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. (Calvisi et al. Gastroenterology 2006; 130: 1117-28.)

- We confirmed the high RAS expression and low let-7a level in HCC tissues
- We confirmed the prediction: let-7 miRNAs are potential regulators of K-Ras and N-ras.
- In vitro: Chol-let-7 exhibited a high transfection rate into HCC cells
- We showed that Chol-let-7a produced satisfactory antitumor effects on HCC cells by inhibiting Ras at a posttranscriptional level (data not shown) in vitro, and function mainly in cytoplasm.
**In vitro: Efficacy effects of Chol-let-7a**

A: Growth curve of HepG2

B: Growth curve of SMMC7721

*Fig.* Living HepG2 and SMMC7721 cells labelled by GFP were identified by green fluorescence. Images taken at the various observation time points are shown. The red fluorescence that indicated *Chol-let-7a* and *Chol-miRCtrl* was primarily focused in the cytoplasm. Through analysis of live images, we found that most of the *Chol-let-7a*-treated cells lost GFP fluorescence earlier than the 2 control groups. Some *Chol-let-7a*-treated cells showed typical features of apoptosis (yellow arrows).

A **Chol-let-7a** promote HCC cell apoptosis

![Graphs showing apoptotic cell population](image)

B **Additional figure** Apoptotic nuclear changes, such as nuclear shrinkage and nuclear fragmentation, were barely observed in *Chol-let-7a*-treated cells.

C Organelle changes after *Chol-let-7a* therapy under transmission electron microscopy

![Images showing organelle changes under TEM](image)

**Fig.** Ultrastucture in *Chol-let-7a*- and *Chol-miRCtrl*-treated cells under TEM at 48 h post-transfection. Sections from *Chol-let-7a*-treated cells revealed the presence of abnormal organelles in the cytoplasm. Increased autophagocytic activity in HepG2 and SMMC7721 cells was observed 48 h after *Chol-let-7a* treatment, as revealed by the presence of abundant lysosomes and phagolysosomes exhibiting heterolysosomes such as phagophores, multivesicular bodies (MVBs), and multilamellar bodies (MLBs) in the cytoplasm, but only slight changes in nuclear morphology were observed. Enlarged irregular mitochondria with disorganized mitochondrial crests and dilated rough endoplasmic reticulum (RER), which are often accompanied by degranulation, were also clearly observed in the *Chol-let-7a*-treated cells. Some mild changes were observed in the *Chol-miRCtrl*-treated HCC cells (data not shown).

**Additional figure**  *Chol-let-7a*-treated cells observed under TEM at 60 h post-transfection.

Long-term treatment produced significant ultrastructure modifications. In the cytoplasm of *Chol-let-7a*-treated cells, mitochondria, heterolysosomes, and RER were *vacuolated* and showed irregular and unclear contours and structures.
Research Aim

• Try to find a potential delivery system for systemic therapy of HCC.
• We confirmed the antitumor efficacy and potential off-target effects of cholesterol-conjugated let-7a mimics (Chol-let-7a) in preclinical models.

• Efficacy and off-target effects-before clinical use
Materials and Methods

Efficacy In vivo

- Subcutaneous and orthotopic xenograft were treated with Chol-let-7a or negative control miRNA through local injections or systemic delivery. Ultrasonography was used to evaluate tumor growth and metastasis.

- Histopathology and ultrastructural features of tumors were used to observe cell changes after systemic treatment.
Materials and Methods

Efficacy In vivo

**Subcutaneous xenograft** nude mice

- Chol-let-7a
  - Intratumoral injections

**Orthotopic xenograft** nude mice

- Chol-let-7a
  - Systemic delivery

**Effects of Chol-let-7a on tumor growth and tumor metastasis of xenografts**

- Ultrasonography
- Tail blood
- CTC examination

- HE:
  - Histopathology
  - Tumor
  - Cell phenotype
  - Mitotic figure
  - Heterogeneity

- Immunohistochemical staining
  - Ki-67
  - RAS

- TEM
  - Ultrastructure
  - Mit; lyso; ERs...
Results and Discussion

Efficacy In vivo
**Subcutaneous xenograft model: Intratumoral injections**

*Chol-let-7a* inhibited HCC growth *(Inhibitory rate, 56.3%)* and tumor invasion when delivered by means of local injection in a subcutaneous xenograft model.

**A** Growth curve of tumor

**B** Max view of tumor

**C** Average volume of tumor

**D** Tumor tissue under LM

**E** let-7a level after treatment

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Fig. 2 Efficacy in vivo—2
—Orthotopic Xenografts model: **Systemic delivery**

**A:** Orthotopic HepG2 xenografts examined by ultrasonography

- **Chol-let-7a** inhibited growth and metastasis of HCC orthotopic xenografts after systemic delivery
- The growth of orthotopic tumours was significantly inhibited following **Chol-let-7a** therapy
- **Chol-let-7a** was shown to effectively carry **let-7a** to the target tumors and produce satisfactory antitumor effects (Inhibitory rate, 66.5%) in an orthotopic xenograft model when administered systemically.

Fig. 2 Efficacy in vivo—Orthotopic Xenografts model: Systemic delivery

B: Xenograft growth curve

C: Max view of the HCC orthotopic tumor and metastasis within liver

Beginning from 2 weeks after Chol-let-7a treatment, inhibition of tumour metastasis was observed by ultrasonography. Metastases within the liver are shown in Fig. 5B. Local invasion and metastasis to the spleen were inhibited in the Chol-let-7a-treated group.

D: Orthotopic HCC tumour under LM

In addition, Chol-let-7a-treated tumor cells showed no significant atypia, and mitoses were very rare per unit of measurement in most areas in comparison with the control groups. Bar: 20µm

**Fig. 2 Efficacy in vivo**
—Orthotopic Xenografts model: **Systemic delivery**

**E: TEM of HCC orthotopic tumours in vivo**

*Chol-let-7a*-treated tumour cells showed no significant atypia, and mitoses were very rare per unit of measurement in most areas as compared to the control groups were confirmed under TEM. Moreover, this feature was more significant near in capillary-rich areas (red arrow).

Effective antitumor efficacy, why?
Fig5. Up-regulated *let-7a* effectively down-regulated all kinds of *ras/RAS* gene expression; mRNAs, proteins——( I )effective inhibition

**A** *Up-regulated let-7a* level in HepG2 orthotopic xenografts

**B** RAS protein expression in xenografts Down-regulated examined by western blotting

* : p<0.05; ** p<0.01
C All ras mRNAs in xenograft tumor tissue down-regulated examined by qRT-PCR

* : \( p<0.05 \); ** \( p<0.01 \)
Materials and Methods—further study
Off–targets: systemic therapy, and why?

• Considering that Chol-let-7a and Chol-miRCtrl may be metabolized in the liver and excreted through the renal system,
• we examined potential damage to the liver and kidney at the culmination of therapy via assessment of histology and ultrastructure.
Results

- Inflammation and necrosis of the liver and kidney were relatively mild in the Chol-let-7a-treated xenograft mice in comparison with the Chol-miRCtrl-treated and blank group.
- *Chol-let-7a* produced some non-specific mild damage to the liver and kidney.
- Mild damage were observed in liver and kidney of normal nude mice.

* (Data not shown)
Conclusions

• Up-regulated *Chol-let-7a* inhibited HCC tumor growth and metastasis in vivo in preclinical models.

• *Chol-let-7a* down-regulated all kinds of *ras/RAS* gene expression; mRNAs, proteins.

• *Chol-let-7a* by systemic delivery, “target” liver and orthotopic tumor in liver; and produce more effective growth inhibition with mild liver and kidney damage.

• *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.
• Further studies
• Systemic side effects
• Effective antitumor efficacy and off-target effects, why?

• Related Articles published in 2014-2015
• Acknowledgements

• For technical support, we thank Huimin Zhao, Wenyu Hao, and Huanxian Cui from the Centre for Experimental Animal Research (CEAR), Institute of Basic Medical Sciences, CAMS/PUMC, as well as Xiao Yang (VisualSonics, Inc., Beijing, China) and Yi Gao (Berthold, Beijing, China). We thank Dr. Wei-Min Tong for constructive suggestions in support of this study, and Dr. Xingyi Hang and Jiahui Liu and Yuxing You for assistance with statistical analysis.

• This work was supported partly by the Scientific Data Sharing Program funded by the Chinese Ministry of Science (2004DKA20240-2013, J G, J C).
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