Humoral immune responses against tumor-associated antigen OVA66 originally defined by serological analysis of recombinant cDNA expression libraries and its potentiality in cellular immunity

Shu Jin,[1,3] Ying Wang,[1,2,3] Yong Zhang,[1,2] Hui-Zhen Zhang,[1] Shu-Jun Wang,[1,2] Jun-Qiao Tang,[1] Hui-Juan Chen[1] and Hai-Liang Ge[1,4]

[1]Shanghai Institute of Immunology, and [2]Institute of Medical Science, Shanghai Jiaotong University School of Medicine, Shanghai, 200025, P.R. of China

(Received January 23, 2008/Revised April 16, 2008/Accepted April 22, 2008/Online publication July 29, 2008)

Immunotherapy for cancer relies on the identification of tumor antigens and efficacy of antitumor immune responses. Serological analysis of recombinant cDNA libraries (SEREX), which is based on the spontaneous humoral responses against potential tumor antigens, has provided a novel strategy for searching novel tumor-associated candidates. Through SEREX analysis, we have identified 24 distinct gene clones by immunoscreening of a cDNA library derived from an ovarian cancer patient. Among these genes, a novel gene, OVA66, was found to be expressed significantly higher in carcinoma samples from cancer patients than in normal controls. Comparing humoral responses to OVA66 between tumor patients and healthy donors, it has been shown that the IgG level against OVA66 was significantly elevated in the serum of cancer patients from different histological types of cancer. To determine whether SEREX-defined OVA66 can trigger promising cytotoxic T lymphocyte (CTL) responses, human leukocyte antigen (HLA)-A*0201-restricted T-cell epitopes were predicted through a computational algorithm. Of four predicted peptides, p306–314 (L235) possesses the ability to induce efficient peripheral blood lymphocyte (PBL)-derived CTL responses capable of specifically recognizing peptide-pulsed T2 cells and lysing carcinoma cell lines expressing both HLA-A2 and OVA66 as determined by cytotoxicity and enzyme-linked immunospot assay (ELISPOT). Taken together, our results demonstrate that the SEREX-defined tumor-associated antigen OVA66 can elicit humoral immunity and may also serve as a potential candidate for T-cell-based immunotherapy for cancer. (*Cancer Sci* 2008; 99: 1670–1678)

The development of efficient immunotherapy for cancer is largely dependent on the identification of tumor antigens that can be used as targets for induction of immune responses. As the presence of infiltrating $CD8^+$ T cells is associated with a better prognosis in tumor patients,[1,2] several strategies have been developed to identify tumor antigens recognized by T cells. The T-cell epitope cloning technique, first introduced by vander Bruggen and colleagues, is the original successful method for the identification of the first human tumor-specific antigen (termed MAGE-1) in the early 1990s.[3] Subsequently, a series of tumor-specific and tumor-associated antigens were determined by other antigen-discovering techniques such as peptide elusion[4] and reverse immunology[5] (deduction of immunogenic peptides by computer algorithms). The identification of these tumor antigens or peptides that elicit spontaneous T-cell responses in cancer patients made it possible to design peptide-based vaccination protocols for clinical studies, largely dedicated to the improved efficacy of cancer therapy.[6]

Although peptide-specific cytotoxic T lymphocyte (CTL) responses play pivotal roles in the defense against cancer[7] the fact that there exist relatively high levels of autoantibodies against tumor antigens in the serum of cancer patients suggests that tumor antigens can also boost humoral responses in patients.[8] Accordingly, serological analysis of recombinant cDNA expression libraries (SEREX) using autologous serum was introduced by Sahin *et al.* in 1995.[9] Following the construction of a cDNA expression library from fresh tumor samples of patients, autologous or allogenic high-titer IgG are adapted to screen for novel tumor antigen candidates. A large family of novel tumor antigens were identified, such as NY-ESO-1,[10] HOM-Mel-40,[11] and HCA587[12] (detailed information can be found through the website http://www2.licr.org/CancerImmunomeDB/). These antigens constitute the repertoire of tumor antigens capable of eliciting humoral responses in cancer patients.

More importantly, the SEREX-derived antigens were also found to be capable of inducing antigen-specific CTL responses against tumor antigens such as NY-ESO-1.[13,14] On the other hand, tumor antigens defined by CTL cloning techniques, such as MAGE-1, can simultaneously trigger efficient humoral responses in cancer patients.[15] The 'cross talking' between CTL responses and humoral responses against the same tumor antigens suggests that the efficiency of immune responses against tumors requires both aspects, although the biological function of antibodies against tumors *in vivo* still needs to be investigated. Therefore, serological screening for tumor antigens provides a new strategy for searching the broad spectrum of peptide-based tumor vaccines inducing CTL responses.

In the present study, we have screened a cDNA expression library derived from a Chinese ovarian cancer patient by using autologous serum. Through immunoscreening, we have identified several novel tumor-antigen candidates. Among them, a novel gene (designated *OVA66*) displayed a tumor-associated expression profile and possessed the ability to arouse humoral responses in tumor patients. Combining reverse immunology techniques, we also provided evidence that OVA66 was able to induce specific CTL responses *in vitro* and potentially is a novel tumor vaccine candidate.

Materials and Methods

Reagents, cell lines, and specimens. Tissue specimens were obtained from Ruijin Hospital (Shanghai, China), including normal tissues (ovary, liver, lung, skeletal muscle, endometrium, heart muscle, kidney, stomach, gallbladder, and testis), benign tumor

[3]Shu Jin and Ying Wang contributed equally to this work.
[4]To whom correspondence should be addressed. E-mail: gehailiang@yahoo.com

doi: 10.1111/j.1349-7006.2008.00860.x

tissues (breast, thyroid gland, uterus, and ovary), and malignant tumor tissues (stomach, liver, esophagus, ovary, and colon). Serum specimens tested included 48 samples from healthy controls and 113 samples from cancer patients who underwent surgical operation. The pathogenic diagnosis was determined by the Department of Pathology at Ruijin Hospital. Tumor cell lines (SGC-7901, BGC823, MKN28, AGS, 582, HepG2, HO8910, Hela, SW480, Jurkat, U-937, Zhang's liver, and BEL-7402) were maintained routinely in the laboratory in RPMI-1640 medium (Gibco, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Gibco). Cell viability was determined using the 0.4% trypan-blue exclusion assay.

Immunoscreening of cDNA expression library. Autologous serum was obtained from donors undergoing diagnostic or therapeutic procedures and was stored at –80°C. To remove the antibodies against antigens related to the vector system, sera: (1 : 10) was preabsorbed to lysates from *Escherichia coli*. The final serum dilution (1:100) was prepared in 0.2% FCS–tris-buffered saline (TBS) and stored at 4°C.

The cDNA expression library from ovarian cancer was constructed according to the manufacturer's instructions (Stratagene, USA). Library screening was carried out as described by Sahin *et al*.[9] with the following modification. Briefly, recombinant phage at a titer of 5×10^3/15-cm plate were amplified for 3 h at 37°C and transferred onto nitrocellulose membranes (S & S Whatman, USA) that were prewet with 10 mmol/L isopropyl β-D-1-thiogalactopyranoside (IPTG) for an additional 4 h incubation at 42°C. Membranes were washed three times with tris-buffered saline with tween-20 (TBST) and then saturated with blocking buffer (Roche, Germany) for 1 h at room temperature. Membranes were then incubated in preabsorbed autologous sera (1:100 dilution) for 1 h. Following serum exposure, filters were incubated in alkaline phosphatase-conjugated sheep antimouse IgG (1:3000 dilution; Roche) for 1 h at room temperature, and processed for 5-bromo-4-chloro-3-indoxyl phosphate (BCIP)/ nitroblue tetrazolium chloride (NBT) color development. A total of 5×10^5 recombinants were screened as described. Positive clones were selected after three rounds of screening and subsequently submitted to *in vivo* excision using ExAssist helper phage following the manufacturer's instructions (Stratagene, USA). The isolated phagemids were propagated in SOLR cells, and phagemid DNA was isolated using the Promega mini-plasmid isolation kit (Promega, USA). The size of the insert was evaluated by Eco*RI*–Xho*I* digestion and gel electrophoresis.

Sequencing and alignment. Nucleotide sequencing was carried out by Genecore Corporation (China). Sequences were aligned using BLAST software in the GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi including the dbEST, nr, and unigene databases).

Preparation of recombinant OVA66 protein in *E. coli*. Full-length cDNA of *OVA66* was cloned using following the following primers: sense, 5′-CGGA*GAATTC*GATGGAGGTGGCGGCTAATTGCTCC-3′; and antisense, 5′-CGGA*CTCGAG*TTAATTCTCTGTATT-TACTTTTATTAA-3′. Recombinant protein was expressed in *E. coli* using the histidine tag-containing vector pET-32a$^+$ (Novagen, USA) through induction with 1 mmol/L IPTG. After a 4-h induction, the bacterial cells were harvested and sonicated. The bacterial cell extract was then purified on a Ni^{2+} column according to the manufacturer's protocol (Qiagen, Germany). The fusion protein was then digested with the thrombin enzyme (Novagen) to exclude the internal leading sequence.

Reverse transcription–polymerase chain reaction. Total RNA was extracted from cells and tissues using Trizol reagent (Invitrogen, USA) and reverse transcription was carried out using the Rever-Aid M-MLV Reverse Transcription kit (Fermentas, Canada). The oligonucleotide primers used were sense 5′-TGCTATTGAGCCT-GATGG-3′ and antisense 5′-CTGGAAGCCGTATGGTTA-3′) for *OVA66*, and sense 5′-TCGTGCGTGACATTAAGGAGAAGC-3′ and antisense 5′-TAGAAGCATTTGCGGTGGACGAT-3′ for *β-actin* gene amplification. Polymerase chain reaction (PCR) products were analyzed on a 1.5% agarose gel. The relative expression ratio was calculated according to the ratio of the density of target genes to the reference gene assayedusing the Gene Genius Bio Imaging System (Fisher Scientific, USA). All experiments were repeated at least three times.

Immunohistochemistry. Paraffin sections from benign and malignant tissues were deparaffinized and incubated in a solution of methanol with 0.3% hydrogen peroxide for 15 min to exclude the intervention of endogenous peroxidase. After rinsing with phosphate-buffered saline (PBS), the slides were incubated with TBS containing 10% FCS for 1 h to block non-specific binding and 0.3% Triton X-100 for 15 min for permeabalization. Slides were incubated in polyclonal rabbit anti-OVA66 IgG (1 μg/mL) overnight at 4°C. Slides incubated with normal rabbit IgG were treated as negative controls. After washing three times with PBS, slides were incubated with biotinylated goat antirabbit IgG (Huamei, China) for 30 min and then horseradish peroxidase-coupled streptavidin (Huamei) for 30 min. Slides were incubated in a substrate solution containing 0.06 mmol/L of the chromogen 3,3′-diaminobenzidine for 5 min, then dehydrated with alcohol and xylene. Finally, slides were mounted with coverslips using neutral resin. The Axioplan 2 imaging system (Zeiss, Germany) was used to capture pictures from the stained sample tissues and analysis was carried out using KS400 (version 3.0) software (Zeiss, Germany).

Enzyme-linked immunosorbent assay. For the enzyme-linked immunosorbent assays (ELISA), 96-well flat plates (Nunc, Denmark) were coated with purified OVA66 protein (150 ng/well) at 4°C overnight. After washing three times with PBS–0.05% Tween-20 (PBST), the plates were blocked with PBS–10% FCS. Then, 100 μL/well of 500-fold diluted sera either from normal controls or tumor patients was then added to each plate. The plates were washed in PBST after 1.5 h incubation at 37°C, and further incubated with 1:1000-diluted horseradish peroxide (HRP)-conjugated goat-antihuman IgG (Huamei) for 1 h at 37°C. After washing, 100 μL/well of tetramethyl-benzidine substrate solution was added, and the reaction was stopped by adding 50 μL of 1 mol/L sulfuric acid. Optical absorbance was measured at 450 nm.

Flow cytometry. Cells (5×10^5) were stained with antibodies against CD14, CD1a, CD83, CD80, and human leukocyte antigen (HLA)-II (BD Pharmingen and Bioscience, USA). T2 cells were stained with affinity-purified monocloncal antibody (BB7.2) against human HLA-A2.1 molecules for 1 h at 4°C and then with goat antimouse IgG–fluorescein isothiocyanate (FITC) (Sigma-Aldrich, USA) for 1 h at 4°C. Cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson, USA). Data acquisition and analysis were carried out using Cellquest software (Becton Dickinson).

Peptide prediction and synthesis. HLA-A*0201-restricted OVA66-derived peptides were predicted using a computer program (http://www-bimas.cit.nih.gov/molbio/hla_bind/). Nonamer peptides were synthesized by Chiron (Australia).

Peptide binding ability and stability assays. For the peptide binding ability assay, T2 cells were incubated with β2-microglobulin and each peptide over a range of concentrations (0.2, 2, 5, and 20 μg/mL) for 4 h. The mean fluorescent intensity (MFI) of HLA-A2 was measured by flow cytometry after staining the cells with mouse anti-HLA-A2 antibody (clone BB7.2) and goat antimouse Ig–FITC (Sigma). T2 cells with β2-microglobulin only were used as a negative control. T2 cells were incubated with each peptide at the optimal concentration for 4 h and the MFI was detected at different time points (0, 2, and 4 h) to determine the binding stability of HLA-A2 molecules with peptides. Binding ability and stability were calculated according to the concentration of peptide at 50% maximum MFI.

Preparation of mature dendritic cells (mDC). Peripheral blood mononuclear cell (PBMC) were isolated from the peripheral blood of HLA-A*0201 healthy donors by Ficoll–Hypaque density separation. Cells were resuspended in RPMI-1640 medium supplemented with 15% FCS, 2 mmol/L L-glutamine, and 1% penicillin–streptomycin (Gibco, USA) and incubated for 2 h at 37°C. Non-adherent cells were removed by gentle pipetting. The remaining cells were cultured in RPMI-1640 supplemented with 15% FCS, 50 ng/mL granulocyte macrophage colony-stimulating factor (GM-CSF) (R & D, USA), and 50 ng/mL interleukin (IL)-4 (R & D). After 6 days' incubation, interferon (IFN)-γ (1000 U/mL) and CD40L (1.5 μg/mL) (R & D) were added for the maturation of cultured DC. Mature dendritic cells (DC) were harvested and used as stimulators for CTL induction after 24 h.

Generation of peptide-specific CTL. DC were pulsed with peptides (40 mg/L) and β_2-microglobulin (3 mg/L) for 2 h at 37°C. Peptide-loaded DC were washed twice to remove excess peptides and irradiated with 30 Gy as a stimulator for CTL induction. Autologous $CD8^+$ T cells from HLA-A*0201 healthy donors were enriched using the Midi MACS sorting technique (Miltenyi Biotec, Germany). Purified $CD8^+$ T cells were cocultured with irradiated autologous peptide-loaded DC at a ratio of 1:10 in RPMI-1640 medium containing 15% FCS, IL-6 (10 ng/mL), and IL-12 (1 ng/mL) (R & D). Restimulation was carried out with peptide-pulsed autologous phytohemagglutinin (PHA) blast weekly plus IL-7 (10 ng/mL) and IL-2 (10 ng/mL) (R & D). Proliferative cellularity was determined before each round of restimulation. After at least four rounds of restimulation, peptide-specific CTL were harvested for functional assays.

Cytotoxicity assay. The peptide-specific cytotoxicity of induced CTL was assessed using the CytoTox 96 non-Radioactive Cytotoxicity Assay kit (Promega). Briefly, target cells (either peptide-pulsed T2 cells or tumor cell lines) were cocultured with CTL for 4 h at effectors (E) : targets (T) ratios of 40:1, 20:1, and 5:1. Culture supernatants were collected for detection of lactate dehydrogenase (LDH) activity. The percentage of specific lysis was calculated as follows:

$$\text{specific lysis } \% = ([OD_{\text{experimental group}} - OD_{\text{spontaneous group}}]/[OD_{\text{maximum group}} - OD_{\text{spontaneous group}}]) \times 100,$$

where OD is the optical density.

Enzyme-linked immunospot (ELISPOT) assay. The Enzyme-linked immunospot (ELISPOT) assay for antigen-specific IFN-γ secretion was established to determine the number of T cells capable of responding to specific stimuli. It was used in accordance with the manufacturer's instructions (U-CyTech, the Netherlands). Briefly, 96-well U-bottom plates were precoated with anti-IFN-γ antibody overnight at 4°C, washed twice with PBS, and blocked with blocking buffer for 2 h. DC-induced peptide-specific CTL (6×10^5) were cocultured with irradiated target cells in the antibody-coated 96-well plates at an E : T ratio of 40:1. Wells with CTL only were set up as blank controls. After 18 h incubation, cells were discarded and biotinylated anti-IFN-γ antibody was added for a further 2 h incubation at 37°C. After washing with PBS, streptavidin-conjugated alkaline phosphatase was added for another 1 h at 37°C. Individual cytokine-producing cells were detected as brown spots after exposure to BCIP/NBT. The number of spots was detected using a Bio-Sys ELISPOT reader (Karben, Germany).

Result

SEREX analysis of ovarian cancer cDNA expression library. A cDNA expression library containing 7.8×10^8 plaque forming units (pfu)/mL amplified clones was prepared from a specimen of ovarian cancer patient. Phage plaques (5×10^5) were immunoscreened using autologous serum. Excluding the false-positive clones, 27 positive clones were identified after three rounds of screening. These clones were subcloned, excised *in vivo*, and converted

Table 1. List of gene fragments obtained by serological analysis of recombinant cDNA libraries from an ovarian cancer cDNA library

No.	Homology	Accession no.	Function
MY-OVA-1	PAIP-I (2768 bp)	NM006451	Translation factor
MY-OVA-2	CCT8-Chaperonin (1821 bp)	NM006585	Cell structure
MY-OVA-3	Nucleolar phosphoprotein p130 KIAA0035 gene (3727 bp)	NM004741	Nucleologenesis
MY-OVA-4	Profilin II (1693 bp)	NM002628	Cell structure
MY-OVA-5	Oligosaccharyltransferase		Modification of protein
MY-OVA-6	Differentiation-related gene 1 (RTP)	NM006096	Control of differentiation
MY-OVA-7	Very early protein ETR101	NM004907	Regulation of cell cycle
MY-OVA-8	Putative RNA binding protein KOC 1	NM006547	Regulatory molecule
MY-OVA-9	Ribosomal protein S4 (RPS4X)	NM001007	Cell structure
MY-OVA-10	Ribosomal protein P1 (RPLP1)	NM001003	Cell structure
MY-OVA-11	Ribosome protein S7 (RPS7)	NM001011	Cell structure
MY-OVA-12	Macrophage inflammatory protein-2α Gro-β cytokine	NM002089	Regulation of cell cycle
MY-OVA-13	Mitotic feedback control protein Madp2 homolog		Regulation of cell cycle
MY-OVA-14	Mitochondrin cytochrome *c* oxidase subunit II gene	AF004339	Energy metabolism
MY-OVA-15	NADH dehydrogenase	NM002628	Energy metabolism
MY-OVA-16	Human mitochondrion gene		Mitochondrial DNA
MY-OVA-17	Human mitochondrion gene		Mitochondrial DNA
MY-OVA-18	Human mitochondrion gene		Mitochondrial DNA
MY-OVA-19	Human mitochondrion gene		Carrier protein
MY-OVA-20	Human mitochondrion gene		Mitochondrial DNA
MY-OVA-21	Unknown		EST 8788
MY-OVA-22	Unknown		EST 1750
MY-OVA-23	Unknown		EST 1753
MY-OVA-24	Unknown		EST 1754
MY-OVA-25	Unknown		EST 1314
MY-OVA-26	Unknown		EST 3533
MY-OVA-27	Adhesion molecule		

doi: 10.1111/j.1349-7006.2008.00860.x

(a)

```
1    ATG GAG GTG GCG GCT AAT TGC TCC CTA CGG GTG AAG AGA CCT CTG TTG GAT
52   CCC CGC TTC GAG GGT TAC AAG CTC TCT CTT GAG CCG CTG CCT TGT TAC CAG
103 CTG GAG CTT GAC GCA GCT GTG GCA GAG GTA AAA CTT CGA GAT GAT CAA TAT
154 ACA CTG GAA CAC ATG CAT GCT TTT GGA ATG TAT AAT TAC CTG CAC TGT GAT
205 TCA TGG TAT CAA GAC AGT GTC TAC TAT ATT GAT ACC CTT GGA AGA ATT ATG
256 AAT TTA ACA GTA ATG CTG GAC ACT GCC TTA GGA AAA CCA CGA GAG GTG TTT
307 CGA CTT CCT ACA GAT TTG ACA GCA TGT GAC AAC CGT CTT TGT GCA TCT ATC
358 CAT TTC TCA TCT TCT ACC TGG GTT ACC TTG TCA GAT GGA ACT GGA AGA TTG
409 TAT GTC ATT GGA ACA GGT GAA CGT GGA AAT AGC GCT TCT GAA AAA TGG GAG
460 ATT ATG TTT AAT GAA GAA CTT GGG GAT CCT TTT ATT ATA ATT CAC AGT ATC
511 TCA CTG CTA AAT GCT GAA GAA CAT TCT ATA GCT ACC CTA CTT CTT CGA ATA
562 GAG AAA GAG GAA TTG GAT ATG AAA GGA AGT GGT TTC TAT GTT TCT CTG GAG
613 TGG GTC ACT ATC AGT AAG AAA AAT CAA GAT AAT AAA AAA TAT GAA ATT ATT
664 AAG CGT GAT ATT CTC CGT GGA AAG TCA GTG CCA CAT TAT GCT GCT ATT GAG
715   CCT GAT GGA AAT GGT CTA ATG ATT GTA TCC TAC AAG TCT TTC ACA TTT GTT
766   CAG GCT GGT CAA GAT CTT GAA GAA AAT ATG GAT GAA GAC GTA TCA GAGAAA
817   ATC AAA GAA CCT CTG TAT TAC TGG CAA CAG ACT GAA GAT GAT TTG ACA GTA
868   ACC ATA CGG CTT CCA GAA GAC AGT ACT AAG GAG GAC ATT CAA ATA CAG TTT
919   TTG CCT GAT CAC ATC AAC ATT GTA CTG AAG GAT CAC CAG TTT TTA GAA GGA
970   AAA CTC TAT TCA TCT ATT GAT CAT GAA AGC AGT ACA TGG ATA ATT AAA GAG
1021 AGT AAT AGC TTG GAG ATT TCC TTG ATT AAG AAG AAT GAA GGA CTG ACC TGG
1072 CCA GAG CTA GTA ATT GGA GAT AAA CAA GGG GAA CTT ATA AGA GAT TCA GCC
1123 CAG TGT GCT GCA ATA GCT GAA CGT TTG ATG CAT TTG ACC TCT GAA GAA CTG
1174 AAT CCA AAT CCA GAT AAA GAA AAA CCA CCT TGC AAT GCT CAA GAG TTA GAA
1225 GAA TGT GAT ATT TTC TTT GAA GAG AGC TCC AGT TTA TGC AGA TTT GAT GGC
1276 AAT ACA TTA AAA ACT ACT CAT GTG GTG AAT CTT GGA AGC AAC CAG TAC CTT
1327 TTC TCT GTC ATA GTG GAT CCT AAA GAA ATG CCC TGC TTC TGT TTG CGC CAT
1378 GAT GTT GAT GCC CTA CTC TGG CAA CCA CAC TCC AGC AAA CAA GAT GAT ATG
1429 TGG GAG CAC ATC GCA ACT TTC AAT GCT TTA GGC TAT GTC CAA GCA TCA AAG
1480 AGA GAC AAA AAA TTT TTT GCC TGT GCT CCA AAT TAC TCG TAT GCA GCC CTT
1531 TGT GAG TGC CTT CGT CGA GTA TTC ATC TAT CGT CAG CCT GCT CCC ATG TCC
1582 ACT GTA CTT TAC AAC AGA AAG GAA GGC AGG CAA GTA GGA CAG GTT GCTAAG
1633 CAG CAA GTA GCA AGC CTA GAA ACC AAT GAT CCT ATT TTA GGA TTT CAG GCA
1684 ACA AAT GAG AGA TTA TTT GTT CTT ACT ACC AAA AAC CTC TTT TTA ATA AAA
1735 GTA AAT ACA GAG AAT TAA
```

(b)

```
MEVAANCSLRVKRPLLDPRFEGYKLSLEPLPCYQLELDAAVAEVKI
RDDQYTLEHMHAFGMYNYLHCDSWYQDSVYYIDTLGRIMNLTVML
DTALGKPREVFRLPTDLTACDNRLCASIHFSSSTWVTLSDGTGRL
YVIGTGERGNSASEKWEIMFNEELGDPFIIIHSISLLNAEEHSIATLL
LRIEKEELDMKGSGFYVSLEWVTISKKNQDNKKYEIIKRDILRGKS
VPHYAAIEPDGNGLMIVSYKSFTFVQAGQDLEENMDEDVSEKIKE
PLYYWQQTEDDLTVTIRLPEDSTKEDIQIQFLPDHINIVLKDHQFLE
GKLYSSIDHESSTWIIKESNSLEISLIKKNEGLTWPELVIGDKQGEL
IRDSAQCAAIAERLMHLTSEELNPNPDKEKPPCNAQELEECDIFF
EESSSLCRFDGNTLKTTHVVNLGSNQYLFSVIVDPKEMPCFCLR
HDVDALLWQPHSSKQDDMWEHIATFNALGYVQASKRDKKFFA
CAPNYSYAALCECLRRVFIYRQPAPMSTVLYNRKEGRQVGQVA
KQQVASLETNDPILGFQATNERLFVLTTKNLFLIKVNTEN
```

Fig. 1. (a) Nucleic acid sequence and (b) deduced amino acid sequence of OVA66.

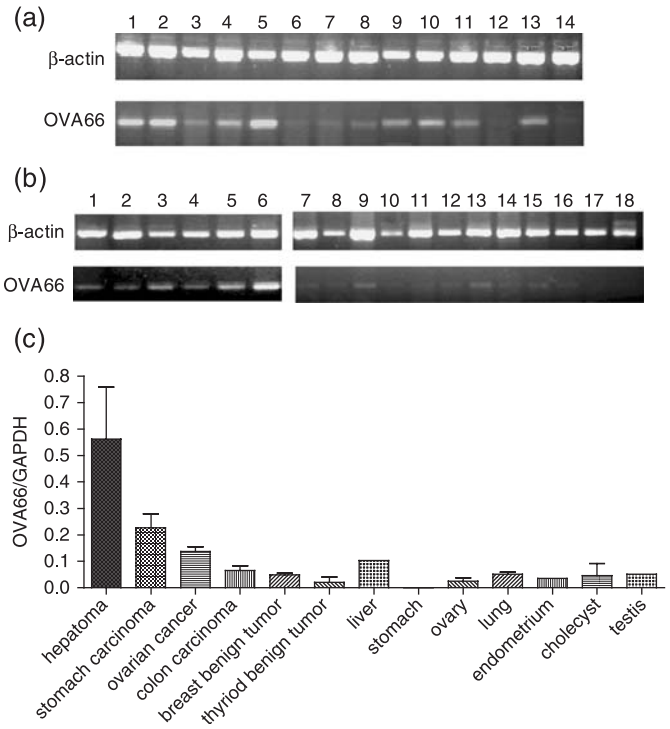

Fig. 2. Expression patterns of OVA66 in cell lines, normal, benign, and malignant tumor tissues. (a) Total RNA was extracted from tumor cell lines (1, SGC-7901; 2, BGC823; 3, MKN28; 4, AGS; 5, 582; 6, HepG2; 7, HO8910; 8, Hela; 9, SW480; 10, Jurkat; 11, U-937; 12, PBMC; 13, Zhang's liver; and 14, BEL-7402) and conventional polymerase chain reaction (PCR) was carried out by using OVA66- and β-actin-specific primers. PCR products were scanned after 1% agarose gel electrophoresis. (b) Conventional PCR was carried out by using samples from malignant and benign tissues (1, ovarian cancer; 2, thyroid benign tumor; 3, colon carcinoma; 4, breast benign tumor; 5, stomach carcinoma; 6, hepatoma; 7, 8, ovary; 9, testis; 10, cholecyst; 11, 12, lung; 13, liver; 14, endometrium; 15, myocardium; 16, kidney; and 17, 18, stomach) as done with tumor cell lines. (c) The OVA66 expression in part of the tissue samples tested was confirmed by real-time PCR. The expression of OVA66 in real-time PCR performance was calculated according to 'Methods and materials'. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

into pBluescriptII phagemids. Results from the restriction mapping showed that the size of the inserts ranged from 0.4 to 2.0 kb. Through sequence alignment, it was found that they were derived from 24 distinct genes. All of the genes were classified as follows: those encoding differential antigens (such as PAIP-1); those encoding structural proteins (such as ribosome protein and chaperonin); and those with unknown properties (Table 1).

OVA66 showed a tumor-associated expression profile. Among the seven unknown expressed sequence tag (EST) sequences, EST1753 (named *OVA66*) is homologous to human chronic myelocytic leukemia antigen *CML66*[16] with a few nonsense alterations of the nucleic acid sequence (Fig. 1). To define the association of OVA66 with tumors, the expression patterns of OVA66 in cell lines, normal, benign and malignant tumor issues were detected by reverse transcription-PCR analysis. Our results indicated that OVA66 was expressed significantly in a panel of tumor cell lines (SGC-7901, BGC823, MKN28, AGS, 582, HepG2, Jurkat, U937, SW480, and HeLa) and normal tissue-derived cell lines (Chang's Liver), but at relatively low levels in BEL-7402 and HO8910 cell lines (Fig. 1a). Among the tissues tested, OVA66 was expressed highly in malignant tumor tissues (such as stomach, liver, esophagus, ovary, colon, breast, and thyroid gland) and testis, but at low levels in other normal tissues and PBMC (Fig. 2b,c).

To further analyze OVA66 protein expression *in situ*, we first generated a rabbit anti-OVA66 polyclonal antibody using prokaryotic-expressing OVA66 protein. Affinity-purified IgG was used to stain paraffin sections from benign and malignant tissues. Our results showed higher cytoplasmic immunoreactivity to OVA66 in malignant tissues, including ovarian, gastric, and colon cancer samples, compared to their normal counterparts (Fig. 3). Remarkably, the expression level of OVA66 protein *in situ* determined by immunohistology was in line with the results from reverse transcription-PCR. These results indicate that OVA66 could serve as a novel tumor-associated antigen that is expressed frequently in multiple carcinomas.

OVA66 elicited specific humoral responses in tumor patients. As OVA66 was obtained from SEREX analysis of the cDNA library using autologous serum screening, we next assessed the frequency of anti-OVA66 humoral immune responses *in vivo*. By setting up an ELISA assay, we determined the IgG level against OVA66 in the sera of 48 healthy donors and 113 tumor patients with different pathogenic types. As shown in Figure 4a, the mean OD_{450}

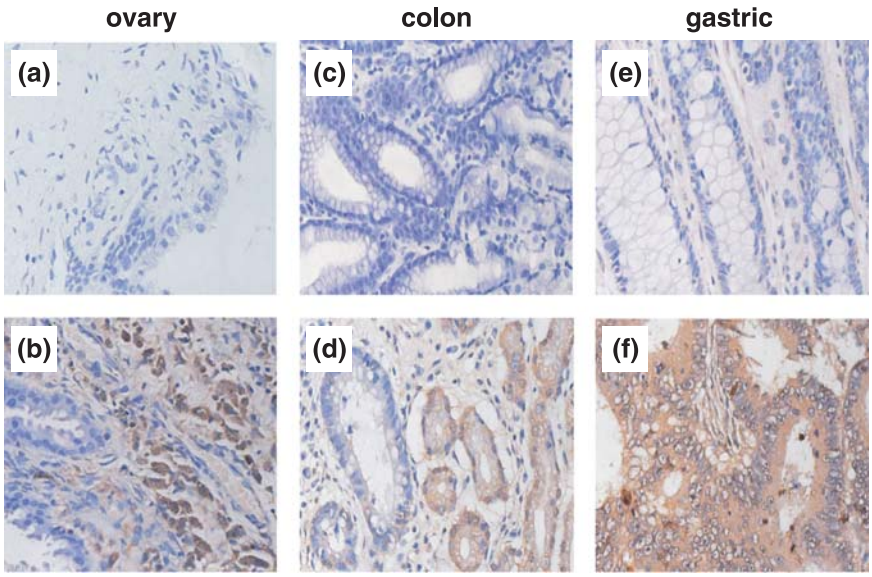

Fig. 3. Immunohistochemical staining of OVA66 antigen in normal and cancer tissues. OVA66 expression was detectable in (b) ovarian, (d) colon, and (f) gastric cancer specimens whereas no or very low reactivity was observed in (a,c) benign and (e) normal tissues. (a–f) Magnification, ×40.

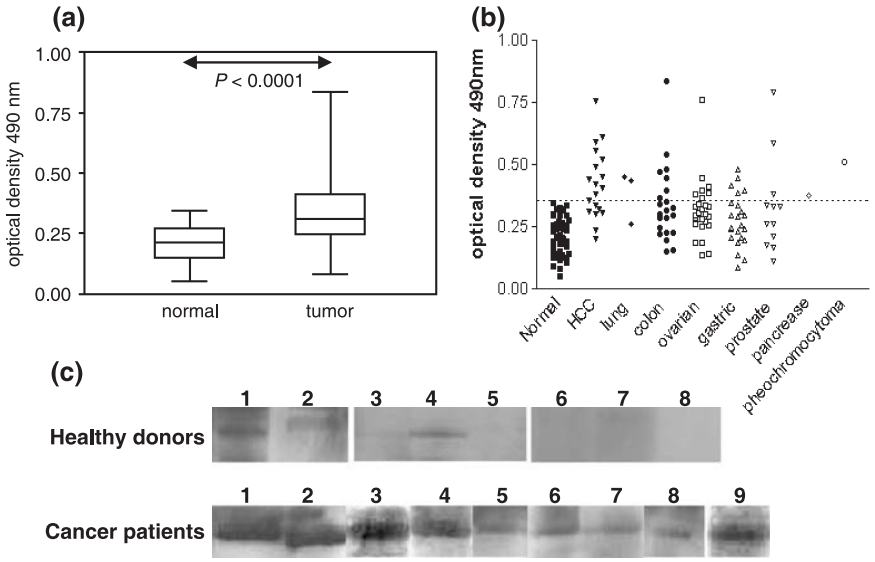

Fig. 4. OVA66-specific antibody responses in healthy donors and cancer patients. (a) Serum samples from healthy donors and tumor patients were diluted at 1:100 and were analyzed by enzyme-linked immunosorbent assay (ELISA) to detect antibodies reactive to recombinant OVA protein. (b) OVA66-reactive sera from healthy donors and tumor patients were evaluated individually. The dashed line indicates a cut-off value ($OD_{mean} \pm 2$ SD) as determined by ELISA assays using sera from healthy individuals. (c) OVA66-specific autoantibodies in the serum from either tumor patients or healthy donors were detected using western blot assays. Sera used in the assays were diluted at 1:1000. The panels of cancer patients represent: 1, gastric cancer; 2, hepatoma; 3, colon carcinoma; 4, lung cancer; 5, 6, colon cancer; 7, ovarian cancer; and 8, 9, esophageal carcinoma. HCC, hepatocellular carcinoma.

Table 2. Predicted HLA-A2 binding peptides derived from the OVA66 sequence

Peptide	Position	Amino acid subsequence	Bioinformatic score
L235	p306-314	FLPDHINIV	1236.981
L236	p33-41	YQLELDAAV	492.089
L237	p568-576	VLTTKNLFL	199.738
L238	p509-517	ALCECLRRV	131.175
L239	HIV Nef180	LMWQFDSRL	827.240

value of anti-OVA66 IgG was 0.21 ± 0.08 in healthy donors and 0.34 ± 0.15 in tumor patients ($P < 0.0001$). After setting the mean OD_{450} plus 2 SD ($OD_{mean} \pm 2SD$) from healthy donors as the cut-off value, the reactivity of serum samples was reevaluated. Although all serum samples from healthy donors tested were negative, OVA66-specific IgG was detectable in 10 out of 19 samples from hepatocellular carcinoma (HCC) patients (52.6%), 6 out of 22 samples from colon cancer patients (27.3%), 2 out of 3 lung cancer samples (66.67%), 5 out of 21 samples from gastric cancers (23.8%), 6 out of 27 samples from ovarian cancer patients (22.2%), and 3 out of 10 samples from prostate cancer patients (30%) (Fig. 4b).

To further determine the existence of OVA66-specific autoantibodies in the serum of cancer patients, western blotting analysis was carried out using purified OVA66 antigen expressed in prokaryotes. Our results indicated that contrary to the few positive results from the healthy donors' serum, most of the serum from cancer patients showed autoantibody reactivity against OVA66 (Fig. 4c). All of the above results indicate that a considerable proportion of tumor patients could mount efficient humoral responses against OVA66 *in vivo*.

Identification and characterization of OVA66-derived HLA-A*0201-binding peptides. The fact that OVA66 could trigger effective humoral responses in tumor patients led us to examine whether it could also serve as a potential inducer for $CD8^+$ CTL responses. The amino acid sequence of the OVA66 protein was first scanned for the most probable HLA-A*0201-specific nonamer peptide epitopes by using a computer program according to the presence of the main HLA-A*0201-specific anchor residues. Of the peptides predicted, four peptides (L235, L236, L237, and L238)

doi: 10.1111/j.1349-7006.2008.00860.x

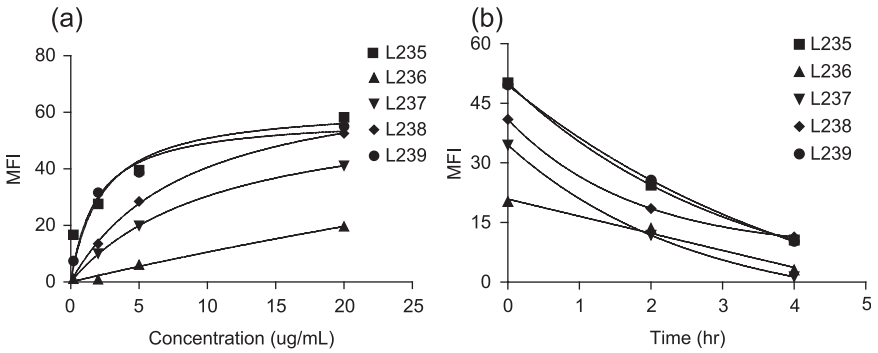

Fig. 5. Binding of OVA66-derived peptides to HLA-A2 molecules. (a) Binding assay of OVA66-derived peptides to HLA-A2 molecules. (b) Binding stability assay of OVA66-derived peptides to HLA-A2 molecules. MFI, mean fluorescence intensity.

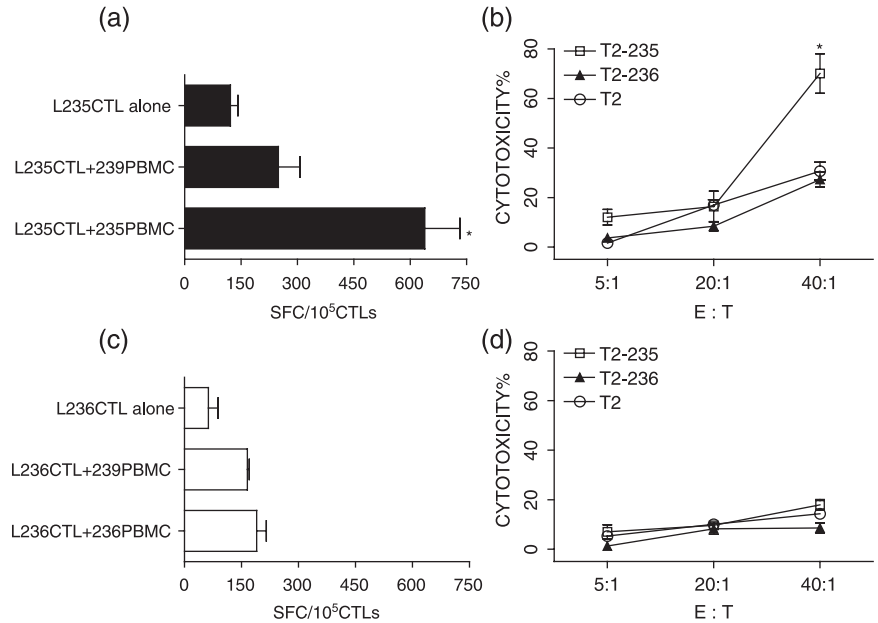

Fig. 6. Specific recognition of peptide-loaded T2 cells by *in vitro*-sensitized cytotoxic T lymphocytes (CTL). The effector CTL were obtained by stimulating $CD8^+$ T cells from healthy donors for 35 days with irradiated autologous dendritic cell (DC) or phytohemagglutinin (PHA)-blasts pulsed with the peptides. The presence of interferon (IFN)-γ producing cells specific to the peptides (a) L235 and (c) L236 was determined in an enzyme-linked immunospot (ELISPOT) assay by coculture with T2 cells pulsed with or without 10 μg/mL cognate and control peptides. The cytotoxic activity of the effector cells against T2 cells pulsed with the peptides (b) L235 and (d) L236 was determined by lactate dehydrogenase (LDH) release assay at different effectors (E) : targets (T) ratios. Values represent the mean of triplicate assays. Data are representative of three independent experiments from three donors. PBMC, peripheral blood mononuclear cell; SFC, spot-forming cells.

derived from OVA66 were selected, based on the highest predicted binding scores, for a further binding assay using a TAP-deficient T2 cell line-based system (Table 2). Peptide (L239) from human immunodeficiency virus (HIV) Nef protein was used as a positive control.[17]

In the T2-mediated binding assay, incubation of T2 cells with each of the four peptides led to a dramatic increase in stable HLA-A2 expression. Of the four peptides tested, L235 displayed equivalent binding affinity to the positive control peptide L239 with the lowest concentration of peptide at 50% MFI_{max} (2 μg/mL), whereas the other three had much lower affinity for HLA-A2 molecules with higher concentrations at 50% MFI_{max} (L236, 8.2 μg/mL; L237, 4.6 μg/mL; and L238, 4.2 μg/mL) (Fig. 5a).

To compare the binding ability of the predicted peptides more precisely, a peptide binding and stability assay was carried out by measuring the disassociation rate of peptide with HLA-A2 molecules at 37°C. T2 cells were pulsed with 40 μg/mL peptide for 4 h incubation. The MFI value of HLA-A2 expression on T2 cells was detected at 0, 2, and 4 h. Consistent with the binding assay, L235 displayed a high peptide–major histocompatibility complex (MHC) binding stability (Fig. 5b). L235 exhibited stronger binding ability and better stability with HLA-A2 molecules than the other peptides.

Induction of OVA66-derived peptide-specific CTL *in vitro*. Based on the aforementioned results, we selected peptides L235 and L236, which represented the peptides with high and low affinity to HLA-A2, respectively, to investigate their ability to induce peptide-specific CTL responses. $CD8^+$ T cells from HLA-A*0201 healthy donors were incubated with autologous mature DC pulsed with synthetic peptides, followed by additional stimulation with peptide-pulsed autologous PHA-blast. The cellularity of T lymphocytes decreased from 2×10^6 to $0.3–0.8 \times 10^6$ during the first week of culture. However, the cells expanded rapidly from the second restimulation. The number of T cells achieved was up to 1×10^8 in total after four stimulations. More than 95% of the expanding CTL were $CD3^+CD8^+$ and those remaining were $CD4^+$ (data not shown). Interestingly, the ability to induce the expansion of peptide-specific CTL specific for L235 and L236 was quite similar.

L235-specific CTL can specifically recognize T2 cells pulsed with cognate peptide. We next tested the specificities of peptide-induced CTL by using ELISPOT assay for the detection of IFN-γ producing T cells. As shown in Figure 5a, consistent with our previous primary results,[18] there was a higher frequency of IFN-γ spot-forming cells in L235-specific CTL upon stimulation with the L235-pulsed stimulators (0.64% ± 0.09%) compared to the L239-loaded PBMC (0.25% ± 0.06%) or CTL alone (0.12% ± 0.02%) (Fig. 6a). In contrast, although L236-specific CTL expanded comparably, there was no significant difference in the average spot numbers in response to T2 cells preloaded with either cognate peptide (0.19% ± 0.02%) or control peptide (0.17% ± 0.05%) (Fig. 6c).

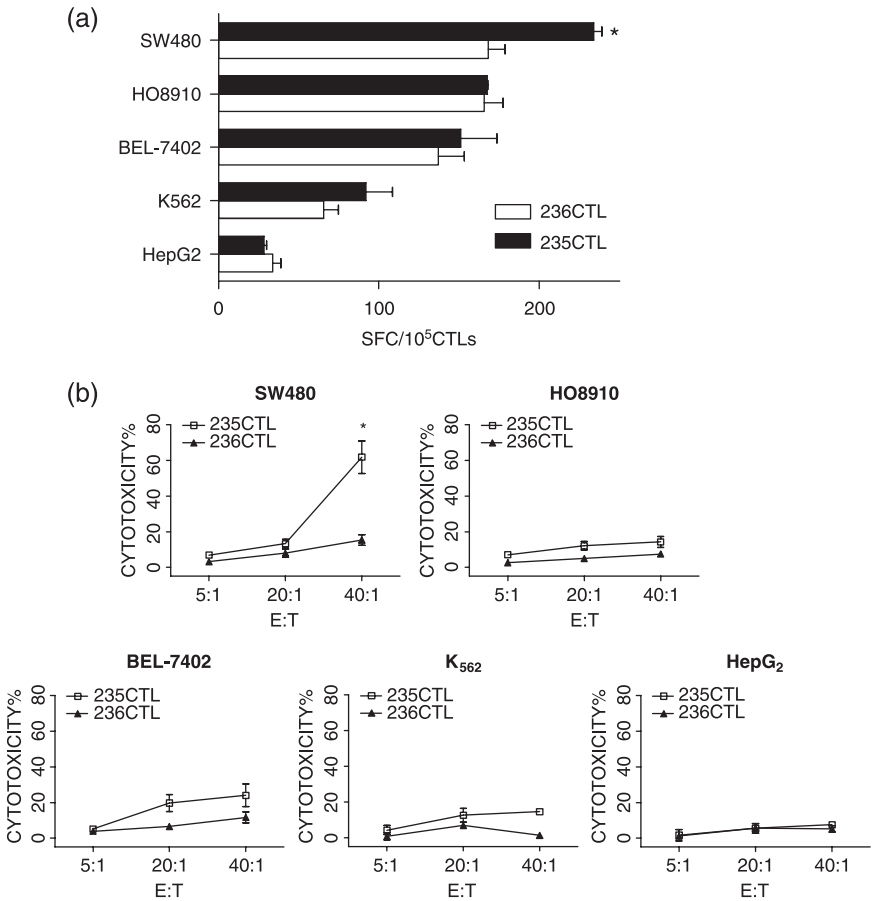

Fig. 7. L235-specific cytotoxic T lymphocytes (CTL) can specifically recognize naturally processing and presenting OVA66-derived peptides. (a) The *in vitro*-sensitized CTL were used to test their capability of inducing interferon (IFN)-γ secretion through coculture with either HLA-A2$^+$ or OVA66$^+$ tumor cells lines. (b) The cytotoxic activity of the CTL induced by peptide L235 or L236 against different target cells (including SW480, HO8910, BEL-7402, K562, and HepG2) was determined by lactate dehydrogenase (LDH) release assay at different effectors (E) : targets (T) ratios. Values represent the mean of triplicate assays. Data are representatives of three independent experiments from three donors. SFC, spot-forming cells.

These *in vitro*-sensitized CTL were further tested for their cytotoxic specificity by detecting the LDH levels in the supernatant of target cells after incubation with CTL at different E : T ratio. As shown in Figure 6b, L235-induced CTL displayed more cytotoxic activity against the T2 cells pulsed with cognate peptide than either L235- or L239-loaded T2, which demonstrated the specificity of CTL raised against L235. Consistent with the ELISPOT results, L236-induced CTL displayed low cytotoxicity against T2 cells pulsed with all three tested peptides (Fig. 6d). These results indicated that the OVA66-derived high-affinity peptide L235 could induce efficient and specific CTL responses *in vitro*.

L235-specific CTL can specifically recognize naturally processing and presenting OVA66-derived peptide. To determine if *in vitro*-primed peptide-specific CTL can also target naturally processed and presented tumor antigens, the immunological effects of these CTL were further measured by using various tumor cell lines, including HO8901 (OVA66$^+$ and HLA-A2.1$^-$), BEL-7402 (OVA66^{low} and HLA-A2.1$^-$), SW480, and HepG2 (both OVA66$^+$ and HLA-A*0201) as target cells. The results from the ELISPOT assays indicated that all tumor cell lines tested could stimulate responding CTL to produce IFN-γ. However, the number of IFN-γ secreting cells was highest among L235-specific CTL when SW480 was used as a stimulator (0.23% ± 0.008%), together with the larger diameter of spots than against L235-pulsed T2 cells (data not shown). There was no comparable difference in spot number among L236-specific CTL when stimulated with different tumor cell lines. The natural killer cells (NK)-sensitive cell line K562 was relatively resistant to IFN-γ production by two peptide-sensitized CTL. Unexpectedly, both L235- and L236-specific CTL showed lower capacity to produce IFN-γ against the HLA-A2$^+$/OVA66$^+$ cell line HepG2 (Fig. 7a).

Moreover, CTL induced by peptide the L235 were able to lyse SW480 (OVA66$^+$ and HLA-A*0201) more efficiently than L236-induced CTL, which further proved that L235 possesses the potential ability to arouse an efficient cellular response. Cytolysis of L235- or L236-specific CTL was less against HO8910 (HLA-A2$^-$ OVA66$^+$), BEL-7402 (HLA-A2$^-$ OVA66^{low}), and NK-sensitive K562 (Fig. 7b). These results have proved that the CTL induced by OVA66-derived L235 *in vitro* could recognize and lyse target cells bearing naturally processing and presenting OVA66-specific peptides specifically. Consistent with the ELISPOT results, the cytotoxicity of CTL induced by L235 was much lower against HepG2, which was both OVA66$^+$ and HLA-A*0201 (Fig. 7b).

Discussion

The identification of tumor-associated antigens recognized by cellular or humoral effectors of the immune system has opened new perspectives for cancer therapy. Different groups of cancer-associated antigens have been described as targets for CTL *in vitro* and *in vivo*.[19,20] SEREX analysis has also aided in the discovery of numerous new tumor antigens. More than 2000 antigens are listed in the SEREX database, which can be classified into several groups: (1) antigens that are overexpressed in malignant tissues; (2) differentiation antigens; (3) point mutations of normal genes; (4) cancer–testis antigens that are expressed in different tumors and normal testis; and (5) viral antigens. The SEREX-defined tumor antigens also facilitate the identification of epitopes (antigenic peptides) recognized by antigen-specific CTL and provide a basis for peptide vaccine and gene therapy in a wide variety of human cancers. SEREX-defined antigens need to be evaluated following an algorithm of several analytical steps

doi: 10.1111/j.1349-7006.2008.00860.x

before they become new target antigens for active immunotherapy: expression pattern analysis to evaluate tumor association; serological analysis with sera from tumor patients and normal individuals to prove tumor-associated immunogenicity; and identification of potential peptide epitopes for CTL responses and evaluation of their potential as vaccine candidates.

Among the positive clones, we have identified EST1753 (*OVA66*) that showed a tumor-associated expression profile. We identified several proteins, including EST1753 (OVA66), that likely showed a tumor-associated expression profile. OVA66 was homologous to the previously reported protein CML66, which was identified from a chronic myelocytic leukemia cDNA library by SEREX analysis. Previous work has shown that it was not expressed in normal tissue, except the heart and testis. Considerable antibody responses from 18 to 38% have been detected in tumor patients with lung cancer, melanoma, and prostate cancer.[16] Consistent with that previous report, our work has further demonstrated that OVA66 expression was low in most normal tissues but increased significantly in tumor tissues such as gastric cancer, HCC, and ovarian cancer, which is accompanied by elevated OVA66 protein expression. The expression level in benign tissue, such as breast and thyroid gland tissues, was relatively low among those tested. These results have strongly proved that OVA66 has tumor-associated properties.

Antibody levels in the serum are remarkably representative of humoral responses. We found that a specific antibody response against OVA66 was evident in 22.2–66.7% of tumor patients with HCC, colon cancer, gastric cancer, ovarian cancer, and lung cancer. Considering the increased OVA66 expression level in tumor tissues, the overexpressed OVA66 seemed to display strong immunogenecity to arouse efficient immune responses, including humoral responses. The correlation between elevated OVA66 protein and antibody levels makes them a possible novel diagnostic pair for clinical applications. Further study will focus on analysis of the protein level of OVA66 in serum for its biomarker potential.

Induction of a CTL response is thought to be essential for effective antitumor defense. The identification of T-cell epitopes from tumor antigens has thus become a critical step in the development of peptide-based immunotherapy for cancer. A major breakthrough in this field was the finding that ligands of a certain MHC molecule carry chemically related amino acids in certain positions,[21] which leads to the definition of a peptide motif for every MHC allele. This method was rapidly adapted to the prediction of potential epitopes from numerous antigens and dedicated to the definition of multiple T-cell epitopes from various tumor antigens,[22] including MAGE-1,[23] NY-ESO-1,[13] HER-2/neu,[24] TRP2,[25] and gp-100.[26] In the present study, we used the same strategy to predict the HLA-A2-restricted T-cell epitopes from the OVA66 antigen. The T2 cell line is HLA-A*0201 and deficient in transporter associated with antigen processing, with the characteristic of presenting exogenous rather than endogenous antigens. The expression level of the HLA-A2 molecule on T2 cells depends on the binding of exogenous antigens to HLA-A2 molecules. In the present study, several predicted HLA-A*0201-restricted epitopes derived from OVA66 protein were pulsed on T2 cells, and the peptide L235 showed a similar HLA-A2 binding ability and stability to the positive control peptide L239. This result is consistent with the computer prediction that L235 has the highest score value (estimate of half time of disassociation of a molecule containing this subsequence) of binding to HLA-A2.

After testing the binding ability and stability of epitope peptides using a T2-dependent cell model, we further induced peptide-specific CTL *in vitro* with autologous antigen presenting cells (APC) and assessed their ability to recognize peptide-pulsed T2 and target cells presenting naturally processed epitopes. From our results, we selected the peptides L235 and L236, which showed putative high and low affinity, respectively, with the HLA-A2 molecule, and could both stimulate the expansion of CD8 T cells from PBMC of HLA-A*0201 healthy donors. However, the peptide L235 was able to elicit stronger peptide-specific CTL cytotoxicity against L235-pulsed target cells. This was also confirmed by the fact that the L235-specific CTL were able to recognize the OVA66$^+$/HLA-A*0201 cell line SW480, but not HLA-A2$^-$ cell lines, suggesting that this peptide is processed naturally and presented on the surface of carcinomas as a potential immunogenic epitope to trigger CTL responses. We noted that another OVA66$^+$/HLA-A*0201 cell line, HepG2, is resistant to the IFN-γ secretion of CTL counterparts, which may be due to the high expression of FasL and transforming growth factor (TGF)-β on HepG2 that could deliver the suppressive signals on CTL and facilitate immunosurveillance escape (data not shown). The lower expression of OVA66 in HepG2 than in SW480, assessed by reverse transcription-PCR, might also contribute to the lower immunogenicity of OVA66 in the HepG2 cell line.

In conclusion, through SEREX analysis we have identified OVA66 as a tumor-associated antigen that could arouse strong humoral responses in tumor patients. Its immunogenic epitope L235 could elicit specific CTL responses *in vitro* and might therefore serve as an attractive component of peptide-based vaccines for cancer immunotherapy.

Acknowledgments

This work was supported by Grants from the National Natural Science Foundation of China (30371598), Shanghai Natural Science Foundation of China (052R14056), and Shanghai Municipal Education Commission (2005JY09).

References

1 Nakano O, Sato M, Naito Y *et al*. Proliferative activity of intratumoral CD8$^+$ T-lymphocytes as a prognostic factor in human renal cell carcinoma: clinicopathologic demonstration of antitumor immunity. *Cancer Res* 2001; **61**: 5132–6.
2 Eerola AK, Soini Y, Paakko P. A high number of tumor-infiltrating lymphocytes are associated with a small tumor size, low tumor stage, and a favorable prognosis in operated small cell lung carcinoma. *Clin Cancer Res* 2000; **6**: 1875–81.
3 van der Bruggen P, Traversari C, Chomez P *et al*. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science* 1991; **254**: 1643–7.
4 GE HL, Wang Y, Wang SJ, Zhang Y. Identification of melanoma-specific peptide epitopes by HLA-A2.1-restricted cytotoxic T lymphocytes. *Acta Biochim Biophys Sin (Shanghai)* 2006; **38**: 110–18.
5 Schultze JL, Vonderheide RH. From cancer genomics to cancer immunotherapy: toward second-generation tumor antigens. *Trends Immunol* 2001; **22**: 516–23.
6 Yang F, Yang XF. New concepts in tumor antigens: their significance in future immunotherapies for tumors. *Cell Mol Immunol* 2005; **2**: 331–41.
7 Van den Eynde BJ, van der Bruggen P. T cell defined tumor antigens. *Curr Opin Immunol* 1997; **9**: 684–93.
8 Old LJ, Chen YT. New paths in human cancer serology. *J Exp Med* 1998; **187**: 1163–7.
9 Sahin U, Tureci O, Schmitt H *et al*. Human neoplasms elicit multiple specific immune responses in the autologous host. *Proc Natl Acad Sci USA* 1995; **92**: 11 810–13.
10 Chen YT, Scanlan MJ, Sahin U *et al*. A testicular antigen aberrantly expressed in human cancers detected by autologous antibody screening. *Proc Natl Acad Sci USA* 1997; **94**: 1914–18.
11 Tureci O, Sahin U, Schobert I *et al*. The *SSX-2* gene, which is involved in the t(X; 18) translocation of synovial sarcomas, codes for the human tumor antigen HOM-MEL-40. *Cancer Res* 1996; **56**: 4766–72.
12 Li B, Qian XP, Pang XW, Zou WZ, Wang YP, Wu HY, Chen WF. HCA587 antigen expression in normal tissues and cancers: correlation with tumor differentiation in hepatocellular carcinoma. *Lab Invest* 2003; **83**: 1185–92.

13 Jager E, Chen YT, Drijfhout JW *et al*. Simultaneous humoral and cellular immune response against cancer–testis antigen NY-ESO-1: definition of human histocompatibility leukocyte antigen (HLA)-A2-binding peptide epitopes. *J Exp Med* 1998; **187**: 265–70.
14 Valmori D, Dutoit V, Lienard D *et al*. Naturally occurring human lymphocyte antigen-A2 restricted $CD8^+$ T-cell response to the cancer testis antigen NY-ESO-1 in melanoma patients. *Cancer Res* 2000; **60**: 4499–506.
15 Stockert E, Jager E, Chen YT *et al*. A survey of the humoral immune response of cancer patients to a panel of human tumor antigens. *J Exp Med* 1998; **187**: 1349–54.
16 Yang XF, Wu CJ, McLaughlin S *et al*. CML66, a broadly immunogenic tumor antigen, elicits a humoral immune response associated with remission of chronic myelogenous leukemia. *Proc Natl Acad Sci USA* 2001; **98**: 7492–7.
17 Boissonnas A, Bonduelle O, Antzack A *et al*. *In vivo* priming of HIV-specific CTLs determines selective cross-reactive immune responses against poorly immunogenic HIV-natural variants. *J Immunol* 2002; **169**: 3694–9.
18 Jin SWY, Wang SJ, Zhang HZ, Li MX, Chen Y, Ge HL. Identification of a novel HLA-A2-restrictive CTL epitope of an ovary cancer-associated antigen OVA66. *Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi* 2005; **21**: 233–236, 242.
19 Novellino L, Castelli C, Parmiani G. A listing of human tumor antigens recognized by T cells: March 2004 update. *Cancer Immunol Immunother* 2005; **54**: 187–207.
20 Jager D, Taverna C, Zippelius A, Knuth A. Identification of tumor antigens as potential target antigens for immunotherapy by serological expression cloning. *Cancer Immunol Immunother* 2004; **53**: 144–7.
21 Falk K, Rotzschke O, Stevanovic S, Jung G, Rammensee HG. Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. *Nature* 1991; **351**: 290–6.
22 Pietersz GA, Pouniotis DS, Apostolopoulos V. Design of peptide-based vaccines for cancer. *Curr Med Chem* 2006; **13**: 1591–607.
23 Mukherji B, Chakraborty NG, Yamasaki S *et al*. Induction of antigen-specific cytolytic T cells in situ in human melanoma by immunization with synthetic peptide-pulsed autologous antigen presenting cells. *Proc Natl Acad Sci USA* 1995; **92**: 8078–82.
24 Baxevanis CN, Sotiriadou NN, Gritzapis AD *et al*. Immunogenic HER-2/neu peptides as tumor vaccines. *Cancer Immunol Immunother* 2006; **55**: 85–95.
25 Parkhurst MR, Fitzgerald EB, Southwood S, Sette A, Rosenberg SA, Kawakami Y. Identification of a shared HLA-A*0201-restricted T-cell epitope from the melanoma antigen tyrosinase-related protein 2 (TRP2). *Cancer Res* 1998; **58**: 4895–901.
26 Walker EB, Haley D, Miller W *et al*. gp100 (209-2M) peptide immunization of human lymphocyte antigen-$A2^+$ stage I–III melanoma patients induces significant increase in antigen-specific effector and long-term memory $CD8^+$ T cells. *Clin Cancer Res* 2004; **10**: 668–80.

doi: 10.1111/j.1349-7006.2008.00860.x