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Gene therapy for hepatocellular carcinoma based on tumour-selective suicide gene expression using the alpha-fetoprotein (*AFP*) enhancer and a housekeeping gene promoter

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Abstract

The aim of this study was to examine whether the human α-fetoprotein (*AFP*) enhancer could be used to induce hepatocellular carcinoma (HCC)-selective expression of the herpes simplex virus thymidine kinase (HSV-*tk*) gene which is under the control of the phosphoglycerate kinase (*pgk*) promotor. The human *AFP* enhancer was linked with the non-tissue-specific, human housekeeping *pgk* promoter in a retroviral vector. *AFP*-producing HCC cells infected with retroviruses carrying the HSV-*tk* gene under the control of the *AFP* enhancer/*pgk* promoter were much more susceptible to the prodrug, ganciclovir (GCV), than those infected with the same retroviruses without the *AFP* enhancer. Non-HCC cells infected with retroviruses carrying the HSV-*tk* gene under the control of the *AFP* enhancer/*pgk* promoter exhibited profoundly increased resistance to GCV compared with those infected with the same retroviruses without the *AFP* enhancer. Northern blot analysis revealed that the *AFP* enhancer caused enhanced HSV-*tk* expression in AFP-producing HCC cells and suppressed HSV-*tk* expression in non-HCC cells. Our results indicate that the *AFP* enhancer could give HCC selectivity to the *pgk* promoter, and that this novel strategy may be useful for HCC-selective cancer gene therapy.

Keywords: Gene therapy; Hepatocellular carcinoma; Herpes simplex virus *thymidine kinase*; Ganciclovir; Acyclovir; α-fetoprotein enhancer; Phosphoglycerate kinase promoter; Tissue specificity

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common causes of cancer-related deaths worldwide, especially in sub-Saharan Africa and Southeast Asia, including China and Japan. In the majority of cases, HCC is found in conjunction with cirrhosis of the liver. Although radical operation is the only curative modality for HCC, patients suitable for surgery are few because of widespread intrahepatic involvement or limited hepatic reserves resulting from coexisting advanced cirrhosis. Furthermore, underlying liver cirrhosis is not only a major obstacle for successful treatment against HCC, but also a factor promoting intrahepatic invasion and metastasis of HCC [1,2]. Thus, despite intensive efforts in the treatment of HCC, there are still no satisfactory treatment modalities for HCC and the prognosis of the disease remains poor.

Gene therapy could provide an innovative therapeutic approach for the treatment of HCC. Whereas many types of transgene have potential tumoricidal effects, genes encoding for prodrug-activating enzymes, the

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so-called suicide genes, have been the most thoroughly investigated [3]. After genetically modifying tumour cells to express such enzymes, systemic prodrug treatment leads to the selective killing of tumour cells. The effectiveness of suicide gene/prodrug strategies against cancer has been shown in animal models with various types of cancer, and the transfer of suicide genes into tumour cells is currently being used in various clinical gene therapy trials for the treatment of cancer [4]. Among several suicide gene prodrug combinations, the herpes simplex virus thymidine kinase (HSV-*tk*) gene is a prototypic suicide gene, because it can effectively phosphorylate antiviral nucleoside analogues, such as acyclovir (ACV) and ganciclovir (GCV) to their monophosphate form, which are further phosphorylated by cellular kinases to DNA polymerase inhibitors [5,6]. Non-tumour-selective, ubiquitous expression of suicide genes may, however, cause unexpected adverse reactions, such as bone marrow suppression [7–9]. Therefore, a pre-requisite for achieving effective and safe gene therapy against cancer is to induce strong, tumour-selective expression of these suicide genes.

Since tumour-selective receptor-mediated vector delivery systems have not been established, the use of tumour-selective promoters, such as the α-fetoprotein (*AFP*) promoter and the carcinoembryonic antigen (*CEA*) promoter, to direct the expression of therapeutic genes is the most promising alternative. It has been shown that the *AFP* promoter isolated from a human genomic library can induce HCC-selective expression of exogenous genes in human HCC cell lines, in which AFP was highly produced [10–12]. However, the *AFP* promoter has been shown to be weak [11,13]. It is critical for successful gene therapy to induce strong expression of the therapeutic exogenous genes. It has been shown that the human *AFP* enhancer and silencer regions located upstream of the *AFP* gene play a critical role in HCC-selective *AFP* expression [13,14]. In this study, we inserted the human *AFP* enhancer including domains A and B into retroviral vectors, and examined whether the regulatory element could induce HCC-selective expression of the HSV-*tk* gene under the control of the non-tissue-specific, housekeeping phosphoglycerate kinase (*pgk*) promoter.

2. Materials and methods

2.1. Cell lines and cell culture

The human HCC cell line HepG2, human cervical carcinoma cell line HeLa, human colorectal carcinoma cell line LoVo, murine embryo fibroblast cell line NIH3T3TK$^-$ and the amphotropic retroviral-packaging cell line PA317 were purchased from the American Type Culture Collection (Rockville, MD, USA). The human HCC cell line SMMC7721 and human renal cell carcinoma cell line RC9406 were established by the Departments of Pathology and Microbiology of the Second Military Medical University (Shanghai, China), respectively. The human HCC cell line PLC/PRF/5 [15] was kindly provided by Y. Liu (Shanghai Eastern Hepatology Institute, Shanghai, China). The cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, 0.3 mg/ml of L-glutamine, 100 units/ml of ampicillin and 100 μg/ml of streptomycin at 37°C in a humidified 95% air/5% CO_2 atmosphere.

Cells were suspended in the conditioned medium at a concentration of 5×10^4 cells/ml and 2 ml of the cell suspension was seeded into a 35-mm tissue culture dish (1×10^5 cells/dish). Following a 1-day accumulation, AFP secretion into the culture media of cells was measured using an enzyme-linked immunosorbent assay (United Biotech, Mountain View, CA, USA) according to the protocol provided by the manufacturer.

2.2. Plasmids

The pGEM7z-*AFPe* plasmid, which includes the core sequence of the human *AFP* enhancer consisting of domains A and B [14], was kindly provided by T. Tamaoki (University of Calgary, Calgary, Canada). The pBPGK-*tk* plasmid, which contains the expression cassette consisting of the human *pgk* promoter and the HSV-*tk* gene, was a gift from X. Qiu (Fudan University, Shanghai, China). The MNSM retroviral vector (Fig. 1) containing the neomycin phosphotransferase (*neo*) gene and the simian virus 40 (*SV40*) early promoter was kindly provided by T. Tsuchiya (Tokyo Medical and Dental University, Tokyo, Japan).

2.3. Construction of retroviral vectors

The MNSM retroviral vector was digested with *Sal*I to delete the *SV40* early promoter fragment and self-ligated with T4 DNA ligase (Promega, Madison, WI, USA). The resulting retroviral vector without the *SV40* early promoter was linearised by digestion with *Bam*HI and ligated with the 2.8-kb expression cassette consisting of the *pgk* promoter and the HSV-*tk* gene excised from the pBPGK-*tk* plasmid by a *Bam*HI digestion. Direction of the inserted expression cassette was determined by a double digestion with *Hind*III and *Eco*RI. The resultant retroviral vector containing the expression cassette in a sense orientation was designated pMN*pg-tk* (Fig. 1). The 727-bp human *AFP* enhancer fragment was released from the pGEM7z-*AFPe* plasmid by an *Eco*RI digestion and blunt-ended with the Klenow fragment. The *Sal*I site is located immediately upstream of the *pgk* promoter/HSV-*tk* expression cassette of the

pMN*pg-tk* retroviral vector. Therefore, the plasmid was digested by *Sal*I, blunt-ended and ligated with the blunt-ended *AFP* enhancer fragment. Orientation of the inserted *AFP* enhancer fragment was confirmed by a double digestion with *Hind*III and *Bam*HI. The constructs containing the *AFP* enhancer fragment in the sense and reverse orientations were designated MN*AFpg-tkα* and MN*AFpg-tkβ* retroviral vectors, respectively (Fig. 1).

2.4. Recombinant retrovirus production

Retroviral vector constructs were converted to the corresponding retroviruses by a lipofection protocol as previously described [16]. The titres of recombinant viral particles were determined by infecting NIH3T3TK$^-$ cells with serial dilutions of the culture medium collected from G418-resistant cells as previously described [17]. The producing cells of MNSM, MN*pg-tk*, MN*AFpg-tkα* and MN*AFpg-tkβ* retroviruses used for the subsequent experiments had titres of approximately 1.6×10^4, 1.4×10^4, 1.0×10^4 and 0.3×10^4 colony-forming units (cfu)/ml, respectively, as assayed by G418 selection of the infected NIH3T3TK$^-$ cells. The culture supernatants of the retroviral-producing cells were confirmed to be free of replication-competent retroviruses by reverse transcription-polymerase chain reaction (RT-PCR) as previously described [8], and served as sources for infectious recombinant retroviruses.

2.5. Gene transfer into cells

Freshly prepared cells were exposed to MNSM, MN*pg-tk*, MN*AFpg-tkα* and MN*AFpg-tkβ* retroviruses at multiplicities of 0.4, 0.3, 0.2 and 0.1 cfu/cell, respectively, for 4–6 h at 37°C, 5% CO_2 in medium containing 8 μg/ml polybrene (Sigma, St Louis, MO, USA) as previously described [18]. Retroviral-infected cells were selected by addition of 400 μg/ml active G418. To avoid the positioning effect of the integrated DNA on transgene expression, uncloned G418-resistant cells, but not cloned ones, were expanded and used as retroviral-infected cells for the subsequent experiments.

2.6. Sensitivity of cells to prodrugs

Retroviral-infected and uninfected parental cells were plated at a density of 10^3 cells/well with various concentrations of ACV (0–1000 μg/ml) or GCV (0–500 μg/ml) in flat-bottomed 96-well culture plates. ACV was purchased from Shanghai Wellcome (Shanghai, China) and GCV was from Roche (Nutley, NJ, USA). After incubation for 4 days, the sensitivity of the cells to the prodrugs was evaluated using the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) conversion assay as previously described [19]. The viability of cells was determined by comparing the number of viable cells with and without prodrugs.

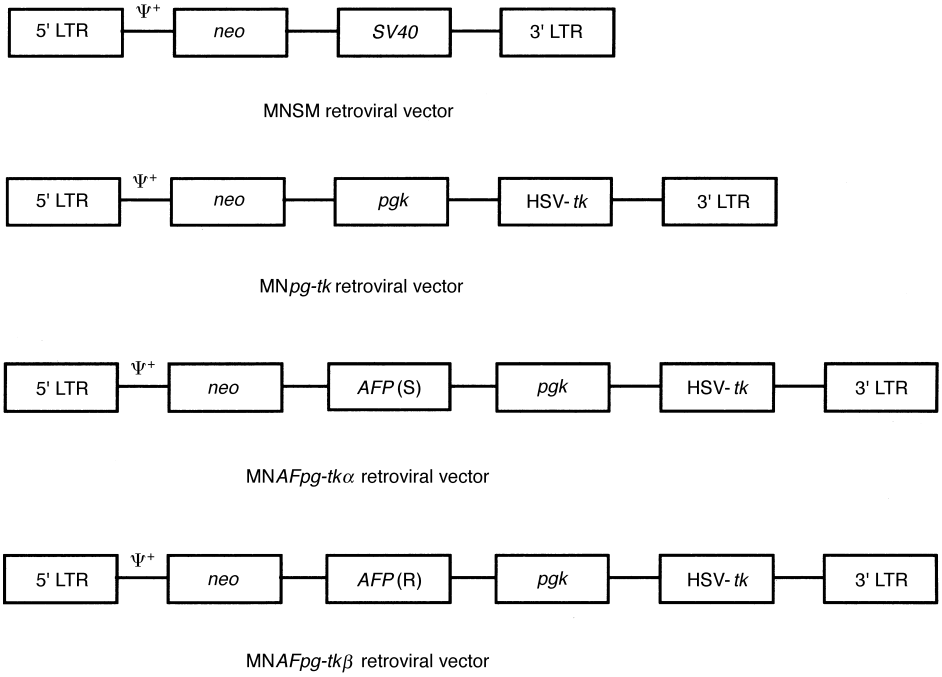

Fig. 1. Structure of retroviral vectors. MN*AFpg-tkα* and MN*AFpg-tkβ* retroviral vectors contain the 727-bp human α-fetoprotein (*AFP*) enhancer element upstream of the *pgk* promoter in the sense (S) and reverse (R) orientations, respectively. Ψ^+ indicates the extended retroviral packaging signal. LTR, long terminal repeat.

2.7. Northern blot analysis

Total RNA was extracted from parental and retroviral-infected cells by RNAzol B (TEL-Test, Friendswood, TX, USA) according to the protocol provided by the manufacturer. Twenty micrograms of the RNA samples were electrophoresed in a 1% agarose/2.2 M formaldehyde gel and transferred to a nylon membrane. Procedures for the Northern blot analysis were performed as previously described [20]. The HSV-*tk* and human glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) genes were used as probes to evaluate the HSV-*tk* expression and to monitor the loading of the RNA samples, respectively. The band densities were measured using the public domain NIH Image Program and HSV-*tk* expression levels were standardised based on the *GAPDH* expression levels.

2.8. Statistics

Results are expressed as means±standard deviation (S.D.). Standard descriptive statistics, Student's *t*-test and Welch's *t*-test, were used according to the distribution of experimental values. P values <0.05 were considered to indicate a significant difference between groups.

3. Results

3.1. AFP levels in the carcinoma cell lines

To estimate the selectivity of the strategy using the human *AFP* enhancer element, it is necessary to identify and rank *AFP*-positive and *AFP*-negative cell lines. Previous study has shown a correlation between AFP secretion into the media and *AFP* transcriptional levels [14]. AFP concentrations in the media were therefore quantified to evaluate the transcriptional levels of the *AFP* gene in the cells used in the experiments. As shown in Table 1, the human HCC lines, HepG2 and PLC/PRF/5, produced large amounts of AFP. Conversely, the human HCC line, SMMC7721, did not produce detectable levels of AFP. The human renal cell carcinoma line, RC9406, human cervical carcinoma line, HeLa, and human colorectal carcinoma line, LoVo, did not produce detectable levels of AFP either.

Table 1
AFP production in human tumour cells

Cell lines	Origin	AFP amount (ng/ml/day)[a]
HepG2	Hepatocellular carcinoma	170±23
PLC/PRF/5	Hepatocellular carcinoma	221±36
SMMC7721	Hepatocellular carcinoma	BT
RC9406	Renal cell carcinoma	BT
HeLa	Cervical carcinoma	BT
LoVo	Colorectal carcinoma	BT

BT, below the threshold of 5.0 ng/ml of AFP; AFP, alpha-fetoprotein.

[a] Results represent means±standard deviation (S.D.) of four separate experiments.

3.2. Sensitivity of genetically modified carcinoma cells to ACV

We first selected ACV as a prodrug and examined the sensitivity of the parental and MN*AFpg-tkα* retroviral-infected HCC cells to ACV. As shown in Fig. 2, AFP-producing HepG2 and non-AFP-producing SMMC7721 HCC cells were shown to be resistant to ACV, and the values of IC_{50}, defined as the dose required for 50% cytotoxicity, for both the parental HCC cells were more than 1000 μg/ml (the highest concentration tested). Although MN*AFpg-tkα* retroviral-infected SMMC7721 cells exhibited a slightly higher sensitivity to ACV compared with the parental cells, the differences were not significant and the IC_{50} value for the MN*AFpg-tkα* retroviral-infected SMMC7721 cells was still also more than 1000 μg/ml. Conversely, MN*AFpg-tkα* retroviral-infected HepG2 cells were susceptible to ACV in a dose-dependent manner and exhibited approximately 30% viability at an ACV concentration of 1000 μg/ml, with the IC_{50} value being approximately 500 μg/ml.

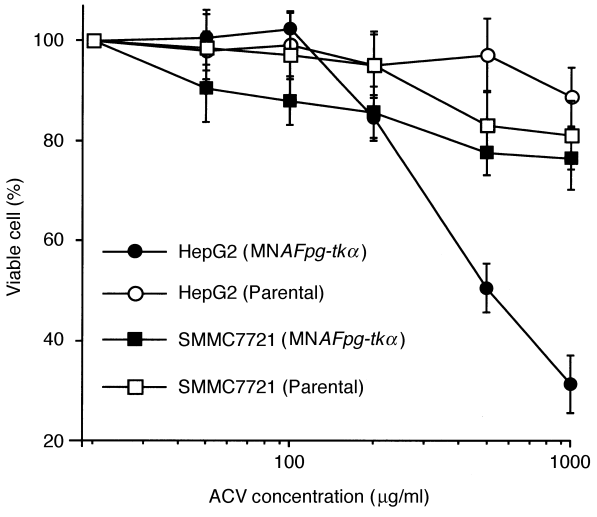

Fig. 2. Sensitivity of hepatocellular carcinoma (HCC) cells to acyclovir (ACV). MN*AFpg-tkα* retroviral-infected HepG2 (●) and SMMC7721 cells (■), as well as parental HepG2 (○) and SMMC7721 cells (□) were cultured with various concentrations of ACV for 4 days and the viability of the cells were estimated by the MTT assay. Values are expressed as means±standard deviation (S.D.) of four separate experiments.

3.3. Sensitivity of the genetically modified carcinoma cells to GCV

As GCV has been shown to be superior to ACV for the effective killing of HSV-*tk*-transduced cells [21], we next examined the sensitivity of the parental and retroviral-infected tumour cells to GCV. As shown in Table 2, all parental and MNSM retroviral-infected tumour cells were shown to be resistant to GCV and the values of IC_{50} to GCV for all parental and MNSM retroviral-infected cells were more than 500 μg/ml (the highest concentration tested). Conversely, all tumour cell lines infected with MN*pg-tk* retroviruses were shown to be susceptible to GCV, and their IC_{50} values ranged from 6.4 to 24.6 μg/ml. Furthermore, AFP-producing HCC cells, HepG2 and PLC/PRF/5, infected with MN*AFpg-tkα* or MN*AFpg-tkβ* retroviruses exhibited significantly higher sensitivity to GCV compared with the corresponding ones infected with MN*pg-tk* retroviruses, resulting in a 4- to 8-fold higher susceptibility to GCV. There were no significant differences in GCV sensitivity between the corresponding MN*AFpg-tkα* and MN*AFpg-tkβ* retroviral-infected cells. Conversely, when non-AFP-producing HCC cells, SMMC7721, were infected with MN*AFpg-tkα* or MN*AFpg-tkβ* retroviruses, the sensitivity of these retroviral-infected cells was significantly lower than that of SMMC7721 cells infected with MN*pg-tk* retroviruses, with the IC_{50} values to GCV being approximately 5- to 10-fold higher. Furthermore, MN*AFpg-tkα* or MN*AFpg-tkβ* retroviral-infected non-HCC cells, RC9406, HeLa and LoVo, exhibited significantly higher resistance to GCV compared with the corresponding cells infected with the MN*pg-tk* retroviruses, with the IC_{50} values to GCV being approximately 9- to 19-fold higher.

3.4. *HSV*-tk expression in tumour cells

We then examined the levels of HSV-*tk* expression in AFP-producing HCC cells and non-HCC cells infected with retroviruses. As shown in Fig. 3, Northern blot analysis revealed that neither parental HCC cell line HepG2 nor parental renal cell carcinoma cell line RC9406 expressed the HSV-*tk* gene, while MN*pg-tk* retroviral-infected HepG2 and RC9406 cells expressed the HSV-*tk* gene. Furthermore, when the levels of HSV-*tk* expression were standardised based on the corresponding *GAPDH* expression levels, it was shown that MN*AFpg-tkα* retroviral-infected HepG2 cells expressed approximately 3-fold higher HSV-*tk* expression compared with MN*pg-tk* retroviral-infected HepG2 cells. Conversely, MN*AFpg-tkα* retroviral-infected RC9406 cells expressed the HSV-*tk* gene much more weakly compared with MN*pg-tk* retroviral-infected RC9406 cells, and the level of HSV-*tk* expression of MN*AFpg-tkα* retroviral-infected cells was approximately 1/5-fold lower than that of the MN*pg-tk* retroviral-infected cells.

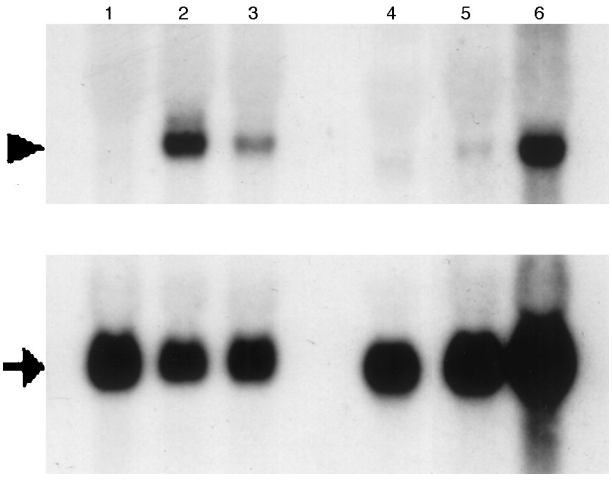

Fig. 3. Herpes simplex virus thymidine kinase (HSV-*tk*) expression in hepatocellular carcinoma (HCC) and non-HCC cells. The arrowhead and arrow indicate the HSV-*tk*-specific and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)-specific transcripts, respectively. Lanes 1–3, parental, MN*AFpg-tkα* retroviral-infected and MN*pg-tk* retroviral-infected HepG2 cells, respectively; lanes 4–6, parental, MN*AFpg-tkα* retroviral-infected and MN*pg-tk* retroviral-infected RC9406 cells, respectively.

Table 2
Ganciclovir (GCV) sensitivity of parental and retroviral-infected cells

Cell lines	IC_{50} to GCV (μg/ml)[a]				
	Parental	MNSM	MN*pg-tk*	MN*AFpg-tkα*	MN*AFpg-tkβ*
HepG2	> 500	> 500	12.8±4.6	1.6±3.2[b]	3.2±4.2[b]
PLC/PRF/5	> 500	> 500	6.4±3.0	0.8±0.2[b]	1.6±1.2[b]
SMMC7721	> 500	> 500	24.6±5.0	240±24[c]	120±24[c]
RC9406	> 500	> 500	6.4±2.5	120±6[c]	60±32[c]
HeLa	> 500	> 500	12.8±8.4	120±3[c]	120±10[c]
LoVo	> 500	> 500	6.4±1.2	120±5[c]	60± 6[c]

[a] Results represent means±standard deviations (S.D.) of four separate experiments.
[b] Values are significantly lower than those of the corresponding MN*pg-tk* retroviral-infected cells.
[c] Values are significantly higher than those of the corresponding MN*pg-tk* retroviral-infected cells.

4. Discussion

We have demonstrated here that the 727-bp human *AFP* enhancer region, including domains A and B, could result in an increased selectivity for HCC by the non-tissue-specific, housekeeping *pgk* promoter. When the *AFP* enhancer region was placed in a sense or reverse orientation upstream of the expression cassette consisting of the *pgk* promoter and the HSV-*tk* gene in a retroviral construct, the sensitivity to GCV of AFP-producing HepG2 and PLC/PRF/5 HCC cells infected with the retroviruses were enhanced 4- to 8-fold compared with the corresponding cells infected with the same retroviruses without the *AFP* enhancer element. Conversely, the sensitivity to GCV of non-AFP-producing SMMC7721 HCC cells infected with retroviruses carrying the HSV-*tk* gene under the transcriptional control of the *AFP* enhancer and *pgk* promoter was markedly decreased compared with those infected with the same retroviruses without the *AFP* enhancer, resulting in a 1/5- to 1/10-fold lower susceptibility to GCV. Importantly, the sensitivity to GCV of the non-HCC cells, RC9406, HeLa and LoVo, infected with retroviruses carrying the HSV-*tk* gene under the transcriptional control of the *AFP* enhancer and the *pgk* promoter was approximately 1/9- to 1/19-fold lower compared with the corresponding cells infected with the same retroviruses without the *AFP* enhancer. Subsequent Northern blot analysis revealed that in AFP-producing HCC cells, HSV-*tk* expression directed by the *AFP* enhancer and the *pgk* promoter was markedly higher than that directed only by the *pgk* promoter. Conversely, in non-HCC cells, HSV-*tk* expression was markedly suppressed when the *AFP* enhancer element was inserted upstream of the expression cassette consisting of the *pgk* promoter and HSV-*tk* gene.

Intensive analyses of the human *AFP* gene and its 5′-flanking region were performed by Tamaoki and his associates [22–24]. The 5.1-kb 5′-flanking region of the human *AFP* gene was shown to contain the 2.0-kb full enhancer regions consisting of the 0.3-kb domain A and 0.4-kb domain B that are located between −4.0 and −3.7 kb and between −3.7 and −3.3 kb, respectively, upstream from the cap site of the human *AFP* gene [13]. The 5.1-kb 5′-flanking region of the human *AFP* gene was also shown to contain the full silencer regions consisting of the strong distal silencer and weak proximal silencer that are located between −1822 and −951 bp and between −402 and −169 bp, respectively, upstream from the cap site of the *AFP* gene [14]. The use of tumour-selective promoters, such as the *AFP* and *CEA* promoters, is an attractive approach for achieving tumour-selective expression of therapeutic genes. However, the use of the weak tumour-selective promoters may be limited, because a low expression level of a therapeutic gene directed by the weak promoter may be insufficient for achieving effective cancer gene therapy. We have shown that stronger intratumoral expression of the *tumour necrosis factor-α* gene resulted in a more effective antitumour activity [20]. Therefore, even if HCC-selective expression of a therapeutic gene is induced, weak transgene expression may result in insufficient antitumour effects.

The *pgk* promoter is recognised as a general, strong promoter and has been used for various gene transfer experiments [25,26]. It has been shown that the *pgk* promoter could induce 2 and 15 times higher transgene expression in myoblasts compared with the metallothionein and cytomegalovirus promoters, respectively [27]. Furthermore, although promoter shut-off is a serious problem for achieving successful gene therapy [28], it has been shown that the *pgk* promoter showed little promoter shut-off in retroviral constructs [29,30]. We demonstrated here that the addition of the 727-bp human *AFP* enhancer fragment to the *pgk* promoter enhanced the promoter activity in AFP-producing HCC cells. Importantly, the addition of the *AFP* enhancer fragment to the *pgk* promoter caused the significant suppression of the *pgk* promoter activity in non-HCC cells and gave HCC selectivity to the non-tissue-specific, housekeeping *pgk* promoter. Therefore, the use of a vector construct carrying a therapeutic gene under the transcriptional control of the *AFP* enhancer fragment and the *pgk* promoter may be a practical and promising strategy for achieving strong, HCC-selective transgene expression. However, it should be noted that more investigations are necessary to put forward the claim that the *AFP* enhancer element can give selective activity to non-tissue-selective housekeeping promoters in AFP-producing HCC. Specifically, it should be examined whether the *AFP* enhancer, in combination with other housekeeping promoters, can induce augmented transgene expression in *AFP*-producing HCC cells and suppress transgene expression in non-HCC cells.

Consistent with our results, Watanabe and colleagues [13] have shown that the activity of the human *AFP* enhancer fragment is cell-selective in that it occurs in HCC cells, but not in non-HCC cells. The stimulatory activity of the *AFP* enhancer element was shown to be independent of the distance to a chloramphenicol acetyltransferase (*CAT*) reporter gene. They showed that the *AFP* enhancer element could direct *CAT* gene expression in conjunction with the *SV40* early promoter in an orientation- and position-independent manner in HCC cells, but not in non-HCC cells. We demonstrated here that the 727-bp human *AFP* enhancer, including domains A and B, could enhance the *pgk* promoter activity in an orientation-independent manner in AFP-producing HCC cells, but not in non-AFP-producing HCC cells and non-HCC cells. With regard to negative control elements of transcriptionally regulatory activities associated with the 5′-flanking region of the human

AFP gene, Nakabayashi and associates [14] have shown that the two silencer regions located between the enhancer and the promoter of the human *AFP* gene play a crucial role in repressing *AFP* expression in human HCC cells. They demonstrated that the lack of *AFP* expression in huH-1/cl-2 HCC cells was due to the action of these silencer regions, while the silencer activity was absent in high *AFP*-expressing HuH-7 HCC cells. They also demonstrated that the silencer regions significantly repressed the *SV40* promoter activity in non-HCC HeLa cells, and that this silencer activity was orientation-independent and not cell type-selective, suggesting that the silencer regions control negative *AFP* expression in non-HCC cells. We demonstrated here that the 727-bp human *AFP* enhancer element significantly suppressed the *pgk* promoter activity not only in non-HCC cells, but also in non-*AFP*-producing HCC cells. This inhibitory effect was induced even when the *AFP* enhancer was placed in a reverse orientation, although the effect was stronger when the *AFP* enhancer was in the sense orientation. It is, therefore, supposed that there are some sequences in the 727-bp *AFP* enhancer region that can suppress the *pgk* promoter activity in an orientation-independent manner in non-AFP-producing HCC cells and non-HCC cells.

Although the *AFP* promoter is promising for achieving AFP-producing HCC-selective transgene expression, the weak promoter activity may limit the utility of the *AFP* promoter for gene therapy strategies targeted against HCC. We demonstrated here that the *AFP* enhancer fragment could give HCC-selective activity to the non-tissue-specific, housekeeping *pgk* promoter. Although the usefulness of this strategy for inducing strong, HCC-selective transgene expression has to be estimated *in vivo*, this novel strategy may provide a practical and promising approach for gene therapy targeted against HCC.

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