

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.**

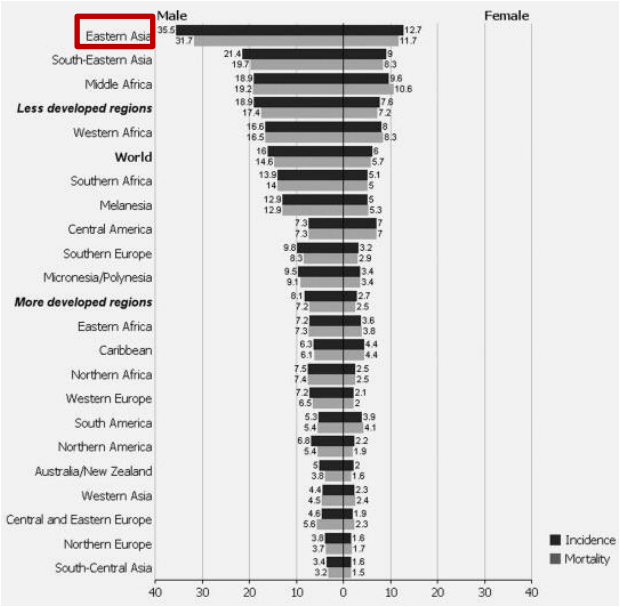


Figure 11. Estimated age-standardized incidence and mortality rates for liver cancer.
Ferlay J, *et al.* Estimates of worldwide burden of cancer in 2008: **GLOBOCAN 2008.** *Int J Cancer. 2010 Dec 15; 127(12):2893-917.*

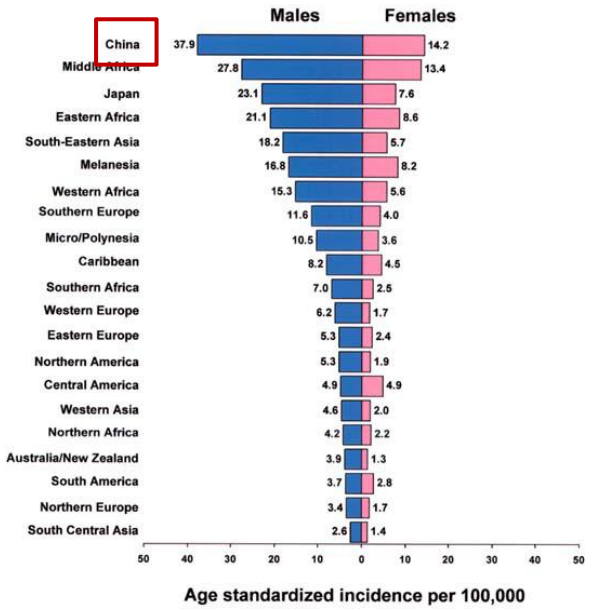
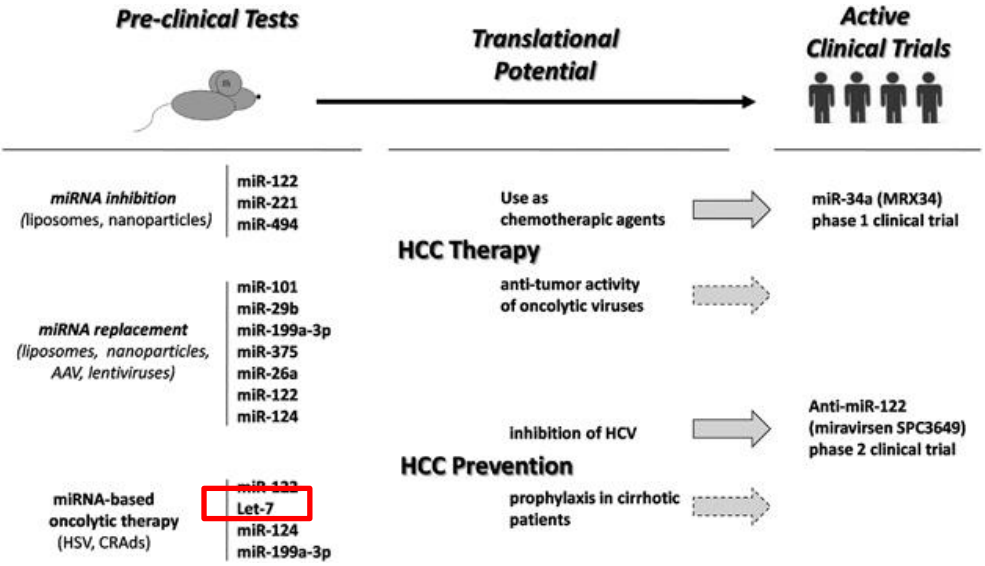


Figure FIGURE 10. Age-standardized Incidence Rates for Liver Cancer. Data shown per 100,000 by sex.
Parkin DM, et al**. Global cancer statistics, 2002.** Cancer J Clin. 2005 Mar-Apr;55(2):74-108.

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.**



Callegari E, et al. MicroRNAs in liver cancer: a model for investigating pathogenesis and novel therapeutic approaches. Cell Death Differ. 2015 Jan;22(1):46-57.

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.**

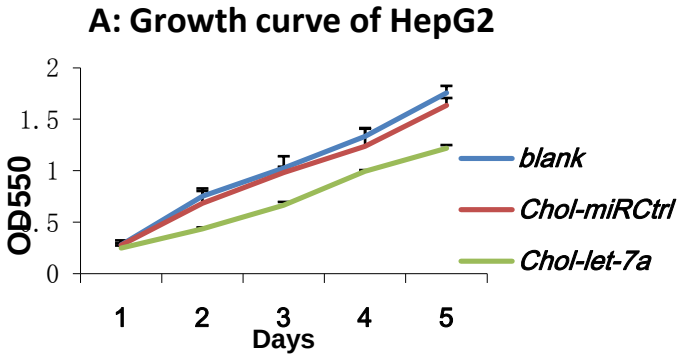
Let-7,
potential therapeutic molecule for HCC?



- Recent studies have suggested that **wild-type Ras activity in human liv 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



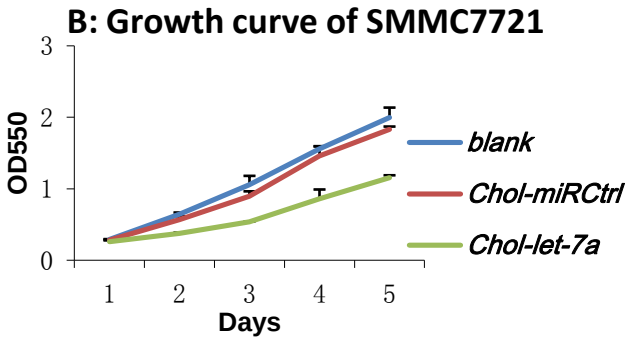


Fig.

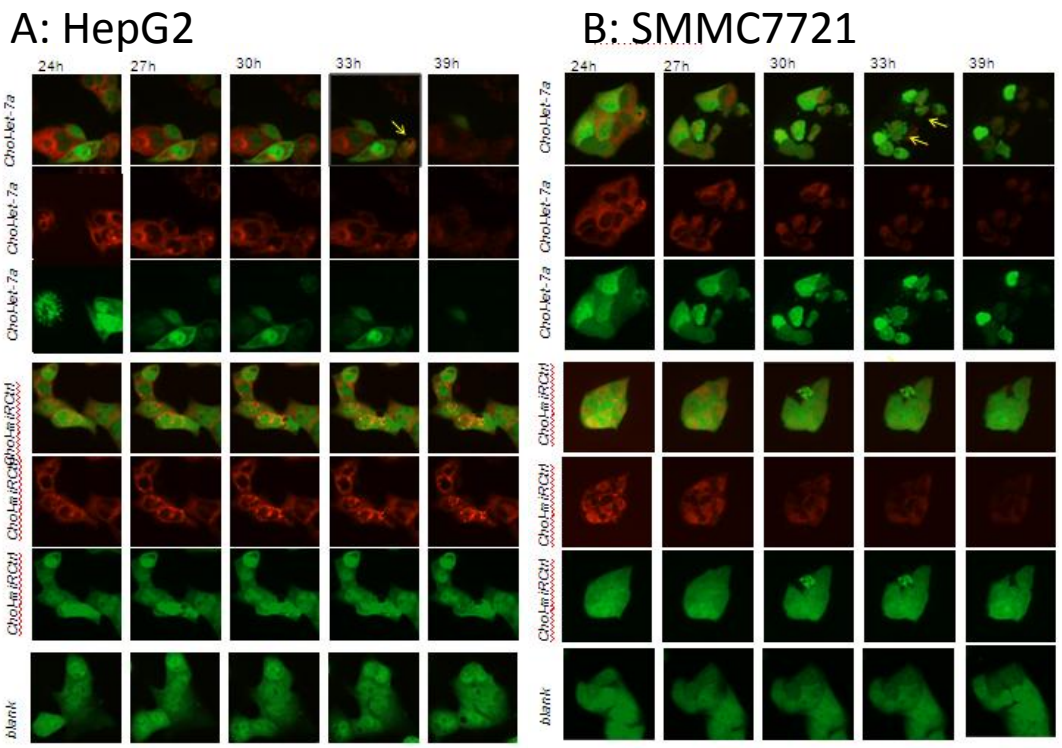


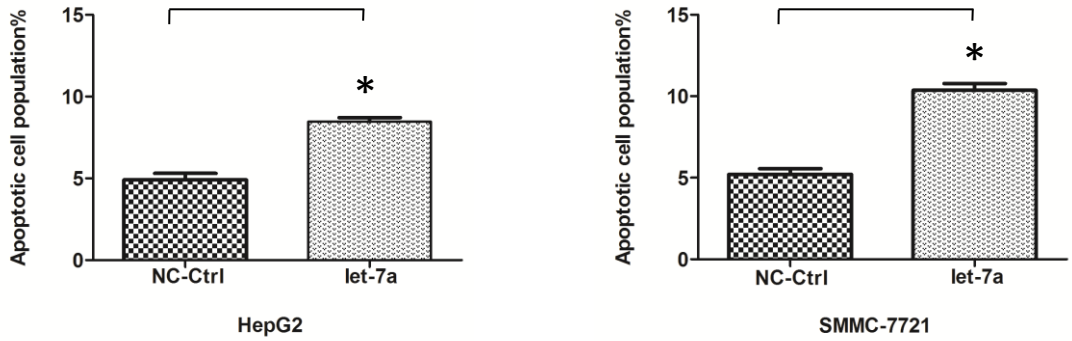
Fig. Living HepG2 and SMMC7721 cells labelled by GFP were identified by green fluorescence**. Images taken at the various observation time points are shown**

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 .

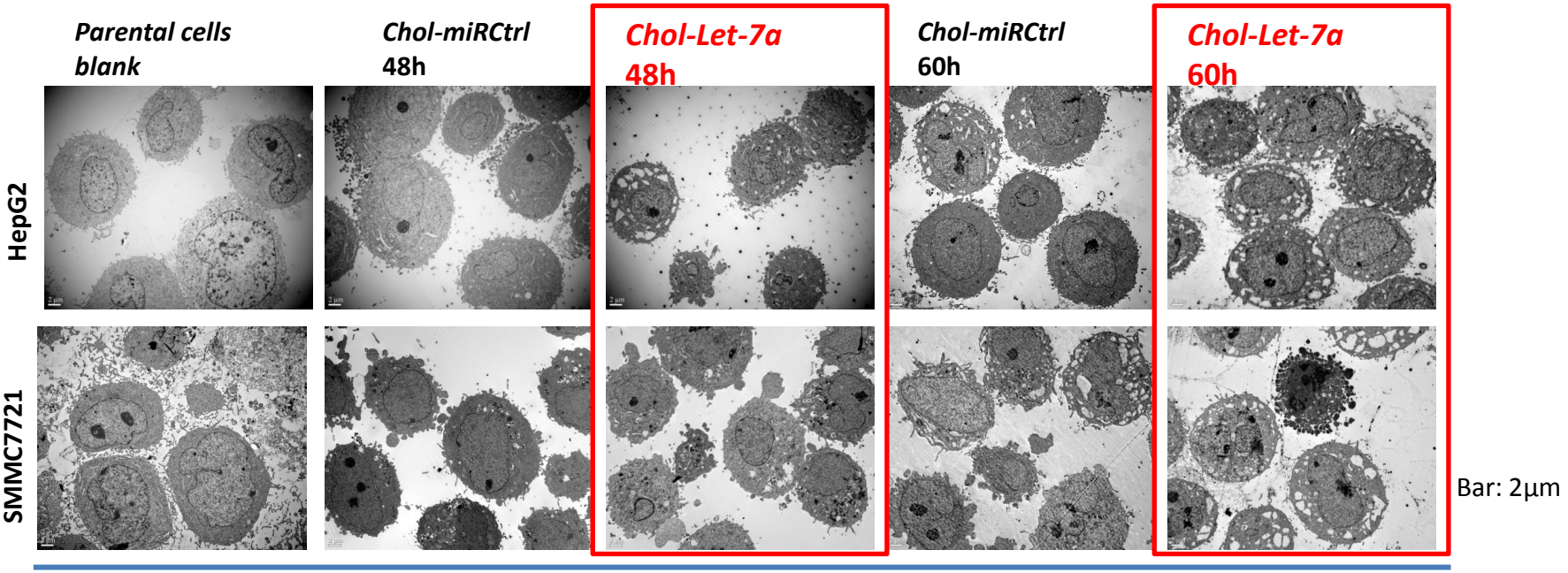
Some *Chol-let-7a*-treated cells showed **typical features of apoptosis (yellow arrows).**

* **Liu MY,… Guan J**. ***BMC Cancer***. 2014;14(1):889. doi:10.1186/1471-2407-14-889

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

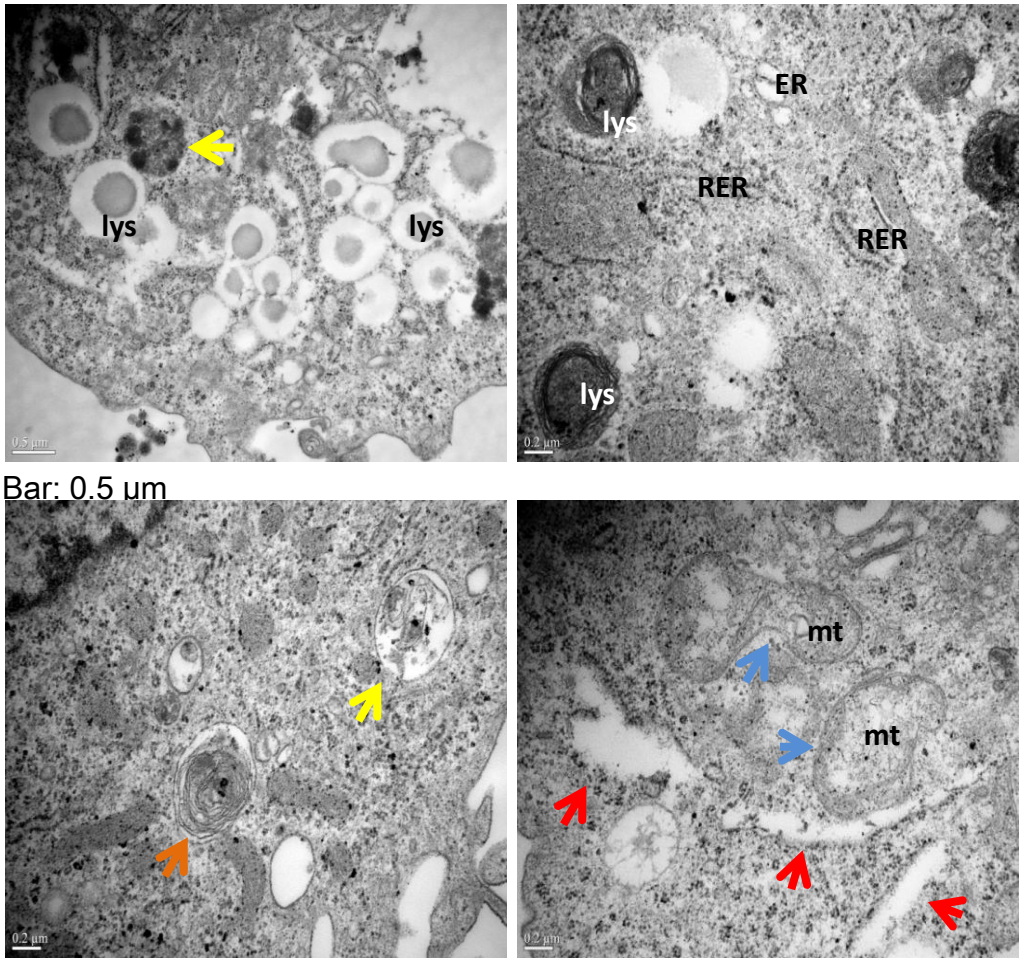


B Additional figure **Apoptotic nuclear changes, such as nuclear shrinkage and nuclear fragmentation, were barely observed in *Chol-let-7a*-treated cells.**



* **Liu YM,… Guan J**. ***BMC Cancer***. 2014;14(1):889. doi:10.1186/1471-2407-14-889

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



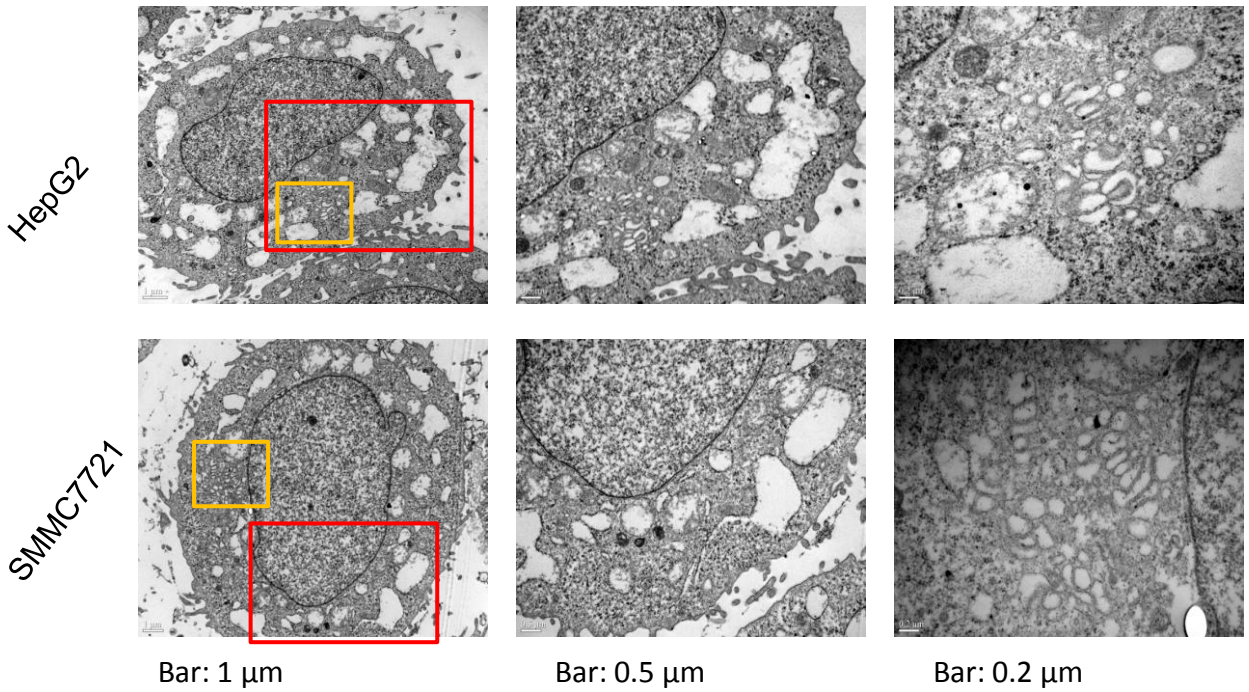
Bar: 0.5 μm

Bar: 0.2 μm

Fig. Ultrustructure 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)

 # Liu MY,… Chen J*, Guan J*. ***BMC Cancer***. 2014;14(1):889. doi:10.1186/1471-2407-14-889

D *Chol-let-7a*-treated HCC cells at 60h



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

Liu MY,… Chen J*, Guan J *. ***BMC Cancer***. 2014;14(1):889. doi:10.1186/1471-2407-14-889

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

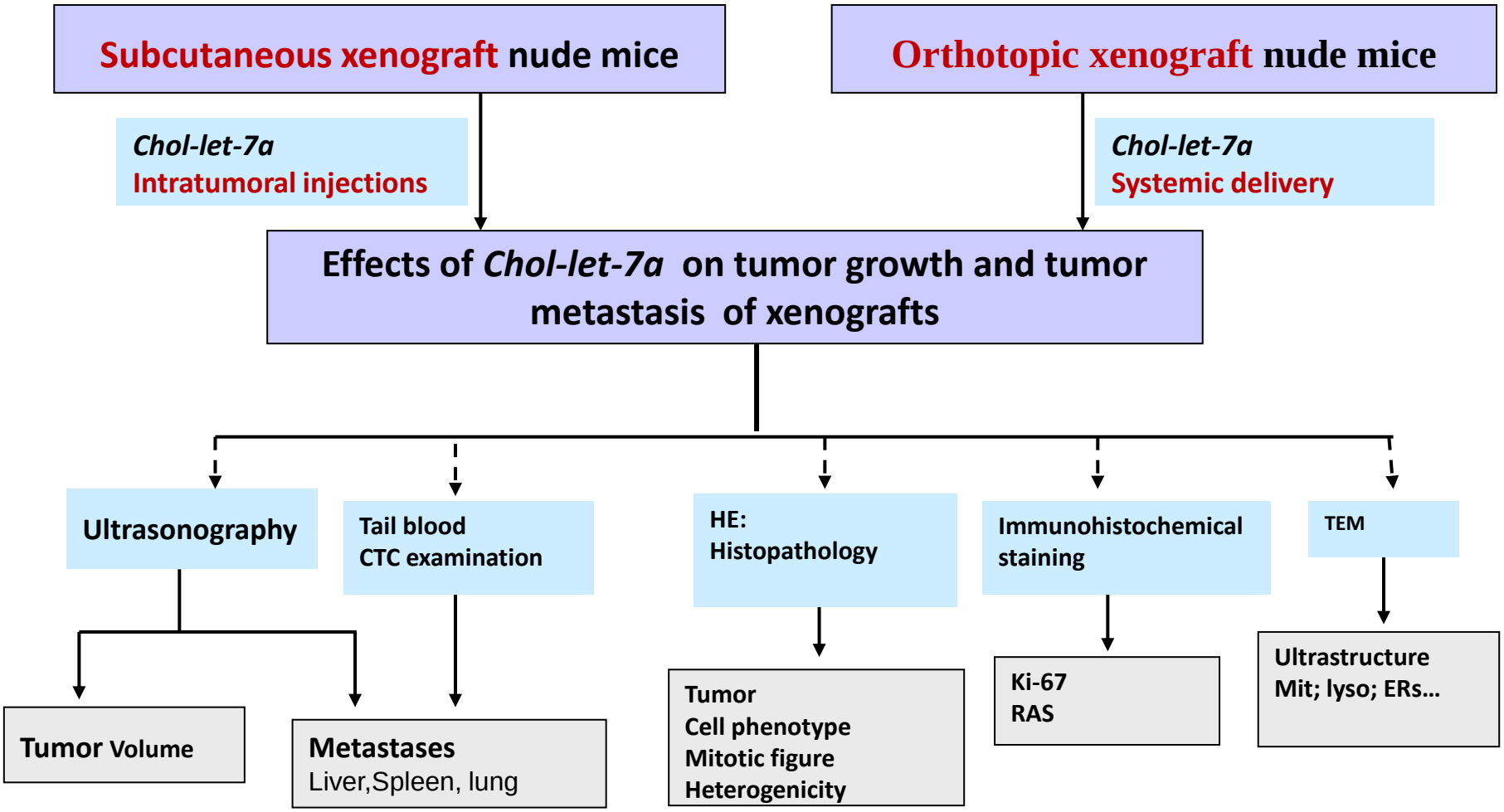
Materials and Methods

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



Results and Discussion

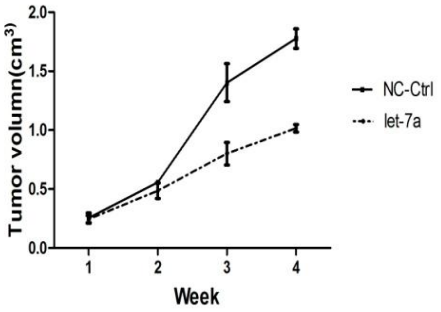
Efficacy In vivo

Fig. 1 Efficacy In vivo-1

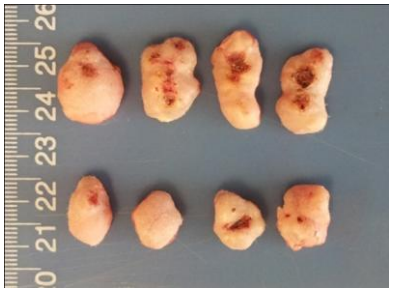
—Subcutaneous xenograft model: Intratumoral injections

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.**

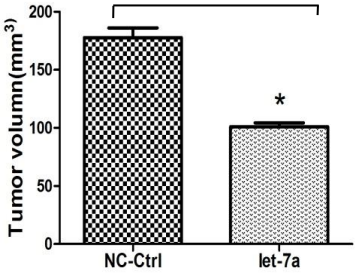
A Growth curve of tumor



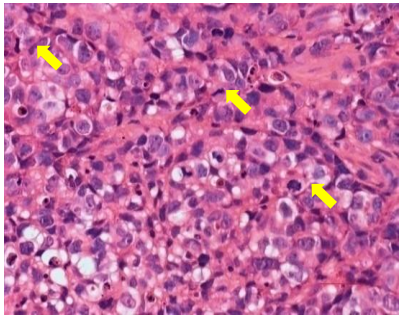
B Max view of tumor



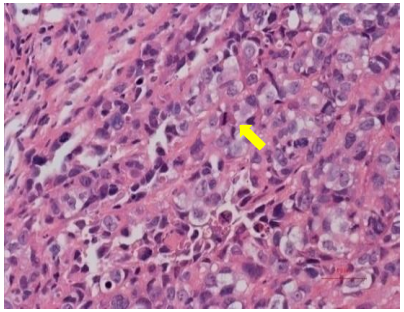
C Average volume of tumor



D Tumor tissue under LM

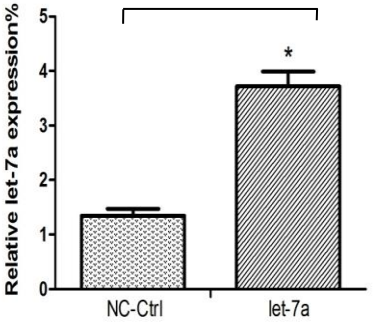


NC-Ctrl



let-7a

E let-7a level after treatment

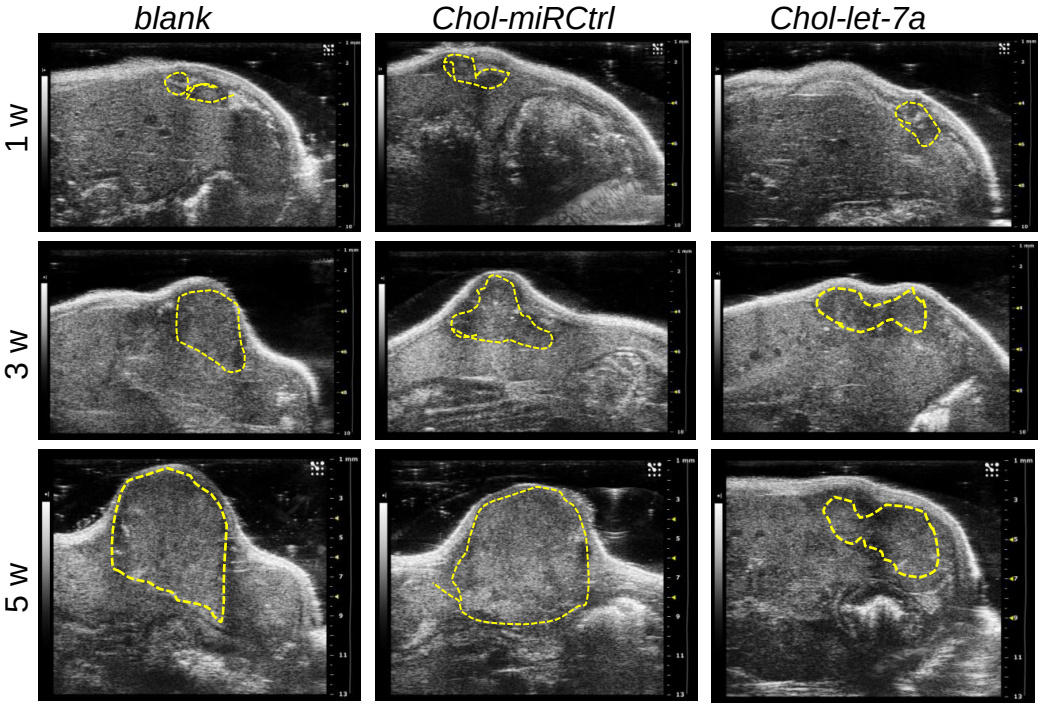


Liu MY, Chen J, Guan J*. ***Med J PUMCH*** 2015; 6:133-139.

Fig. 2 Efficacy in vivo-2

—Orthotopic Xenografts model: Systemic delivery

A: Orthotopic HepG2 xenografts examined by ultrasonograpy

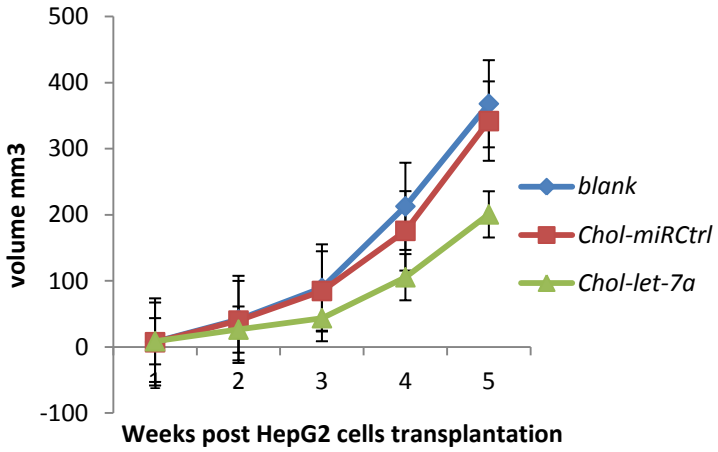


- ***Chol-let-7a* inhibited growth and metastasis of HCC orthotopic xenografts after systemic delivery**
- The growth of orthotopic tumours was significantly inhibited following *Chol-let-7a* therapy
- ***Chol-let-7a* was shown to effectively carry *let-7a* to the target tumors and produce satisfactory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

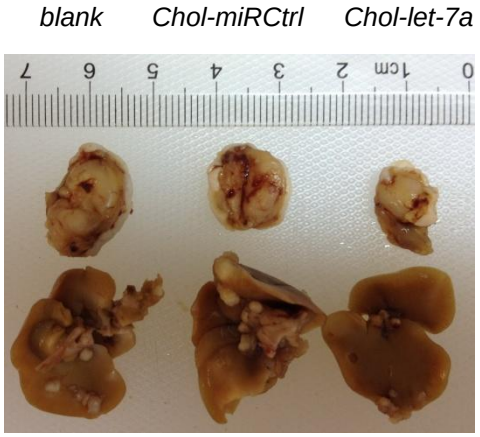
Liu MY,… Chen J*, Guan J*. ***BMC Cancer***. 2014;14(1):889. doi:10.1186/1471-2407-14-889

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

B: Xenograft growth curve

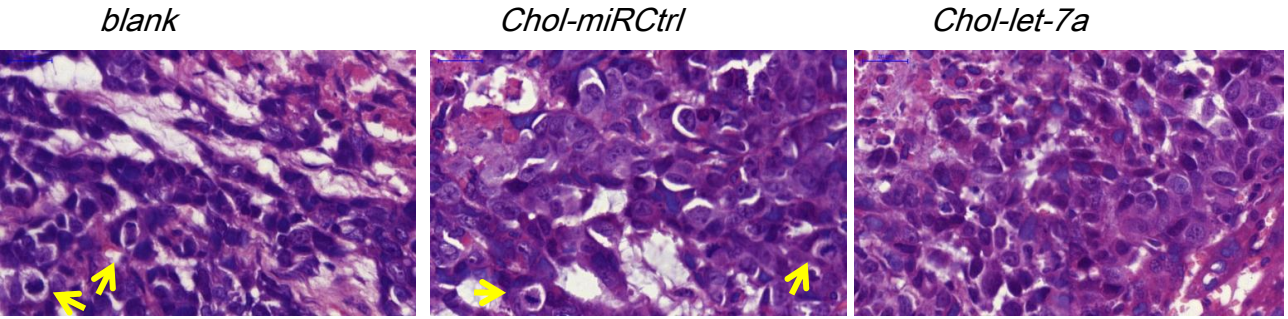


C: Max view of the HCC orthotopic tumor and metastasis within liver



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

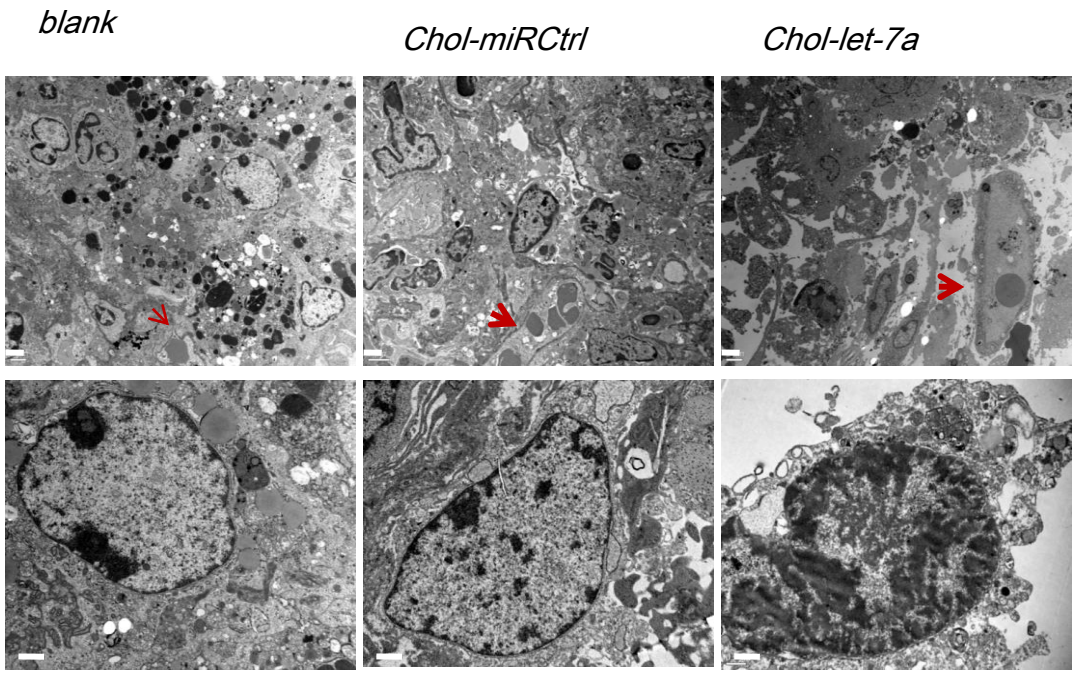


In addition, *Chol-let-7a*-treated tumor cells showed no significant atypia, and mitoses were very rare per unit of measurement in most areas in comparison with the control groups Bar: 20μm

Liu MY,… Chen J*, Guan J*. ***BMC Cancer***. 2014;14(1):889. doi:10.1186/1471-2407-14-889

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

E: TEM of HCC orthotopic tumours in vivo



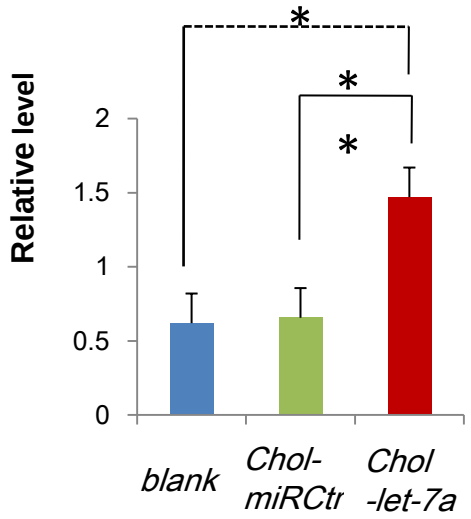
Chol-let-7a-treated tumour cells showed no significant atypia, and mitoses were very rare per unit of measurement in most areas as compared to the control groups **were confirmed under TEM.**
Moreover, this feature was more significant near in capillary-rich areas(red arrow)

Liu MY,… Chen J*, Guan J*. ***BMC Cancer***. 2014;14(1):889. doi:10.1186/1471-2407-14-889

Effective antitumor efficacy, why?

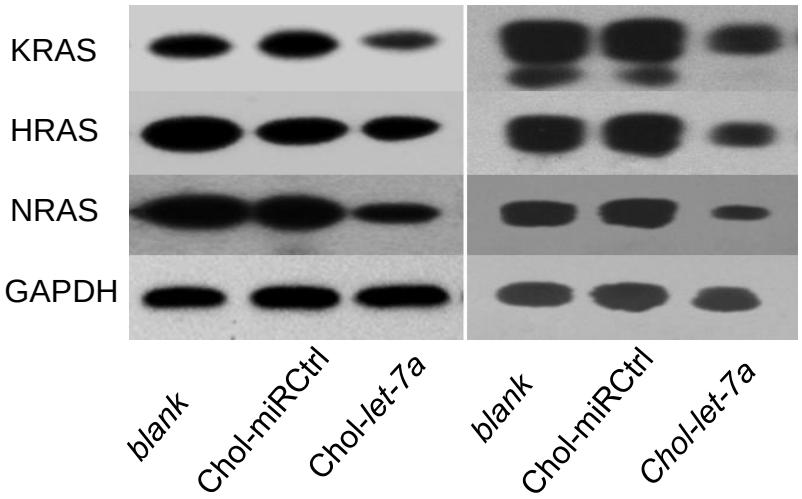
Fig5. Up-regulated *let-7a* effectively down-regulated all kinds of *ras*/RAS gene expression; mRNAs, proteins——(Ⅰ)effective inhibition

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

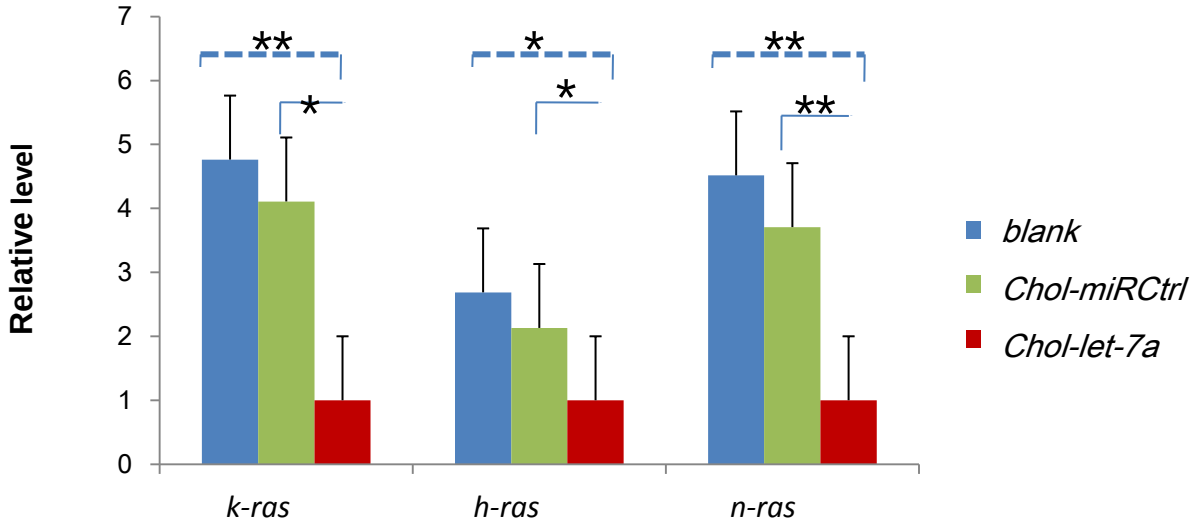


* : $p<0.05$; ** $p<0.01$

B <u>RAS protein expression in xenografts Down-regulated</u> examined by western blotting



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**

- 1. Liu YM, Xia Y, Dai W, Han HY, Dong YX, Cai J, Zeng X, Luo FY, Yang T, Li YZ, Chen J, Guan J(Corresponding author). **Cholesterol-conjugated let-7a mimics: antitumor efficacy on hepatocellular carcinoma in vitro and in a preclinical orthotopic xenograft model of systemic therapy**. ***BMC Cancer***. 2014;14(1):889. doi:10.1186/1471-2407-14-889
- 2. Liu YM, Li X, Xia Y, Dai W, Han HY, Cai J， Dong YX, Zeng X, Luo FY, Yang T, Chen J, Guan J(Corresponding author). **Cholesterol-conjugated *let-7a* miRNA mimics: promising tools for HCC systemic therapy**. ***RNA & DISEASE***. 2015; 2(2) doi: 10.14800/rd.630
- 3. Liu YM, Chen J, Guan J. **Local injections of cholesterol-conjugated let-7a mimics inhibit growth of HCC in a subcutaneous xenograft model by targeting all 3 human ras/Ras**. ***Med J PUMCH*** 2015; 6:133-139.

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