Potential therapeutic value of dendritic cells loaded with NY-ESO-1 protein for the immunotherapy of advanced hepatocellular carcinoma

YUQING CHEN, AIMIN HUANG, MEIQIN GAO, YONGQIN YAN and WENMIN ZHANG

Department of Pathology, Fujian Medical University, Fuzhou, Fujian 350004 P.R. China

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Abstract. NY-ESO-1 is one of the most immunogenic cancer-testis (CT) antigens. Cancer vaccine trials based on NY-ESO-1 are currently ongoing. Dendritic cells (DCs) are the most potent antigen-presenting cells. The immune functions of DCs in a number of tumors have been identified; however, the potential therapeutic value of DCs pulsed with NY-ESO-1 in hepatocellular carcinoma (HCC) has not been extensively investigated. The objectives of the present study were to evaluate T cell response following stimulation with DCs pulsed with the recombinant NY-ESO-1 protein (rESO) and to establish a correlation between NY-ESO-1 expression and clinicopathological features in HCC patients. DCs were generated with granulocyte/macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) from human peripheral blood mononuclear cells. A mixed T cell reaction with DCs loaded with recombinant NY-ESO-1 protein (rESO-DCs) was evaluated by MTT assay. T cell responses against HCC cell lines were analyzed by measuring lactate dehydrogenase (LDH) activity. The protein levels of NY-ESO-1 were detected by immunohistochemistry (IHC) in a tissue microarray (TMA) containing 190 HCC samples. NY-ESO-1 transcript abundance was determined by reverse transcriptase-polymerase chain reaction (RT-PCR) in 54 out of the 190 HCC samples. The results revealed that mature DCs were induced and that rESO-DCs significantly stimulated T cell proliferation. The specific lysis of T cells stimulated with rESO-DCs was significantly higher in the NY-ESO-1-positive HCC cells compared with the NY-ESO-1-negative cells and the other controls ($p<0.01$). NY-ESO-1 was expressed in 15.8% (30/190)of the HCC samples, as shown by IHC and in 24.1% (13/54) of the samples, as shown by RT-PCR. The frequency of NY-ESO-1 expression was significantly higher in HCC patients with portal vein tumor thrombosis (24.6%) compared with those without thrombosis (11.2%, $p=0.013$). Our data suggest that DCs loaded with NY-ESO-1 protein stimulate antigen-specific T cell responses against HCC cells *in vitro*. NY-ESO-1 may thus be used as a potential target for immunotherapy in advanced HCC.

Correspondence to: Professor Wenmin Zhang, Department of Pathology, Fujian Medical University, 88 Jiao Tong Road, Fuzhou, Fujian 350004, P.R. China
E-mail: wminz@163.com

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Introduction

Hepatocellular carcinoma (HCC) is the fifth most common type of cancer worldwide and is particularly prevalent in China (1,2). Currently, treatments for HCC include surgical resection, chemotherapy and liver transplantation (3). However, the outcomes remain dismal. The 5-year survival rate for patients with HCC has been reported to be 30-50% (4,5). Thus, novel, alternative therapeutic options for HCC are urgently required. Immunotherapy for cancer has received much attention in recent years. A number of tumor antigens, such as human telomerase-reverse transcriptase (hTERT) or alpha-fetoprotein (AFP) have been identified as immunotherapeutic targets, and a number of immunotherapeutic trials have been performed to evaluate the potential therapeutic value of HCC immunotherapy (6).

Cancer-testis (CT) antigens have been considered attractive targets for cancer immunotherapy due to their restricted expression patterns in a variety of tumors and normal tissues (7). To date, >150 genes or gene families encoding CT antigens have been identified (8). However, only a limited number of CT antigens have been shown to elicit both humoral and cellular responses. NY-ESO-1 (cancer/testis antigen 1B), also known as CTAG1, is one of the most immunogenic CT antigens (9). It was originally found in esophageal cancer by serological recombinant cDNA expression cloning (SEREX) (10) and is expressed in several tumors, including HCC (11-14). NY-ESO-1 expression is associated with a poor tumor outcome and is recognized as a potential biomarker for the prediction of tumor recurrence and treatment outcomes in patients with gastrointestinal stromal tumors and cutaneous melanomas (15).

Due to its expression patterns and strong immunogenicity, NY-ESO-1 has emerged as one of the most attractive targets for cancer vaccines (7,9). The NY-ESO-1 vaccine, based on peptide or protein, has been tested in the treatment of patients with tumors expressing NY-ESO-1, including lung cancer, ovarian cancer, esophageal cancer and melanoma (16-20). The majority

of the patients showed enhanced immune responses and disease stabilization was achieved in some patients (20). Furthermore, these studies illustrated the safety of various preparations of NY-ESO-1 vaccines. The use of *in vitro*-generated, autologous dendritic cells (DCs) as a cellular adjuvant for vaccine delivery has been widely tested in cancer patients (21). The potential efficacy of induced immunity against HCC has been supported by a report that the immunization of 2 HCC patients with autologous HCC lysate-loaded DCs resulted in the prolonged survival of >3 years in 1 patient (22). However, whether DCs pulsed with NY-ESO-1 protein can induce antigen-specific immune responses against HCC remains unclear. In addition, the correlation between NY-ESO-1 expression and clinical parameters has not been extensively investigated.

In a previous study, we purified the recombinant NY-ESO-1 protein (rESO) (23). In this study we aimed to evaluate T cell response against HCC cell lines following incubation with DCs loaded with rESO. Furthermore, we assessed the mRNA and protein abundance of NY-ESO-1 in HCC samples and determined the correlation between NY-ESO-1 expression and clinical parameters.

Table I. Clinicopathological characteristics of HCC patients.

Characteristic	Paraffin-embeded HCC specimens	Fresh-frozen HCC specimens
No. of patients	190	54
Average age (years)	49	50
Age range (years)	21-75	32-70
Male/female	162/28	44/10
HBsAg-positive	155	44
AFP (≥20 ng/ml)	132	39
With cirrhosis	172	48
With portal vein tumor thrombosis	65	18
Edmondson's classification (grades I-II/III-IV)	103/87	29/25

HBsAg, hepatitis B surface antigen; AFP, alpha-fetoprotein. HCC, hepatocellular carcinoma.

Materials and methods

Patients and samples. A total of 190 paraffin-embedded HCC specimens and their adjacent non-cancerous tissues were collected at the Center for Liver Disease, the First Affiliated Hospital, Fujian Medical University, Fuzhou, China, between 2007 and 2009. Frozen tissues were available in 54 cases. They were frozen immediately in liquid nitrogen after removal from surgical resection and stored at -80˚C until use. Informed consent was obtained from all patients. The clinicopathological parameters of these cases are summarized in Table I. Tissue microarrays (TMAs) of the HCC and adjacent non-cancerous liver samples were prepared according to standard procedures (Beecher Instruments Inc., Silver Spring, MD, USA). The study was approved by the Ethical Review Board of Fujian Medical University.

HCC cell lines. The HCC cell lines, H4M and H2P, were kindly provided by Dr JianMing Wen at the Department of Pathology, the First Affiliated Hospital, Sun Yat-sen University, Guangzhou, China. HCC cells were cultured in RPMI-1640 medium with 10% fetal bovine serum (FBS), L-glutamine, penicillin (100 IU/ml) and streptomycin (100 μg/ml) at 37˚C.

Preparation of DCs. Peripheral blood mononuclear cells (PBMCs) from healthy volunteers were isolated from blood by Ficoll-Hypaque density gradient centrifugation (Amersham Biosciences, Uppsala, Sweden). The cells were then seeded on 6-well plates for 2 h at a density of 2-3x10^6 cells/ml. Non-adherent cells were removed and adherent cells were incubated in RPMI-1640 medium supplemented with 20% FBS, 1,000 U/ml of granulocyte macrophage colony-stimulating factor (GM-CSF) and 500 U/ml of interleukin-4 (IL-4; PeproTech Inc., Rocky Hill, NJ, USA). After 3 days of incubation, the old medium with floating cells was gently removed and replaced with fresh medium. After 5 days of incubation, 1/3 of the cells were collected as immature DCs (imDCs). The remaining cells were treated with rESO or IL-4 at a concentration of 50 μg/ml for 24 h. The cells were then incubated with 10 ng/ml tumor necrosis factor-α (TNF-α) for 48 h to induce the formation of mature DCs (mDCs). ImDCs and mDCs were assayed by flow cytometry.

Detection of T cell response. T cells (4x10^5 cells/well) were incubated with imDCs or mDCs at a ratio of 20:1 at 37˚C for 72 h in RPMI-1640 medium with 20% FBS, 100 U/ml interleukin-2 (IL-2) and 20 μg/ml phytohaemagglutinin (PHA). Cell proliferation was measured by MTT assay as previously described by Li *et al* (24). Absorbance was measured at 570 nm using a Multi-Well Plate Reader (Beckman Coulter Inc., Brea, CA, USA). The proliferation index (PI) of the T cells was calculated using the following equation: PI = mixed lymphocyte reaction/lymphocyte reaction. The experiment was conducted 3 times.

Flow cytometry. The level of surface molecules on DCs was determined by flow cytometry using anti-human antibodies: FITC-CD83, PE-CD86 and APC-HLA-DR (BioLegend, San Diego, CA, USA) as previously described (25). Negative controls were fluorochrome-conjugated isotype-matched irrelevant antibodies (Invitrogen, Carlsbad, CA, USA). Briefly, cells suspended in PBS were incubated with antibodies at room temperature for 30 min in the dark. The cells were then analyzed by a BD FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA).

Cytotoxicity assays. Allogeneic T cells were collected as effector cells, and H4M/H2P cells were used as the target cells. Effector cells included the rESO-DC-T group (T cells stimulated with DCs loaded with rESO), the IL-4-DC-T group (T cells stimulated with DCs treated with IL-4) and the T cells group (T cells without stimulation). Effector cells and target cells were incubated at effector/target ratios of 20:1 or 50:1 for

4 h at 37˚C in 96-well plates. The activity of T cells against the target tumor cells was measured as previously described in a standard lactate dehydrogenase (LDH) release assay (26). The cytotoxicity of the T cells was calculated as a percentage of specific lysis using the following formula: % specific lysis = (effector/target release - spontaneous release)/(maximal release - spontaneous release) x100%. Data are presented as the means ± standard deviation.

Immunohistochemistry (IHC). Formalin-fixed slides from TMAs were deparaffinized by xylene and rehydrated by a series of graded alcohol. Endogenous horseradish peroxidase activity was blocked by treatment with 3% (v/v) H_2O_2. Antigen retrieval was achieved by heating the samples in a microwave in 10 mM citrate buffer (pH 6.0) for 20 min. Non-specific binding was blocked by incubation with 1% (w/v) BSA in phosphate-buffered saline (PBS) for 1 h at room temperature. The slides were then incubated with 1:200 monoclonal anti-NY-ESO-1 antibody (clone E978; Zymed Laboratories, Inc., South San Francisco, CA, USA) overnight at 4˚C. The slides were then incubated with HRP-labeled anti-mouse secondary antibody for 1 h. Immunoreactivity was visualized by diaminobenzidine (DAB). The sections were counterstained with hematoxylin. PBS was used for rinsing between each step. Negative controls were created by omitting the primary antibody. NY-ESO-1 expression was scored by 2 independent observers. The level of NY-ESO-1 expression was described semi-quantitatively using a 4-grade scoring system: -, no staining or focal staining (<5% total); +, 5-<25%; ++, 25-50%; and +++, >50%.

Reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was extracted from the 54 frozen samples and HCC cell lines using TRIzol reagent (Gibco-BRL, Gaithersburg, MD, USA) according to the manufacturer's instructions. The reverse transcription reaction was performed using the First Strand cDNA Synthesis kit (MBI Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions. Amplification was carried out using the following primers: ESO-1F (exon 1), 5'-cgcctgcttgagttctacctc-3'; and ESO-1R (exon 3), 5'-agggaaagctgctggagacag-3'. The reaction was conducted under the following conditions: 5 min at 95˚C, followed by 30 sec at 94˚C, 1 min at 60˚C and 45 sec at 72˚C for 35 cycles, with a 10 min elongation step at 72˚C. β-actin was used as the positive control. The expected PCR product sizes of NY-ESO-1 and β-actin were 219 and 120 bp respectively. Bands were visualized by ethidium bromide staining after separation on a 1.5% agarose gel. The assay was carried out at least 2 times.

Statistical analysis. Statistical analyses were carried out using SPSS version 13.0 software (SPSS, Chicago, IL, USA). Fisher's exact test or the χ^2 test were used to analyze categorical data. Variance analysis was used to determine the statistical significance of the differences between 2 groups. A p-value <0.05 was considered to indicate a statistically significant difference.

Results

DC induction and identification. We obtained 2±0.31x10^7 mononuclear cells from 50 ml PBMCs following Ficoll separation. The cells were then treated as described in Materials and methods. After 5 days of incubation with GM-CSF and IL-4, 2.2±0.49x10^6 dendritic-like cells were obtained based on morphology. Flow cytometry analysis revealed that 38.90±2.43% of the imDCs were positive for HLA-DR (Fig. 1). In addition, 3.67±0.49% and 17.23±1.24% of the imDCs were positive for CD83 and CD86, respectively. After rESO or IL-4 induction for 24 h and the addition of TNF-α for 48 h, the rESO-DCs or IL-4-DCs showed a typical branch-like appearance. These cells exhibited a significantly higher expression of HLA-DR, CD83 and CD86 ($p<0.05$) (Fig. 1). Compared with the imDCs, positivity for HLA-DR, CD83 and CD86 increased by 2- to 21-fold in the rESO-DCs. Similarly, the percentage of HLA-DR, CD83 and CD86 positivity increased by 2- to 17-fold in the IL-4-DCs compared with the imDCs.

To determine the potential of DCs to stimulate T cell proliferation, we performed a mixed T lymphocyte reaction by MTT assay. The PI of the T cells mixed with rESO-DCs was higher than that of those mixed with IL-4-DCs and the imDCs (3.80±0.66 vs. 2.99±0.26 and 1.44±0.36). A statistically significant difference was observed when comparing the rESO-DCs with the imDCs ($p<0.05$) (Fig. 2). These data illustrated that DCs pulsed with rESO protein were more effective in stimulating T lymphocyte proliferation compared with the control cells.

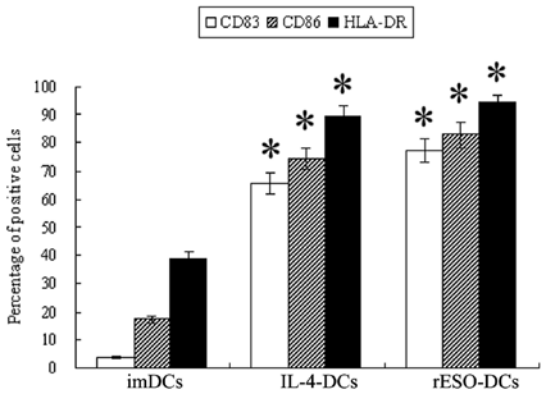

Figure 1. The expression of surface molecules on DCs was assayed by flow cytometry. Data are expressed as the means ± SD of the percentage positive cells for triplicate assays. The expression of CD83, CD86 and HLA-DR in the rESO-DCs and IL-4-DCs was significantly upregulated compared with the imDC group (*$p<0.05$). imDCs, immature dendritic cells (DC), IL-4-DCs, DCs streated with interleukin-4 (IL-4); rESO-DCs, DCs pulsed with recombinant NY-ESO-1 protein (rESO).

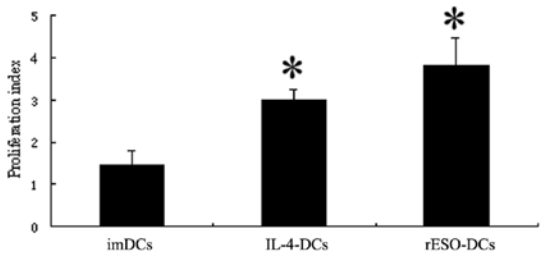

Figure 2. Proliferation index of T lymphocytes following coculture with different dendritic cells (DCs). *$p<0.05$ vs. imDC group. imDCs, immature DCs; IL-4-DCs, DCs treated with interleukin-4 (IL-4); rESO-DCs, DCs pulsed with recombinant NY-ESO-1 protein (rESO).

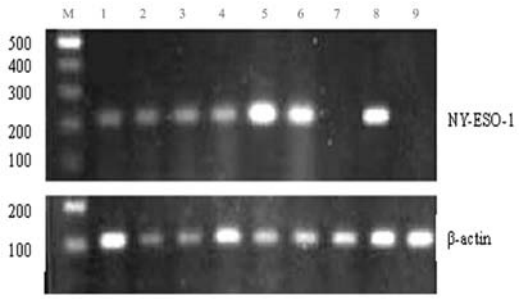

Figure 3. Representative gels of NY-ESO-1 mRNA in hepatocellular carcinoma (HCC) samples and cell lines as determined by reverse transcription-PCR (RT-PCR). The size of the expected product for NY-ESO-1 was 219 bp. HCC tissue (lanes 1-6) and H4M cells (lane 8) were positive for NY-ESO-1 expression. Non-tumorous liver tissue (lane 7) and H2P cells (lane 9) were NY-ESO-1-negative. M, 100 bp molecular weight marker.

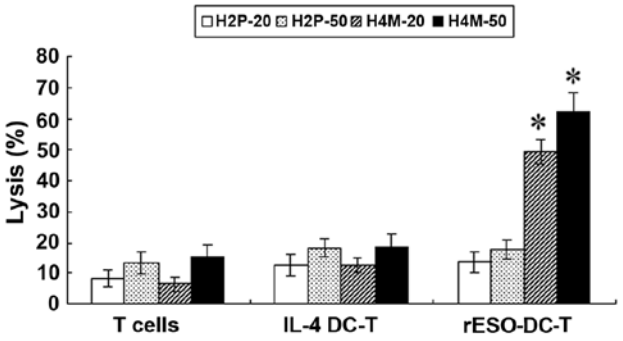

Figure 4. Cytotoxic activity of different T cells stimulated with dendritic cells (DCs). Following stimulation with DCs [(pulsed with NY-ESO-1 protein or interleukin-4 (IL-4)] or no stimulation, T cells were collected as the effector cells. The HCC cell lines, H4M or H2P, were used as the target cells. Following incubation with effector to target cell (E:T) ratios of 1:20 or 1:50, the cytolytic activity was assessed by measurement of lactate dehydrogenase (LDH) release. Percentage lysis of recombinant NY-ESO-1 protein (rESO)-DC-T against H4M cells was significantly higher than that of the other T cell groups or against H2P cells (*p<0.01). rESO-DC-T, T cells stimulated with DCs pulsed with rESO; IL-4-DC-T, T cells stimulated with DCs treated with IL-4; T cells, T cells without stimulation; H4M-50 or H4M-20, T cells to H4M at ratios of 50:1 or 20:1; H2P-50 or H2P-20, T cells to H2P at ratios of 50:1 or 20:1.

Cytotoxicity of NY-ESO-1-specific T lymphocytes. The cytotoxicity of T cells against 2 HCC cell lines was determined by LDH release assay. Allogeneic T cells were collected as the effector cells, and H4M and H2P cells were used as the target cells. H4M cells were NY-ESO-1-positive and H2P cells were NY-ESO-1-negative, as shown by RT-PCR (Fig. 3). T cells and HCC cells were incubated at a ratio of 50:1 or 20:1. When the H4M cells were used as the target cells, the specific NY-ESO-1 T cells lysed the cancer cells effectively in a dose-dependent manner (62.13±5.89% for ratio 50:1 and 49.23±3.78% for ratio 20:1), significantly higher than that of the IL-4-DC-T and T cells group (p<0.01) (Fig. 4). By contrast, the specific lysis among the rESO-DC-T, IL-4-DC-T and T cells did not

Table II. NY-ESO-1 expression in the HCC patients.

	IHC			RT-PCR		
Group	N	Positive	(%)	N	Positive	(%)
Gender						
Male	162	25	15.4	44	10	22.7
Female	28	5	17.9	10	3	30.0
Tumor size						
<5 cm	52	7	13.5	13	3	23.1
>5 cm	138	23	16.7	41	10	24.4
Portal vein tumor thrombosis						
Positive	65	16	24.6[a]	18	6	33.3
Negative	125	14	11.2	36	7	19.4
Edmondson's classification						
Grades I-II	103	14	13.6	29	6	20.7
Grades III- IV	87	16	18.4	25	7	28
HBsAg						
Positive	155	24	15.5	44	10	22.7
Negative	35	6	17.1	10	3	30.0
AFP						
<20 ng/ml	58	9	15.5	15	3	20.0
≥20 ng/ml	132	21	15.9	39	10	25.6
Total	190	30	15.8	54	13	24.1

[a]p<0.05 compared with HCC patients without portal vein tumor thrombosis. HCC, hepatocellular carcinoma; IHC, immunohistochemistry; AFP, alpha-fetoprotein.

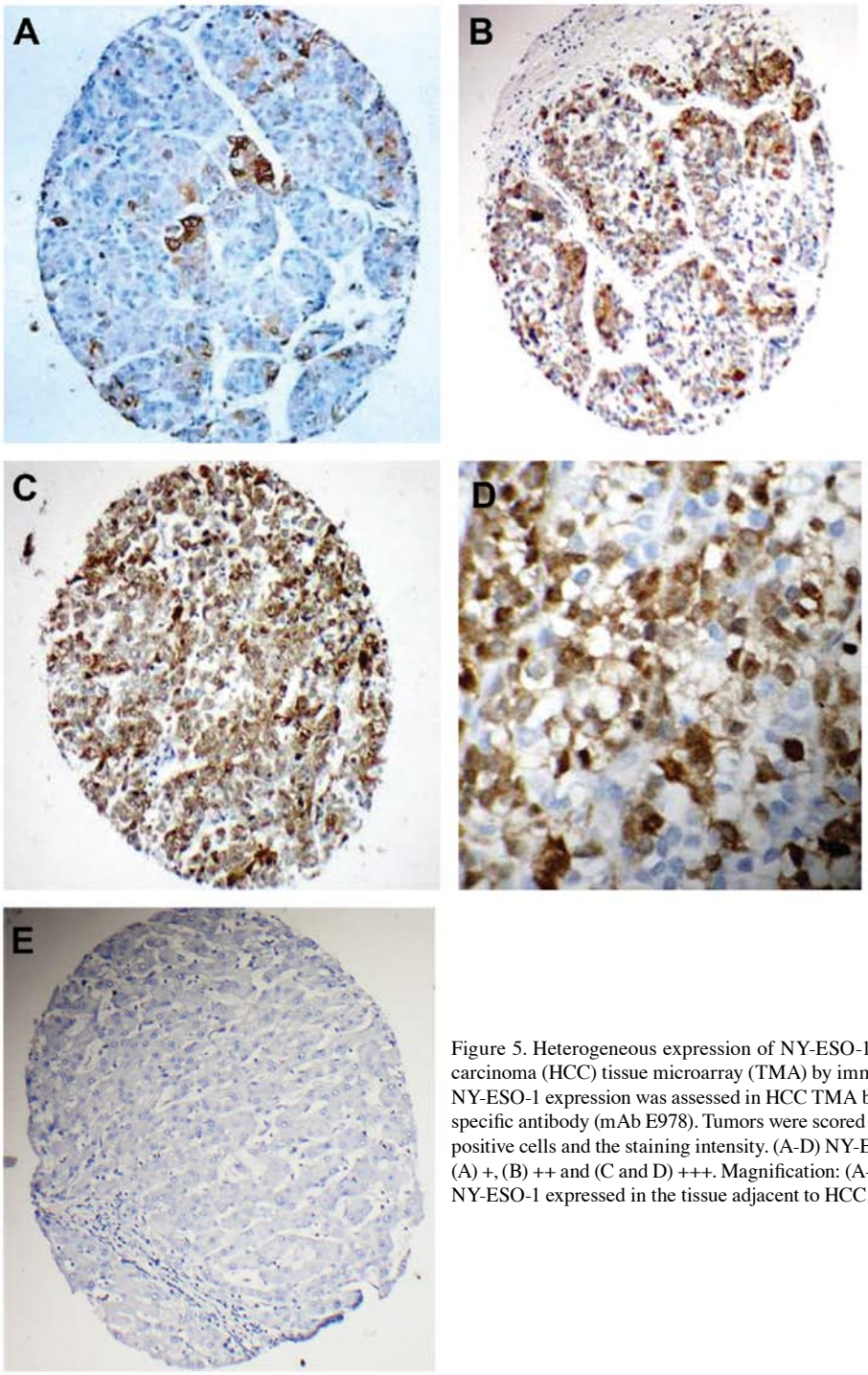

Figure 5. Heterogeneous expression of NY-ESO-1 protein in hepatocellular carcinoma (HCC) tissue microarray (TMA) by immunohistochemistry (IHC). NY-ESO-1 expression was assessed in HCC TMA by IHC using the NY-ESO-specific antibody (mAb E978). Tumors were scored based on the percentage of positive cells and the staining intensity. (A-D) NY-ESO-1 expression scored as (A) +, (B) ++ and (C and D) +++. Magnification: (A-C) x100; (D) x400. (E) No NY-ESO-1 expressed in the tissue adjacent to HCC. Magnification, x100.

differ significantly in the H2P cells, which did not express NY-ESO-1. These results demonstrate that T cells stimulated with DCs pulsed with rESO exert significant antigen-specific lysis on HCC cells which express NY-ESO-1.

NY-ESO-1 expression in HCC patients. IHC analyses of NY-ESO-1 protein indicated that a total of 30 out of the 190 HCC specimens expressed NY-ESO-1 (15.8%). Among these, 8 (4.2%) were graded as +++, 12 (6.3%) as ++, and 10 (5.3%) as + (Fig. 5A-C). NY-ESO-1 was located predominantly in the cytoplasm, although nuclear staining was observed in a few cells (Fig. 5D). No staining was observed in the tissue adjacent to HCC (Fig. 5E). We also determined the expression profile of NY-ESO-1 mRNA in 54 tumors by RT-PCR. A detectable NY-ESO-1 transcript was observed in 10 tumors (18.5%). Representative results are shown in Fig. 3.

Correlation between NY-ESO-1 expression and clinical parameters. Table II summarizes the correlation between NY-ESO-1 expression and the clinicopathological characteristics of the tumor samples. In the HCC patients with portal vein tumor thrombosis, the frequency of NY-ESO-1 positivity was 24.6% (16/65). By contrast, the frequency of NY-ESO-1 positivity in the HCC patients without portal vein tumor thrombosis

Table III. Expression of NY-ESO-1 in 54 HCC samples detected by IHC and RT-PCR.

	IHC				
RT-PCR	+ (n=5)	++ (n=2)	+++ (n=3)	Negative (n=44)	Total
Positive	4	2	3	4	13
Negative	1	0	0	40	41
Total	5	2	3	44	54

HCC, hepatocellular carcinoma; IHC, immunohistochemistry; RT-PCR, reverse transcriptase-polymerase chain reaction.

was 11.2% (14/125), which was significantly lower compared with the HCC patients with portal vein tumor thrombosis (p=0.013). Statistical analysis also revealed that the frequency of the detectable NY-ESO-1 transcript was higher in HCC patients with portal vein tumor thrombosis as compared with the HCC patients without portal vein tumor thrombosis (33.3 vs. 19.4%, p=0.21). Statistical analysis did not reveal a correlation between NY-ESO-1 expression and age, gender, tumor size, histological grade or hepatitis B surface antigen (HBsAg)/AFP status in the HCC samples.

Correlation between NY-ESO-1 mRNA and protein levels in HCC patients. A total of 54 HCC samples were examined for both the transcript and protein levels of NY-ESO-1. A total of 10 cases were positive for NY-ESO-1 protein, as shown by by IHC and 13 tumors expressed NY-ESO-1 mRNA, as shown by RT-PCR. As presented in Table III, 4 cases were positive for NY-ESO-1 mRNA expression, as shown by RT-PCR, but were shown to be negative by IHC. Out of the 10 tumors with positive immunostaining, 1 tumor was shown to be negative by RT-PCR. This case displayed positive IHC staining for NY-ESO-1.

Discussion

The survival of HCC patients is poor despite advancements in HCC therapy (3). In recent years, immunotherapy has become a promising strategy for tumor therapy. A variety of immunotherapy regimens have emerged for HCC patients, including HCC lysates (27), tumor cell-DC fusion (28) and cytokines (29). However, the effects of these methods have been limited. The major barrier to antigen-specific immunotherapy in HCC is a lack of well-defined immunogenic tumor antigens. It has been shown that NY-ESO-1 is one of the most immunogenic CT antigens (7). Immune responses against NY-ESO-1 have been induced in a number of tumors, such as melanoma (30), ovarian cancer (31) and lung cancer (12). The NY-ESO-1 vaccine has been investigated in clinical trials of melanoma and ovarian cancer (32). Recently, a NY-ESO-1 vaccine has also been examined in esophageal and prostate cancer patients (33). Patients bearing NY-ESO-1-expressing tumors displayed an effective induction of NY-ESO-1 antibody and CD4/CD8 T cell responses. Although only a few studies have reported the immune responses induced by NY-ESO-1 in HCC patients (34), NY-ESO-1-specific immune responses in HCC are not yet well understood. In our study, our results demonstrated that NY-ESO-1-specific T cell responses were induced, which significantly increased the lysis of NY-ESO-1-expressing HCC cells *in vitro*. These data provide evidence supporting the use of NY-ESO-1-based immunotherapy and suggest that NY-ESO-1 may be a useful target for the immunotherapy of HCC patients.

For cancer clinical trials targeted against defined antigens, a detailed knowledge of the antigen expression is crucial. Our data indicated that the positive rate of NY-ESO-1 protein was 15.8% and the NY-ESO-1 mRNA expression was 24.1% in the HCC samples, which was higher than that found in the study by Luo *et al* (35), and is comparable to the results of Wang *et al* (13). We also illustrated a high concordance between RT-PCR and IHC for NY-ESO-1 expression in the HCC samples. The HCC patients displayed moderate or high levels of NY-ESO-1 and as shown by IHC, they were positive for the NY-ESO-1 transcript. However, we detected positivity for the NY-ESO-1 transcript in 4 HCC samples without a detectable NY-ESO-1 protein expression. A likely explanation for this is that RT-PCR has a higher sensitivity to detect NY-ESO-1 than IHC. In this study, we also observed heterogeneity for NY-ESO-1 expression by IHC in a few cases, and this information could not be achieved by RT-PCR. It may therefore be desirable to use both IHC and RT-PCR to obtain information regarding the expression level and antigen distribution.

NY-ESO-1 has been speculated to be a prognostic marker in gastrointestinal stromal tumors, melanoma and HCC (15,36,37). NY-ESO-1 expression is associated with a worse tumor outcome. The present study also revealed that NY-ESO-1 expression in HCC patients with portal vein tumor thrombosis had a significantly higher intensity and positivity compared with that in HCC patients without portal vein tumor thrombosis, as shown by IHC and RT-PCR. Thus, it can be hypothesized that the NY-ESO-1 gene may play an important role in tumor invasion and progression. Although the mechanisms involved are unclear, a NY-ESO-1 vaccine may play an important role in the advanced stages of disease, as also found in esophageal carcinoma by Bujas *et al* (38).

In conclusion, we identified specific T cell responses stimulated with DCs pulsed with NY-ESO-1 in HCC cell lines. We also demonstrate that NY-ESO-1 is heterogeneously expressed in HCC patients, specifically in advanced HCC patients with portal vein tumor thrombosis. This suggests that a DC-based NY-ESO-1 vaccine may prove to be effective for immunotherapy in advanced HCC. Moreover, a combination of demethylation or preparation of multiple CT antigens may increase the efficacy of HCC immunotherapy.

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