Regulation of HSVtk gene by endogenous microRNA-122a in liver cell lines as suicide gene therapy

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ABSTRACT

Aim: Here, we use miR-122a that exhibits liver-specific expression for developing a post-transcriptional regulative system mediated by microRNAs.

Background: Gene therapy with adenovirus (Ad) vectors that express herpes simplex virus thymidine kinase (HSVtk) and ganciclovir (GCV) have been suggested as a therapeutic strategy to cancer. However, Ad vectors injected into tumors are dispersed into the systemic circulation and transduce liver cells, resulting in severe hepatotoxicity. To be effective, the delivery and expression of suicide genes to cancer treatment ought to be specific to tumor cells, and avoid death of healthy cells. Researchers have demonstrated that expression of transgene could be suppressed in healthy cells with use of vectors that are reactive to microRNA regulation.

Methods: We constructed an Ad vector carrying four tandem copies of target sequences of miR-122a that were incorporated into 3'-UTR of HSVtk gene. The expression level of miR-122a was quantified in HepG2 and Huh7 cell lines.

Results: Quantitative RT-PCR analysis demonstrated that Huh7 cells express large amounts of miR-122a compared to HepG2 cells. The viability of Huh7 cells and HepG2 cells after infection by Ad-tk-122aT vector was 83% and 23.5%, respectively. The viability of Huh7 cells was not reduced in the presence of GCV after infection by Ad-tk-122a vector. In contrast, cytotoxicity of HSV-tk/GCV was similar in Huh7 cells and HepG2 cells by Ad-tk vector, with 35.3% and 27% viability, respectively.

Conclusion: Inclusion of the miR-122a target sequences in the HSVtk expression cassette yielded a feasible strategy for reducing cytotoxicity of suicide gene in a liver cell line with high miR-122a expression

Keywords: microRNA regulation; miR-122a; HSVtk gene; ganciclovir; cancer therapy

Introduction

Of various approaches to cancer gene therapy, suicide cancer gene treatments using the herpes simplex virus thymidine kinase (HSVtk) gene and ganciclovir (GCV) have been demonstrated in clinical and preclinical researches (1, 2). The therapeutic effect of the HSVtk/GCV consists of transduction of the HSVtk gene in tumor cells and change of nontoxic GCV to highly toxic phosphorylated GCV by HSVtk gene. Following that, phosphorylated GCV inhibits DNA replication and causes cell death. Several gene delivery systems, including retrovirus vectors, adenovirus vectors, and liposomes, have been used in HSVtk/GCV gene therapy. Adenovirus vectors have several advantages over other viral and nonviral vectors, because they have high transduction efficacy and can be grown to high titers (3, 4).

Regulation of virus host range is of particular importance. For best therapeutic effects, gene therapy...
vehicles should target cancer cells while avoiding other organs and normal cells (5, 6). Suicide gene therapy is associated with hepatic damage, because Ad vectors have high tropism to liver cells. In order to increase the safety and efficacy of suicide gene therapy by HSVtk gene that is transduced by Ad vectors, unwanted effects caused by Ad vector in the liver should be diminished, without reducing transgene expression in the tumor cells. Lately, researchers have focused on post-transcriptional regulatory systems mediated by microRNAs (miRNAs) (7, 8). MicroRNAs are noncoding small RNAs that contribute to the regulation of their cognate target genes, usually by incomplete base-pairing with the 3′-untranslated region (UTR) of the target mRNA, which results in cleavage/degradation of the mRNA and translational repression (8, 9).

Insertion of miRNA target sequences into the 3′-UTR of a gene decreases the expression level of the gene, with the amount of reduction dependent on the cellular expression levels of the miRNA (8, 10, 11). Because miR-122a is highly expressed in liver cells, we hypothesized that insertion of sequences complementary to miR-122a into the 3′-UTR of HSVtk gene in Ad vectors will reduce HSVtk expression in liver cell without affecting HSVtk expression in cancer cells (12).

To test this hypothesis, we constructed an Ad vector containing four tandem copies of miRNA-binding sites for miR-122a in 3′-UTR of HSVtk gene and used the hepatoma cell lines, Huh7 and HepG2, as models for suicide gene therapy with this Ad vector. We showed that endogenous miR-122a regulated HSVtk expression in Huh7 cell line with high level of endogenous miR-122a in comparison to HepG2 cell line with low level of endogenous miR-122a.

**Methods**

Fetal bovine serum (FBS) and Dulbecco’s modified Eagle’s medium (DMEM) were purchased from Gibco. The human hepatoma cell lines HepG2 and Huh7, and human embryonic kidney cell line AD-293 were obtained from the Groningen research institute of pharmacy (Groningen, The Netherlands). The replication-defective recombinant adenovirus vector, AdEasy™ Adenoviral Vector System, was obtained from Agilent Technologies.

TriPure Isolation Reagent was purchased from Roche. Universal cDNA Synthesis kit, LNA™-enhanced microRNA qPCR primer sets and SYBR® Green Master Mix kits were purchased from Exiqon. MTT Assay Kit was purchased from Promega. All experiments were performed at least three times and their average values are reported here.

**Cell culture**

The human hepatoma cell lines HepG2 and Huh7, and human embryonic kidney cell line AD-293 were grown in high-glucose Dulbecco's modified Eagle's medium supplemented with 2 mM l-glutamine, 100 U of penicillin/mL, 100 μg of streptomycin/mL, and 10% fetal bovine serum. Cells were grown in 25 cm² polystyrene tissue culture flasks in a humidified atmosphere of 5% CO₂ at 37 °C.

**Plasmids and Ad vectors**

Recombinant adenovirus vector was generated from adenovirus type 5, and the E1 and E3 regions were deleted to prevent virus replication. The pSELECT-zeo-HSV1tk was purchased from Invivogen Company. We amplified tk gene with forward (F XhoI: 5′-AATCTCGAGATGGCCTCGTACCCCGGCCA -3) and reverse (R EcoRI: 5′-CCGGAATTCTTATCCTACGGACCCGGAACGCA -3) primers containing Xho1 and EcoR1 site, respectively, in a PCR from pSELECT-zeo-HSV1tk. Briefly, a 1.7-kb XhoI/EcoRI fragment was purified and subcloned into pAd track shuttle, to form the pAd track-tk shuttle.

Ad vectors containing miR-122a target sequences were established by in vitro ligation. A pAd track-tk/122a shuttle plasmid with four tandem copies of perfectly complementary sequences to miR-122a in the 3′-UTR of the HSVtk expression cassette, was established as follows: A EcoRI/BamHI fragment of miR-122a target sequences (5′-AAATTCACAAACCCATTGTCACACTCCACAGC ACAAAACCATTGTCAACTCCTATTGCAAAACA AACCATTGTCAACTCCAGGACAAACAC CATTGTCAACTCCAA -3) (perfect complementary sequences of miR-122a are underlined) was ligated to pAd track-tk, resulting in pAd track-tk/122a. For generating Ad vectors (Ad, Ad-tk-122aT, and Ad-tk), the resultant plasmids were linearized by digesting with restriction endonuclease PmeI, and were subsequently
co-transformed into E. coli (BJ5183 cells) with the pAdEasy-1 adenoviral backbone plasmid. Recombinants were selected for kanamycin resistance, and recombination was confirmed by restriction endonuclease analyses. Finally, the linearized recombinant plasmids were transfected into AD-293 cells with FuGENE® HD Transfection Reagent (Promega, USA), in accordance with the manufacturer’s instructions. Biological titers were measured using the Adeno-X rapid titer kit (Clontech, Mountain View, CA) according to the manufacturer’s instructions.

miRNA expression analysis

Total RNA was extracted from HepG2 and Huh7 cell lines using TriPure Isolation Reagent (Roche) following the manufacturer's protocol. Residual DNA was removed with use of RNase-free DNase I (Fermentase) following the manufacturer's protocol. To quantify miR-122a expression, real-time PCR was performed using the TaqMan miRNA assay kit for miR-122a by the Applied Biosystems 7500 Sequence Detection System (Applied Biosystems). The expression of miRNA was defined from the threshold cycle (Ct), and relative expression levels were calculated after normalizing with reference to expression of U6 small nuclear RNA.

In vitro gene expression analysis

HepG2 and Huh7 cells were seeded at 1000 cells/well on 96-well plates. On the following day, the cells were infected with Ad, Ad-tk and Ad-tk-122aT for 1.5 hours at 400 VP/cell. Following 24-hour incubation, 20µL of GCV was added to cells. After 3 days, the viability of cells was measured using a MTT Assay Kit (Promega). Briefly, MTT 10.0 µL (5.0 mg/mL) was added to each well and the cells were incubated at 37 °C for 4 hours. Then, the MTT-containing medium was removed and the formazan crystals formed by living cells were dissolved in 200.0 µL DMSO. Absorbance at 570 nm was determined by a microplate reader (ELx800™, BioTek, Winooski, VT) at test and reference wavelengths of 570 nm (13).

Statistical analysis

For calculation of miR-122a expression fold change, the expression level of miR-122a in each cell line was normalized to that of U6 snRNA, as the internal control. Then, miR-122a expression in Huh7 cell line was compared to the HepG2 cell line (2^-ΔΔCt).

We used samples t-test to calculate statistical significance and data were considered statistically significant if P-values < 0.05.

Results

Establishment of an Adeno vector that carry tandem copies of miR-122a-target sequences

To test the effect of endogeneous miR-122a on transgene expression in hepatic cells, Ad vector was constructed containing miRNA-binding sites for miR-122a (9). HSVtk gene was inserted into the Xhol and EcoRI sites of the Ad vector. Then, four tandem copies of sequences with perfect complementarity to miR-122a were inserted into the EcoRI and BamHI sites of the Ad-tk vector.

Fig. 1B shows that Ad-tk-122aT vector carries HSVtk gene with four tandem copies of miR-122a complementary target sequences in its 3'-UTR. Ad-tk vector containing no miRNA target sequences was used as the control vector in this study (Fig. 1A).

Endogenous level of miR-122a in Huh7 and HepG2 cell lines

miR-122a is highly expressed in the liver. Expression level of miR-122a in two human hepatoma cell lines, Huh7 and HepG2, were evaluated by quantitative RT-PCR. Quantitative RT-PCR analysis
demonstrated that Huh7 cells express higher expression level of miR-122a compared to HepG2 cells (P = 0.000; Fig. 2).

**Figure 2.** Relative expression levels of miR-122a in Huh7 and HepG2 cells as assessed by quantitative reverse transcriptase-PCR. The ratios of expression levels of miR-122a to U6 are shown as relative miR-122a expression levels. Data are presented as mean ± S.E. (n = 3).

**Regulation of HSVtk gene in liver cell lines by miR-122a**

We evaluated the Ad vector containing the miR-122a target sequences for HSVtk/GCV suicide gene therapy in treating cancer. Ad-tk-122aT vector was constructed (Fig. 1B), and the cytotoxicity effect of HSVtk gene was measured in Huh7 and HepG2 cells that express various levels of miR-122a. Huh7 and HepG2 cells were infected with Ad, Ad-tk and Ad-tk-122aT vectors at 400 VP/cell, and exposed to 20 µL/mL of GCV for 3 days. The viability of cells in each group was measured by MTT assay. As shown in Fig. 3, HepG2 cells, but not Huh7 cells, were sensitive to GCV after infection with Ad-tk-122aT. The viability of Huh7 cells and HepG2 cells after infection with Ad-tk-122aT vector was 83% and 23.5%, respectively (P = 0.003; Fig. 3). The cytotoxicity effect of HSVtk/GCV was similar in Huh7 cells and HepG2 cells infected with Ad-tk vector, with 35.3% and 27% viability, respectively (P = 0.796; Fig. 3). No cytotoxicity effect was demonstrated in either cell type after infection with the Ad vector (P = 0.862; Fig. 3).

**Figure 3.** Cytotoxicity of HSVtk/GCV in Huh7 and HepG2 cells transfected by different Ad vectors (Ad, Ad-tk and Ad-tk-122aT). Cytotoxicity was measured by MTT assay kit. Ad: adenovector; Ad-tk: adenovector with herpes simplex virus thymidine kinase gene; Ad-tk-122aT: adenovector with miR-122a target sequences in 3′-UTR region of herpes simplex virus thymidine kinase gene. Data are presented as mean ± SE (n = 3).

**Discussion**

The potential of HSVtk/GCV suicide gene therapy by Ad vectors has been widely reported (4). However, an important problem of this kind of therapy is the toxicity of the suicide gene to healthy tissue. So, regulation of suicide gene expression is required to increase the efficacy and safety of gene therapy (5, 9). Our study was conducted to evaluate the efficacy of using a miRNA-regulated transgene expression network for HSVtk gene in hepatoma cell lines infected with Ad vectors. Much consideration has been focused on using microRNAs to control gene expression. miRNA regulation has several advantages over transcriptional targeting, and a recent strategy consists of engineering of the vector that contains miRNA target (miRT) sequences which can be identified and regulated by...
cellular miRNAs (5, 9, 14). Because of the highly differential expression of many miRNAs between normal and malignant tissues, cellular miRNAs have been used to target gene therapy vectors with use of tumor suppressor miRNAs which are down-regulated in cancer cells (8, 15-17). Thus, insertion of tumor suppressor target sequences into a vector can limit the expression of transgene in normal cells, while the expression of transgene remain in tumors that lack these miRNAs; so, targeting by microRNAs could reduce toxicity to normal tissues and provide a new way of giving specificity to cancer tissues (4, 18, 19). The miR-122a is a liver specific miRNA, and down-regulated in liver cancer cells (12). Based on these detections, four tandem copies of miR-122a target elements were inserted into the 3′-UTR region of HSVtk gene (Fig. 1B) to regulate HSVtk gene in hepatoma cell lines by Ad vectors (20, 21).

Human hepatoma cell lines, Huh7 and HepG2, expressing different levels of miR-122a (Fig. 2) were chosen as models in this study. The copy number of miRNA binding sites in the 3′-UTR region was based on previously published paper, which showed that almost four copies of miRNA binding sites work better than two or one copies. Insertion of the miR-122a target sequences remarkably decreased the cytotoxicity of HSVtk/GCV in Huh7 cell line (Fig. 3); however, no suppression cytotoxicity was observed in the HepG2 cell line. Differences in miRNA expression pattern have been shown to separate transgene expression between intimate cellular lineages; however, the extension of specific applications of these elements to cell and gene therapy will require further tests in suitable experimental models. A main relevance in using the miR-122a-regulated expression system is its potential effect on endogenous targets of miR-122a such as the cationic amino acid transporter-1 gene (17, 22, 23). The effect of miR-122a on levels of endogenous targets of miR-122a were not determined in hepatoma cell lines in this study; however, it is possible that employment of miR-122a to the target sequences incorporated into the HSVtk gene causes a loss of regulation of natural miR-122a targets. Recent studies propose that miRNA-regulated expression of natural targets is not disturbed by large amounts of miRNA target (21). In addition, a feedback loop may be present that increases the expression of miRNAs in response to increasing target elements (22, 24). Further studies will be needed to distinguish this effect. Nevertheless, it is clear that miRNAs can provide a strong method to regulate the expression of a transgene. Here, we demonstrated a model in suicide gene therapy for cancer mediated by Ad vector. In addition, this method has the potential to raise the safety and efficacy of Ad vectors but also as part of other therapeutic strategies.

Acknowledgment
The authors would like to acknowledge Hidde Haisma. This investigation was supported by Baqyatallah University of Medical Sciences, (Grant No. 44-1-145-3597).

Conflict of interests
The authors declare that they have no conflict of interest.

References
1. Zarogoulidis P, Chatzaki E, Hohenforst-Schmidt W, Goldberg EP, Galaktidou G, Kontakiotis T, et al. Management of malignant pleural effusion by suicide gene therapy in advanced stage lung cancer: a case series and literature review. Cancer gene ther 2012;19:593-600.
2. Eager RM, Nemunaitis J. Clinical development directions in oncolytic viral therapy. Cancer gene ther 2011;18:305-17.
3. Khare R, Y Chen C, A Weaver E, A Barry M. Advances and future challenges in adenoviral vector pharmacology and targeting. Curr Gene Ther 2011;11:241-58.
4. Ginn SL, Alexander IE, Edelstein ML, Abedi MR, Wixon J. Gene therapy clinical trials worldwide to 2012-an update. J Gene Med 2013;15:65-77.
5. Duarte S, Carle G, Faneca H, De Lima MCP, Pierrefrite-Carle V. Suicide gene therapy in cancer: where do we stand now? Cancer Lett 2012;324:160-70.
6. Kelly EJ, Russell SJ. MicroRNAs and the regulation of vector tropism. Mol Ther 2008;17:409-16.
7. Bader AG, Brown D, Stoudemire J, Lammers P. Developing therapeutic microRNAs for cancer. Gene Ther 2011;18:1121-6.
8. Gentner B, Naldini L. Exploiting microRNA regulation for genetic engineering. Tissue Antigens 2012;80:393-403.
9. Amendola M, Giustacchini A, Gentner B, Naldini L. A double-switch vector system positively regulates transgene expression by endogenous microRNA expression (miR-ON vector). Mol Ther 2013;21:934-46.
10. Cheng CJ, Slack FJ. The duality of oncomiR addiction in the maintenance and treatment of cancer. Cancer J 2012;18:232.

11. Geisler A, Jungmann A, Kurreck J, Poller W, Katus HA, Vetter R, et al. microRNA122-regulated transgene expression increases specificity of cardiac gene transfer upon intravenous delivery of AAV9 vectors. Gene Ther 2011;18:199-209.

12. Bandiera S, Pfeffer Sh, Baumert TF, Zeisel MB. miR-122-A key factor and therapeutic target in liver disease. J Hepatol 2015;62:448-57.

13. Ghanbari Safari M, Hosseinkhani S. Lipid composition of cationic nanoliposomes implicate on transfection efficiency. J Liposome Res 2013;23:174-86.

14. Suzuki T, Sakurai F, Nakamura S-i, Kouyama E, Kawabata K, Kondoh M, et al. miR-122a-regulated expression of a suicide gene prevents hepatotoxicity without altering antitumor effects in suicide gene therapy. Mol Ther 2008;16:1719-26.

15. Kutay H, Bai S, Datta J, Motiwala T, Pogribny I, Frankel W, et al. Downregulation of miR-122a in the rodent and human hepatocellular carcinomas. J Cell Biochem 2006;99:671-8.

16. Akerblom M, Sachdeva R, Quintino L, Wettergren EE, Chapman KZ, Manfre G, et al. Visualization and genetic modification of resident brain microglia using lentiviral vectors regulated by microRNA-9. Nat Commun 2013;4:1770.

17. Brown BD, Venneri MA, Zingale A, Sergi LS, Naldini L. Endogenous microRNA regulation suppresses transgene expression in hematopoietic lineages and enables stable gene transfer. Nat Med 2006;12:585-91.

18. Jopling C. Liver-specific microRNA-122: Biogenesis and function. RNA Biol 2012;9:137.

19. Xie J, Xie Q, Zhang H, Ameres SL, Hung J-H, Su Q, et al. MicroRNA-regulated, systemically delivered rAAV9: a step closer to CNS-restricted transgene expression. Mol Ther 2011;19:526-35.

20. Brown BD, Gentry B, Cantore A, Colleoni S, Amendola M, Zingale A, et al. Endogenous microRNA can be broadly exploited to regulate transgene expression according to tissue, lineage and differentiation state. Nat Biotechnol 2007;25:1457-67.

21. Safari MG, Baesi K, Hosseinkhani S. An alternative approach in regulation of expression of a transgene by endogenous miR-145 in carcinoma and normal breast cell lines. Biotechnol Appl Biochem 2017;64:244-50.

22. Chang J, Nicolas E, Marks D, Sander C, Lerro A, Buendia MA, et al. Research Paper miR-122, a Mammalian Liver-Specific microRNA, is Processed from mRNA and May Downregulate the High Affinity Cationic Amino Acid Transporter CAT-1. RNA Biol 2004;1:106-13.

23. Girard M, Jacquevin E, Munnich A, Lyonnet S, Henrion-Cau de A. miR-122, a paradigm for the role of microRNAs in the liver. J Hepatol 2008;48:648-56.

24. Iino I, Kikuchi H, Miyazaki S, Hiramatsu Y, Ohta M, Kamiya K, et al. Effect of miR-122 and its target gene cationic amino acid transporter 1 on colorectal liver metastasis. Cancer Sci 2013;104:624-30.