

*Featured Article***Spontaneous Tumor-Specific Humoral and Cellular Immune Responses to NY-ESO-1 in Hepatocellular Carcinoma**

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ABSTRACT

Purpose: Hepatocellular carcinoma (HCC) is the fifth most common cancer around the world. Although several therapeutic approaches for treatment of HCC are available, survival rates for HCC patients are still very poor because of inefficient treatment options. For HCC, as well as other tumors, antigen-specific immunotherapy remains a viable approach that is dependent on the definition of tumor-associated antigens. NY-ESO-1, a member of the cancer testis antigen family, is one possible candidate for a tumor-specific antigen in HCC. The aim of this study was to show the relevance of NY-ESO-1 in hepatocellular carcinoma.

Experimental Design: Sera samples from 189 HCC patients were analyzed for NY-ESO-1-specific antibodies. Forty-nine HCC patients were screened for NY-ESO-1 mRNA expression in HCC tissue. Selected patients were followed for up to 3 years to correlate their immune response with their clinical course of events. NY-ESO-1-specific CD4+ and CD8+ T-cell responses from NY-ESO-1 seropositive patients were analyzed and a NY-ESO-1-specific cytotoxic T-cell line was generated.

Results: Twelve of 49 analyzed tumor samples expressed NY-ESO-1 mRNA and 23 of 189 patients showed NY-ESO-1-specific antibody responses. These humoral immune responses were accompanied by NY-ESO-1-specific functional CD4+ and CD8+ T-cell responses. Finally, NY-

ESO-1 humoral responses were dependent on the presence of NY-ESO-1-expressing tumors.

Conclusions: This is the first report of a spontaneous immune response in HCC patients to a known tumor-specific antigen, NY-ESO-1 protein. Our data favor the possibility of immunotherapeutic strategies for the treatment of HCC.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide with increasing incidence in Western countries (1). Current treatment options include surgical resection and liver transplantation (2), as well as local ablative therapy, which are effective only in localized tumors (3). In addition, HCC responds poorly to systemic chemotherapy; therefore, identifying and establishing novel approaches for the treatment of HCC is quite a challenge.

One approach that has shown promise in other tumors is immunotherapy (4). However, immunotherapeutic approaches for treatment of HCC have not been established thus far (5). In addition, the immunological mechanisms underlying the progression and immunogenicity of HCC are not clearly understood (6). Therefore, identification of immunologically relevant and tumor-specific antigens is necessary because these antigens can serve as important tools both for diagnosis and for therapeutic approaches (7).

Recently, a family of tumor-specific antigens (cancer-testis antigens) has been identified, that is, antigens the expression of which is limited to tumor tissue and testis (8, 9). mRNA expression of one of the cancer-testis antigens, NY-ESO-1 (10), has been detected in 20–30% of cancers (11). NY-ESO-1 mRNA has also been detected in 30% of HCC tumors by reverse transcription-PCR (RT-PCR; Ref. 11). However, until today, NY-ESO-1-specific immune responses have not been studied in HCC patients. Because NY-ESO-1 is expressed only in tumors but not in normal tissue, it would be an ideal candidate for the development of antigen-specific immunotherapy in HCC, as well as a useful marker for monitoring immune responses in HCC patients.

In this study, we have analyzed the immunological significance of NY-ESO-1 in HCC. We show that HCC patients develop humoral and cellular responses to NY-ESO-1, a tumor-specific antigen. Our data suggest that HCC is immunogenic enough to induce spontaneous tumor-specific immune responses *in vivo* and that NY-ESO-1 could be used as a useful marker for monitoring immune responses in the course of an immunotherapeutic treatment of HCC patients.

MATERIALS AND METHODS

Patients. Blood and tumor tissue samples were collected from a total of 189 HCC patients seen at the Department of Gastroenterology, Hepatology and Endocrinology, Hannover

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Note: Supplementary data for this article can be found at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org>); F. Korangy and L. Ormandy contributed equally to the work.

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Table 1 Patient characteristics and disease classification

No. of patients	189
Average age (years)	64
Age range (year)	32–85
M/F	158/31
HBsAg ^a positive (%)	27
Anti-HCV positive (%)	21
HBsAg and anti-HCV positive (%)	8
Hemochromatosis	2
Okuda I/II/III ^b	90/76/23
Child A/B/C ^c	134/45/10

^a HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus infection.

^b Okuda K, Ohtsuki T, Tomimatsu M, et al., *Cancer*. 1985 Aug 15;56(4):918–28.

^c Pugh RN, Murray-Lyon IM, Dawson JL, et al., *Br J Surg* 1973 Aug;60(8):646–9.

Medical School, Hannover, Germany. Patient characteristics and disease classification are shown in Table 1. HCC was diagnosed according to the diagnostic guidelines of the European Association for the Study of the Liver (12). α -Fetoprotein (AFP) levels were determined at the central clinical chemistry laboratory of Hannover Medical School using a commercially available electrochemiluminescence immunoassay (Roche). Tumor tissues were obtained during routine surgical procedures or by ultrasound-guided needle biopsy and were immediately frozen in liquid nitrogen. Written consent was obtained from all of the patients before blood and/or tissue sampling, and the Ethics Committee of Hannover Medical School approved the study protocol. Peripheral blood mononuclear cells (PBMCs) were routinely tested for HLA-A2 expression using a panel of HLA-A2-specific monoclonal antibodies: MA2.1; BB7.2, and PA 2.1 (American Type Culture Collection) as described previously (13).

Generation and Purification of Recombinant NY-ESO-1 protein. The NY-ESO-1 cDNA was kindly provided by Yao-Tseng Chen (Cornell University, New York, NY) and was cloned into the prokaryotic expression vector pTrc-his (Invitrogen). Full-length NY-ESO-1 protein and an irrelevant control protein [interleukin-4 (IL-4)] were expressed in *Escherichia coli* with a six-histidine (his) tag at their NH₂ termini and were purified over nickel column. The purified proteins were detected in a Western blot with anti-his antibody (Qiagen, Hilden, Germany), and purity was estimated to be >85% by SDS-PAGE analysis.

Peptides. Peptides NY-ESO-1_{157–165} (SLLMWITQC) and influenza virus matrix M1_{58–66} (GILGFVFTL) were obtained from Eurogentec, Belgium. The purity (>95%) of each peptide was confirmed by mass-spectral analysis and high-performance liquid chromatography.

RT-PCR Analysis of NY-ESO-1 Expression. Total RNA was isolated from tumor samples, from liver tissue, and from MEL373 (kindly provided by Dr. Pierre Coulie, Ludwig Institute for Cancer Research, Brussels, Belgium) using the RNAeasy kit (Qiagen). The primers used for the reverse transcription reaction were: 5'-CCCCACCGCTTCCCGTG-3' and 3' primer: 5'-CTGGCCACTCGTGCTGGGA-3'. Amplification was done with 35 cycles at an annealing temperature of 60°C.

β -Actin was used as the positive control. PCR products were visualized by agarose gel electrophoresis. The expression of NY-ESO-1 in tumors was counted as positive, only if the RT-PCR reaction repeated at least twice.

Immunoscreening of Patients' Sera by ELISA. NY-ESO-1 recombinant protein was bound to nickel-coated 96-well plates (Qiagen). Serum dilutions (1:100 to 1:100,000) of the HCC patients' sera were added to the plates and were incubated for 2 h at room temperature. The plates were then washed and were incubated with antihuman IgG-horseradish peroxidase, followed by 3,3',5,5' tetramethylbenzidine substrate solution (Bio-Rad Laboratories). The cutoff for the ELISA was made so that the response to NY-ESO-1 was at least 2-fold above the response to the control protein.

Immunoblot Analysis. Serum antibody responses against recombinant NY-ESO-1 protein were tested by Western Blot analysis. Briefly, 1 μ g of recombinant NY-ESO-1 protein (or tumor lysate when indicated) was electrophoresed along with an irrelevant recombinant protein (IL-4), on a 12% SDS gel. The gel was blotted onto nitrocellulose filter and was blocked with 3% BSA PBS-Tween 20. The blots were then incubated with patient sera or with anti-his antibody (Qiagen). Binding of the antibodies was visualized using goat antihuman IgG-horseradish peroxidase (Sigma) followed by ECL detection reagent (Amersham/Pharmacia Biosciences).

Generation of Dendritic Cells. Monocytes were isolated from PBMCs by CD14+ magnetic separation (Miltenyi, Bergisch-Gladbach, Germany). The CD14+ cells were cultured in AIM-V medium supplemented with 1000 units/ml human granulocyte macrophage colony-stimulating factor (Leukomax; Sandoz) and 1000 units/ml IL-4. After 7 days, cells were matured using 1 μ g/ml PGE₂, TNF- α and 500 units/ml IL-6. Dendritic cells were loaded with 30 μ g/ml peptide for 1 h in the presence of 10 μ g/ml β 2-microglobulin. For the detection of antigen-specific CD4+ T cells, dendritic cells were generated from peripheral blood. Briefly, PBMCs from HCC patients were cultured in IL-4 (1000 units) and granulocyte macrophage colony-stimulating factor (1000 units) for 6 days. On day 6, the dendritic cells were pulsed with full-length recombinant NY-ESO-1 protein or with an irrelevant control protein (IL-4). The quality of the dendritic cell preparation was confirmed by flow cytometry (CD80, CD86, and MHC class II expression).

Detection of NY-ESO-1-Specific CD4+ and CD8+ T Cells. Frequency of CD4+ and CD8+ T cells was analyzed by detection of γ -IFN secreting cells on antigen-specific stimulation using γ -IFN secretion assay (Miltenyi Biotech, Bergisch Gladbach, Germany; Ref. 14) as well as using HLA-A2-IgG dimeric molecules as described previously (13). Briefly, for the detection of CD8+ T-cell responses to NY-ESO-1 in HCC patients, PBMCs of the HLA-A2 patients were incubated with the NY-ESO-1_{157–165} peptide and 10 units/ml IL-2 (Proleukin; Chiron). On day 7, cytokine secretion of T cells was tested against T2 pulsed with the NY-ESO-1 peptide or an irrelevant control peptide and was analyzed by fluorescence-activated cell sorter (FACS) on a FACS Calibur (Becton Dickinson). Live gating was performed, and a minimum of 50,000 gated events were analyzed. In parallel, NY-ESO-1_{157–165} peptide-pulsed HLA-A2-immunoglobulin dimeric molecules were used to identify peptide-specific T cells as described previously (13). For

detection of CD4⁺ T-cell responses to NY-ESO-1 protein, *in vitro* generated dendritic cells (as described above) were pulsed with NY-ESO-1 protein or control protein and were used to stimulate autologous PMBCs in a 20-h assay. γ -IFN secretion was detected by FACS analysis according to the manufacturer's instructions (Miltenyi Biotech), and live gating with a minimum of 100,000 gated events was done.

Generation of Peptide-Specific CD8⁺ T-Cell Bulk Cultures. CD8⁺ T lymphocytes were enriched from CD14-depleted PBMCs using a panel of antibodies (anti-CD4, anti-CD11b, anti-CD16, anti-CD19, anti-CD36, anti-CD56) to deplete CD8⁺ cells from the PBMCs using the CD8 isolation-kit (Miltenyi Biotech). The resulting population, consisting of >90% CD8⁺ T cells, was used as responder cells and stimulated with peptide-pulsed autologous dendritic cells. 1.25×10^6 CD8⁺ T cells were plated with 5×10^5 peptide-loaded autologous dendritic cells in a 96-well plate in the presence of 10 units/ml IL-2. On day 7, the cells were restimulated with peptide-pulsed T2 cells or peptide-loaded autologous PBMCs. CTL analysis was done 5 days later using peptide-loaded T2 cells as target cells.

⁵¹Cr-Release Assay. ⁵¹Cr-release assays were performed as described previously (15). Briefly, 5 days after the last stimulation, CTL cells were harvested from *in vitro* cultures and used as effectors in the cytotoxicity assay against peptide-pulsed radiolabeled T2 cells at different E:T ratios. After 4 h at 37°C, supernatants were harvested and were counted on a gamma counter. CTL activity was calculated as the percentage of specific ⁵¹Cr release using the following equation: % specific lysis = (sample release - spontaneous release) ÷ (maximal release - spontaneous release) × 100%.

RESULTS

NY-ESO-1 mRNA Expression in HCC Patients. Tumor tissue samples from HCC patients were tested for NY-ESO-1 mRNA expression by RT-PCR. A total of 49 biopsy and tissue HCC samples were tested. Twelve samples were positive for NY-ESO-1 mRNA expression. Fig. 1A shows a representative RT-PCR of some of the tumor samples tested for NY-ESO-1 mRNA expression (Lanes 2, 4, 5, 6, 7, 8, 12, and 13 are positive for mRNA expression). No NY-ESO-1 mRNA expression was found in control nontumor liver tissue (Lane 17) and tissue from patients with other liver diseases (viral hepatitis, liver cirrhosis, primary sclerosing cholangitis and autoimmune hepatitis; Fig. 1B).

Detection of NY-ESO-1-Specific Antibody Responses in HCC Patients. Because NY-ESO-1 mRNA is expressed in HCC tumor tissue, we wondered whether there is a humoral response to this antigen in HCC patients. Therefore, we tested sera of 189 HCC patients for NY-ESO-1 antibody responses by ELISA and Western blotting. Fig. 2A shows a representative ELISA from 54 HCC patients. As a control, an irrelevant protein (IL-4) was tested with every serum sample. A total of 23 HCC patients [23 (~ 12%) of 189] had NY-ESO-1-specific antibodies in their serum. No responses were seen in healthy individuals or in serum from patients with nonmalignant liver disease (chronic Hepatitis B virus or Hepatitis C virus infection and nonviral liver cirrhosis; data not shown). Table 2 lists all 23 HCC patients with NY-ESO-1 antibody responses. No correlation was found with stage of disease and presence of viral infection. In some HCC patients (8 of 23), the antibody responses were very strong and still detectable at serum dilutions of up to 25,000 (Fig. 2B).

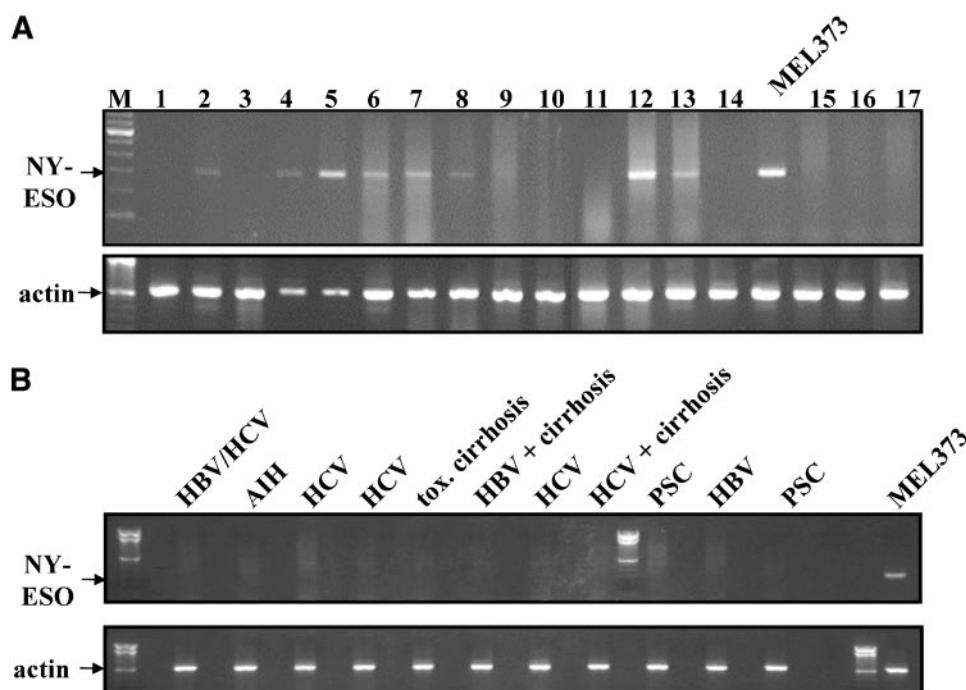
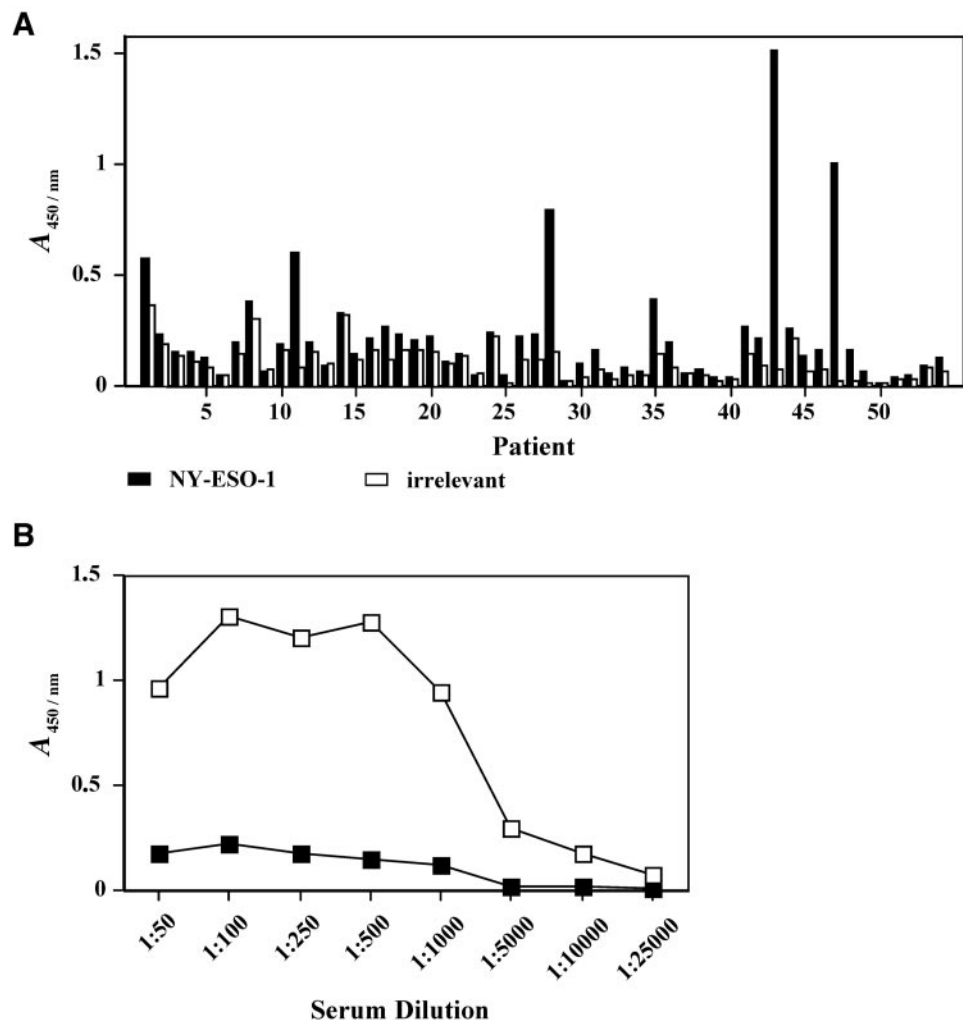


Fig. 1 Expression of NY-ESO-1 mRNA in hepatocellular carcinoma (HCC) patients as determined by reverse transcription-PCR (RT-PCR). **A**, tumor samples from HCC patients were analyzed for NY-ESO-1 mRNA expression by RT-PCR. Arrow, the expected product for NY-ESO-1. *M*, the 100-bp molecular weight marker. *Mel373*, a melanoma tumor cell line that is positive for NY-ESO-1 expression. *Lanes 2, 4, 5, 6, 7, 8, 12, and 13* are positive for NY-ESO-1 expression. No NY-ESO-1 expression is seen in nontumorous liver (*Lane 17*). **B**, RT-PCR analysis of tissue samples from patients with different nonmalignant liver diseases (*HBV*, hepatitis B virus infection; *HCV*, hepatitis C virus infection; *AIH*, autoimmune hepatitis; *PSC*, primary sclerosing cholangitis); *tox. cirrhosis*, toxic cirrhosis.

Fig. 2 Humoral responses of hepatocellular carcinoma (HCC) patients to NY-ESO-1 protein. **A**, sera of HCC patients were assayed for NY-ESO-1 antibody by ELISA. Recombinant NY-ESO-1 protein (*black column*) and an irrelevant control protein (*white column*) were used in an ELISA to determine antibody responses in HCC patients as described in "Materials and Methods." Sera from HCC patients were diluted 1:100 to 1:200. Representative 54 of 189 patients are shown. Patients shown in *Lanes 1, 11, 28, 35, 43, and 47* are positive. **B**, detection of NY-ESO-1 antibody in the serum of HCC164 at dilution 1:25,000. Serum from HCC164 patient was diluted at 1:500 to 1:25,000 and was tested in an ELISA against NY-ESO-1 protein (\square) or irrelevant control protein (*IL-4*, \blacksquare) as described in "Materials and Methods."



To confirm the specificity of NY-ESO-1 antibody responses in HCC patients, every positive serum was also tested by Western blot analysis against recombinant NY-ESO-1 protein. Fig. 3A shows a representative Western blot analysis of serum from one healthy donor and one HCC patient (HCC patient 164) against NY-ESO-1 and an irrelevant protein (IL-4 protein). The healthy donor showed no response against NY-ESO-1 protein in his serum, whereas HCC patient 164 had a strong response when tested against NY-ESO-1 and no response against the control protein. Fig. 3B is a Western blot of the dilution of the serum of the same patient (HCC 164) as shown in Fig. 2B. The Western blot analysis of the serum dilutions proved further that the responses observed are due to NY-ESO-1 protein and no other contaminants.

Finally, we tested whether endogenously processed NY-ESO-1 protein from the HCC tumor tissue is recognized by the patients' serum. This would be further proof that the humoral responses detected are tumor specific and are due to the presence of NY-ESO-1 antigen on the tumor. As shown in Fig. 3C, serum from an HCC patient responded to NY-ESO-1-expressing HCC tumor lysate in an immunoblot analysis. As expected, no

response was seen when the lysate was tested against the serum of a healthy donor.

We were able to analyze matched serum and tissue samples from nine patients with NY-ESO-1-expressing tumors. Six of these patients also had a positive antibody response, whereas three did not (Table 3). There was also one patient with NY-ESO-1-specific antibody responses but no mRNA expression in the tumor. However, the tumor sample was taken after chemotherapeutic treatment of the tumor, when the tissue was already necrotic as shown by histopathology (data not shown).

Correlation of NY-ESO-1 Antibody Titers and Course of Disease. It has been described for patients with melanoma that NY-ESO-1-specific immune responses depend on the presence of NY-ESO-1-expressing tumors and correlate with the progression of the disease (16). We, therefore, decided to analyze serum samples from patients with NY-ESO-1 antibody responses during the course of their disease. One patient, HCC164, was followed for antibody responses over a course of 18 months (Fig. 4A). Decreasing serum antibody responses to NY-ESO-1 were observed in this patient in parallel with gradual regression of his tumor after chemoembolization treatment. The

Table 2 HCC^a patients (n = 23) with NY-ESO-1 antibody responses

Age (yr)	Gender	Etiology	Okuda stage ^b	Child classification ^c	AFP (ng/ml)
78	F	tox.	1	A	33
78	M	HCV	1	A	77,680
57	F	HCV	2	A	19
63	M	HBV	2	B	384,600
69	F	HBV, HCV, tox.	2	B	430
70	M	HCV	3	B	5,660
45	M	HCV	2	B	35
54	F	tox.	2	A	2
67	M	tox.	1	A	187
65	M	tox.	3	A	21,240
54	M	HCV	1	A	29
72	M	tox.	3	A	26
58	M	HBV, HCV	2	A	296
81	M	HCV	2	A	5,240
46	M	Hemochr.	1	A	4,003
74	M	tox.	2	A	300
32	M	No cirrhosis	1	A	2,879
63	M	HBV	2	A	14,940
54	M	HCV	1	A	29
60	F	Unknown	1	A	n.d.
72	M	HBV	1	A	n.d.
52	M	HCV, tox.	1	A	6,224
36	F	HBV, HCV, tox.	2	B	149,500

^a HCC, hepatocellular carcinoma; AFP, α -fetoprotein; tox., toxic; HCV, hepatitis C virus infection; HBV, hepatitis B virus infection; Hemochr., hemochromatosis.

^b Okuda K, Ohtsuki T, Tomimatsu M, et al., *Cancer* 1985;56:918–28.

^c Pugh RN, Murray-Lyon IM, Dawson JL, et al., *Br J Surg* 1973;60:646–9.

patient's antibody titers dropped after the first and second *trans*-arterial chemoembolization of his tumor. Three months after the second *trans*-arterial chemoembolization, his tumor was surgically removed and shown to be completely necrotic by histopathological examination. In parallel, NY-ESO-1 antibody responses were no longer detectable and remained negative up to 9 months after surgery (three time points after surgery tested) at which time the patient remained free of disease. This decrease in NY-ESO-1 antibody levels was also tested by immunoblot analysis of the patient's sera at all seven time points (Fig. 5). Patient HCC42, in contrast, showed an increase in antibody titer over a course of 3 years (Fig. 4B). Because of the unfavorable course of the disease, repetitive percutaneous ethanol injections after an initial *trans*-arterial chemoembolization were required. Parallel to progression of disease, NY-ESO-1 antibody titers, as well as AFP levels, increased in the serum of this patient. AFP is a tumor marker widely used to screen patients with liver cirrhosis for the development of HCC but also to monitor patients after therapy (17). However, <50% of all HCC patients show increased AFP levels in their serum (18), so that tumors can progress without an increase in AFP levels as seen in patient HCC 355, whose NY-ESO-1 antibody responses were analyzed over a period of 9 months. This patient also showed increasing NY-ESO-1 antibody titers in parallel with the progression of the disease (Fig. 4C). In addition, seven more patients showed similar responses over time (data not shown).

Detection of NY-ESO-1-Specific CD4+ and CD8+ T-Cell Responses in HCC Patients. Presence of humoral responses to NY-ESO-1 in HCC patients prompted us to look for CD4+ and CD8+ T-cell responses as well. PBMCs from sev-

eral HCC patients with positive antibody responses against NY-ESO-1 were tested for cellular T cell responses in their blood. We decided to screen for NY-ESO-1-specific responses against the NY-ESO-1_{157–165} peptide, which is presented by the HLA-A2 MHC molecule, because the HLA-A2 haplotype is the most common HLA restriction element in Caucasians. NY-ESO-1-specific CD8+ T cells were found by FACS analysis after one *in vitro* stimulation of PBMCs in five of six HCC patients using either HLA-A2 immunoglobulin dimeric molecules or peptide-specific cytokine secretion. Fig. 6A shows a representative FACS analysis using both methodologies. When NY-ESO-1_{157–165}-pulsed HLA-A2 immunoglobulin dimeric molecule was incubated with *in vitro* stimulated PBMCs, 0.16% of all lymphocytes stained positive (upper right quadrant). In a separate experiment, PBMCs were also analyzed by γ -IFN cytokine secretion assay. Fig. 6A shows 0.95% of the lymphocytes responded to the NY-ESO-1 peptide, whereas only 0.17% of the cells responded to the irrelevant control. As shown in Fig. 6B, a range of 250–6500 NY-ESO-1-specific CD8+ T cells/100,000 CD8+ T cells were detected in five of six HLA-A2 positive HCC patients tested using dimeric MHC molecules after one *in vitro* stimulation. These results were confirmed in two patients using cytokine secretion analysis. In contrast, no CD8+ T cell responses were detected in NY-ESO-1-antibody-negative and healthy individuals. As shown in Fig. 4A, patient HCC164 had significant NY-ESO-1 antibody responses, which were no longer detectable after the removal of his tumor. Therefore, we also analyzed his CD8+ T-cell responses before surgery and 9 months postsurgery. In parallel with the loss of his

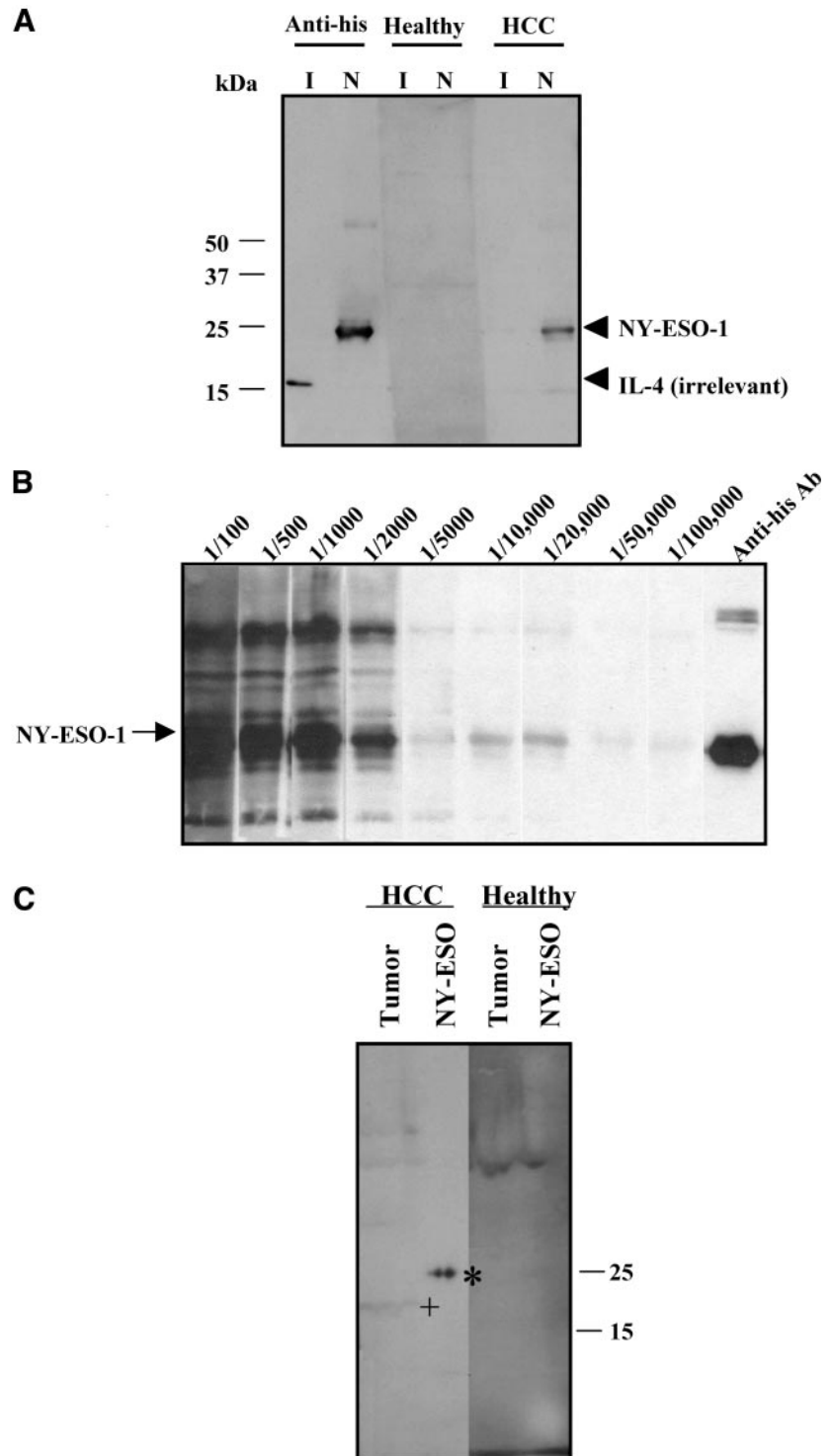


Fig. 3 Western Blot analysis of hepatocellular carcinoma (HCC) patient's serum antibody reactivity. **A**, recombinant NY-ESO-1 protein and irrelevant protein were loaded onto a 12% SDS gel and were probed with diluted serum (1:100) of HCC patient and a healthy donor. The band with the apparent M_r of 24,000 corresponds to the NY-ESO-1 protein. Anti-His antibody (against the histidine tag of the protein) was also used to show the expression of NY-ESO-1 (N) and interleukin 4 (I; IL-4). *kDa*, M_r in thousands. **B**, Western Blot analysis of serum dilution. Serum from HCC164 patient was diluted and was tested in a Western blot analysis as described in "Materials and Methods." A control antibody against the histidine tag of NY-ESO-1 protein (*Anti-his Ab*) was used as a positive control. The band with the apparent M_r of 24,000 corresponds to the NY-ESO-1 protein. **C**, recognition of endogenously processed NY-ESO-1 by the patient's serum by Western Blot analysis. Recombinant NY-ESO-1 or lysate of NY-ESO-1-expressing tumor was loaded onto a 12% SDS gel and was probed with sera from HCC patient and a healthy donor. *, NY-ESO-1 his; +, native NY-ESO-1.

antibody responses, no NY-ESO-1-specific CD8+ T cell responses were detected (data not shown).

Because we were able to detect strong NY-ESO-1-specific humoral responses, we decided to also look for NY-ESO-1-specific CD4+ T cells in peripheral blood from serum-positive

HCC patients. Autologous dendritic cells were generated from PBMCs of HCC patients, pulsed with full-length NY-ESO-1 protein, and used for stimulation of autologous CD4+ T cells. γ -IFN secretion was measured by cytokine secretion assay as described in "Materials and Methods." NY-ESO-1-specific

Table 3 NY-ESO-1 expression and antibody (Ab.) responses

mRNA positive (12)	Ab. response negative	3
	Ab. response positive	6
	n.d. ^a	3
mRNA negative (37)	Ab. response negative	22
	Ab. response positive	1 ^b
	n.d.	14

^a n.d., not determined, because no serum sample was available.

^b Tumor was tested by reverse transcription-PCR (RT-PCR) after chemoembolization and when complete necrosis in the tumor was diagnosed by histopathology.

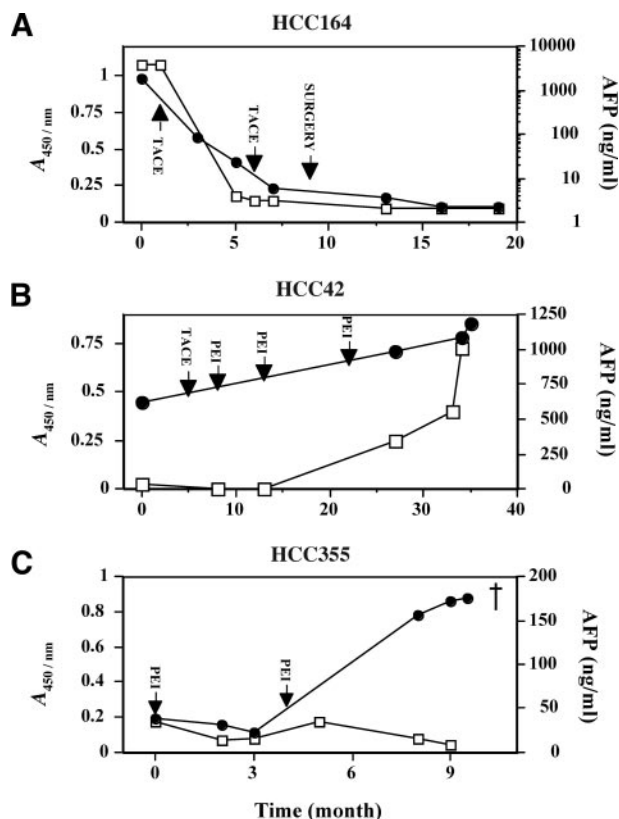


Fig. 4 Correlation of NY-ESO-1 antibody titers and course of disease. Changes of NY-ESO-1 serum antibody (NY-ESO-1 Ab) over time in hepatocellular carcinoma (HCC) patients 164 (A), 42(B) and 355(C). The sera from different time points were diluted from 1:100 to 1:100,000 and tested against recombinant NY-ESO-1 protein in an ELISA. Patient HCC164 underwent *trans*-arterial chemoembolization (TACE) and surgical resection of the tumor. Patient HCC42 underwent TACE and repeated [percutaneous ethanol injection (PEI)] procedures. Patient HCC355 underwent two PEI procedures. ●, NY-ESO-1 Ab; □, α -fetoprotein (AFP).

CD4⁺ T cells were seen in all 5 patients analyzed (although at various levels), but not in healthy or serum-negative patients (data not shown). A representative FACS analysis is shown in Fig. 7A, in which 0.21% of the lymphocytes from an HCC patient responded to NY-ESO-1 protein with a background of 0.08% of the lymphocytes responding to the control protein. The number of NY-ESO-1-specific CD4⁺ T cells in 5 HCC patients

ranged from 300 to 1200/100,000 CD4⁺ T cells (Fig. 7B). In addition, this analysis was confirmed by enzyme-linked immunospot (ELISPOT) assay for two of the patients, and similar results were observed (data not shown). The CD4⁺ T-cell responses observed were dependent on the presence of antigen on tumor, because when a patient's tumor was surgically removed (HCC 164, Fig. 4A; $t = 16$ months), his CD4⁺ T cell responses also disappeared (data not shown).

Generation of NY-ESO-1-Specific CD8⁺ T Cells from HCC Patients by *in Vitro* Peptide Stimulation. To verify cytotoxic activity of NY-ESO-1-specific T cells, a NY-ESO-1-specific CD8⁺ T cell bulk culture from one of the serum-positive patients was generated using peptide-loaded autologous dendritic cells or PBMCs as stimulators. After three stimulations, the T-cell bulk culture was tested in a cytotoxicity assay against T2 cells pulsed with the NY-ESO-1 peptide or an irrelevant control peptide in a 4-h ⁵¹Cr-release assay (Fig. 8). Significant cytolytic activity was observed against NY-ESO-1₁₅₇₋₁₆₅ pulsed T2 cells, but not against T2 cells pulsed with an HLA-A2-binding-control peptide. 50% and 23% lysis was detected at E:T ratios of 100:1 and 33:1 with less than 10% nonspecific lysis. Because no HLA-A2-positive NY-ESO-1-expressing HCC cell line was available, we also used a human NY-ESO-1-expressing melanoma cell line as a target in this assay. However, this target was not specifically recognized possibly because of the low affinity of T cells as seen earlier with other tumor-antigen-derived peptides (19).

DISCUSSION

HCC is one of the most common cancers in the world with a survival rate of less than 5% in symptomatic patients and a rapid increase in incidence (20). Until today, treatment options have been limited to surgery and local ablative therapy (12). It is, thus, paramount to develop novel strategies for cure of this disease. Tumor immunotherapy is an alternative approach that has not yet been studied in detail in HCC. Indeed, data available from animal models (6) and a few human studies suggest a role for the immune system in development and outcome of this disease (21–23).

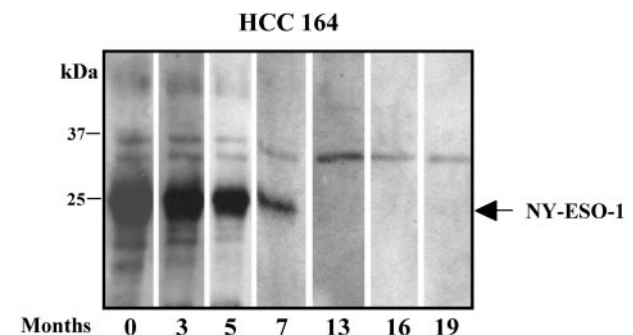


Fig. 5 Decrease of NY-ESO-1 serum antibody over time in hepatocellular carcinoma (HCC) patient 164 before and after surgical removal of the tumor, as assessed by Western Blot analysis. Recombinant NY-ESO-1 protein was loaded onto 12% SDS gel and was probed with serum of HCC164 (dilution 1:200) collected at different time points. The surgery was performed in month 9. *kDa*, M_r in thousands.

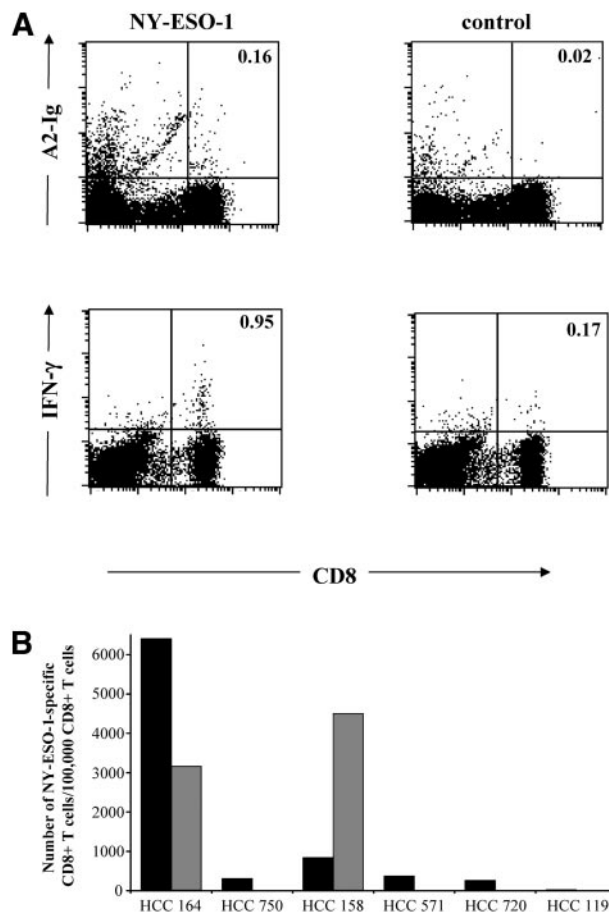


Fig. 6 Analysis of CD8⁺ T-cell responses in hepatocellular carcinoma (HCC) patients. **A**, detection of NY-ESO-1-specific CD8⁺ T cells in peripheral blood mononuclear cells after one *in vitro* stimulation using A2-IgG molecule loaded with NY-ESO-1 or a control peptide (*top panels*). γ -IFN release by CD8⁺ T cells against peptide-loaded T2 cells was detected using cytokine secretion assay as described in “Materials and Methods” (*bottom panels*). **B**, frequency of CD8⁺ T cells/100,000 CD8⁺ T cells in 6 HLA-A2-positive HCC patients as measured by using NY-ESO-1-loaded A2-immunoglobulin molecule (*black columns*) and confirmed in two HCC patients (*HCC 164, HCC 158*) by cytokine secretion assays (*gray columns*).

In this study, we show that HCC can be naturally immunogenic with NY-ESO-1 as a tumor-specific antigen that elicits both humoral and cellular responses in HCC patients. NY-ESO-1, a member of the cancer-testis antigens family is expressed in a variety of tumor types, including HCC (10, 24). Both humoral and cellular immune responses against NY-ESO-1 have been detected in melanoma patients (25), but no data on the immunological role of NY-ESO-1 in HCC is available.

Here, we show for the first time robust cellular (CD4⁺ and CD8⁺) and humoral tumor-specific immune response in a significant number of HCC patients against NY-ESO-1. To evaluate the immunogenicity of HCC, sera from 189 patients were analyzed. NY-ESO-1-specific antibodies were detected in 12% of HCC patients. This frequency is similar to what has been found for NY-ESO-1 in other tumors such as melanoma, breast,

ovary, lung, bladder, and neuroblastoma patients (26, 27). We also found NY-ESO-1 mRNA in 25% of HCC tumors by RT-PCR. Because 12% of all analyzed HCC serum samples contained NY-ESO-1-specific antibodies and six of nine patients with NY-ESO-1-expressing tumors were serum positive (Table 3), this would suggest that more than 50% of all HCC patients develop spontaneous tumor-specific immune responses against NY-ESO-1. In addition, our data suggests an association between the presence of tumor and the prevalence of NY-ESO-1-specific antibodies, which further proves the specificity of the responses seen in these patients.

Because a significant number of patients demonstrated a clear tumor-specific immune response against NY-ESO-1, we extended our studies to an analysis of cellular immune responses. For analysis of CD4⁺ T cell responses, *in vitro* generated dendritic cells, pulsed with NY-ESO-1 protein, were used to stimulate autologous T cells in HCC patients. Strong CD4⁺ T-cells responses were detected in five of five patients. In melanoma patients, there have been extensive studies regarding CD4⁺ T-cell responses against different MHC class II-restricted NY-ESO-1 epitopes (28–31). We are in the process of an identification of epitopes recognized by CD4⁺ T cells in HCC patients that might prove useful for vaccination studies.

NY-ESO-1-specific CD8⁺ T cells were detectable in five of six patients tested after one *in vitro* stimulation, which usually

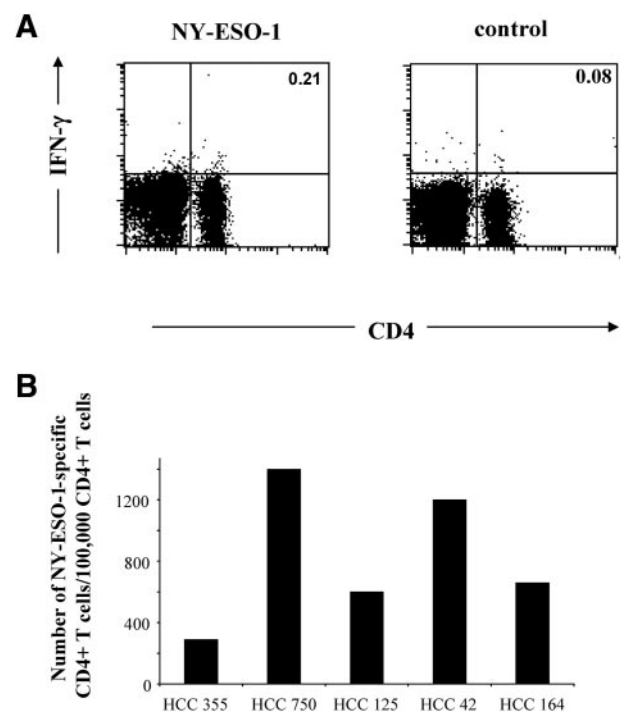


Fig. 7 Analysis of CD4⁺ T-cell responses in 164 hepatocellular carcinoma (HCC) patients and healthy donor. **A**, *in vitro* generated dendritic cells were pulsed with recombinant NY-ESO-1 or with control protein (*control*) and were used to stimulate autologous peripheral blood mononuclear cells in a 20-h incubation. γ -IFN release by CD4⁺ T cells was detected using cytokine secretion assay as described in “Materials and Methods.” **B**, frequency of CD4⁺ T cells/100,000 CD4⁺ T cells in five HCC patients as measured by cytokine secretion assays.

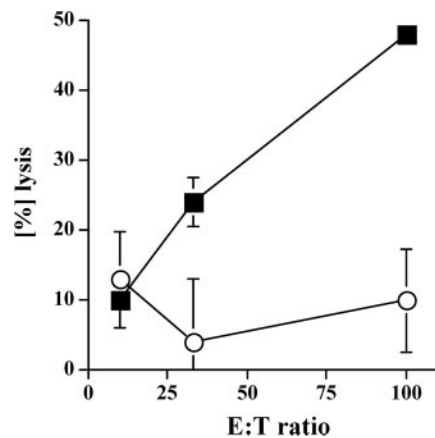


Fig. 8 Cytolytic activity of a NY-ESO-1-specific CD8⁺ T-cell line from a hepatocellular carcinoma (HCC) patient. CD8⁺ T cells from an HLA-A2-positive patients lyse specifically NY-ESO-pulsed target cells. CD8⁺ T cells were analyzed after the third stimulation against T2 cells pulsed with an irrelevant peptide (MI, ○) or NY-ESO-loaded cells (NY-ESO, ■) at different E:T ratios as indicated.

does generate specific T cells from naive precursors (32). T cells were detected by two independent assays, peptide loaded HLA-A2 dimeric molecules and cytokine secretion analysis. In one patient, more cells were detected using cytokine secretion analysis possibly because the multivalent MHC molecules sometime fail to detect low-affinity antigen-specific T cells (19, 33). Another HCC patient with positive NY-ESO-1 serum response did not show any CD8⁺ T-cell responses. This might be due to the very advanced stage of the tumor at the time of testing. Another possibility could be that in this patient, there were NY-ESO-1 CD8⁺ T-cell responses directed against a different epitope than tested in our assays. No responses were seen in NY-ESO-1 serum-negative or healthy donors. In addition, as with CD4⁺ T cell and humoral responses in HCC 164, his CD8⁺ T-cell responses also disappeared after the removal of NY-ESO-1-expressing tumor. Finally, the development of NY-ESO-1-specific antibody titers in HCC patients correlated well with clinical persistence of NY-ESO-1-expressing tumors. Several patients were followed over a course of 3 months up to 3 years, during which time NY-ESO-1 antibody levels correlated with stage of disease. Interestingly, in one patient, a decrease in NY-ESO-1 antibody levels correlated with tumor regression and finally tumor resection. This patient's antibody titers fell rapidly after tumor resection, showing that the antibody response to NY-ESO-1 in HCC patients is dependent on the level and persistence of antigen on tumor. This finding agrees with another study done in melanoma patients, in which NY-ESO-1-specific antibody titers correlate with clinical events (16).

To our knowledge, this is the first demonstration of integrated humoral and cellular immune responses in HCC patients to a tumor-specific antigen. AFP has also been suggested as a possible HCC-associated antigen. AFP is expressed in fetal liver and re-expressed in 60% of HCC patients (34). However, its expression is not limited to HCC and can also be detected in serum from patients with liver cirrhosis (35). Recent data ob-

tained in mice and in healthy donors demonstrate that AFP-specific CD8⁺ T cell responses can be generated (36). Another report observed AFP-specific T-cell responses in patients with liver cirrhosis, with or without HCC, suggesting that AFP immune responses are not tumor specific (37). Finally, vaccination experiments in mice using an AFP-expressing DNA vaccine demonstrate the feasibility of inducing AFP-specific immune responses, which protect mice from growth of s.c. injected AFP-positive tumors (38, 39). However, there is also evidence that DNA vaccination in mice using AFP-expressing plasmid can induce a mild autoimmune hepatitis (40). Therefore, because AFP is a self-antigen and its expression pattern is not limited to tumor, the risk of autoimmunity using AFP-specific immunotherapeutic approaches might be high. In contrast, NY-ESO-1 has the advantage of its expression pattern limited to testis and the tumor itself, which has important implications for the design of antigen-specific cancer vaccines. Finally, it has been shown recently that MAGE-specific CD8⁺ T-cell responses can be found in HCC patients (41). However, because of technical limitations, this study is restricted to only one patient with unusually high tumor-specific lymphocytes (42). II

In conclusion, our data provide direct evidence that patients with HCC can develop robust tumor-specific humoral and cellular immune responses. We show that NY-ESO-1 is an immunologically relevant antigen for HCC and might potentially be used as a target in an immunotherapeutic setting against HCC in selected patients. Finally NY-ESO-1 with its tumor-restricted expression and immunogenicity may prove to be a suitable HCC tumor antigen for immune monitoring using NY-ESO-1-specific cancer vaccines.

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