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ORIGINAL ARTICLE

A phase I clinical trial of thymidine kinase-based gene therapy in advanced hepatocellular carcinoma

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The aim of this phase I clinical trial was to assess the feasibility and safety of intratumoral administration of a first-generation adenoviral vector encoding herpes simplex virus thymidine kinase (*HSV-TK*) gene (Ad.TK) followed by systemic ganciclovir to patients with advanced hepatocellular carcinoma (HCC). Secondarily, we have analyzed its antitumor effect. Ten patients were enrolled in five dose-level cohorts that received from 10^{10} to 2×10^{12} viral particles (vp). Ad.TK was injected intratumorally and patients received up to three doses at 30-day intervals. Positron emission tomography was used to monitor TK gene expression. Ad.TK injection was feasible in 100% of cases. Treatment was well tolerated and dose-limiting toxicity was not achieved. Cumulative toxicity was not observed. Hepatic toxicity was absent even in cirrhotic patients. Fever, flu-like syndrome, pain at the injection site and pancytopenia were the most common side effects. No partial responses were observed and 60% of patients showed tumor stabilization of the injected lesion. Importantly, two patients who received the highest dose showed signs of intratumoral necrosis by imaging procedures. One of them achieved a sustained stabilization and survived for 26 months. In conclusion, Ad.TK can be safely administered by intratumoral injection to patients with HCC up to 2×10^{12} vp per patient.
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Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer and the third leading cause of cancer-related death in the world, with > 500 000 deaths per year.[1] Unfortunately, the incidence and mortality associated with HCC is increasing steadily in the United States and Europe.[2,3] Current curative options, such as hepatic resection, liver transplantation and local ablative therapies, can be applied to a minority of patients in referral medical institutions.[4,5] Thus, in the majority of advanced HCC cases the prognosis is dismal because of underlying cirrhosis and poor tumor response to chemotherapeutic agents.[3] Recently, treatment with sorafenib in patients with advanced disease increased their median survival

from 7.9 to 10.7 months when compared with patients receiving placebo, despite objective responses were only found in 2% of sorafenib-treated patients.[6] Therefore, new therapeutic options are urgently needed for the treatment of advanced HCC. As an experimental strategy currently explored to generate alternative HCC treatments, gene therapy is receiving much attention.[7,8] Up to now, more than 1300 gene therapy clinical trials have been performed worldwide (http://www.wiley.co.uk/genmed/clinical) and 9% were based on the transfer of the so-called suicide genes. This strategy, also called prodrug-activating gene therapy, offers the potential for selective tumor destruction without inducing significant systemic toxicity.[9,10] The thymidine kinase (TK) from herpes simplex virus (HSV) is the best characterized suicide gene.[11] Expression of functional *HSV-TK* in transduced cells has the ability to transform a nontoxic prodrug such as ganciclovir (GCV) into a toxic phosphorylated (GCV-triphosphate) compound that competes with triphosphate as a substrate for DNA polymerase.[12,13] This causes inhibition of both nuclear and mitochondrial DNA synthesis leading to cellular death.[12,13] A characteristic of the suicide genes is the so-called bystander effect, which is to some extent caused by diffusion of the toxic

drug metabolite from transduced cells resulting in the death of neighboring tumor cells.[9,14] The bystander effect also involves the generation of local inflammation, attraction of dendritic cells and induction of immunity.[15,16] By these mechanisms, gene transduction of a fraction of tumor cells is able to lead to the extensive tumor response.

Several groups including ours have demonstrated the efficacy of HCC treatment with the *HSV-TK*/GCV system in different animal models.[17–19] Although this therapeutic approach rises expectations, one of the main concerns that could limit its clinical usefulness is related to toxic side effects affecting nontumoral tissue, especially liver parenchyma in case of liver tumors.[20] These side effects can be avoided by directly injecting vectors within tumor nodules or by engineering them to express *HSV-TK* under tumor-specific promoters, such as α-fetoprotein.[21] In this study, we report the results of a phase I clinical trial consisting in the intratumoral injection of escalating doses of Ad.TK followed by systemic GCV in patients with advanced HCC.

Patients and methods

Ad.TK construction

Ad.TK is a first-generation, replication-defective adenoviral vector that expresses the *TK* from HSV type 1 under the control of the strong, nonselective cytomegalovirus (CMV) promoter. To produce Ad.TK, an expression cassette was constructed by inserting the 2.8-kb *Bgl*II/*Bam*HI fragment of pMK containing the *HSV1-tk* gene into a transient expression vector pMV100 under the control of the CMV major immediate early promoter and upstream of a polyadenylation signal (polyA). The orientation was checked by restriction mapping. The CMV immediate early promoter/*HSV-TK* cassette was then excised from the transient expression vector and inserted into the adenovirus transfer vectors pMV60 to generate pMV60/TK. For construction of Ad.TK, pJM17 (containing the backbone of adenovirus serotype 5) and pMV60/TK were co-transfected into 293 cells (from the American Type Culture Collection, Manassas, VA) and plaques were screened to obtain Ad.TK, which was then propagated in 293 cells, purified by CsCl density gradient, dialyzed and stored at −80 °C. For human use, clinical grade lots of adenovirus were produced by Molecular Medicine LLC (Los Angeles, CA) and tested for titer, sterility and general safety. The virus titer was determined by plaque assay for plaque forming units (pfu) and the number of viral particles (vp) was calculated by measuring the optical density of the viral DNA content. The ratio vp/pfu was 130.

Study design

We performed an open-label, nonrandomized, dose-escalation phase I trial in which intratumoral Ad.TK followed by systemic GCV (intravenous GCV or oral valganciclovir) was administered to patients with advanced HCC.

Objectives

The primary end point of the study was to assess the feasibility and safety of single and repeated direct intratumoral injections of Ad.TK followed by systemic GCV, and to determine the maximal tolerated dose and the dose-limiting toxicity of Ad.TK. A secondary end point was antitumor activity.

Patient selection and enrollment

To be eligible, patients had to meet all the following criteria: (i) age between 18 and 80 years; (ii) unequivocal diagnosis of HCC either histological or noninvasive as for the European Association for the Study of the Liver (EASL) criteria; (iii) a Karnofsky Index $\geqslant 50\%$; (iv) tumor not amenable to standard curative or palliative therapies; (v) an accessible tumor mass; (vi) a life expectancy beyond 2 months; and vii) the ability to give signed informed consent. Exclusion criteria included (i) pregnancy or lactation; (ii) a neutrophil count $\leqslant 0.5$ hpl or a platelet count $\leqslant 20$ hpl; (iii) anti-human immunodeficiency virus antibodies; (iv) an active bacterial, fungal or viral infection; and (v) participation in another clinical trial or any sort of antitumor therapy in the previous month.

Permission for this clinical trial was obtained from the Institutional Ethical Committee, the Local Government's Ethical Committee for Clinical Investigation, the National Biosafety Commission and the Spanish Agency for the Evaluation of Medicinal Products. Informed consent was also obtained from all patients before enrollment. All patients were treated at the Liver Unit in Clínica Universitaria de Navarra. The trial has been registered in clinicaltrials.gov database with the number NCT00844623.

Patients were enrolled consecutively in five cohorts of two patients with the following dose-escalation plan: Cohort 1, 2×10^{10} vp; Cohort 2, 10^{11} vp; Cohort 3, 2×10^{11} vp; Cohort 4, 10^{12} vp; Cohort 5, 2×10^{12} vp. Originally the trial was designed to administer ganciclovir as a twice-daily intravenous infusion of 5 mg kg^{-1} of body weight every 12 h for 14 days starting 2 days after Ad.TK injection. However, after its oral derivative valganciclovir was approved for human use, the protocol was amended and patients were given twice-daily oral valganciclovir at an equivalent dose of 900 mg for 14 days starting 2 days after Ad.TK injection.[22]

Ad.TK preparation and injection

Ad.TK was administered in one single tumor location using a 22-gauge fine-needle placed under ultra sound or computed tomography guidance. The viral dose corresponding to each cohort was thawed shortly before injection and diluted in saline to a final volume of at least 20% of the volume of the lesion to be injected. Tumor volume was calculated using the following formula: $4/3\pi r^3$, where r is half the maximal tumor diameter. The solution containing Ad.TK was very slowly injected into the tumor at different sites so that one injection was performed every 2 cm in diameter when possible, in order to maximize vector delivery within the tumor mass.

Table 1 Flowchart of the trial

Procedures	Screening	Day										
		0	1	2	3	4	5	6	9	10–15	16	30
Blood tests	X	X	X			X						X	X
HIV antibodies	X											
Pregnancy test	X											
Chest X-ray	X	X					X						X
Abdominal Ultrasound	X	X					X						X
Thoracoabdominal CT	X											
Bone scintigraphy	X											
Brain MRI	X											
DHT	X			X					X			
Viral shedding		X	X	X	X	X	X					
PET				X					X		X	X
Adverse Events		X	X	X	X	X	X	X	X	X	X	X
Ad.TK		X										
Valganciclovir or ganciclovir				X	X	X	X	X	X	X	X	

Abbreviations: CT, computed tomography; DHT, delayed hypersensitivity test; HIV, human immunodeficiency virus; MRI, magnetic resonance imaging; PET, proton emission tomography.

Patient evaluation

Table 1 summarizes the evaluation of the patients throughout the trial. Patients were closely followed during the first 16 days by daily evaluation of toxicity and a comprehensive set of laboratory tests (including blood cell count, serum glucose, triglycerides, cholesterol, calcium, amylase, urea, creatinine, electrolytes, D-dimer, fibrinogen, lactate, ammonia and liver function tests) performed at days 0, 1, 4, 5, 9 and 16. At day 30, toxicity was re-evaluated and response to therapy was assessed using the World Health Organization criteria.[23] If at that time tumor disease was stable or responding and no serious adverse reactions had been observed, a second dose was administered into the same nodule. The whole procedure was repeated but no more than three doses of Ad.TK could be administered to the same patient. Patients were then followed monthly with computed tomography scan and blood tests for the evaluation of response and assessment of adverse events.

The maximal tolerated dose was defined as the one lower than the dose at which two patients experienced a dose-limiting toxicity. Dose-limiting toxicity was defined as grade 4 toxicity of any duration related to Ad.TK or nonreversible grade 3 toxicity related to Ad.TK. Toxicity was assessed throughout the study using the National Cancer Institute Common Toxicity Criteria version 3.0.[24] Adverse reactions observed were classified as definite, probable or possible according to the Karch and Lasagna criteria.[25]

Imaging studies

After the first three patients had been recruited, the protocol was amended to include noninvasive monitoring of gene expression using proton emission tomography (PET). A PET scan was obtained at day 2 using [18F]FHBG (a fluorine-18 labeled penciclovir analog) as

a radiotracer as described.[26] If the PET scan was positive for TK gene expression, then the study was repeated at day 9 after withdrawing valganciclovir for 24 h.

Results

Patients characteristics

Table 2 summarizes the characteristics of treated patients. Ten patients were enrolled. Median age was 65.5 years and 70% were males. All patients had an underlying chronic liver disease, in most cases advanced HCV-related liver cirrhosis. However, all patients were fully ambulatory and had an Eastern Cooperative Oncologic Group score of 0–2. Regarding tumor burden, most patients had multiple tumor nodules, three had portal vein thrombosis and two had extrahepatic metastases to the abdominal lymph nodes and to the peritoneum, respectively. Accordingly, six patients were in Barcelona Clinic Liver Cancer (BCLC) stage B and were treated after they had progressed to arterial embolization. The remaining four patients were in BCLC C stage and received Ad.TK plus GCV as the first specific therapy. None of the patients received antineoplastic chemotherapy or immunosuppressant drugs in the month previous to Ad.TK injection.

Treatment procedure

Intratumoral injection of Ad.TK was feasible in 100 % of cases. As shown in Table 3, 16 intratumoral injections were administered to the 10 patients using US guidance. Ad.TK was injected into a liver tumor nodule in nine patients and into a peritoneal metastasis in one patient. Five patients received a single dose of Ad.TK, four patients received two doses and only one patient received the three doses that were the maximal allowed. It was not necessary to reduce the dose of GCV or vanganciclovir in any of the patients treated.

Table 2 Baseline characteristics of the patients

Age (years)	65.5 (range 51–77)
Males	70%
Etiology of chronic liver disease	
HCV/HBV/alcohol/others	6/1/2/1
Hemoglobin (g per 100 ml)	12.8 (11.35–13.47)
ALAT (UI ml^{-1})	42 (25–102)
Total bilirubin (mg per 100 ml)	1.98 (1.51–2.81)
Serum albumin (g per 100 ml)	2.97 (2.25–3.22)
Ascites	40%
Child-Pugh stage A/B/C	4/6/0
Tumor nodules	
1–3	60%
>10	40%
Portal vein thrombosis	30%
Extrahepatic metasases	20%
BCLC stage A/B/C/D	0/6/4/0

Abbreviations: ALAT, alano aminotransferase; BCLC, Barcelona Clinic Liver Cancer; HBV, hepatitis B virus; HCV, hepatitis C virus.
All variables are expressed as median (interquartile range) unless otherwise explained.

Table 3 Tumor response and TK expression

Dose (vp)	Patient number	Treatment courses	Response injected nodule	Response distant nodule	PET scan
2×10^{10}	01	1	PD	NE	Negative
	02	1	SD	PD	Negative
10^{11}	03	3	SD	SD	Negative
	04	2	PD	PD	Negative
2×10^{11}	05	1	PD	PD	Negative
	06	1	SD	SD	Negative
10^{12}	07	2	SD	NE	Positive
	08	2	PD	PD	Positive
2×10^{12}	09	2	SD[a]	PD	Positive
	10	1	SD[a]	PD	Positive

Abbreviations: NE, not evaluable; PD, progressive disease; PET, proton emission tomography; SD, stable disease; TK, thymidine kinase.
[a]Presence of tumor necrosis on imaging procedures.
Response was evaluated by measuring the maximum diameter of the injected tumor nodule and the most representative distant nodule when present and measurable. Tumor response was evaluated after the last treatment according to the World Health Organization criteria. TK expression was evaluated by PET scan using [^{18}F]FHBG (a fluorine-18 labeled penciclovir analog) as radiotracer.

Toxicity

A total of 49 treatment-emergent agent-related adverse events were recorded throughout the follow-up period (Table 4). Overall, Ad.TK administration was well tolerated and dose-limiting toxicity was not observed. Mild to moderate fever and a flu-like syndrome, both responsive to common antipyretics were observed 24–48 h

Table 4 Adverse events possibly related to Ad.TK administration observed between days 1 and 30 after injection of Ad.TK in 16 treatment courses

Adverse event	Percentage of courses	Relation to Ad.TK
Fever	62	Definite
Flu-like syndrome	62	Definite
Lymphopenia	44	Definite
Injection site pain	12	Definite
Abdominal pain	38	Probable
Leukopenia	38	Probable
Thrombocytopenia	19	Probable
Anemia	19	Probable
Vomiting	12	Probable
Encephalopathy	31	Possible
Edema	31	Possible
Diarrhea	12	Possible
Ascites	12	Possible
Hyperbilirubinemia	6	Possible
Hypertransaminasemia	6	Possible
Itching	6	Possible

Relation to Ad.TK was classified as definite, possible or unlikely according to Karch and Lasagna.
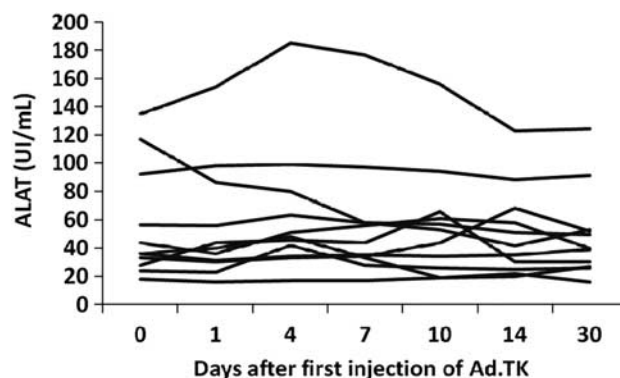
Figure 1 Changes in transaminases after intratumoral injection of Ad.TK followed by systemic ganciclovir.

after Ad.TK injection in 62% of the courses, irrespective of the dose of Ad.TK, and were occasionally associated with profuse sweating and malaise. Pain at the site of injection lasting for 1–3 days after treatment was experienced in nearly half the courses. In 12% of the courses, vomiting occurred the day of treatment that responded to antiemetics.

Hematological toxicity was also frequently observed. Lymphopenia, leukopenia, thrombocytopenia and anemia appeared in 7–38% of all patients. Both Ad.TK injection and GCV or valganciclovir treatment could have contributed to this decrease in blood cell counts. There was an apparent direct relationship between the adenoviral dose and the intensity of lymphopenia.

Regarding liver toxicity, most patients had altered liver function tests of varying degrees before treatment, but consistent, relevant changes were not observed after injection of Ad.TK. However, one patient had a transient, modest rise in serum transaminases after treatment

(Figure 1). It is noteworthy that none of the cirrhotic patients experienced significant liver toxicity, even at the highest dose level. Among patients receiving multiple doses, side effects usually recurred but cumulative toxicity was not observed. Also, there was no suggestion of long-term toxicity among patients followed for more than 6 months.

Transgene expression

A biological response to therapy was examined by evaluating transgene expression. *TK* expression in the tumor as detected by PET was dependent on the injected dose of the adenovirus being detectable in all patients who received a dose of $\geqslant 10^{12}$ vp.[22] Beyond that threshold, a dose–expression relationship was not found. Also, no expression could be observed when the study was repeated 9 days after vector injection. And importantly, when patients received a second injection of the same amount of viral particles one month after the first one, transgene expression could not be detected by PET scan.

Antitumor activity

Overall median survival was 5 months (95% confidence interval, 0.00–11.88 months). However, one patient treated with 10^{12} vp had a remarkable long-term outcome. Both the injected tumor and a distant, noninjected tumor showed a slight increase in size for 3 months after injection, and then tumor growth was arrested for 18 months. No other therapy was given and she finally died 26 months after Ad.tk therapy due to impaired liver function secondary to progression of her liver cirrhosis and to some extent of her liver tumors.

Although clinical efficacy was not a primary end point, response to therapy was evaluated in both the injected nodule and a distant nodule at day 30 after the last dose of Ad.TK in all but two patients who had no other measurable disease than the injected nodule. No partial responses were observed and 40% of the patients showed tumor progression in the injected nodule after treatment. Although three patients showed stable disease under the threshold of 10^{12} vp, objective responses were observed only in patients treated with a dose of the vector $\geqslant 10^{12}$ vp. Accordingly, two patients who received 2×10^{12} vp of Ad.TK showed signs of intratumoral necrosis on imaging procedures (one patient is shown in Figure 2). The size of the injected tumor nodule did not change significantly, whereas distant nodules had progressed by the time of evaluation. On the basis of the World Health Organization criteria, there were no partial responses. However, when we consider the extent of necrosis following the EASL[27] guidelines, this patient had a partial response.

Discussion

Adenovirus-based vectors have been extensively employed for gene transfer in anticancer strategies because of their high capability to infect both dividing and nondividing cells.[28] We have previously demonstrated
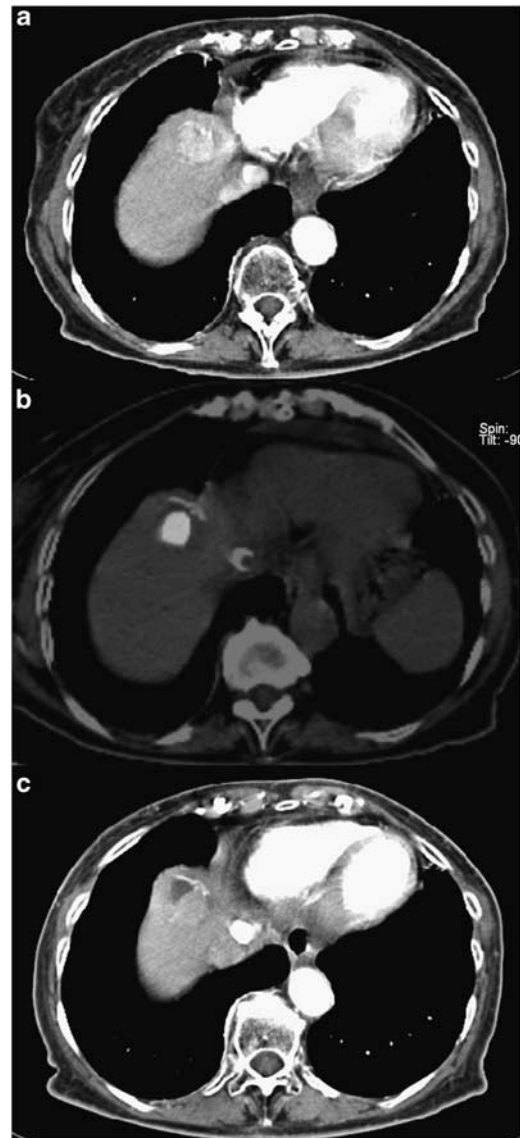
Figure 2 Response to treatment. Patient number 9 received 2×10^{12} viral particles into a liver nodule (**a**). Intense TK expression was noted on PET scan 2 days later (**b**), and tumor necrosis was observed after valganciclovir administration for 14 days (**c**).

the efficacy of intratumoral and intrahepatic artery delivery of Ad.TK in rodents with single and multicentric HCC, including a very challenging model developed in rats chronically intoxicated with diethylnitrosamine.[17,18,29] Dose-limiting toxicity in rodents derives from the induction of liver damage due to mitochondrial dysfunction after TK-mediated GCV activation in normal nondividing hepatocytes.[20] Thus, the primary goal of this phase I trial was to evaluate the feasibility and safety of this gene therapy approach in a population of patients with coexisting liver cirrhosis. We found that the US-guided administration of Ad.TK was feasible in 100% of cases and no complications associated with the procedure were reported. Importantly, no relevant side effects were observed even among patients with impaired

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liver function. Although most patients had advanced liver disease, all patients remained compensated after the administration of Ad.TK. This absolute lack of liver toxicity could be due to a restricted expression within the neoplastic nodule after intratumoral injection of the adenoviral vector, or to a subtoxic level of TK-mediated GCV activation in hepatocytes, or even to a low sensitivity of transduced hepatocytes to mitochondrial dysfunction.

On the other side, a therapeutic effect was insinuated albeit modestly. No relevant tumor responses were observed using the World Health Organization criteria that only consider changes in tumor diameter but not tumor necrosis. This system might underestimate the efficacy of Ad.TK treatment because two patients treated with the highest dose of Ad.TK showed tumor necrosis and lack of growth in the injected nodule while progressing in noninjected lesions. This is congruent with the results observed in other clinical studies that had employed Ad.TK/GCV for the treatment of different cancers such as ovarian, prostate, mesothelioma, glioma and metastatic colorectal carcinoma.[11] The experience using Ad.TK/GCV in patients with advanced HCC is scarce,[30] and for secondary liver tumors the results from a single clinical trial in patients with metastatic colorectal carcinoma have been published.[31] In the latter, authors demonstrated the safety of direct injection of up to 10^{13} vp of an adenovirus coding for *TK* into metastatic colorectal liver tumors followed by GCV treatment; nevertheless no objective antitumor responses were reported.

Transduction efficiency of treated lesions and the possibility to infect nondesired tissue is a challenge for clinical application of gene therapy vectors. It will probable be necessary to consider novel systems for visualization of gene expression *in vivo* using PET to allow a precise data of transgene expression of treated tumor lesions as well as biodistribution.[32] In this regard, it would be of interest to consider the threshold of 10^{12} vp previously achieved by PET imaging in patients with HCC to maximize intratumor delivery and enhance the efficacy of the combination.[22,33] On the other hand, improvements in vector technology introducing specific promoters, such as α-fetoprotein gene, to restrict TK expression to tumor tissue could increase the safety of the strategy.[34] Better clinical efficacy would probably be seen in patients with less advanced disease or applying TK/GCV in combination with radiotherapy,[35] immunostimulatory cytokines[29,36] or antiangiogenic molecules.[6,37] Further improvements in the construction of adenoviral capside could help to overcome the pre-existing anti-adenovirus immune response[38] that precludes repeated injection of this type of vectors. In summary, our results show that prodrug activating gene therapy of HCC using the TK/GCV system is feasible, safe and able to produce an antitumor effect. However, relevant clinical efficacy was not observed most likely as a result of short transgene expression because of the use of a first-generation adenoviral vector for gene transfer and also because of the failure to achieve repeated HCC transduction with repeated vector injection. Improved gene transfer tools

should be developed in order to progress in the clinical development of this therapeutic strategy.

Conflict of interest

The authors declare no conflict of interest.

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