Abstract of Doctoral Dissertation

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Design of Novel Targeted Co-delivery System for Combined Chemotherapy and Gene Therapy of Hepatocellular Carcinoma
（肝細胞癌に対する化学療法と遺伝子治療の新規併用療法の確立）

Introduction:

Hepatocellular carcinoma (HCC), is the 5th most common cancer worldwide and the third most common cause of cancer-related deaths. Treating advanced stages of HCC is difficult and the response rate to most chemotherapeutics is still low (10-15%) mainly due to the emergence of multidrug resistance (MDR). These drawbacks necessitate looking for novel approaches such as gene therapy that can effectively cure the disease by targeting its primary cause at the molecular level. Sorafenib (SOR) is a multiple kinase inhibitor designated by FDA as the drug of choice for resistant HCC. However, it remains the last therapeutic approach and there are no effective solutions if the tumor develops resistance to it. In addition, its systemic administration is restricted by numerous toxicities and side effects. Meanwhile, Midkine (MK) is a growth cytokine overexpressed in HCC with anti-apoptotic, mitogenic, angiogenic and chemoresistant roles. In the current study, we propose that combining SOR with anti-Midkine nucleic acid therapy (MK-siRNA) would exert a synergistic therapeutic activity against HCC. Furthermore, we developed novel lipid nanoparticles (LNPs) for the co-delivery of such payload to HCC with high efficiency and selectivity. The selected main lipid was YSK05, a novel pH-sensitive lipid designed in our laboratory. LNPs were modified with a novel highly-selective HCC-targeting peptide, SP94.

Results and discussion:

1. In vitro study

In the first part of this study, we tested our hypothesis in vitro. We optimized various aspects regarding the formulation and composition of these LNPs to control their efficiency and selectivity. Our results revealed significant uptake in human and murine HCC (HepG2 and Hepa 1-6, respectively) compared to other cancerous cells (HeLa) or normal hepatocytes (FL83B). Evaluation of the cytotoxicity and MK gene knockdown activity confirmed the same pattern of selectivity. In addition, we showed the first evidence that MK-siRNA increases the sensitivity of HCC cells to SOR as suggested from its effect on SOR dose-response curve which significantly differed when MK-siRNA was replaced by a control siRNA (siCntrl).

2. In vivo optimization

The promising in vitro results triggered us to extend the applicability of our system to the in vivo level. Preliminary in vivo evaluation of the system after intravenous administration to HCC-bearing mice indicated that it could not accumulate in the tumor or exert MK knockdown activity there which reflected the challenges of the in vivo delivery to HCC. To overcome this, we designed a step-wise optimization process considering the possible challenges restricting the delivery of LNPs to the tumor; the pharmacokinetic performance, the stroma-rich tumor microenvironment hindering the penetration of LNPs to HCC cells, and the inadequate endosomal escape capability upon shifting from the in vitro to the in vivo situation. Controlling PEG-SP94 density in the LNPs dramatically affected their pharmacokinetic performance which lead to improvement in MK knockdown activity in the tumor, but the selectivity remained poor as indicated upon comparing the activity in the tumor versus the healthy liver. We applied a microfluidic device (iLiNP) and optimized diverse factors related to the operation or lipid composition to tune the particle size. Interestingly, reducing the particle size to 70 nm dramatically improved the gene knockdown activity and increased the selectivity to the tumor

indicating successful penetration through the stroma barrier. However, further reduction in the particle size by increasing YSK05 ratio above 50 mol% sensitized Apolipoprotein E (ApoE) transporter in the serum to redirect the LNPs to the healthy liver, therefore LNPs with 70 nm size (50 mol% YSK05) were considered to be the optimum. To avoid significant effect on the healthy liver, we attempted masking ApoE recognition of our LNPs by filling the interspaces in their surface by additional DSPE-PEG_{2K}-MA. Surprisingly, at 3 mol% ratio, the LNPs showed strong knockdown activity in the tumor compared to limited activity in the healthy liver. Moreover, the particle size of the new LNPs was reduced to 60 nm by the effect of the additional PEG. Eventually, we screened various helper lipids to maximize the endosomal escape performance which revealed the superiority of DOPE. The optimized system was referred to as ultra-small lipid nanoparticles (usLNPs).

3. Therapeutic evaluation

We evaluated the therapeutic activity of these usLNPs for the treatment of HCC-bearing mice. SOR and siRNA doses were adjusted to 2.5 mg/Kg and 0.5 mg/Kg, respectively. Treatment was given on days 7,10,13,16,19,22 and 25 post tumor inoculation and the mice were terminated on the 28^{th} day. Surprisingly, SOR+MK-siRNA combination showed ~85% eradication of the tumor compared with SOR+siCntrl (~40%) or MK-siRNA (~18%) which suggested the synergism between SOR and MK-siRNA. The treatment did not result in significant body weight loss commonly seen with ordinary chemotherapeutics suggesting the biosafety of our usLNPs. Furthermore, treatment with SOR+MK-siRNA combination resulted in 3-fold down regulation of the characteristic HCC marker, Alpha fetoprotein (AFP), and 4-fold down regulation of Osteopontin (OSP) and vascular endothelial growth factor 1 (VEGF-1).

We raised the challenge and attempted the treatment of SOR-resistant tumor. In vitro results suggested the ability of MK-siRNA to reverse the resistance to SOR in established SOR-resistant HepG2 cells. The upregulation of MK gene by 3-folds in the resistant cell line suggested the role of MK pathway in acquiring the resistance to SOR. Surprisingly, SOR+MK-siRNA combination could eradicate SOR-resistant tumor in mice by ~70% which was almost insensitive to SOR+siCntrl at the same dose.

Conclusions:

In conclusion, we designed LNPs for efficient and selective co-delivery of SOR and MK-siRNA to HCC both in vitro and in vivo. We reported the synergism between SOR and MK-siRNA for the first time. According to our knowledge, our system is the most efficient for siRNA delivery to HCC so far (ED_{50}=0.1 mg/Kg). We could eradicate HCC in mice using the lowest dose of SOR so far (2.5 mg/Kg). We challenged SOR-resistant HCC for the first time. We believe that our system holds a great promise for potential clinical application in HCC treatment.