

Development of HVJ-Liposome Mediated Gene Therapy Using HSV-Thymidine Kinase Gene for Hepatocellular Carcinoma

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The suicide gene therapy for hepatocellular carcinoma (HCC) has given some promise, but the toxicity of adenovirus-mediated gene delivery using the cytomegalovirus promoter (CMV) region linked to herpes simplex virus thymidine kinase gene (HSV-TK) in combination with ganciclovir (GCV) therapy was reported. In this study, to improve the technical issue of conventional gene therapy, we examined the usefulness of hemagglutinating virus of Japan (HVJ)-anionic-liposome-mediated CMV-TK/GCV as an introductory target gene and development the selective application of alpha-fetoprotein (AFP) enhancer/promoter or heat shock protein (HSP) promoter for gene therapy.

By the luciferase reporter gene assay, both HVJ-liposome transfection and the transcriptional activation of AFP enhancer/promoter or that of the HSP promoter by heat treatment were found to be quite effective *in vivo* and *in vivo/in vitro* models respectively. The toxicity of HVJ-anionic liposome-mediated gene therapy was smaller than that of adenovirus-driven approach as judged by histopathological examination of experimental animals' liver and GPT blood test. Thus, the HVJ-liposome-mediated AFP-TK/GCV or HSP-TK/GCV technique may be a potent and useful strategy of the gene therapy of HCC.

Key Words: suicide gene therapy, HVJ-anionic-liposome, cytomegalovirus thymidine kinase, toxicity, hepatocellular carcinoma, targeting

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Introduction

Hepatocellular carcinoma (HCC) is one of major malignancies with poor prognosis and accounts for approximately 1,250,000 annual cancer deaths in the world^{1,2)}. For patients with advanced HCC, gene therapy may provide a potential treatment modality to prolong survival and improve quality of life, because of the limited efficiency of therapy and present therapeutics^{3–5)}.

In cancer gene therapy, HSV-tk converts the nontoxic GCV to a highly toxic phosphorylated-GCV that acts as a chain terminator of DNA synthesis and an inhibitor of DNA polymerase^{6–8)}. Advantages of the HSV-tk system include lower toxicity for normal non-dividing cells and a bystander effect, in which HSV-tk-negative tumor cell death may be caused by neighboring HSV-tk-positive tumor cells exposed to GCV⁹⁾. Retrovirus- and adenovirus-mediated HSV-tk/GCV treatment has been studied in HCC^{10–13)}, and the adenovirus approach was found to exhibit greater gene transfer efficiency than retrovirus-mediated one. However, it may provoke acute hepatitis and severe hepatotoxic side effects during the course^{14–17)}.

Therefore, molecular targeting and safety vectors are utmost importance for the purpose of side effects reduction in suicide gene therapy¹⁸⁾. Tissue- or tumor-specific promoters offer an attractive approach from this point of view as such promoters are able to express a target gene in cell-type specific manner^{19–21)}. Gene transduction efficiency using HVJ-liposome system in various tissues has been reported, and its associated low immunogenicity facilitates further investigation^{22,23)}.

In this view, we estimated the toxicity of CMV-TK/GCV therapy using HVJ-anionic-liposome method *in vivo* based on our experimental results, and further discuss the applicability of AFP-enhancer/promoter or HSP promoter for gene therapy of HCC.

Materials, methods and results

Cell lines and animals

Three hepatoma cell lines with different AFP-producing characteristics, HuH7 is high AFP producer, HepG2-moderate, PLC/PRF/5-low and Lovo cells (colon carcinoma cell line, does not produce AFP) were used in the study. Experimental animals were male BALB/C-nu/nu mice.

Preparation of HVJ-liposome

Hemagglutinating virus of Japan (HVJ)-liposome for *in vivo* experiment was prepared as described before²⁴. Phosphatidylserine (Avanti Polar Lipids, Inc., Alabama), phosphatidylcholine (Sigma Chemical Co., St. Louis, MO) and cholesterol (Sigma) were mixed in a weight ratio of 1:4.8:2. The lipid mixture (10 mg) was transferred into a glass tube and dried to form a fine layer in a rotary evaporator (Iwaki Glass, Tokyo, Japan), and then hydrated in 200 μ l of balanced salt solution (BSS; 137 mM NaCl, 5.4 mM KCl, 13 mM Tris-HCl, pH 7.6) containing DNA-high mobility group I complex, preincubated at 20 $^{\circ}$ C for 1 h. The mixture was vortexed for 30 sec and then left to stand for 30 sec, the procedure was repeated eight times, and liposomes were finally formed by sonication, then incubated at 37 $^{\circ}$ C for 30 min with shaking at 120 per minute. In the next step, 10,000 hemagglutinating units (HAU) of purified HVJ inactivated by ultraviolet irradiation (1980 J/m²) were slowly added to the liposome suspension at 4 $^{\circ}$ C during 10 min, then incubated at 37 $^{\circ}$ C for 60 min with shaking. Free HVJ was removed from the HVJ-liposome solution by gradient sucrose density cen-

trifugation. The second layer of sucrose containing the HVJ-liposome was collected.

Promoter assay *in vitro*

To estimate the efficiency of human AFP-enhancer/promoter, its transcriptional activity was examined *in vitro* by means of dual luciferase reporter assay in three hepatoma cell lines and Lovo cells using the renilla luciferase/cytomegalovirus promoter system as a reference. The AFP-enhancer/promoter activity in all cell lines was increased compared to that in Lovo cells: 858-fold in HuH7, 486-fold in HepG2, and 76-fold in PLC/PRF/5 (Fig. 1(A)) (Miki et al., unpublished data).

The transcriptional activation of humane HSP promoter was measured 6 h after heat treatment cells (43 $^{\circ}$ C for 30min) and found to be elevated to 156-, 662-, and 59- times compared to in PLC/PRF/5, HepG2 and HuH7 cells, respectively (Fig. 1(B)).

In vitro gene therapy

AFP-tk gene was transferred into three different AFP producing hepatoma cell lines and Lovo cells using the HVJ-cationic-liposome²⁴. Each cell line was subjected to the GCV-sensitivity test using 10 μ M using mock gene-transfected cells as a control. The survival of Lovo, PLC/PRF/5, HepG2 and HuH7 cells was 100%, 72%, 44%, and 35%, respectively (Fig. 2A) (Miki et al., unpublished data). The killing effect of AFP-tk gene therapy was increased in prorated manner in cell lines according to their ability to produce AFP. Experiments with transferred HSP-tk gene were performed similarly to describe above. Cell survival of PLC/PRF/5, HepG2

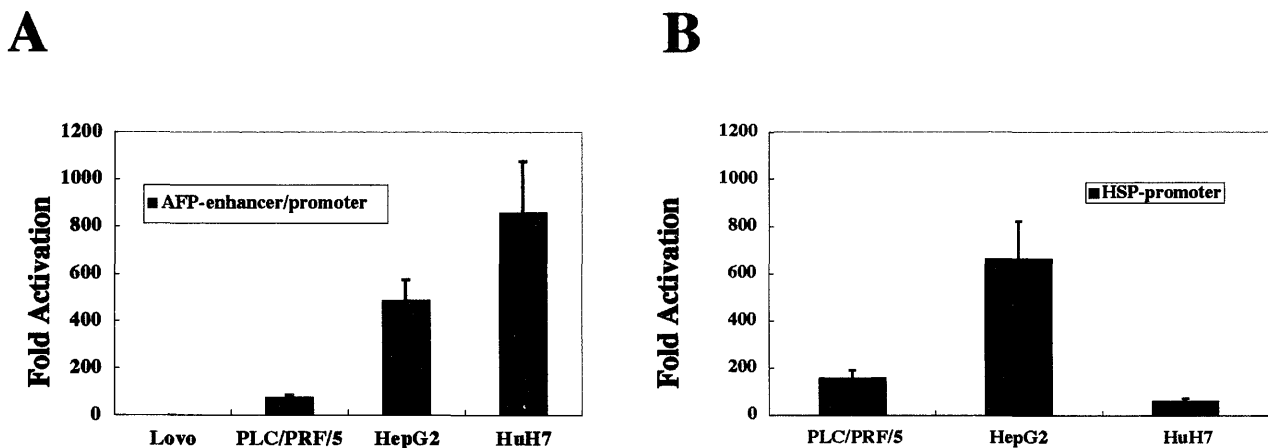


Fig. 1. (A) AFP-enhancer/promoter activity in three-hepatoma cell lines and Lovo cells. Data represent fold activation of transcription activity compared to AFP-enhancer/promoter activity in Lovo. (Mean \pm SEM, n=4).

(B) HSP-promoter activation in three-hepatoma cell lines by heat shock at 43 $^{\circ}$ C for 30min. After heat shock, cells were incubated for 6h at 37 $^{\circ}$ C and harvested for luciferase assay. Fold activation is expressed as a relative luciferase activity for each cell line without heat treatment. (Mean \pm SEM, n=3).

and HuH7 were 59%, 35%, and 64%, respectively (Fig. 2B).

Promoter assay in vivo

To estimate the transfectional efficiency of HVJ-anionic-liposome, the renilla luciferase gene driven by the cytomegalovirus promoter was used. Fig. 3A demonstrated that the reporter gene was transfected not only into the tumor tissue but also into organs. However, after the heat treatment (43°C for 30min), significant induction of HSP-promoter activity *in vivo* was observed mostly in the tumor, and less efficiency in other organs (Fig. 3B). And significant induction of AFP-enhancer/promoter activity *in vivo* was observed mostly in the tumor, and less efficiency in other organs (data not shown).

In vivo experiment

The nude mice implanted with HCC were prepared as previously described²⁵. These animals implanted with HCC (n=2) and without HCC (n=3) were injected intraperitoneally with HVJ-anionic-liposome containing CMV-tk (40µg) on day 0. GCV administration (20 mg/kg injected intraperitoneally) was initiated daily for 10 days.

Number of white blood cell (WBC) count, serum level of blood urea nitrogen (BUN) and serum glutamic-pyruvic transaminase (GPT) activity were measured at day 0, day 4, day 7 and day 10. To evaluate the toxicity of gene therapy, animals were sacrificed at 10 days after the end of GCV treatment and examined histopathologically.

One of two male nude mice implanted with HuH7 cells developed bloody ascites. WBC count was 1,000/µl, BUN

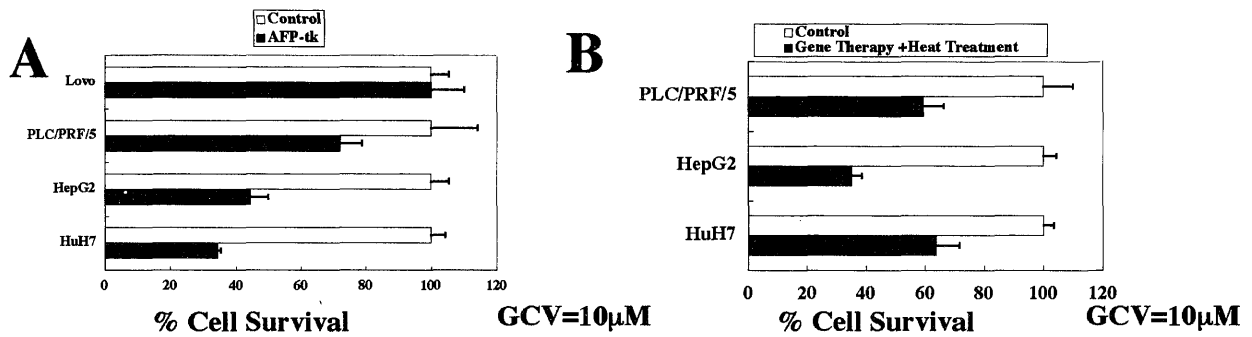


Fig. 2. Cell survival studies: (A) Lovo, PLC/PRF/5, HepG2, and HuH7 treated with GCV=10µM. In vitro gene therapy was carried out as follows. Group I; mock gene transfection as a control, Group II; AFP-tk transfection. Mock gene or AFP-tk plasmids were introduced into the cells using the HVJ-cationic liposome. (B): PLC/PRF/5, HepG2, and HuH7 treated with GCV=10µM. Group I, mock gene-transfected cells as a control; Group II, HSP-tk-transfected cells by heat treatment (43°C 30min). With 10µM GCV, percent survival in each group was standardized to cell number of Group I as a control (100%). (Mean±SEM, n=3)

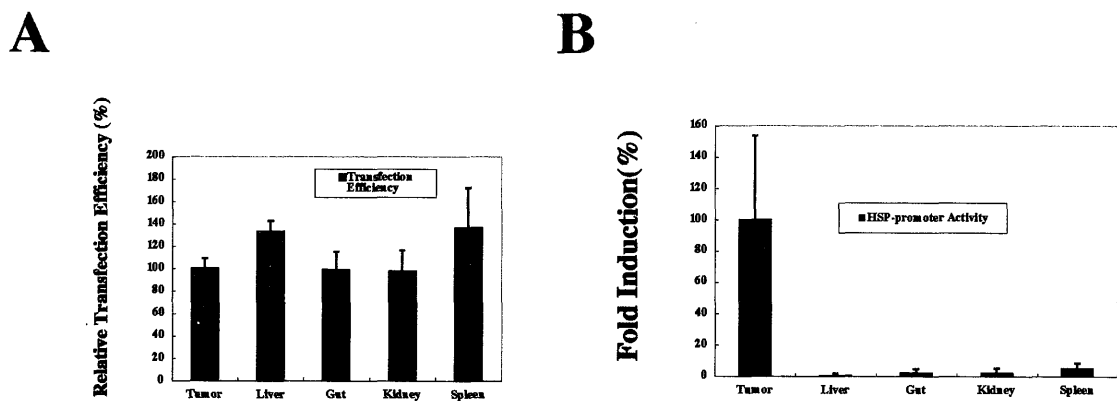


Fig. 3. (A): The transfection efficiency by HVJ-anionic-liposome. Fold activation was standardized to the luciferase activity in tumor (100%). Reporter gene expression in liver, gut, kidney, and spleen was 133%, 99%, 98%, and 136%, respectively. (B): HSP-promoter activation *in vivo* treated with heat shock at 43°C 30min. Six hour after heat shock, luciferase assay was performed (n=3). Fold induction was standardized to the luciferase activity in tumor (100%). Each data point represents mean±SEM

was 32 (mg/dl) and serum GPT activity was 79 (I.U./l). The animal died 4 days after the beginning of GCV treatment. Pathological examination showed that liver had HCC tumor invasion and several areas of inflammation (Figure 5A).

Blood test data from another mouse implanted with HCC cells are shown in Fig. 4A. WBC count and serum level of BUN were slightly increased after the gene therapy, while serum GPT activity increased up to 200 (I.U./l) 7 days after GCV treatment. Blood test data from control mice are shown in Fig. 4B. Changes of WBC count and serum levels of BUN were not remarkable, and serum GPT activities were not elevated after the gene therapy.

Histological pictures of liver tissue of a HCC implanted nude mouse, a nine-month-old nude mouse, and a five-week-old nude mouse is shown in Figure 5B, 5C, and 5D, respectively. In all areas examined, inflammation was moderate as shown in Figure 5B or minimal Figure 5C, 5D. The observations were consistent with the toxic effect of GCV rather than with the consequence of transfection.

AFP-tk gene therapy was effective as same as the CMV promoter-mediated HSV-tk approach. Both of methods suppressed tumor growth of same extent (Table 1) (Miki et al., unpublished data). This facility suggests the limitation of the enhancement of promoter activity in suicide gene therapy of HCC.

Discussion

In the present study, we estimated the AFP-tk or HSP-tk suicide gene therapy for HCC, examples of molecular targeting, under the control of human AFP gene enhancer/promoter or human HSP gene promoter. In the initial part of the study, we confirmed the transcriptional activity of AFP-enhancer/promoter and HSP-promoter. Transcriptional activity of human AFP-enhancer/promoter in hepatoma cell lines was increased to 76- to 858-fold more than that in Lovo non-AFP producing cells. The transcriptional activation of humane HSP promoter found to be elevated to 59- to 662-times 6 h after heat treatment cells (43°C for 30min) compared to in PLC/PRF/5, HepG2 and HuH7 cells, respectively.

Our results of gene therapy studies demonstrated that AFP-tk or HSP-tk transduced hepatoma cells were sensitive to GCV corresponding to transcriptional activation of human AFP-enhancer/promoter or humane HSP promoter, respectively. Thus, HSP promoter or AFP-enhancer/promoter seems to be feasible for molecular targeting of HCC.

Serum glutamic-pyruvic transaminase (GPT) activity was measured as an indicator of liver dysfunction, since GPT is found primarily in the liver and released from damaged hepacytes. The recent study²⁶⁾ have demonstrated that serum transaminase levels were elevated 8- to 16-fold above normal 7 days after the beginning of Adenovirus- β -gal (Ad β -gal) administration. Moreover, liver histopathology showed that severe hepatocellular necrosis with a markedly inflammatory

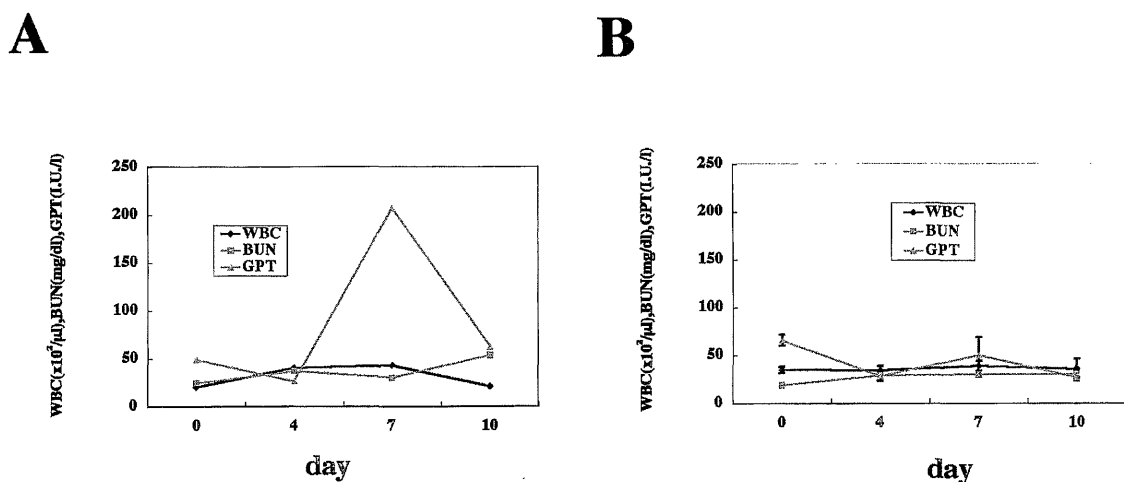


Fig. 4. (A): Blood test data from the mouse implanted with HuH7 cells. WBC count and BUN level were slightly elevated after the gene therapy, serum GPT activity increased up to 200 (I.U./l) 7 days after the CMV/GCV treatment. (B): Blood test data from eleven-month male nude mice (n=3). WBC counts, serum levels of BUN, and serum GPT activity were not found to be elevated after the gene therapy. Male nude mice were injected intraperitoneally with HVJ-anionic-liposome containing CMV-tk (40 μg) (day 0). Ganciclovir was administered intraperitoneally 20 mg/kg daily. To evaluate the side effects of the therapy, WBC count, BUN level and serum GPT activity were measured on day 0, day 4, day 7 and day 10. Each data point represents the mean ± SEM.

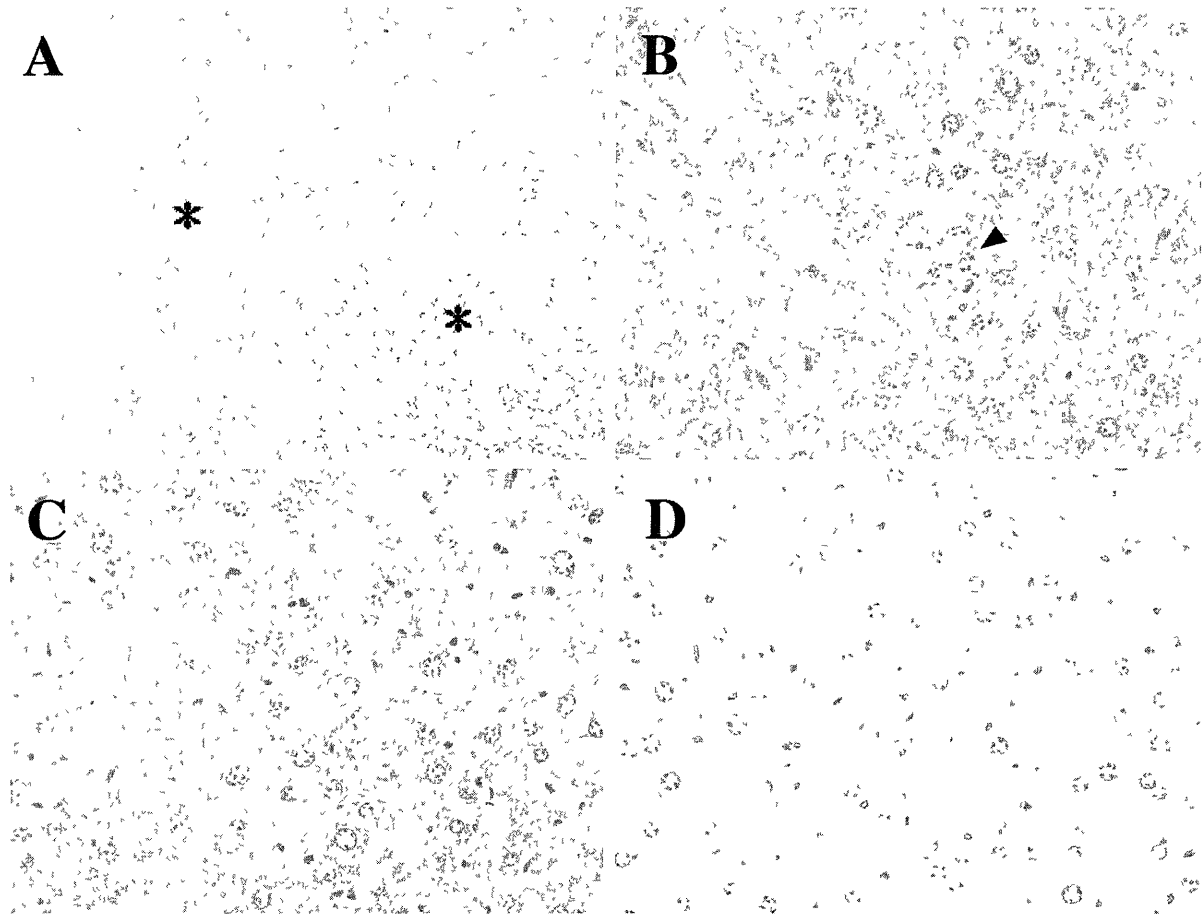


Fig. 5 Nude mice implanted with HuH7 cells had bloody ascites and died 4 days after the beginning of CMV/GCV treatment. Hematoxylin-eosin stained liver (A) sections (x100). Some inflammatory cells and many of HuH7 cells were observed (*) in liver section. Hematoxylin-eosin stained liver section of eleven-month-old nude mouse implanted with HCC cells (B) and of normal eleven-month-old nude mouse (C) (x400). The animals were sacrificed 10 days after the CMV/GCV treatment. Hematoxylin-eosin stained liver section in five-week-old nude mouse without HuH7 tumor (D) (x400). The animal implanted with HCC cells had some inflammatory cells (arrow in B) in liver, whereas control animals did not (C and D).

Table 1. Tumor growth in nude mice implanted with HuH7. Mice used in these experiments were divided into three groups: Group I, mock gene transfection (n=8), Group II, AFP-tk transfection (n=11), and Group III, CMV-tk transfection (n=7).

% Original Tumor Size

Time(Days)	0	9	18
Control	100	488 ± 140	2793 ± 743
AFP-tk	100	121 ± 14	239 ± 49
CMV-tk	100	140 ± 15	206 ± 39

*, **, P < 0.001

infiltrate in mice treated with Adβ-gal²⁶⁾. On the other hand, after HVJ-liposome-mediated β-gal administration, GPT activities were not elevated²⁷⁾. In this study, after HVJ-liposome-mediated CMV TK/GCV gene therapy, GPT activities were not elevated and liver histopathology showed inflammation was small. Thus, the toxicity of

HVJ anionic liposome mediated gene therapy is lower than that of adenovirus mediated one.

Adenovirus-mediated gene therapy may be accompanied by the development of immunity, besides side effect. In accordance with this, its effect becomes restricted to regression of local tumor in clinical trials for cancer patients. In contrast, HVJ-liposome is constructed from inactivated envelopes of the Sendai virus and liposome, therefore HVJ-liposome delivery system has low immunogenicity and is enabling repeated administrations^{22, 23)}.

Gene transfer using HVJ-liposome transfects not only tumor tissue but also various normal organ tissues. However, significant induction of HSP-promoter or AFP-enhancer/promoter activity *in vivo* was observed mostly in the tumor, and less efficiency in other organs. AFP-tk gene therapy with HVJ liposome delivery system has

similar efficiency as CMV/HSV-tk in terms of tumor suppression.

Hence, hepatoma specific promoter, seems to be applicable for gene therapy of HCC in humans. This strategy, represents an improvement of conventional gene therapy, and contributes to an inducible and cell-specific gene therapy for advanced HCC.

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