Simultaneous Cellular and Humoral Immune Response against Mutated p53 in a Patient with Lung Cancer[1]

Yoshinobu Ichiki, Mitsuhiro Takenoyama,[2] Makiko Mizukami, Tetsuya So, Masakazu Sugaya, Manabu Yasuda, Tomoko So, Takeshi Hanagiri, Kenji Sugio, and Kosei Yasumoto

We recently identified several Ags recognized by tumor-infiltrating B lymphocyte-derived Ab using SCID mice and a xenografted non-small cell lung cancer system. One of these identified Ags was mutated p53 with a point mutation resulting in the alteration of codon 158 from Arg to Leu. The aim of this study was to ascertain whether cellular immunity against mutated p53 exists in the same patient together with humoral immunity. Two different nona peptides (mutated $p53_{150}$ and $p53_{155}$ peptides), including a mutated amino acid derived from p53, were synthesized according to the binding motif of HLA class I of the established cancer cell line A904L from the patient. Mediastinal lymph node lymphocytes of the patient were stimulated weekly with the peptides. The mutated $p53_{155}$ peptide-stimulated lymphocytes showed specific cytotoxicity against both autologous EBV-transformed B cells pulsed with mutated $p53_{155}$ peptide and A904L. The mutated $p53_{155}$ peptide-specific CTL clone in an HLA-Cw*0702 restriction was established and analyzed for its TCR usage. Clonotypic PCR using CDR3-specific primers was applied to the tumor tissue containing the tumor-infiltrating lymphocytes. The specific amplification of PCR was found in the tumor tissue. These results demonstrated that not only B lymphocytes producing specific Ab against the p53 protein, but also CTL against mutated p53, expressed in autologous lung cancer cells exist in the tumor tissue. This approach may allow us to better understand the mechanisms of T and B cell immunity against the same tumor Ag in cancer patients. ***The Journal of Immunology*****, 2004, 172: 4844–4850.**

The development of approaches to analyze both humoral and cellular immune reactivity to cancer has led to the molecular characterization of tumor Ags recognized by CTL (1, 2) and Abs using a serological analysis of recombinant cDNA expression cloning (SEREX)[3] (3–5). However, the relationship between the CTL and B lymphocytes of cancer patients in the responses against one particular Ag has only been observed to date in a limited number of Ags, including MAGE-1, tyrosinase, NY-ESO-1, and coactosin-like protein (CLP) (6–9). MAGE-1 and tyrosinase were initially identified by the use of specific CTL (1, 2), and Abs against these Ags have also been detected by a serological analysis of cancer patients (SEREX) (3, 6, 7). NY-ESO-1 and CLP were first identified by the SEREX method (4, 5), and they have also been demonstrated to be recognized by CTLs (8, 9).

We previously reported that 1) fresh human lung cancer tissues engrafted in SCID mice produced human IgG; 2) IgG derived from autologous tumor-infiltrating B lymphocytes (TIB) highly bound to the intra- and extracellular Ags of autologous lung cancer cells; and 3) less reactivity was observed against normal lymphocytes by IgG derived from autologous TIB than from peripheral blood B cells (10–12). These results indicate that TIB recognize lung cancer cell lines, including the autologous lung cancer cell line, and produce tumor-specific antibodies. We identified 37 Ags recognized by the Abs derived from TIB engrafted in SCID mice. One of these identified Ags was mutated p53 with a point mutation resulting in the alteration of codon 158 from Arg to Leu (10). The production of Ab against p53 protein by TIB indicates that specific B lymphocytes recognizing p53 protein accumulate around the tumor microenvironment. The TIB-derived Ab also showed a same level of reactivity against wild-type p53 protein. The aim of the present study was to ascertain whether cellular immunity against mutated p53 may exist in the regional lymph nodes and also in the primary tumor tissue of the same patient together with humoral immunity against the same Ag.

Materials and Methods

The study protocol was approved by the human and animal ethics review committee of University of Occupational and Environmental Health, Japan, and a signed consent form was obtained from each subject before obtaining the tissue samples used in this study.

Patient

Patient A904, a 51-year-old male, presented in September 1996 with large cell carcinoma of the lung. After the first operation, a metastatic tumor appeared in the right adrenal gland, which was subsequently resected in October 1997. Thereafter, no recurrence was observed for 6 years.

SCID mice

Female SCID mice (BALB/c · C57BL/Ka-Igh-1^b/ICR [N17F34] scid/acid, 6 wk old) were obtained from Charles River (Tokyo, Japan) and maintained in specific pathogen-free conditions throughout the study. Surgically resected specimens from the patient with lung cancer (A904) were used to prepare tissue specimens for implantation as described previously (11, 12). Engrafted SCID mice were bled by a retro-orbital venipuncture once a week after the xenotransplantation of the cancer tissue, and serum was collected and pooled for analysis of human Ig. The human Ig titers were measured by the latex agglutination method (11).

Second Department of Surgery, School of Medicine, University of Occupational and Environmental Health, Kitakyushu, Japan

Received for publication July 21, 2003. Accepted for publication February 18, 2004.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan.

[2] Address correspondence and reprint requests to Dr. Mitsuhiro Takenoyama, Second Department of Surgery, School of Medicine, University of Occupational and Environmental Health, 1-1 Iseigaoka, Yahatanishi-ku, Kitakyushu 807-8555, Japan. E-mail address: m-take@med.uoeh-u.ac.jp

[3] Abbreviations used in this paper: SEREX, serological analysis of Ags by recombinant cDNA expression cloning; CLP, coactosin-like protein; CM, culture medium; CT Ag, cancer-testis Ag; EBV-B, Epstein-Barr virus-transformed B cells; RLNL, regional lymph node lymphocyte; TIB, tumor-infiltrating B lymphocyte; TIL, tumor-infiltrating lymphocyte.

0022-1767/04/$02.00

Table I. *Mutated and wild-type p53-derived peptides predicted by a binding motif of the A904L-HLA alleles*

Type	Sequences	Residues	Predicted Binding HLA Alleles
Mutated $p53_{150}$ (m-$p53_{150}$)	TPPPGTRVL	150–158	HLA-A*2402 and B*0702
Mutated $p53_{155}$ (m-$p53_{155}$)	TRVLAMAIY	155–163	HLA-Cw*0702
Wild $p53_{155}$ (w-$p53_{155}$)	TRVRAMAIY	155–163	HLA-Cw*0702

Culture medium (CM)

The CM we used consisted of RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% heated-inactivated FCS (Life Technologies), 10 mM HEPES, 100 U/ml penicillin G, and 100 mg/ml streptomycin sulfate.

Cell lines

A904L is a lung large cell carcinoma cell line derived from patient A904. B cells transformed by EBV (EBV-B) were produced from patient A904 by infecting PBMC with the supernatant from the EBV producer line B95.8. K562 is an erythroleukemia cell line that is sensitive to NK cell cytotoxicity.

Immunohistochemical staining for p53 protein

A904L was stained with Abs against p53 protein using the DO-1 Ab (Oncogene Science, Cambridge, MA) (13).

HLA typing

The tumor cell line genotypes of HLA alleles were determined by PCR, performed by Shionogi Biomedical Laboratories (Osaka, Japan). The genotypes of A904 EBV-B were HLA-A*2402, 2603, -B*0702, 3901, and -Cw*0702, 0702. In contrast, the genotypes of A904L were HLA-A*2402, -B*0702, and -Cw*0702 with a loss of the haplotype.

Peptides

From the reported data concerning the HLA binding motif of peptides (14), the following peptides including the point mutation were selected as Ag peptide candidates to induce CTL. The HLA-A*2402 and -B*0702 binding peptides, the same TPPPGTRVL (mutated $p53_{150}$ is underlined), and the HLA-Cw*0702 binding peptides, TRVLAMAIY (mutated $p53_{155}$ is underlined) and TRVRAMAIY (wild-type $p53_{155}$), were synthesized (Table I).

Generation of CTL using synthetic peptides

Peptide-specific CTL were induced by the method reported by Coulie et al. (15), with a slight modification. In brief, the regional lymph node lymphocytes (RLNL) of the patient were first pulsed with the synthetic mutated p53 peptides at a final concentration of 20 μg/ml for 1 h at room temperature, and then were cultured in the presence of 20 U/ml IL-2 (donated by Takeda Chemical Industries, Osaka, Japan), 5 ng/ml IL-4 (Serotec, Oxford, U.K.), and 5 ng/ml IL-7 (Genzyme, Cambridge, MA). The initial number of RLNL to induce CTL against each peptide was 2.4×10^7. These RLNL were independently divided into eight wells (3×10^6 cells/well) of a 24-well culture plate (Iwaki Glass, Tokyo, Japan). On day 7 the cultured cells were restimulated in the manner described above. On day 14 they were stimulated by irradiated autologous EBV-B prepulsed with the synthetic mutated p53 peptides at a final concentration of 20 μg/ml for 1 h at room temperature. The stimulation was conducted at a responder/stimulator cell ratio of 10:1. CTL activity was assessed by a standard 4-h ^{51}Cr release assay 1 wk after the third stimulation.

To obtain T cell clones, a limiting dilution was performed from the bulk CTL line by the following method. The cells were seeded at 0.1, 0.3, 1, 3, 10, 50, or 500 cells/well in 96-well, U-bottom plates (Iwaki Glass) and stimulated under the following culture conditions: CM with irradiated autologous EBV-B prepulsed with the mutated p53 peptide (5×10^3/well) as stimulator cells and EBV-B established from allogenic PBMC (5×10^4/well) as feeder cells in the presence of IL-2 (50 U/ml), IL-4 (5 ng/ml), and IL-7 (5 ng/ml). Next, the irradiated stimulator cells and feeder cells were added to each well for restimulation of the lymphocytes once per week with CM containing IL-2, IL-4, and IL-7.

Phenotypic analysis

The CTLs were doubly labeled with FITC- and PE-conjugated mAbs to CD3, CD4, CD8, and CD56. These cells were tested, using a two-color analysis, on a flow cytometer.

Monoclonal Ab

Hybridomas (HB-145 and HB-95) were purchased from American Type Culture Collection (Manassas, VA). C7709.A2.6 (anti-HLA-A24) and B1.23.2 (anti-HLA-B and -C) were donated by Dr. P. G. Coulie (Cellular Genetics Unit, Universite Catholique de Louvain, Brussels, Belgium). The supernatants of American Type Culture Collection HB-145 (IVA 12; anti-HLA-DR, -DP, and -DQ), HB-95 (W6/32; anti-HLA-A, -B, and -C), C7709.A2.6, and B 1.23.2 were used to analyze the HLA restriction of the CTLs.

Cytokine production

The cultured lymphocytes (3×10^4/ml) were cocultured with autologous tumor cells or autologous EBV-B (3×10^5/ml) pulsed with peptides for 4 h. Next, the amount of IFN-γ in the culture supernatant was measured using an IFN-γ ELISA test kit (Life Technologies) according to the instruction manual. To confirm the restriction element of HLA for CTL to recognize the p53 Ag, an HLA transfection experiment using COS-7 cells was performed. The cDNA of HLA-A*2402, -B*0702, and -Cw*0702 was obtained from autologous tumor cells by RT-PCR and cloned into the pcDNA3. The cDNA plasmid of 100 ng of HLA-A*2402, -B*0702, or -Cw*0702 was suspended in 50 μl of OptiMEM (Life Technologies) mixed with Lipofectamine reagent (Life Technologies), and samples were cultured at room temperature for 1 h at a final volume of 100 μl for a duplicate assay. A 50-μl aliquot of the mixture was then added to the COS-7 cells (5×10^3 cells/well), which were then incubated for 4 h at a final volume of 50 μl. Thereafter, the COS-7 cells were pulsed with the mutated $p53_{155}$ peptide at a final concentration of 20 μg/ml. The COS-7 cells were cultured for 1 day in RPMI 1640 medium supplemented with 10% FCS containing IL-2 (final concentration, 25 U/ml), followed by the addition of CTL (3×10^3 cells/well). After 18-h incubation, 100 μl of the culture supernatant was collected and assayed in duplicate for IFN-γ production by ELISA.

TCR usage analysis

TCR Vβ usage of the CTL clone was assessed by RT-PCR and sequencing. RNA extracted from the CTL clone using RNeasy Mini Kit (Qiagen, Valencia, CA) was converted to cDNA using a random primer. The cDNA served as a template for PCR amplification using a panel of Vβ-specific forward primers and reverse Cβ primers. The PCR products were sequenced with the BigDye Terminator Cycle Sequence kit (PerkinElmer, PE

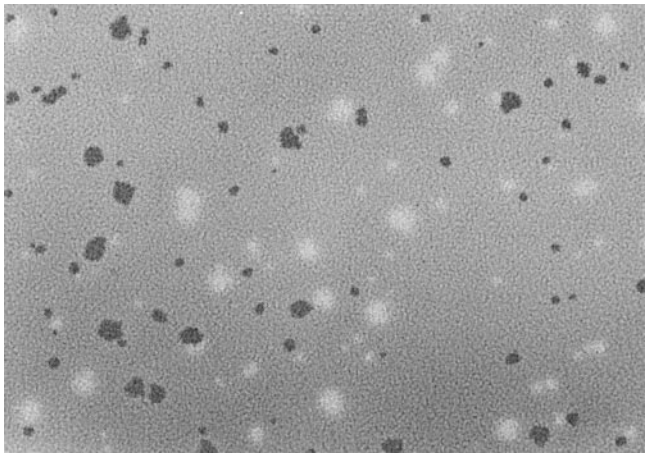

FIGURE 1. The serum from SCID mice engrafted with the lung cancer tissue was also diluted to 2×10^{-5}. The reactivity of the serum against the wild-type p53 protein is shown.

Applied Biosystems, Foster City, CA). The products of the sequence reaction were analyzed on an ABI 310 sequencer (PerkinElmer).

PCR analysis

To prove that the CTL clone expressing the same TCR was present in the primary tumor tissue, regional lymph node collected at the surgery (1×10^5 cells), and the peripheral blood of patient A904 collected 3 and 5 years after the operation, RNA was extracted from each sample and converted to cDNA. The cDNA served as template for PCR amplification using Vβ-specific forward primer (OKY56, 5′-GCACAACAGTTCCCTGACTTGC-3′) and the TCR-D-J joint region-specific reverse primer (OKY198, 5′-CCAAAATACTGCGTATCTATCACTCC-3′). The second PCR was performed using the diluted first PCR products (1/100) as a template with the same forward and newly designed TCR-D-J joint region-specific reverse primer (OKY199, 5′-TATCACTCCAAGCCCAAG-3′).

Results

Identification of the p53 mutation as a tumor-specific Ag using the modified SEREX method

As we reported previously, one of 37 distinct cDNA clones identified using the modified SEREX method, which exhibited strong reactivity, corresponded to the gene encoding p53 with one base substitution at position 473 (CGC to CTC) that altered codon 158 from Arg to Leu (10). Titration of the p53 Ab of the SCID mouse serum was performed by a dilution method. The serum was thus shown to have a very strong reactivity with p53 protein up to 2×10^{-5} dilution (data not shown). Probably this mutation resulted in an overexpression of the p53 protein, as the p53 protein was accumulated in the nuclei of the tumor cell line A904L by immunohistochemistry using staining for DO-1 antibody. To determine whether the mutated amino acid in p53 is the epitope recognized by TIB-derived antibody, cDNA coding for wild-type p53 was cloned from autologous EBV-B and tested for the reactivity of TIB-derived antibody. The serum also showed a strong reactivity against wild-type p53 protein, thus indicating that the epitope in p53 recognized by the Ab does not involve the mutated amino acid (Fig. 1).

Induction of bulk CTLs specific for mutated p53

The cytotoxic activity was detected in two of eight wells stimulated with mutated $p53_{155}$ peptide binding to HLA-Cw*0702. The cytotoxic activities against autologous EBV-B pulsed with mutated $p53_{155}$ peptide and against the autologous tumor cell line (A904L) were detected after three peptide stimulations (data not shown). However, cytotoxic activity could not be induced by stimulation with a mutated $p53_{150}$ peptide which has a binding affinity to HLA-A*2402 or -B*0702.

Establishment of CTL clone specific for mutated $p53_{155}$ peptide

A limiting dilution of these bulk CTLs was performed to obtain a mutated $p53_{155}$ peptide-specific CTL clone. Consequently, one CTL clone ($CD3^+$, $CD8^+$) was obtained.

As shown in Fig. 2, the cytotoxic activities of this CTL clone against autologous EBV-B pulsed with mutated $p53_{155}$ peptide and against the autologous tumor cell line (A904L) were detected in a dose-dependent manner. However, the cytotoxic activity against autologous EBV-B pulsed with wild-type $p53_{155}$ peptide could not be detected. These results suggested that the autologous tumor cell line

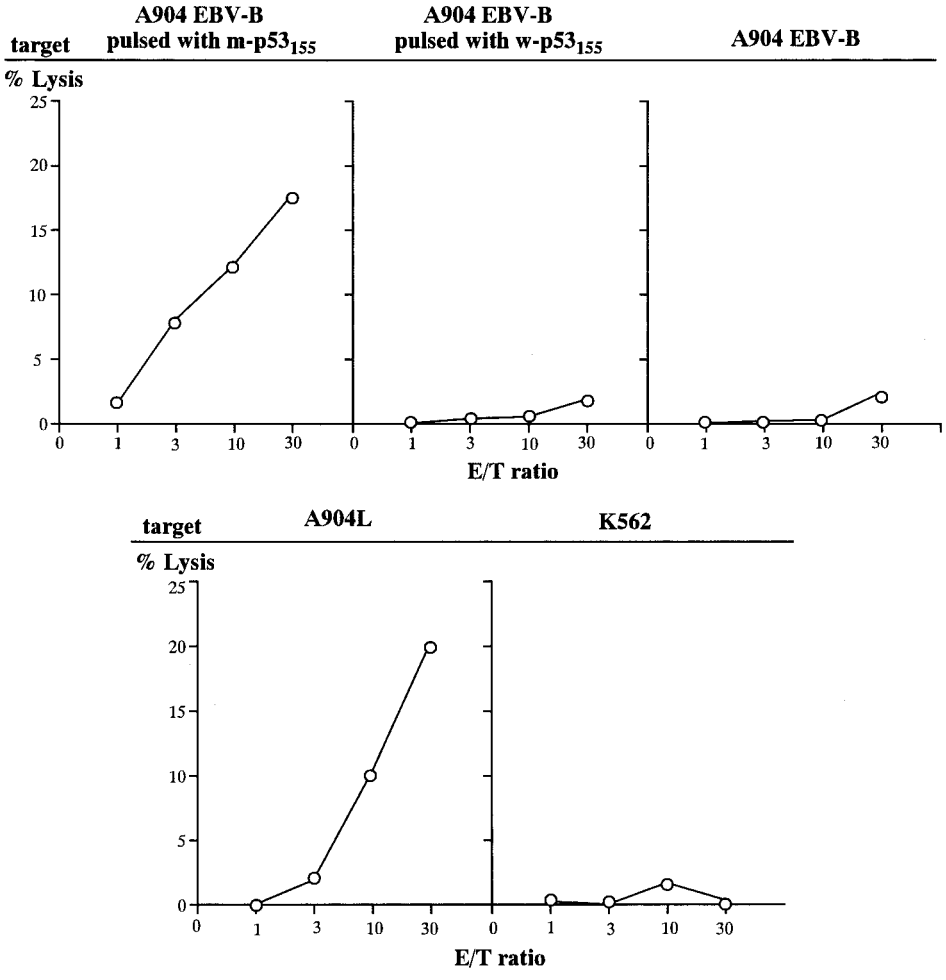

FIGURE 2. The cytotoxicity of the mutated $p53_{155}$ peptide-specific CTL clone. The cytotoxic activities of the mutated $p53_{155}$ peptide-specific CTL clone against autologous EBV-B pulsed with mutated $p53_{155}$ peptide and the autologous tumor cell line (A904L) were assessed by a standard 4-h ^{51}Cr release assay.

(A904L) endogenously presented the mutated $p53_{155}$ peptide recognized by the CTL clone that was derived from the RLNL of the patient.

Demonstration of the restriction element of mutated $p53_{155}$ peptide-specific CTL clone

A blocking assay for the production of IFN-γ by the CTL was performed using various mAbs. The CTL clone produced IFN-γ in response to mutated $p53_{155}$ peptide-pulsed autologous EBV-B (Fig. 3*A*) and an autologous tumor cell line (Fig. 3*B*). The production of IFN-γ was partially inhibited by both anti-MHC class I and anti-HLA-B/C antibodies. The same CTL responses were observed against autologous A904L cells. This CTL clone produced a significant level of IFN-γ in response to COS-7 cells transfected with HLA-Cw*0702, but not to cells transfected with either HLA-A*2402 or -B*0702, which was pulsed with mutated $p53_{155}$ peptide, as shown in

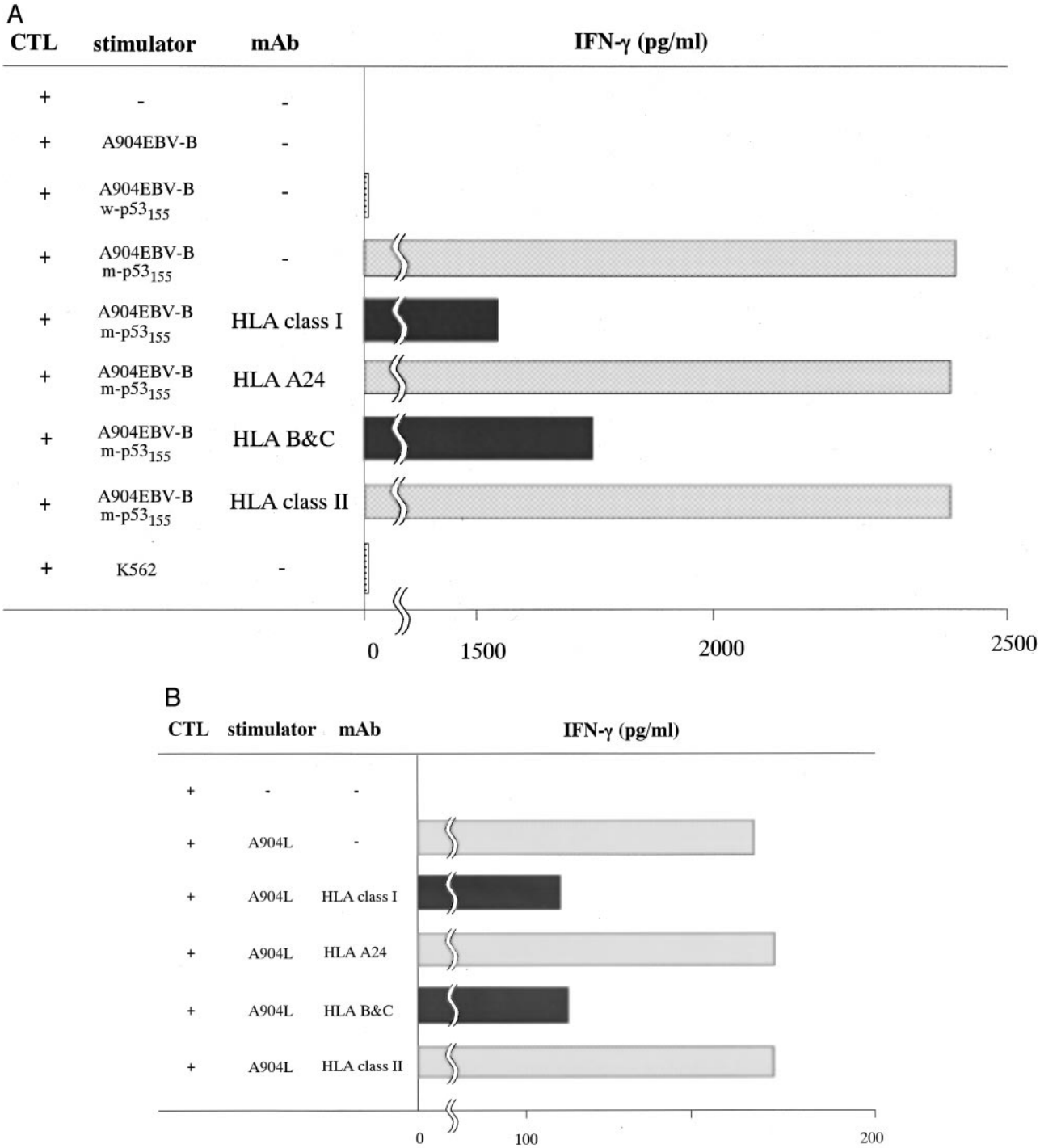

FIGURE 3. IFN-γ production of the mutated $p53_{155}$ peptide-specific CTL clone. *A*, The CTL clone (3×10^3/well) was cocultured with autologous EBV-B (3×10^4/well) pulsed with the mutated $p53_{155}$ peptide for 4 h, and the amount of IFN-γ in the culture supernatant was measured using an IFN-γ ELISA test kit. To analyze the HLA restriction of the CTL clone, anti-MHC class I mAb, anti-HLA-A24 mAb, anti-HLA-B and -C mAb, and anti-MHC class II mAb were used. *B*, The CTL clone (3×10^3/well) was cocultured with the autologous tumor cell line (3×10^4/well) for 4 h in the presence of the indicated mAbs. The supernatants were assessed for IFN-γ by ELISA.

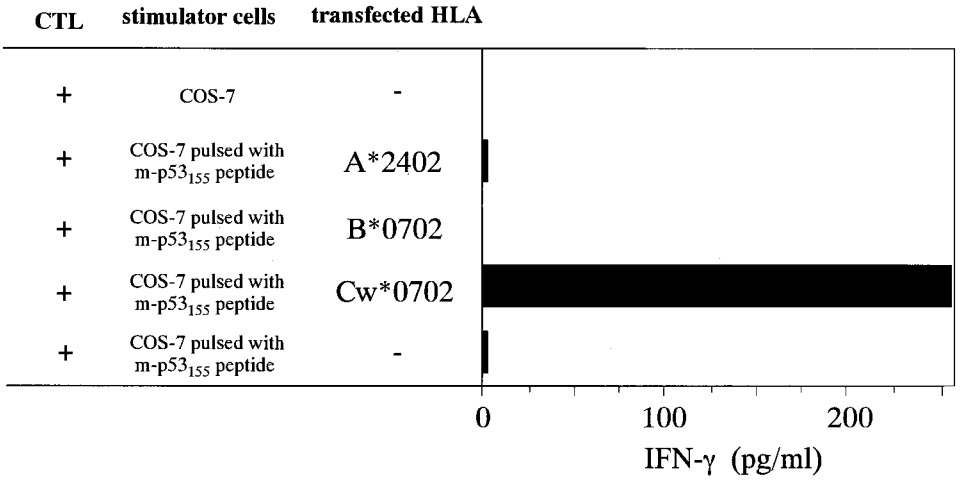

FIGURE 4. HLA restriction of the mutated $p53_{155}$ peptide-specific CTL clone. The cDNA plasmid of 100 ng of HLA-A*2402, -B*0702, or -Cw*0702 was suspended in 50 μl of OptiMEM, mixed with Lipofectamine reagent, and samples were cultured at room temperature for 1 h at a final volume 100 μl for a duplicate assay. A 50-μl aliquot of the mixture was then added to the COS-7 cells (3×10^4 cells/well), which were then incubated for 4 h at a final volume of 50 μl. Thereafter, the COS-7 cells were pulsed with the mutated $p53_{155}$ peptide at a final concentration of 20 μg/ml. The COS-7 cells were cultured for 1 day in RPMI 1640 medium supplemented with 10% FCS containing IL-2 (final concentration, 25 U/ml), followed by addition of CTL (3×10^3 cells/well). After 18-h incubation, 100 μl of the culture supernatants were collected and assayed in duplicate for IFN-γ production by ELISA.

Fig. 4. Therefore, this CTL clone recognized mutated $p53_{155}$ peptide with restriction by HLA-Cw*0702.

TCR analysis

The mutated $p53_{155}$ peptide-specific CTL clone was proved to express the Vβ9 gene segment, and it possessed Vβ9-Dβ1-Jβ2-Cβ2 (Fig. 5*A*). To determine whether lymphocytes expressing the same TCR as the CTL clone were present in the tumor tissue, regional lymph node collected at surgery, and peripheral blood collected 3 and 5 years after the operation, clonotypic PCR was performed using Vβ-specific forward primer and TCR-D-J joint region-specific reverse primer. The cDNA from each sample served as a template for PCR amplification. As shown in Fig. 5*B*, the same TCR as the mutated $p53_{155}$ peptide-specific CTL clone existed in 20 mg of primary tumor tissue, but not in 1×10^5 of lymphocytes from the regional lymph node and not in 1×10^6 of the lymphocytes from the peripheral blood of the patient collected 3 and 5 years after operation. We further analyzed 8×10^6 of PBMC from the patient collected 3 years after operation for TCR usage, and the same TCR could not be detected (data not shown). These results indicated that the mutated $p53_{155}$ peptide-specific CTL clone accumulated in the tumor tissue.

Discussion

Recently, a large number of tumor-specific Ags have been identified with autologous CTLs and Abs in cancer patients. They can be classified into five groups as follows: 1) cancer-testis (CT) Ags, which are expressed in a range of different tumor types, but not in normal tissue except for the testis; 2) melanocyte differentiation Ags, expressed in melanomas and normal melanocytes; 3) Ags encoded by mutated normal genes; 4) self-Ag overexpressed in malignant tissues; and 5) viral Ags. However, only four (MAGE-1,

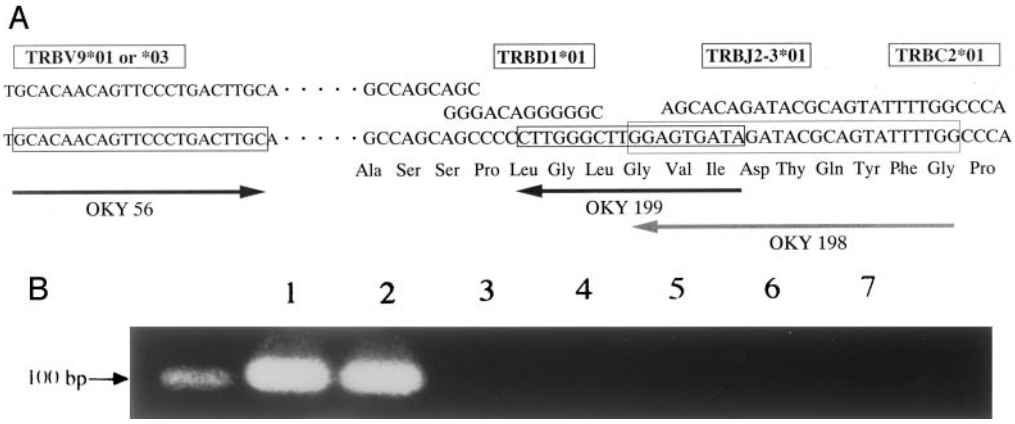

FIGURE 5. The existence of the mutated $p53_{155}$ peptide-specific CTL clone in the tumor tissue by TCR analysis. *A*, An analysis of TCR usage of the mutated $p53_{155}$ peptide-specific CTL clone. The CTL clone possessed Vβ9-Dβ1-Jβ2-Cβ2. *B*, Clonotypic PCR using CDR3-specific primers of the mutated $p53_{155}$ peptide-specific CTL clone. RNA was extracted from the mutated $p53_{155}$ peptide-specific CTL clone (*lane 1*), 20 mg of the primary tumor tissue (*lane 2*), 1×10^5 of lymphocytes from the regional lymph node (*lane 3*), 1×10^6 of lymphocytes from the peripheral blood of the patient collected at 3 years (*lane 4*) and 5 years (*lane 5*) after operation, and 1×10^6 of lymphocytes from the peripheral blood of two healthy donors (*lanes 6* and *7*) and was converted to cDNA. The cDNA served as templates for PCR amplification using a Vβ-specific forward primer (OKY56) and TCR-D-J joint region-specific reverse primer (OKY198). A second PCR was performed using the diluted first PCR products (1/100) as a template with the same forward and another designed TCR-D-J joint region-specific reverse primer (OKY199).

tyrosinase, NY-ESO-1, and CLP) (9) have been reported to have the ability to elicit both cellular and humoral immune responses. The CT Ag MAGE-1 was identified with the CTL clone derived from a melanoma patient (1), and an mAb against MAGE-1 protein was produced in rabbit sera immunized with the MAGE-1 fusion protein (6). The melanocyte differentiation Ag tyrosinase was also identified with the CTL clone derived from a melanoma patient (2), and Abs against tyrosinase protein were detected in melanoma patients (7). The CT Ag NY-ESO-1 was identified with SEREX in a patient with esophageal cancer (4), and CTL reactivity against the autologous NY-ESO-1-positive tumor cell line was detected in the melanoma patient with a high titer Ab response against the NY-ESO-1 (8). NY-ESO-1-specific $CD8^+$ T cell responses have been reported in >90% of NY-ESO-1 Ab-positive patients, whereas NY-ESO-1 Ab-negative patients showed no detectable NY-ESO-1-specific T cell reactivity (8, 16). The self-Ag overexpressed in malignant tissues CLP was identified with SEREX in a patient with pancreatic cancer (5), and CTL and IgG specific to CLP peptides were detected in patients with pancreatic and colon cancer (9). In these tumor Ags, only two (NY-ESO-1 and CLP) have been reported to elicit both cellular and humoral immune responses in the same patient (8, 9). In this study we demonstrated that one point mutation of p53 can simultaneously elicit not only a humoral immune response against overexpressed p53 protein, but also a specific CTL response against the mutated epitope of the protein in the same patient. This is the first report of the existence of both cellular and humoral immune responses against the same tumor Ag in tumor tissue.

p53 plays a key role in the G_0-G_1 cell cycle regulation, and mutations in the p53 genome are found in >50% of human cancer diseases (17, 18). The p53 mutations lead to changes in p53 protein turnover and subsequent p53 protein accumulation in the tumor cells (19). The accumulated p53 is recognized by B cells and also CTLs. To date, five MHC class I-restricted, naturally presented, human wild-type sequence p53 peptides have been identified. They have been shown to be able to induce epitope-specific CTL from PBMC obtained from healthy individuals (20–26). The $p53_{125–134}$ peptide is HLA-A24 restricted (26), whereas the other four, $p53_{65–73}$, $p53_{149–157}$, $p53_{217–225}$, and $p53_{264–272}$, are HLA-A2.1 restricted. In this study we identified a new mutated $p53_{155–163}$ peptide restricted by HLA-Cw*0702, which was recognized by autologous CTL. An analysis of 1961 Caucasian individuals showed HLA-Cw*0702 to be present in 658 of 3922 alleles, thus demonstrating a phenotype frequency of ~31% (27). In Japanese, the phenotype frequency of HLA-Cw*0702 is 24%, and it is predominantly found in HLA-Cw alleles (28).

A mutation of the p53 suppressor gene has been observed in ~47% of non-small cell lung cancer (29). Mutated p53 at codon 158 has been reported in four of 65 patients (6.2%) with non-small cell lung cancer (30), and the International Agency for Research on Cancer TP 53 Mutation Data indicated that 38 cases (2.0%) showed the same mutation in 1893 lung tumors (31). Although the mutated p53 at codon 158 is found in a small population, HLA-Cw*0702 is predominantly found in the HLA-Cw alleles of cancer patients. Therefore, the mutated $p53_{155}$ peptide could be a potential target for cancer vaccination.

In this study because no TIL of A904 were available, clonotypic PCR using CDR3-specific primer was performed to identify mutated $p53_{155}$ peptide-specific CTLs that infiltrated into tumor tissue. Consequently, the same TCR as the mutated $p53_{155}$ peptide-specific CTL clone could be detected in the tumor tissue containing TIL. However, the presence of CTL could not be identified by the clonotypic PCR in 1×10^5 of RLNL. The mutated $p53_{155}$ peptide-specific CTL clone could be elicited from two of eight wells (each well comprised 3×10^6 RLNL). The reason why the CTL could not be detected in 1×10^5 RLNL is because the frequency of the CTL clone is estimated to be ~1 cell/1×10^7 cells of RLNL.

Anti-p53 Abs were detected in the sera of patients with an accumulation of p53 protein due to p53 gene mutation in the tumor. Most epitopes of p53 Abs have been shown to be located in the immunodominant N- and C-terminal region outside the core DNA binding domain (32). The secondary structure of these regions remains unchanged in the mutant forms of the protein, suggesting that the immune response is more likely to be due to the accumulation and overexpression of the protein, rather than the appearance of novel antigenic determinants induced by such a mutation. Whereas intratumoral p53 protein accumulation appears to be a prerequisite for the production of anti-p53 Abs, the exact mechanisms of the self-immunization process have not yet been clarified, and whether this type of gene mutation may influence the production of anti-p53 Abs remains a matter of debate. In our results, p53 protein overexpressed in the A904L tumor might have thus induced a strong humoral immunity in this patient.

The identification of tumor-specific Ags in SEREX requires high titrated IgG Ab selectively responsive to tumor-specific Ags, and the development of such Abs requires the help of $CD4^+$ T cells. We also speculate that mutated p53-specific $CD4^+$ T cells may exist in the tumor tissue, thereby causing IgM to IgG conversion.

Acknowledgments

We thank Yumiko Hase and Kahoru Noda for their technical expert help.

References

1. Bruggen, P., C. Traversari, P. Chomez, C. Lurquin, E. D. Plaen, B. Eynde, A. Knuth, and T. Boon. 1991. Gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science 254:1643.*
2. Brichard, V., A. Van Pel, C. Wolfel, E. De Plaen, B. Lethe, P. Coulie, and T. A. Boon. 1993. The tyrosinase gene codes for an antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. *J. Exp. Med. 254:489.*
3. Sahin, U., O. Tureci, H. Schmitt, B. Cochlovius, T. Johannes, R. Schmits, F. Stenner, G. Luo, I. Schobert, and M. Pfreund. 1995. Human neoplasms elicit multiple specific immunne responses in the autologous host. *Proc. Natl. Acad. Sci. USA 92:11810.*
4. Chen, Y. T., M. S. Scanlan, U. Sahin, O. Tureci, A. O. Gure, S. Tsang, B. Williamson, E. Stockert, M. Pfreundschuh, and L. J. Old. 1997. A testicular antigen aberrantly expressed in human cancers detected by autologous antibody screening. *Proc. Natl. Acad. Sci. USA 94:1914.*
5. Nakatsura, T., S. Senju, K. Yamada, T. Jotsuka, M. Ogawa, and Y. Nishimura. 2001. Gene cloning of immunogenic antigens overexpressed in pancreatic cancer. *Biochem. Biophys. Res. Commun. 256:75.*
6. Chen, Y. T., E. Stockert, Y. Chen, Y. C. Chesa, W. J. Rettig, P. D. Bruggen, T. Boon, and L. J. Old. 1994. Identification of the MAGE-1 gene product by monoclonal and polyclonal antibodies. *Proc. Natl. Acad. Sci. USA 91:1004.*
7. Fishman, P., O. Merimski, E. Baharav, and Y. Shoenfeld. 1997. Autoantibodies to tyrosinase. *Cancer 79:1461.*
8. Jäger, E., Y. T. Chen, J. W. Drijfhout, J. Karbach, M. Ringhoffer, D. Jäger, M. Arand, H. Wada, Y. Noguchi, E. Stockert, et al. 1998. Simultaneous humoral and cellular immune response against cancer-testis antigen NY-ESO-1: detection of human histocompatibility leukocyte antigen (HLA)-A2-binding peptide epitopes. *J. Exp. Med. 187:265.*
9. Nakatsura, T., S. Senjyu, M. Ito, Y. Nishimura, and K. Itoh. 2002. Cellular and humoral immune responses to a human pancreatic cancer antigen, coactosin-like protein, originally defined by the SEREX method. *Eur. J. Immunol. 32:826.*
10. Yasuda, M., M. Takenoyama, Y. Obata, M. Sugaya, T. So, T. Hanagiri, K. Sugio, and K. Yasumoto. 2002. Tumor-infiltrating B lymphocytes as a potential source of identifying tumor antigen in human lung cancer. *Cancer Res. 62:1751.*
11. Imahayashi, S., Y. Ichiyoshi, I. Yoshino, R. Eifuku, M. Takenoyama, and K. Yasumoto. 2000. Tumor-infiltrating B-cell-derived IgG recognized tumor components in human lung cancer. *Cancer Invest. 18:530.*
12. Williams, S. S., F. A. Chen, H. Kida, S. Yokota, K. Miya, M. Kato, M. P. Barco, H. Q. Wang, T. Alosco, T. Umemoto, et al. 1996. Engraftment of human tumor-infiltrating lymphocytes and the production of anti-tumor antibodies in SCID mice. *J. Immunol. 156:1908.*
13. Gu, C. D., T. Osaki, T. Oyama, M. Inoue, M. Kodate, K. Dobashi, T. Oka, and K. Yasumoto. 2002. Detection of micrometastatic tumor cells in pN0 lymph nodes of patients with completely resected nonsmall cell lung cancer. *Ann. Surg. 235:133.*
14. Parker, K. C., M. A. Bednarek, and J. E. Coligan. 1994. Scheme for ranking potential HLA-A2 binding of individual peptide side-chains. *J. Immunol. 152:163.*

15. Coulie, P. G., V. Karanikas, D. Colau, C. Lurquin, C. Landry, M. Marchand, T. Dorval, V. Brichard, and T. Boon. 2001. A monoclonal cytolytic T-lymphocyte response observed in a melanoma patient vaccinated with a tumor-specific antigenic peptide encoded by gene MAGE-3. *Proc. Natl. Acad. Sci. USA 98: 10290.*
16. Jäger, E., S. Gnjatic, Y. Nagata, E. Stocker, D. Jäger, J. Karbach, A. Neumann, J. Rieckenberg, Y. T. Chen, G. Ritter, et al. 2000. Induction of primary NY-ESO-1 immunity: $CD8^+$ T lymphocyte and antibody responses in peptide-vaccinated patients with NY-ESO-1^+ cancers. *Proc. Natl. Acad. Sci. USA 97:12198.*
17. Soong, R., P. D. Robbins, B. R. Dix, F. Grieu, B. Lim, S. Knowles, K. E. Williams, G. R. Turbett, A. K. House, and B. J. Iacopetta. 1996. Concordance between p53 protein overexpression and gene mutation in a large series of common human carcinomas. *Hum. Pathol. 27:1050.*
18. Hainaut, P., and M. Hollstein. 2000. p53 and human cancer: the first ten thousand mutations. *Adv. Cancer Res. 77:81.*
19. Lane, D. P. 2000. p53 and human cancers. *Br. Med. Bull. 50:582.*
20. Houbiers, J. G., H. W. Nijiman, S. H. van der Burg, J. W. Drifthout, P. Kenemans, C. J. van de Velde, A. Brand, F. Momburg, W. M. Kast, and C. J. Melief. 1993. In vitro induction of human cytotoxic T lymphocyte responses against peptides of mutant and wild-type p53. *Eur. J. Immunol. 23:2072.*
21. Ropke, M. J., J. Hald, P. Guldberg, J. Zeuthen, J. Norgaard, L. Fugger, A. Svejgaard, S. van der Burg, W. Nijiman, M. J. C. Melief, et al. 1996. Spontaneous human squamous cell carcinoma are killed by a human cytotoxic T lymphocyte clone recognizing a wild-type p53-derived peptide. *Proc. Natl. Acad. Sci. USA 93:12704.*
22. Chikamatsu, K., K. Nakano, W. J. Storkus, E. Appella, M. T. Lotze, T. L. Whiteside, and A. B. DeLeo. 1999. Generation of anti-p53 cytotoxic T lymphocytes from human peripheral blood using autologous dendritic cells. *Clin. Cancer Res. 5:1281.*
23. Gnjatic, S., Z. Cai, M. Viguier, S. Chouaib, J. G. Guillet, and J. Choppin. 1998. Accumulation of the p53 protein allows recognition by human CTL of a wild-type p53 epitope presented by breast carcinoma and melanomas. *J. Immunol. 160:328.*
24. Barford, M. A., R. T. Petersen, F. A. Kirkin, P. T. Straten, H. M. Claesson, and J. Zeuthen. 2000. Cytotoxic T-lymphocyte clones, established by stimulation with the HLA-A2 binding $p53_{65–73}$ wild type peptide loaded on dendritic cells in vitro, especially recognize and lyse HLA-A2 tumor cells overexpressing the p53 protein. *Scand. J. Immunol. 51:128.*
25. Mc Ardle, S. E. B., C. R. Rees, A. K. Mulcahy, J. Saba, A. C. Mclntyre, and K. A. Murray. 2000. Induction of human cytotoxic T lymphocytes that preferentially recognize tumor cells bearing a conformational p53 mutant. *Cancer Immunol. Immunother. 49:417.*
26. Eura, M., K. Chikamatsu, F. Katsura, A. Obata, Y. Sabao, M. Takiguchi, Y. Song, E. Appella, L. T. Whiteside, and B. A. DeLeo. 2000. A wild type-sequence p53 peptide presented by HLA-A24 induces cytotoxic T lymphocytes that recognize squamous cell carcinoma of the head and neck. *Clin. Cancer Res. 6:979.*
27. Benettinotti, M. P., M. C. Panelli, E. Ruppe, S. Mocellin, G. Q. Phan, D. E. White, and F. M. Marincola. 2003. clinical and immunological evaluation of patients with metastatic melanoma undergoing immunization with the HLA-Cw*0702-associated epitope MAGE-A12:170–178. *Int. J. Cancer. 105:210.*
28. Hashimoto, M., T. Kinoshita, M. Yamashita, H. Tanaka, T. Imanishi, H. Ihara, Y. Ichikawa, and T. Fukunishi. 1994. gene frequencies and haplotypic associations within the HLA region in 916 unrelated Japanese individuals. *Tissue Antigens 44:166.*
29. Wild, C. P., M. Ridanpaa, S. Anttila, R. Lubin, T. Sousi, K. Husgafvel-Pursiainen, and H. Vainio. 1995. p53 antibodies in the sera of lung cancer patients: comparison with p53 mutation in the tumor tissue. *Int. J. Cancer 64:176.*
30. Miyake, M., M. Adachi, C. Huang, M. Higashiyama, K. Kodama, and T. Taki. 1999. A novel molecular staging protocol for non-small cell lung cancer. *Oncogene 18:2397.*
31. Hernandez-Boussard, T., P. Rodriguez-Tome, R. Montesano, and P. Hainaut. 1999. IARC p53 mutation database: a relational database to compile and analyze p53 mutations in human tumors and cell lines, International Agency for Research on Cancer. *Hum. Mutat. 14:1.*
32. Schlichtholz, B., Y. Legros, D. Gillet, C. Gaillard, M. Marty, D. Lane, F. Calvo, and T. Soussi. 1992. The immune response to p53 in breast cancer patients is directed against immunodominant epitopes unrelated to the mutational hot spot. *Cancer Res. 52:6380.*