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Targeted retardation of hepatocarcinoma cells by specific replacement of alpha-fetoprotein RNA

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Abstract

Although hepatocellular carcinoma (HCC) is one of the world-wide common malignancies, development of more specific and controlled therapeutic methods should be warranted. In this study, we describe a novel approach to HCC therapy that is based on *trans*-splicing ribozyme-mediated replacement of HCC-associated specific RNAs. We have developed a specific ribozyme that can target and replace human alpha-fetoprotein (AFP) RNA, which is highly expressed in HCC, with new transcript exerting therapeutic activity selectively in AFP-expressing liver cancer cells. The RNA replacement was employed via a high-fidelity *trans*-splicing reaction with the targeted residue in the AFP-expressing cells. Noticeably, the ribozyme could selectively deliver activity of suicide gene, herpes simplex virus thymidine kinase gene, into the liver cancer cells expressing the AFP RNA and thereby specifically and effectively retarded the survival of these cells with ganciclovir treatment. Simultaneously with the specific induction of therapeutic gene activity, the ribozyme reduced expression level of the targeted AFP RNA in the cells. These results suggest that the AFP RNA-targeting *trans*-splicing ribozyme could be a useful genetic agent for HCC-targeted efficient gene therapy.

Keywords: Alpha-fetoprotein; Hepatocarcinoma; Group I intron; *Trans*-splicing ribozyme; RNA replacement; Cancer gene therapy

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies in the world, generally followed by liver cirrhosis or chronic infection with hepatitis B or C virus (Hussain et al., 2001). A number of strategies of such as surgery, chemotherapy, radiation, and liver transplantation have been utilized for the HCC treatment, however, most of the therapeutic treatments are related to significant side effects (Bruix, 1997). To overcome these clinical problems, gene therapy has been considered as a new therapeutic approach to treat the HCC.

Alpha-fetoprotein (AFP) is classified as a member of the albuminoid gene family consisting of albumin (ALB), vitamin D protein, AFP, and alpha-ALB. AFP is a tumor-associated fetal protein produced by the fetal liver and yolk sac. The biological function of AFP is known to bind and transport of numerous ligands such as bilirubin, fatty acids, retinoids, steroids, heavy metals, dyes, flavonoids, phytoestrogens, dioxin, and various drugs (Mizejewski, 2001). However, the expressional level of AFP is highly elevated in HCC and germ cell tumors (Otsuru et al., 1988; Abelev and Eraiser, 1999). Therefore, AFP has been considered as one of the most important markers in the diagnosis and targeting of HCC (Taketa, 1990). Moreover, AFP may function as a direct or indirect factor associated with hepatoma growth (Wang et al., 2001). Thus, a number of attempts to develop specifically AFP-targeting cancer therapeutics such as AFP-anisense, immunogene therapy and AFP-promoter system have recently been proposed (Vollmer et al., 1999; Wang et al., 2001; Uch et al., 2003). However, specific targeting of AFP-expressing tumor cells combined with AFP inhibition will be one of more effective anti-HCC approaches.

Tetrahymena group I intron could target and cleave a substrate RNA and *trans*-splice an exon attached at its 3′ end onto the cleaved target RNA in mammalian cells as well as in bacteria (Sullenger and Cech, 1994; Jones et al., 1996). The *trans*-splicing ribozyme has been developed to revise mutant transcripts associated with several human genetic and malignant diseases (Lan et al., 1998; Phylactou et al., 1998; Rogers

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et al., 2002; Kastanos et al., 2004; Shin et al., 2004). Moreover, we demonstrated that the ribozyme could selectively induce antiviral gene activities in HCV RNA-containing cells through the specific RNA replacement of the HCV RNA (Ryu et al., 2003). Furthermore, we have recently shown that group I-based ribozymes could replace cancer-specific transcripts such as human telomerase reverse transcriptase RNA or carcinoembryonic antigen transcript with RNA exerting cytotoxic activity, and thus the ribozymes could specifically regress cancer cells expressing the target RNAs (Kwon et al., 2005; Jung and Lee, 2006). This RNA replacement approach would be a more attractive approach for cancer therapy because it should inhibit or reduce the expression of the target RNA and simultaneously produce therapeutic gene activity selectively in the target RNA-associated cancer cells.

In this study, we describe the AFP-targeting specific *trans*-splicing ribozyme as a new HCC-specific targeting agent, which triggers the expression of therapeutic gene, and thus induces retardation of cell survivability, effectively and selectively in AFP-expressing liver cancer cells through the targeted RNA replacement.

2. Materials and methods

2.1. Mapping AFP transcript

The mapping ribozyme library was constructed by randomizing the internal guide sequence (IGS) as previously described (Lan et al., 1998; Kwon et al., 2005). For *in vitro* mapping, 50 nM of the ribozyme library was incubated with full length AFP transcripts (500 nM) that were *in vitro* transcribed from an AFP cDNA clone in a splicing reaction buffer at 37 °C for 3 h. For intracellular mapping, 1 pmole of the ribozyme library was cotransfected with 1 pmole of the full length AFP RNAs into non AFP-expressing MCF7 cells using 4 μl of DMRIE-C reagent (Invitrogen). The resulting *trans*-splicing products were then reverse-transcribed and amplified with a 3′ tag primer specific for the ribozyme's 3′ exon and a 5′ primer specific for the target AFP RNA (5′-CCGGAATTCTAATACGACTCACTATAGGGATATTGTGCTTCCACCACTGC-3′), and sequenced to identify sequences around the spliced Us of the substrate.

2.2. Ribozyme construction

Rib−20, Rib−8, Rib+2, Rib+11, Rib+94, Rib+162 ribozymes were constructed by *in vitro* transcription of DNA templates generated from pT7L-21 encoding a slightly shortened version of the natural *Tetrahymena* group I intron (Sullenger and Cech, 1994) using PCR with a 5′ primer harboring the T7 promoter and each ribozyme's IGS and with a 3′ primer specific for the 3′ exon sequence. The IGS on the L-21 *trans*-splicing ribozyme (5′-GGAGGG-3′) was exchanged with 5′-GTTGGC-3' in Rib−20, 5′-GGTTGT-3′ in Rib−8, 5′-GTGGTT-3′ in Rib+2, 5′-GCCCAC-3′ in Rib+11, 5′-GTATGG-3′ in Rib+94, or 5′-GTTCTG-3′ in Rib+162. Inactive control was generated by deletion of the catalytic center of each ribozyme, as described (Sullenger and Cech, 1994).

To construct pRib+2-Fluc, the Rib+2 was modified by insertion of synthesized complementary oligonucleotides containing an extended P1 plus a 6-nt-long P10 helix upstream to the ribozyme's IGS and introduction of firefly luciferase (Fluc) cDNA in frame as the 3′ exon of the ribozyme. The DNA fragment consisting of Rib+2 sequence with the extended IGS plus Fluc cDNA was inserted between the *Hin*dIII and *Not*I sites of pcDNA3.1(−) encoding transgene under the CMV promoter (Clontech). The pRib+2AS-Fluc was constructed via the insertion of a 300-nt-long antisense sequence complementary to the downstream region (+11 to +310 residue) of the targeted uridine of the AFP RNA into the *Hin*dIII site of pRib+2-Fluc. An inactive control [R(d)+2AS-Fluc] was generated. pRib+2-TK and pRib+2AS-TK were created by replacement of Fluc cDNA present in pRib+2-Flu and pRib+2AS-Fluc, respectively, with herpes simplex virus thymidine kinase (HSV-*tk*) cDNA. pCMV-Fluc and pCMV-TK encode the cDNA of Fluc and the HSV-*tk* gene, respectively, under the control of the CMV promoter.

2.3. Cell cultures

Human hepatocarcinoma Huh7 cells and gastric adenocarcinoma AGS cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS). Human hepatocarcinoma HepG2 cells were maintained in EMEM (Invitrogen) supplemented with 10% FBS. Human breast adenocarcinoma MCF cells were grown in RPMI 1640 (Invitrogen) with 10% FBS. All cells were purchased from ATCC (American Type Culture Collection).

2.4. Analysis of trans-splicing reaction in cells

For the analysis of *trans*-splicing products in cells, total RNA was isolated from ribozyme vector-transfected cells 24 h after transfection with guanidine isothiocyanate supplemented with 20 mM EDTA. RNA (5 μg) was reverse transcribed with a oligo(dT) primer in the presence of 10 mM L-argininamide, and the resulting cDNA was amplified with a 5′ primer specific for the AFP RNA and with a 3′-primer specific for the 3′ exon Fluc sequence (5′-GCGCAACTGCAACTCCGATAA-3′) or specific for the 3′ exon for TK sequence (5′-CAGTAGCGTGGGCATTTTCT-3′). The amplified cDNA was then cloned, and sequenced.

2.5. Ribozyme assay in cells

For the assay of suicide HSV-*tk* gene activity, AFP+ HepG2 and Huh7 cells or AFP− MCF7 and AGS cells were mock-transfected or transfected with 200 ng of pEGFR-N1 (Clontech) along with 3 μg of pRib+2AS-Fluc, pRib+2-TK, pRib+2AS-TK, or pCMV-TK. The transfection reagent for each cell is as follows: HepG2, MCF7, and AGS cells, ExGen^{TM} 500 (Fermentas); Huh7, WelFect-M^{TM} GOLD (JBI). The next day after transfection, the cells were treated with 100 μM of ganciclovir (GCV; Cymevene®, Roche). After 2 days of GCV treatment,