Induction of cytotoxic T-lymphocyte responses using dendritic cells transfected with hepatocellular carcinoma mRNA

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ABSTRACT

This study aims to induce an efficient expansion of cytotoxic T-lymphocytes (CTL) from peripheral blood mononuclear cells (PBMCs) using dendritic cells (DC) transfected with hepatocellular carcinoma (HCC) messenger RNA (mRNA) for adoptive immunotherapy of HCC. Dendritic cells are generated from PBMCs. HCC mRNA is isolated either from HepG-2 cells or from tumour tissue from three HCC patients, and then amplified using the polymerase chain reaction (PCR). Expansion of CTLs is achieved from PBMCs induced by DCs transfected with HCC mRNA and cytotoxicity is measured using a crystal violet staining assay. The proportion of CD3+, CD4+ and CD8+ cells is determined using flow cytometry. Dendritic cells transfected with the total HCC mRNA stimulated antigen-specific cytotoxic T-cell responses that are capable of recognising and killing autologous tumour cells *in vitro*. The cytotoxic activity was inhibited by treatment with anti-CD3, anti-CD8 and anti-MHC class I monoclonal antibodies, but not with anti-CD4 and MHC class II antibodies. In conclusion, HCC mRNA-transfected DCs may represent a broadly applicable vaccine strategy to induce potentially therapeutic CTL responses in HCC.

KEY WORDS: Carcinoma, hepatocellular.
Dendritic cells.
T lymphocytes, cytotoxic.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide and is especially prevalent in East Asia and Africa.[1-3] Although early diagnosis and treatment improves survival rate, the prognosis is still very poor,[4] and many patients lose the chance of surgical resection when they present late in the course of the disease. Although chemotherapy, microwave ablation, ethanol injection and chemoembolisation offer the only chance of treatment in these patients, long-term results are disappointing.[5] Thus, immunotherapy has emerged as an attractive alternative.[6]

Cytotoxic T lymphocyte (CTL) responses play a pivotal role in antitumour immunity. Immunisation with antigens recognised by tumour-specific CTLs may represent an effective strategy for cancer immunotherapy.[7] However, CTL responses are generally not strong enough to kill tumour cells and are not always tumour-specific.[8-10] Therefore, the key problems for tumour immunotherapy are how to increase the toxicity of CTL and its specificity toward tumour cells.

Dendritic cells (DC) are the most potent antigen presenting cells and are able to stimulate naïve and quiescent CD4+ and CD8+ T-cell responses *in vivo* and *in vitro*.[11-16] Recently, studies have shown that immunisation with DCs containing autologous tumour-derived antigens in the form of tumour lysates, peptides or DNA is capable of priming tumour-specific CTL responses[17,18] against melanoma,[19] colorectal cancer,[20-23] prostate cancer[24] and paediatric solid tumours.[25]

Although these experiments provide us with exciting results, problems remain. As the number of known tumour antigens is relatively small and generally only a limited amount of tumour tissue is available, the application of T-cell function studies is often restricted to a small number of patients. The introduction of a DNA-pulsed DC vaccine can circumvent this problem, but the possible integration of tumour cell genome into normal tissues is a concern.

Recently, it was shown that DCs transfected with RNA coding for whole tumour messenger RNA (mRNA) are able to induce potent antigen and tumour-specific T-cell responses directed against multiple epitopes. This technique does not require definition of the specific tumour antigen or haplotype of the patient and has the potential of broad clinical application. Such a polyvalent vaccine may reduce the probability of clonal tumour escape and elicit CTL responses directed against a broad range of tumour antigens.

Through *in vitro* retrotranscription, adequate mRNA can be obtained from a limited amount of tumour tissue, which means that an RNA-transfected DC vaccine is applicable even in patients with small tumours. In addition, mRNA has no risk of integration into the host genome and eliminates dependence on transcription for production of the gene product. Owing to its short half-life, transfection of DCs with total mRNA tends to be much safer.[26]

Study shows that RNA amplified from a small number of tumour cells can be used for DC transfection and is very effective in stimulating CTLs, and antitumour immunity

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Table 1. Clinical data of three HCC patients.

Patient	Gender	Age	Disease duration (days)	AFP (ng/L)	Tbil (μmol/L)	DBil μmol/L)	Alb (g/L)	AST (U/L)	ALT (U/L)	Tumour differentiation
1	male	50	20	1200	20.1	14.5	38	45	35	Moderate
2	male	39	16	860	18.5	13.6	41	38	24	Moderate
3	male	54	30	<20	16.8	11.9	39	26	23	Moderate

would allow treatment of patients with low tumour burden.[13] Thus, a DC vaccine transfected with total mRNA is a promising modality for adoptive immunotherapy.

This study aims to induce HCC-specific CTLs using DCs transfected with HCC mRNA *in vitro* and to observe the cytotoxic activity of tumour-specific CTLs against HCC.

Materials and methods

Patients, cell lines and reagents

Peripheral blood and HCC tissue were obtained from three patients (Table 1) who were diagnosed with HCC and who underwent surgical resection. Patients 1 and 2 were serum α-fetoprotein positive (AFP+)[27] and patient 3 was serum AFP negative (AFP–). The cell line HepG-2 (human HCC, AFP+), HCC cells of patient 1, SGC-7901 (human gastric adenocarcinoma) and TuHR3TKB (human renal carcinoma) were obtained from the Institute of Physical and Chemical Research (RIKEN) Cell Bank in Japan. Granulocyte macrophage-colony stimulating factor (GM-CSF) and interleukin-4 (IL-4) were obtained from Genzyme, Japan. The Trizol kit was obtained from Invitrogen, USA, and DOTAP from Biontex, USA.

Preparation of DCs **in vitro**

Dendritic cells were generated from peripheral blood mononuclear cells (PBMCs), as described previously.[18] Briefly, PBMCs were separated by Ficoll-Paque density gradient centrifugation of heparinised blood obtained from the patients. The PBMCs were suspended at 6-7 x 10^6 cells/mL in 30 mL AIM V medium and transferred to tissue culture flasks for adherence. After 1 h, the non-adherent cells were removed and 30 mL AIM V medium with 100 ng/mL GM-CSF and 500 u/mL IL-4 were added. The DCs were harvested after seven days and pulsed with antigen. The DCs were cryopreserved in 90% autologous plasma at 5x10^6 cells/mL and PBMCs were cryopreserved at 5x10^7 cells/mL.

Isolation and amplification of HCC mRNA **in vitro**

Total RNA was isolated from the autologous tumour tissue of all three patients, as well as the HepG-2 cell lines using the Trizol kit. Quantity and purity was determined by ultraviolet (UV) spectrophotometry. One μg of total tumour RNA was reverse-transcribed in a final volume of 20 μL using the SuperScript II TMRT kit.

First-strand complementary DNA (cDNA) synthesis was primed with 10 pmol modified oligo-dT primer (5′-AAGCAGTGGTATCAACGCAGAGTACT [30] VN-3′), in which V is G, A or C and N is G, A, T or C. The reaction system was incubated at 42°C for 30 min prior to the addition of 10 pmol T7 strand switch primer (5′-CTAATACGACTCACTATAGGGCGGG-3′). Following a further 30-min incubation, the reaction was stopped by being placed on ice.

The cDNA was amplified by placing 2 μL RT reaction in a 50-μL PCR reaction containing 5 μL 10x buffer, 1 μL 2.5 mmol/L MgCl2, 1 μL 10 pmol/L primer, 1μl KOD HiFi polymerase, 1.7 mmol/L dNTP and 2 μL dimethyl sulphoxide (DMSO). Cycling parameters were: 94°C for 30 sec, 66°C for 30 sec, 72°C for 6 min, and a final extension at 72°C for 10 min. A total of 20 cycles were performed.

Subsequently, 1 μg cDNA was placed in a standard *in vitro* transcription reaction using the T7 mMessage mMachine kit, following the manufacturer's instructions. The reaction was carried out at 37°C for 2-4 h, followed by addition of DNase I and further incubation for 15 min. Then RNA was isolated by phenol/chloroform extraction and isopropanol precipitation. Following precipitation, a sample was dissolved in DEPC-treated water and verified for size and integrity on 1% agarose gel.[28]

Transfection of DCs with HCC mRNA

Dendritic cells were washed (x2) in phosphate-buffered saline (PBS) and in Opti-MEM medium (x1) and then resuspended to a concentration of 1◊106 cells/mL in Opti-MEM medium. The liposomal transfection reagent (DOTAP, Biontex) was used to deliver RNA to cells.

Briefly, 10 μg total mRNA in 100 μL Opti-MEM medium was added to 30 μL DOTAP in 100 μL Opti-MEM medium and incubated at room temperature for 20 min in polystyrene tubes. The RNA /DOTAP complex was added to 2x10^6 DCs in a final volume of 2 mL and incubated at 37°C for 4 h. The cells were washed (x2) in PBS and incubated in RPMI 1640 supplemented with 10% FBS, 100 ng/mL GM-CSF and 500 u/mL IL-4 for seven days.

The transfected DCs were classified according to the different stimulators used, as follows: DCs transfected with HepG-2 HCC mRNA, DCs transfected with HCC mRNA from AFP+ patients, DCs transfected with HCC mRNA from AFP- patients, Opti-MEM medium-transfected DCs and DOTAP-transfected DCs.

Induction of CTL

RHAMa medium originally developed for human lymphocytes culture,[29] was used for induction of CTL after supplementation with 5% autologous plasma and recombinant human interleukin 1β (IL-1β, Otsuka Pharmaceutical, Japan; 167 u/mL), IL-2 (Takeda Chemical Industries, Osaka., Japan; 67 u/mL), IL-4 (Ono Pharmaceutical, Osaka, Japan; 67 u/mL) and IL-6 (Ajinomoto, Tokyo, Japan; 134 u/ml).

Briefly, PBMCs were suspended at a concentration of 1x10^6 cells/mL in the culture medium and mixed at a

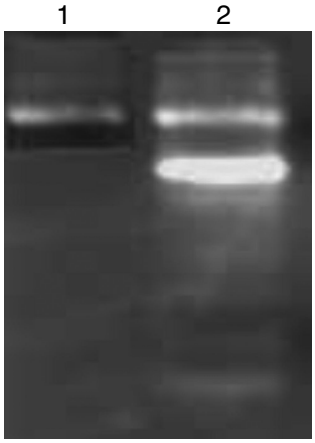


Fig. 1. Final amplified HCC mRNA products (agarose gel electrophoresis). Biochemical analysis of RNA amplified from the autologous tumour tissue of patients 1, 2 and 3 and the HepG-2 cell lines. Lane 1: amplified HCC mRNA *in vitro*; lane 2: total RNA extracted from HCC tissue.

PBMC:DC ratio of 10:1 in the well of a 24-well culture plate. Half of the culture medium and cytokines was changed every two days until the lymphocytes began to grow. Then, the CTL preparation was maintained at a suitable dilution with the remaining transfected DCs in the culture medium. After approximately 10 days, the CTLs were transferred to a six-well culture plate and restimulated with different transfected DCs. The CTLs were then harvested.

Cytotoxic activity

The target tumour cells were seeded in 96-well culture plates at a density of $5x10^3$ cells/well in 100 µL RHAMa medium containing 5% autologous plasma and were precultured overnight. Appropriate numbers of CTL, which had been adjusted to the desired effector-to-target (E/T) ratio, were added to the wells with 100 µL RHAMa medium containing 5% autologous plasma. The plates were incubated for 24 h at 37°C in a humidified CO2 incubator and then the wells were washed (x1) gently with an appropriate amount of PBS.

Adherent target cells were counted as the number of surviving cells using the crystal violet (CV) staining assay, as described previously.[30] The CV assay in the present study is as accurate and sensitive for assessment of the killing activity of CTL against adherent target cells as is the standard ^{51}Cr-release cytoxicity assay for E/T ratios of ≤10. HCC cells of the AFP+ patients, HepG-2, SGC-7901 and TuHR3TKB cell lines were treated as the target cells.

The number of adherent cells was expressed as the absorbance (*A*) at 450 nm. The killing activity (%) was calculated as follows: (1-[A-AL] / [AT-B]) x100, where A is the absorbance in each well; AL is the mean of absorbance in the wells containing the lymphocytes without target cells; AT is the mean absorbance in the wells containing target cells without lymphocytes; and B is the mean absorbance of blank wells. All measurements were performed in triplicate for each observation point.

Table 3. Phenotype of the CTL induced by DCs transfected with HCC mRNA either from HepG-2 cells (experiment 1) or from tumour tissues from patients 1, 2 or 3 (percentage of CD3+ and CD8+ cells to CTL).

Group	CD83(%)	CD86 (%)	HLA-DR
DCs	51.1	73.6	99.7
DCs+mRNA of HepG-2	80.0	97.4	99.9
DCs+mRNA of HCC-1	82.3	96.5	99.7
DCs+mRNA of HCC-2	78.7	93.6	99.5

P<0.05 for CD83 and CD96.

P>0.05 for HLA-DR

Table 2. Phenotype of DCs transfected with HCC mRNA

	Before induction	After induction
Experiment 1	27.8	89.3
Patient 1	26.5	73.6
Patient 2	29.6	86.8
Patient 3	25.4	53.6

Flow cytometry

Suspended cells ($1x10^6$ cells/mL) were washed (x3) in PBS, reacted with monoclonal antibodies for 30 min and then stained with fluorescence isothiocyanate (FITC)-labelled goat anti-mouse IgG polyclonal antibody for 5 min. The cells were washed with PBS containing 4% fetal bovine serum (FBS), resuspended in the same buffer at a concentration of $1x10^6$ cells/mL and analysed immediately by Fluorescence Activated Cell Sorter (FACS, Becton-Dickinson), as described previously.[31] Surface phenotypes CD40, anti-CD80, anti-CD83 and anti-CD86 of DCs and the proportions of CD3+, CD4+, CD8+, CD16+ and CD56+ cells were determined using mouse anti- human monoclonal antibodies.

Statistical analysis

Student's *t*-test was used to compare the difference. The significance level was $P<0.05$. SPSS 10.0 software was used.

Results

***Induction and culture of DCs* in vitro**

After two days of culture in medium with 100 ng/mL GM-CSF and 500 u/ml IL-4, PBMCs began to show cellular fission and cell colonies. Cell colony size increased and reached a peak on day 5, but disappeared by day 7. The cells showed the typical morphological DC features (large size, irregular, dendritic, veil-like shape).

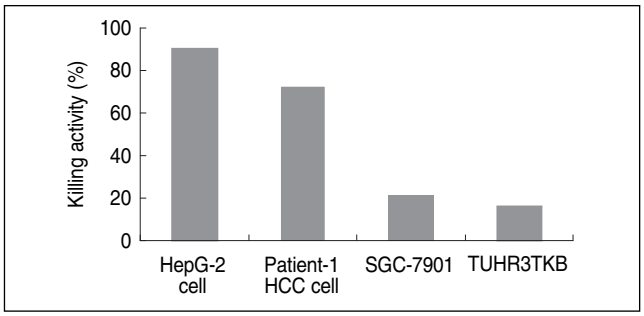
Amplification of HCC mRNA

The HCC mRNA was amplified from HCC tumour tissue. The primary amplified HCC mRNA product was 174 bp in size and was divided into two fragments (102 bp and 72 bp) using the restriction enzyme. The final product was 101 bp in size and was divided into two fragments (60 bp and 41 bp) using the restriction enzyme (Fig. 1).

Phenotype of DCs transfected with HCC mRNA

After transfected with HCC mRNA, DCs were identified using flow cytometery. The percentage of CD83- or CD86-

Fig. 2A. Specific cytotoxicites of the CTL induced by DC transfected with AFP+ HepG-2 cells. CTL killed most of the autologous HepG-2 cells and HCC cells of the AFP+ patient but did not kill any allogeneic tumour cells (SGC-7901 and TUHR3TKB cells).



positive DCs transfected with the mRNA of the HepG-2 cell line or of the AFP+ patients' HCC tissue were significantly higher than those of the other DC groups ($P<0.05$; Table 2).

Characteristics of CTLs

After PBMCs from patient 1 were co-cultured with DCs transfected with AFP+ HepG-2 cell mRNA for three weeks, CD3+ and CD8+ cells increased from 27.8% to 89.3% of the total CTL. In all three patients, the major population of CD3+ and CD8+ cells in the CTL increased from 26.5%, 29.6%, and 25.4% to 73.6%, 86.8% and 53.6%, respectively. The percentage of CD3+ and CD8+ cells of CTL from DCs transfected with HCC mRNA from the AFP- patient 3 was lower than that of CTL from DCs with transfected HCC mRNA from the AFP+ HepG-2 cells and the two AFP+ patients (Table 3).

Cytotoxic activity

The CTLs from DCs transfected with AFP+ HepG-2 cells' HCC mRNA killed 91% of HepG-2 cells and 72% of HCC cells from patient 1 at an E/T ratio of 8 after 24 h. In contrast, they killed only 21% of SGC-7901 and 16% of TUHR3TKB cells (Fig. 2A). The CTLs from DCs transfected with AFP+ patient 1's HCC mRNA killed 61%, 75% and 90% of HepG-2 cells, while the CTL from DCs transfected with AFP + patient 2's HCC mRNA killed 75%, 89% and 93% of HepG-2 cells at E/T ratios of 2, 4 and 8, respectively, after 24 h (Fig. 2B). However, the CTL from DCs transfected with AFP- patient 3's HCC mRNA killed only 15%, 23% and 46% of HepG-2 cells at E/T ratios of 2, 4 and 8, respectively, after 24 h (Fig. 2C).

As shown in Figure 3, inhibition of cytotoxicity of CTL by monoclonal antibodies against surface proteins was observed in patient 1. The effector cells were pretreated with monoclonal antibodies against CD3, CD4 and CD8 (Nichirei, Nu-T3 clone [5 μg/mL], Nu-Th/I clone [5 μg/mL], Nu-Ts/c clone [5 μg/mL]) at 4°C for 30 min. The target cells were pretreated with antibodies against MHC-class I and class II (Dako, W6132 clone [16.5 μg/mL] and CR3143 clone [20.3 μg/mL], respectively) at 37°C for 4 h and incubated with the effector cells at an E/T ratio of 8 at 37° for 4 h. The killing activity of the CTL from patient 1 was inhibited by pretreatment with monoclonal antibodies against CD3, CD8 and anti-MHC-class I, but not by antibodies against CD4 and MHC-class II at an E/T ratio of 8.

Discussion

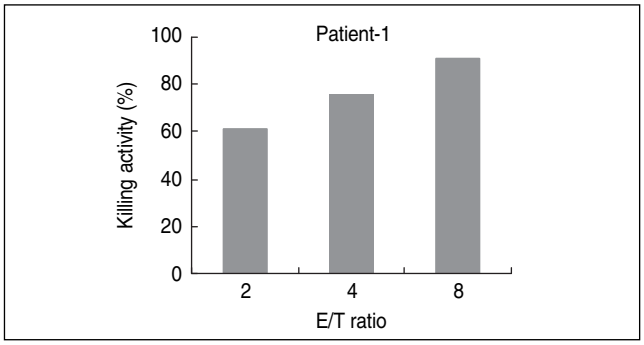
Reported tumour vaccines have been used to enhance T-cell responses in many forms (e.g., as whole tumour cells, tumour cell lysates, genetically modified tumour cells and peptide, and autologous dendritic cells).[32-34] Tumour cells themselves could be used as immunogens, as they are thought to be able to express a set of tumour-specific peptide-MHC complexes recognised by CTLs.

Although many reports have described the establishment and characterisation of tumour-specific CTLs and their possible therapeutic effects, the induction of human CTLs against HCC has not been easy. One of the key obstacles is the preparation of sufficient tumour antigen for repeated stimulation of CTL growth over a prolonged culture period. Therefore, vaccination with the patient's own repertoire of tumour antigens may offer a superior strategy to elicit protective immunity. However, this approach is currently limited to a select group of patients from whom sufficient tumour tissue can be obtained for antigen preparation.

Active immunotherapy could induce tumour-specific CTLs and achieve a long-term antitumour immune response.[35] An HCC-associated antigen, AFP, has served as a target for T-cell immunotherapy in animals,[36,37] but so far only patients carrying matched MHC alleles have benefited from a tumour-associated, antigen-based vaccine.[38] However, antigen preloaded DCs have elicited a strong antitumour immune response.[39,40]

The present study shows that HCC mRNA isolated from HepG-2 cells and from HCC tumour tissues obtained by microdissection can be amplified without loss of function. It provides the foundation for the establishment of a tumour cDNA library and the preparation of mRNA containing the total tumour genetic information. After the DCs are transfected with the tumour mRNA, they become potent

Fig. 2B. Specific cytotoxicites of the CTL induced by DC transfected with HCC mRNA from AFP+ patients 1 and 2.



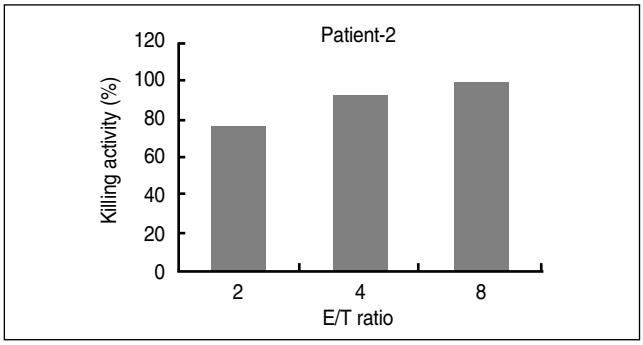
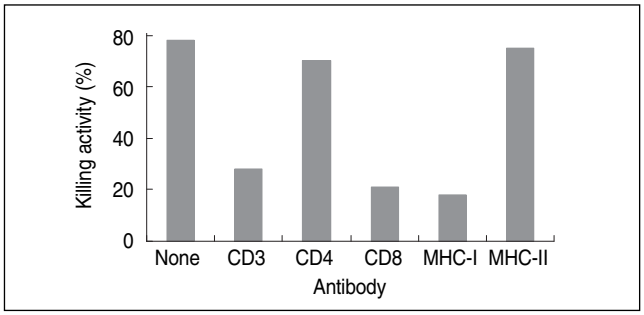


Fig. 3. Inhibition of cytotoxity of CTL by monoclonal antibodies against surface proteins in patient 1.



specialised tumour antigen presenting cells, and the effect is similar to that of a DC hybridoma.

Boczkowski[41] reported that mouse DCs pulsed with *in vitro* synthesised chicken ovalbumin (OVA) mRNA can stimulate primary OVA-specific CTL responses *in vitro* or *in vivo*, and inhibit metastasis *in vivo*. Nair[7] indicated that human DCs incubated with carcinoembryonic antigen (CEA) mRNA induce CEA-specific CTLs that possess powerful antitumour effect.

Dendritic cell vaccination using mRNA as the source of tumour antigen may have several advantages.[42-44] First, unlimited amounts of mRNA can be obtained through amplication of small amounts of mRNA extracted from tumour cells. Second, RNA-transfected DCs have the potential to present a variety of antigen epitopes to cytotoxic lymphocytes through the MHC class I pathway, and probably also through the MHC class II pathway. However, there are concerns about safety, but as virus vector-based retrotranscription is not involved in the transfection of DC with mRNA, there is neither risk of viral gene or tumour mRNA integration into the host genome nor expression of oncogenic proteins.

Studies show that murine and human DCs transfected with tumour mRNA can stimulate potent CTL responses *in vitro* and *in vivo*, leading to a significant reduction in metastases and survival benefit in tumour-bearing mice.[41,45,46] Takayama *et al*.[47] reported that adoptive immunotherapy lowers post-surgical recurrence rates of HCC. Furthermore, autologous dendritic cell-based immunotherapy in 10 patients with unresectable primary liver cancer has been reported in phase I clinical trials.[48]

The present study shows that when PBMCs from HCC patients are co-cultured with DCs transfected with AFP+ HepG-2 cell mRNA for 3 weeks, CD3+ and CD8+ cells increased from 27.8% to 89.3% of the total CTL. In all three patients, the major CTL population comprised CD3+ and CD8+ cells. The CTL induced by DCs transfected with AFP+ HCC mRNA killed 91%, 90%, and 93% of HepG-2 cells at an E/T ratio of 8, but killed only 21% of SGC-7901 and 16% of TUHR3TKB cells. The CTLs induced by DCs transfected with mRNA from patient 3 killed only 46% of HepG-2 cells at an E/T ratio of 8.

Cytotoxic activity was inhibited by treatment with anti-CD3, anti-CD8, and anti-MHC class I monoclonal antibodies but not with anti-CD4 and anti-MHC class II antibodies. These results indicate that the CTLs induced by DCs transfected with AFP+ HCC mRNA showed specific lysis of AFP+ HepG-2 target cells and AFP+ patient 1's HCC cells in an MHC class I-restricted manner.

The results of the present results suggest that HCC mRNA-transfected DCs represent a broadly applicable vaccine strategy to induce potentially therapeutic CTL responses in HCC. The HCC mRNA antigen can be obtained easily from very small amounts of tumour tissue. The HCC mRNA DC vaccine would appear to be a promising adjunctive modality in the treatment of human HCC. □

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