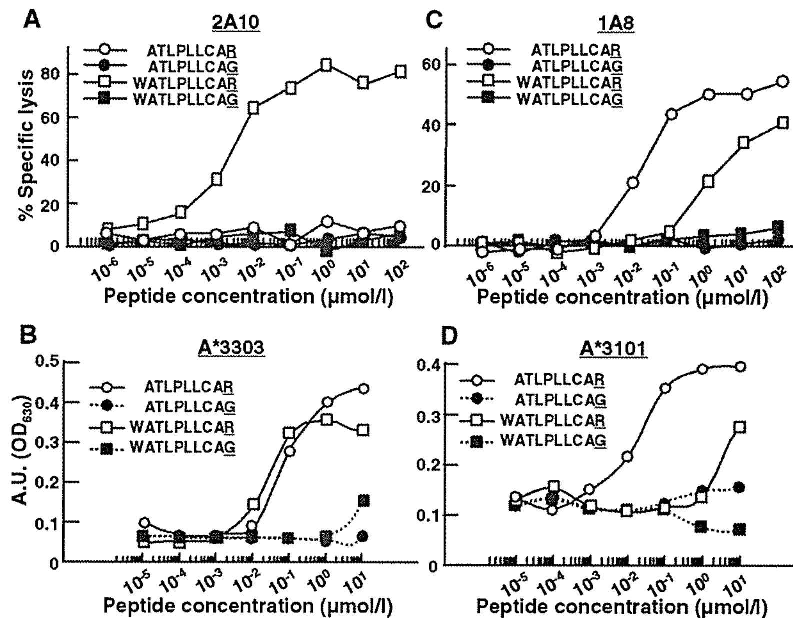

Fig 4. Epitope reconstitution and peptide–human leucocyte antigen (HLA)-binding assays. Donor B lymphocyte cell lines (B-LCLs) were labelled with ^{51}Cr and distributed in 96-well round-bottomed plates, pulsed with serial dilutions of the indicated peptides for 30 min at room temperature, and then used as targets for cytotoxic T lymphocyte clones 2A10 (A) and 1A8 (C) in a standard ^{51}Cr release assay (E:T ratio 10:1). All experiments were performed at least in duplicate. Peptide–HLA-binding assays were carried out as previously described (Sylvester-Hvid *et al*, 2002) with some modifications (see *Materials and methods*) (B and D). Purified HLA and β2m molecules were folded in folding buffer containing the serially diluted peptides indicated for 48–72 h. Amounts of properly folded HLA-A*3303 (B) and -A*3101 (D) molecules were assessed by enzyme-linked immunosorbent assay using plate-coated, conformation-dependent W6/32 and horseradish peroxidase-tagged anti-β2m monoclonal antibodies. Folding efficiency is expressed in arbitrary units (AU) corresponding to OD_{630}.

except for normal monocytes and AML FAB M4 and M5 subtypes; four of four M4 and three of three M5 primary leukaemic cells were strongly positive, but B-ALL cells from one patient were only weakly stained (Fig 5 and data not shown). Contrary to our expectations, dermal fibroblasts, normal renal epithelial cells, renal carcinoma cell lines and keratinocytes were diffusely stained (Fig 5 and data not shown), suggesting that not only CTSH variants 1 encoding the isoform a, but also variant 2 are expressed in most kinds of cells.

No apparent correlation of preferential CTL cytotoxicity against haematopoietic cells in proportion to CTSH protein expression

Cytokine-pretreated dermal and bone marrow fibroblasts, which expressed relatively low levels of CTSH protein, were not lysed by the CTLs (Figs 1A,B and 5). We then investigated whether or not dermal fibroblasts from UPN 028, unrelated renal normal epithelial cells and carcinoma cell lines (possessing HLA-A*3101 and $CTSH^R$ allele confirmed by genotyping) expressing high levels CTSH protein (Fig 5) were susceptible to lysis. Surprisingly, these non-haematopoietic cell lines were not lysed by the CTLs even after cytokine pretreatment (Fig 6A). These cells were, however, not resistant to cytolysis as they became susceptible when cognate $CTSH^R$/A31peptide was pulsed exogenously (Fig 6B).

Activated haematopoietic cells are known to express immunoproteasomes containing LMP2, LMP7 and/or PA28α and to alter proteasome cleavage specificity (Kloetzel, 2001), which thus may account for preferential generation and presentation of the CTSH mHags. However, except for keratinocytes, cytokine-pretreated non-haematopoietic cells expressed these immunoproteasome components at levels comparable with those of haematopoietic cells tested (Fig 6C).

Discussion

Minor histocompatibility antigens are known to play an important role as alloantigens inducing GVHD and/or GVL effects after HLA-identical allogeneic HSCT. Thus, it is still necessary to identify novel mHags and to study the potential clinical significance and mechanisms involved in the generation of antigenicity. This study demonstrated that splice variant 1 of CTSH mRNA transcribed from the *CTSH* gene, located on chromosome 15q24–q25, encodes two novel mHag epitopes restricted by two different HLA-A alleles but belonging to the same HLA-A3 supertype. Nonameric peptide ATLPLLCAR ($CTSH^R$/A31) was presented by HLA-A*3101, while decameric peptide with one N-terminal aa extension,

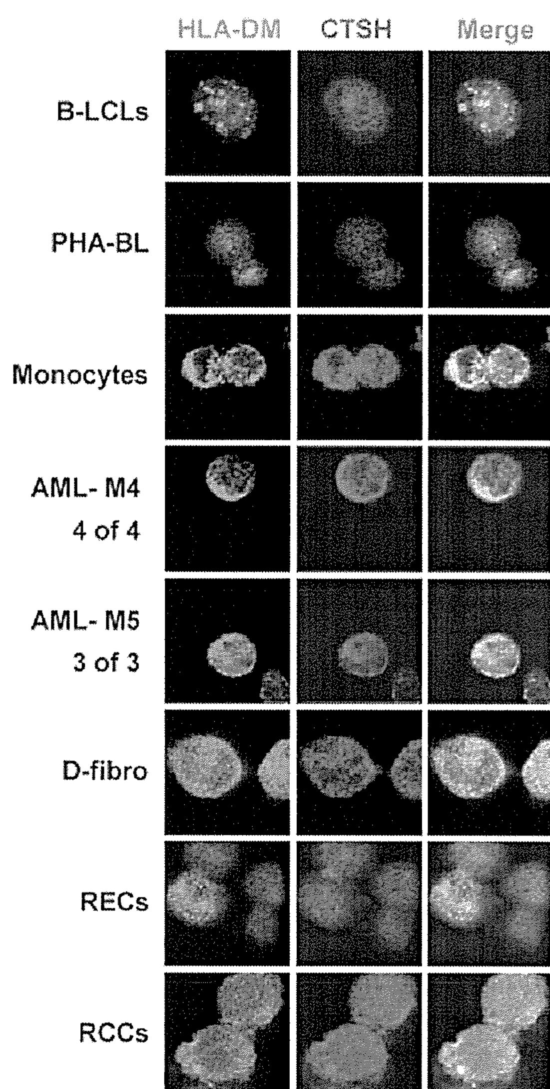

Fig 5. Confocal microscopic analysis of cathepsin H (CTSH) protein expression and its intracellular localisation in various normal tissues. Cells were fixed and permeabilised and then incubated for 60 min with goat anti-CTSH monoclonal and mouse anti-human leucocyte antigen (HLA)-DM antibodies. After washing, bound antibodies were detected with fluorescence-labelled second antibodies. Finally, stained cells were washed with phosphate-buffered saline, cytocentrifuged and analysed by laser scanning confocal microscopy. Representative laser-scanning confocal micrographs demonstrating the distribution of CTSH (red), HLA-DM (green) and co-localisation of CTSH with HLA-DM (yellow) are shown. The numbers of samples that were positive for CTSH among acute myeloid leukaemia (AML) samples tested are indicated under the AML French–American–British subtypes. PHA-BL, phytohaemagglutinin-blasts; D-fibro, dermal fibroblasts; RECs, normal renal epithelial cells; RCC, renal carcinoma cell lines.

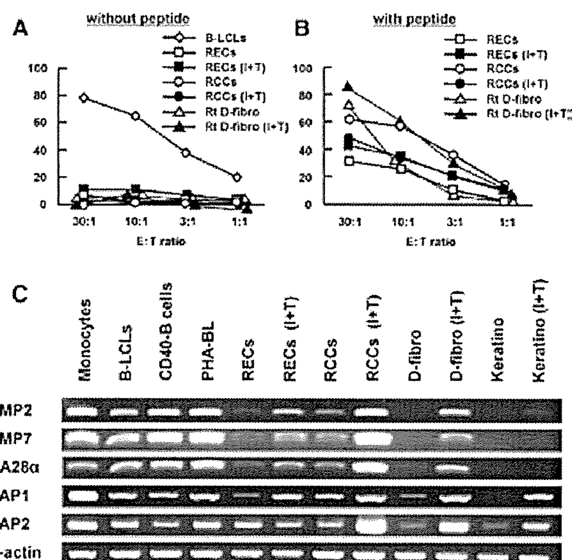

Fig 6. Cytotoxic activity of cytotoxic T lymphocyte (CTL) clone 1A8 against non-haematopoietic cells and effects of cytokine treatment on antigen processing and presenting molecules. (A) Recipient unique patient number 028 B lymphocyte cell lines (B-LCLs) and dermal fibroblasts (D-fibro), unrelated normal renal epithelial cells (RECs) and renal carcinoma cell (RCC) lines obtained from an human leucocyte antigen (HLA)-A*3101-positive and cathepsin H $(CTSH)^R$-positive RCC patient were tested for cytolysis by 1A8 in a standard ^{51}Cr release assay. CTSH expression of the D-fibro, RECs and RCC was confirmed as in (Fig 5. Target cells pretreated with interferon (IFN)-γ (500 U/ml) and tumour necrosis factor (TNF)-α (10 ng/ml) for 48 h before ^{51}Cr labelling are indicated as (I + T). This treatment resulted in 3·8-fold increase of HLA-class I expression in dermal fibroblasts (data not shown). (B) As a control experiment, the same target cells were also incubated with 10 μmol/l $CTSH^R$/A31 peptide for 30 min before adding 1A8 CTL. (C) Expression levels of genes encoding immunoproteasome-associated molecules LMP2, LMP7 and PA28α and those encoding peptide transporters TAP1 and TAP2 in target cells tested in the current studies were analysed by semiquantitative reverse transcription polymerase chain reaction and prepared as previously described (Ito *et al*, 2006). Non-haematopoietic cells were examined with or without IFN-γ and TNF-α pretreatment (I + T) for 48 h.

WATLPLLCAR ($CTSH^R$/A33), was presented by HLA-A*3303. The non-synonymous coding SNP located on *CTSH* exon 1 composed the C-terminal anchor motif of both epitopes. The epitope reconstitution assay and peptide–HLA-binding assay indicated that peptides with Gly instead of Arg at the C terminus, which is the sole anchor position for both HLA-A*3101 and -A*3303 (Falk *et al*, 1994), were unable to bind to these HLA molecules at all, suggesting that lack of HLA-binding peptide in donors homozygous for $CTSH^G$ may be attributable to allo-responses against recipients carrying $CTSH^R$ allele following allo-HSCT.

To date, various mechanisms involved in mHag generation have been reported as follows: peptide binding to MHC observed in HA-1/A2 (den Haan *et al*, 1998) and HA-2 (Pierce *et al*, 2001); proteosomal cleavage in HA-3 (Spierings *et al*, 2003); peptide transport in HA-8 (Brickner *et al*, 2001); recognition of MHC-peptide complex by cognate T cells in SMCY/B7 (Wang *et al*, 1995), DFFRY/A1 (Pierce *et al*, 1999), HB-1 (Dolstra *et al*, 2002) and HA-1/B60 (Mommaas *et al*, 2002); and differential protein expression in UGT2B17 (Mu-

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rata et al, 2003), PANE1 (Brickner et al, 2006) and LRH-1 (Rijke et al, 2005). Among those, our mHags were considered to be generated by differential peptide binding to MHC, and the substitution of Gly for Arg at the C-terminus, resulting in the complete loss of binding, was sufficient to account for the difference in recognition of CTSH/A31^{+} or CTSH/A33^{+} cells from their negative counterparts. The differential binding mechanism is similar to the cases of HA-1/A2 and HA-2, but seems to be more significant because there are only 12- to 15-fold differences in peptide binding in HA-1/A2 and HA-2, respectively, although binding affinity to HLA-A2 was evaluated by competition-based assays (den Haan et al, 1998).

Initially, to identify the gene encoding the mHag recognised by CTL 2A10, two-point linkage analysis (Akatsuka et al, 2003b; Rijke et al, 2005) was conducted. Coincidentally, not only 2A10 but also 1A8 CTL clones showed an identical lytic pattern, suggesting that the polymorphic genes controlling the expression of the two mHags were located on a narrow chromosomal region or, alternatively, that a single gene encoded both mHags, as seen with the BCL2A1 mHags (Akatsuka et al, 2003a,b). However, the mapped region was found to contain many genes whose characteristics had not yet been fully elucidated, so we did not further pursue candidate gene(s) by this *in silico* approach. Rijke et al (2005) successfully applied quantitative PCR to identify the *P2X5* gene by comparing the expression pattern of candidate genes in three cell types (B-LCL, monocytes and fibroblasts) with the lytic pattern of these cells by their CTL. Considering the fact that we were indeed very close to the target gene, *CTSH*, by linkage analysis, extensive quantitative PCR analyses tailored for the candidate genes may have identified of *CTSH* as the gene encoding mHag recognised by our CTL clones.

The primary AML cells of UPN 028 were recognised by the CTL clones. Thus we speculate that CTSH may function as a target of a GVL response. Indeed, the two patients with high-risk AML from whom CTSH-specific CTLs were isolated continue to be disease-free after more than two and a half years. However, one concern is that CTSH is expressed not only in haematopoietic cells (Greiner et al, 2003) but also some epithelial cells including type II pneumocytes (Brasch et al, 2002), suggesting that it may also be a target for GVHD and targeting this molecule with CTLs might carry the risk of toxicity. For example, mild bronchiolitis obliterans organising pneumonia observed in patient UPN 027 might be associated with chronic GVHD (Afessa et al, 2001), and it is conceivable that immune responses to CTSH expressed in type II pneumocytes could be a factor. Unfortunately, we could not exclude this possibility because bronchoalveolar lavage, which might contain T cells that could be tested with CTSH^{R} tetramers, was not performed.

It was unexpected, but quite encouraging, that our CTSH^{R}-specific CTLs demonstrated lytic activity against haematopoietic cells including leukaemic cells but not fibroblasts or renal normal epithelial and carcinoma cells, although the latter non-haematopoietic cells did express CTSH^{R} proteins. It has been shown that the splicing variant 1 of *CTSH* encoding the mHag epitopes results in a longer protein (isoform a) which is likely to be localised to lysosomes, and variant 2 produces a shorter protein (isoform b) which is more likely to be a secreted protein (Waghray et al, 2002). Thus, to find differences between CTL-sensitive and -resistant target cells, we studied the expression of CTSH mRNA, intracellular distribution of CTSH protein, and also the expression of genes involved in antigen processing and presentation. However, no clear evidence to explain the differential recognition of target cells with high CTSH expression by the CTLs was obtained. In line with this, it is of note that an HLA-B8-restricted line specific for the mHag encoded by *UTY* was reported to lyse only haematopoietic cells, irrespective of the relatively ubiquitous expression of the gene in male cells (Warren et al, 2000). Because our mHags were derived from the leader sequence of CTSH isoform a precursor, more active translation in haematopoietic cells which accompany the production of excised leader peptide, compared with stable mature CTSH detectable by the antibody used in this study, may account for the differential susceptibility to the CTLs. In any event, determination of mechanisms involved in differential antigen expression between haematopoietic and non-haematopoietic cells is a high priority in future studies.

Finally, the restriction molecules of our CTLs, HLA-A*3303 and -A*3101, are known to belong to the HLA-A3 supertype (Falk et al, 1994; Sette & Sidney, 1999), which is found in 46% of Japanese and 38% of Caucasians (Sette & Sidney, 1999). Accordingly, the A3 supertype now includes A*0301, A*1101, as well as A*6801. HLA alleles in the A3 supertype use a similar anchor motif of Arg or Lys at their C-termini. Thus, it may be worthwhile to examine whether nonameric CTSH^{R}/A31 or decameric CTSH^{R}/A33 peptides would also bind to HLA molecules belonging to A*0301 and A*6801, because these HLA alleles are common in Caucasians and may create an opportunity to evaluate the significance of CTSH^{R}-encoded mHags. The results obtained in this study suggest that CTSH may be used as a target for GVL responses after allogeneic HSCT if potential GVHD induction can be manipulated by suitable techniques, such as suicide gene introduction into CTLs. Animal models to determine the susceptibility of CTSH-positive leukaemic progenitor cells and other tissue cells to CTSH^{R}-specific CTLs *in vivo* are now being developed using immune-deficient mouse systems.

Acknowledgements

The authors thank Drs S. Riddell, P. Martin (Fred Hutchinson Cancer Research Center) and W. Ho for critically reading the manuscript, and Yoshinori Ito, Ayako Demachi-Okamura, Michiyo Nakayama, Yasue Matsudaira, Keiko Nishida, and Hiromi Tamaki for their expert technical assistance. This study was supported in part by grants-in-aid for Scientific Research (C) (no. 17591025) and Scientific Research on Priority Areas (B01) (no. 17016089), from the Ministry of Education,

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Culture, Science, Sports, and Technology, Japan; Research on Human Genome, Tissue Engineering Food Biotechnology (to Y.A.) and the Second and Third Team Comprehensive 10-year Strategy for Cancer Control (no. 30), from the Ministry of Health, Labour, and Welfare, Japan; and grants from Daiko Foundation (to Y.A.) and Japan Leukaemia Research Fund (to Y.A.).

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Cytotoxic T Cell Responses to Human Telomerase Reverse Transcriptase in Patients With Hepatocellular Carcinoma

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Human telomerase reverse transcriptase, hTERT, has been identified as the catalytic enzyme required for telomere elongation. hTERT is expressed in most tumor cells but seldom expressed in most human adult cells. It has been reported that 80% to 90% of hepatocellular carcinomas (HCCs) express hTERT, making the enzyme a potential target in immunotherapy for HCC. In the current study, we identified hTERT-derived, HLA-A*2402–restricted cytotoxic T cell (CTL) epitopes and analyzed hTERT-specific CTL responses in patients with HCC. Peptides containing the epitopes showed high affinity to bind HLA-A*2402 in a major histocompatibility complex binding assay and were able to induce hTERT-specific CTLs in both hTERT cDNA-immunized HLA-A*2402/K^b transgenic mice and patients with HCC. The CTLs were able to kill hepatoma cell lines depending on hTERT expression levels in an HLA-A*2402–restricted manner and induced irrespective of hepatitis viral infection. The number of single hTERT epitope-specific T cells detected by ELISPOT assay was 10 to 100 specific cells per 3 × 10^5 PBMCs, and positive T cell responses were observed in 6.9% to 12.5% of HCC patients. hTERT-specific T cell responses were observed even in the patients with early stages of HCC. The frequency of hTERT/tetramer$^+$CD8$^+$ T cells in the tumor tissue of patients with HCC was quite high, and they were functional. In conclusion, these results suggest that hTERT is an attractive target for T-cell–based immunotherapy for HCC, and the identified hTERT epitopes may be valuable both for immunotherapy and for analyzing host immune responses to HCC. (HEPATOLOGY 2006;43:1284-1294.)

Hepatocellular carcinoma (HCC) is the most frequent primary malignancy of the liver and has gained much clinical interest because of its increasing incidence.[1-3] Although current advances in therapeutic modalities have improved the prognosis of HCC patients,[4-6] the survival rate is still not satisfactory. One of the reasons for the poor prognosis is the high rate of recurrence after treatment. To protect against recurrence, tumor antigen-specific immunotherapy is an attractive strategy. Although many tumor-specific antigens have been identified in various cancers, the number of HCC-specific antigens known is still limited.

Human telomerase reverse transcriptase, hTERT, has been identified as the catalytic enzyme required for telomere elongation.[7-10] Recently, several results regarding hTERT-specific cytotoxic T cell (CTL) responses were reported for humans and mice.[11-20] These reports revealed that hTERT-specific CTLs induced by stimulation with peptides or DNA-based immunization kill cancer cell lines that have high levels of hTERT, suggesting that hTERT-reactive T cell clones are not deleted from the human T cell repertoire and that hTERT may be a useful tumor-specific antigen as a target for T-cell–based immunotherapy for cancers. However, the existence of hTERT-specific CTLs and the relationship between immunological

Abbreviations: HCC, hepatocellular carcinoma; hTERT, human telomerase reverse transcriptase; CTL, cytotoxic T cell; HIV, human immunodeficiency virus; PBMC, peripheral blood mononuclear cell; AFP, alpha-fetoprotein; CMV, cytomegalovirus; FCS, fetal calf serum; TIL, tumor infiltrating lymphocyte; PCR, polymerase chain reaction; TRAP, telomerase repeat amplification protocol; IFN-γ, interferon gamma.

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Received August 8, 2005; accepted March 20, 2006.

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Published online in Wiley InterScience (www.interscience.wiley.com).
DOI 10.1002/hep.21203
Potential conflict of interest: Nothing to report.

responses and clinical factors have not been well characterized in patients with HCC.

In the current study, we first attempted to identify HLA-A*2402–restricted T cell epitopes derived from hTERT and then analyzed hTERT-specific immunological responses in HCC patients.

Patients and Methods

Patient Population. The study examined 72 HLA-A24–positive patients with HCC who were admitted to Kanazawa University Hospital between January 2002 and December 2004, consisting of 48 men and 24 women ranging from 46 to 81 years of age with a mean age of 67 ± 9 years. HCCs were detected by imaging modalities such as dynamic computed tomography (CT) scan, magnetic resonance imaging, and abdominal arteriography. The diagnosis of HCC was histologically confirmed by taking ultrasound-guided needle biopsy specimens in 29 cases, surgical resection in four cases, and autopsy in four cases. For the remaining 35 patients, the diagnosis was based on typical hypervascular tumor staining on angiography in addition to typical findings, which showed hyperattenuated areas in the early phase and hypoattenuation in the late phase on dynamic CT.[21] All subjects were negative for antibodies to human immunodeficiency virus (HIV) and gave written informed consent to participate in this study in accordance with the Helsinki declaration. Eleven healthy blood donors with HLA-A24, who did not have a history of cancer and were negative for hepatitis B surface antigen and anti-HCV antibody, served as controls.

Laboratory and Virologic Testing. Blood samples were tested for hepatitis B surface antigen and HCV antibody by commercial immunoassays (Fuji Rebio, Tokyo, Japan). HLA-based typing of peripheral blood mononuclear cells (PBMCs) from patients and normal donors was performed using complement-dependent microcytotoxicity with HLA typing trays purchased from One Lambda.

The serum alpha-fetoprotein (AFP) level was measured by enzyme immunoassay (AxSYM AFP, Abbott Japan, Tokyo, Japan), and the pathological grading of tumor cell differentiation was assessed according to the general rules for the clinical and pathological study of primary liver cancer.[22] The severity of liver disease (stage of fibrosis) was evaluated according to the criteria of Desmet et al.,[23] using biopsy specimens of liver tissue, where F4 was defined as cirrhosis.

Synthetic Peptides. To identify potential HLA-A24–binding peptides within hTERT, the sequence was reviewed using a computer-based program, which was employed by accessing the World Wide Web site BioInformatics and Molecular Analysis Section for HLA peptide binding predictions (available from http://bimas.cit.nih.gov). The HLA-A24–restricted epitopes derived from HIV envelope protein,[24] cytomegalovirus (CMV) pp65,[25] and HCV NS3 were used as control peptides to test for T cell responses, and the HLA-A2–restricted epitope derived from AFP[26] was used as a control peptide for HLA-A24 stabilization assay as previously described. Peptides were synthesized at Mimotope (Melbourne, Australia) and Sumitomo Pharmaceuticals (Osaka, Japan). They were identified using mass spectrometry, and their purities were determined to be greater than 80% by analytical high-pressure liquid chromatography.

Cell Lines. Three human hepatoma cell lines, HepG2, HuH6, and HuH7, were cultured in Dulbecco's minimum essential medium (Gibco, Grand Island, NY) with 10% fetal calf serum (FCS) (Gibco).

T2-A24 cells, which were T2 cells transfected with HLA-A*2402,[25] were cultured in RPMI 1640 medium containing 10% FCS and 800 μg/mL G418 (GibcoBRL, Grand Island, NY). The HLA-A*2402 gene-transfected C1R cell line (C1R-A24)[27] was cultured in RPMI 1640 medium containing 10% FCS and 500 μg/ml of hygromycin B(Sigma, St Louis, MO), and K562 was cultured in RPMI 1640 medium containing 10% FCS. All media contained 100 U/mL penicillin and 100 μg/mL streptomycin (GibcoBRL, Grand Island, NY).

Plasmid Construction. The plasmid which contains hTERT cDNA was subcloned as previously described.[28] In brief, the EcoRI-SalI fragment containing the hTERT cDNA was subcloned from pCI-Neo-hTERT, which was provided by Dr. Seishi Murakami (Cancer Research Institute, Kanazawa University). The fragment was subcloned into the EcoRI-SalI sites of the plasmid pNKZ-FLAG (pNKZ-FLAG-hTERT).

Injection of hTERT cDNA Into HLA-A*2402/K^b Transgenic Mice. Transgenic mice expressing the α1 and α2 domains from the HLA-A*2402 molecule and the α3 domain from the murine H-$2K^b$ molecule,[29] kindly provided by Sumitomo Pharmaceuticals (Osaka, Japan), were bred in a specific-pathogen–free environment at the animal facility in Kanazawa University. For immunization with the hTERT cDNA, mice were injected with 50 μL cardiotoxin (Latoxan, Rosans, France) (10 μmol/L) per leg into the tibialis anterior muscles on both sides. Five days after injection of the cardiotoxin, the vector pNKZ-FLAG-hTERT containing the hTERT cDNA was injected into the same part of the muscle. Mice immunized with the plasmid pNKZ-FLAG were also used as negative controls. Splenocytes harvested on day 7 after the

Table 1. Characteristics of the Patients Studied

Clinical Diagnosis	No. of Patients	Sex M/F	Age (yr) Mean ± SD	ALT (IU/L) Mean ± SD	AFP (ng/mL) Mean ± SD	Etiology (B/C/Others)	Child-Pugh (A/B/C)	Diff. degree* (Wel/Mod/Por/ND)	Tumor size** (Large/Small)	Tumor multiplicity (Multiple/Solitary)	Vascular Invasion (+/−)	TNM Stage (I/II/IIIA/IIIB/IIIC/IV)
HCC patients	72	48/24	67 ± 9	66 ± 36	1722 ± 7029	9/59/4	43/25/4	15/21/1/35	44/28	39/33	15/57	30/26/9/1/2/4
Normal donors	11	8/3	35 ± 2	ND	ND	ND	ND	ND	ND	ND	ND	ND

*Histological degree of HCC; wel: well differentiated, mod: moderately differentiated, por: poorly differentiated, ND: not determined.
**Tumor size was divided into either "small" (≤2 cm) or "large" (> 2 cm).

injection of cDNA were tested directly *ex vivo* for IFN-γ production using an ELISPOT assay.

Preparation of PBMCs and Tumor-Infiltrating Lymphocytes. PBMCs were isolated as previously described.[30,31] Fresh PBMCs were used for the CTL assay, and the remaining PBMCs were resuspended in RPMI 1640 medium containing 80% FCS and 10% dimethyl sulfoxide (Sigma, St. Louis, MO) and cryopreserved until used. Tumor-infiltrating lymphocytes (TILs) were isolated by mechanical homogenization of tumors, which were resected by surgical treatment and cryopreserved as described until used.

Major Histocompatibility Complex Binding Assay. Peptide binding assays were performed as previously described.[31,32] The data were expressed as % mean fluorescence intensity (MFI) increase, which was calculated as follows: Percent MFI increase = (MFI with the given peptide − MFI without peptide)/(MFI without peptide) × 100.

ELISPOT Assay. ELISPOT assays were performed as previously described[31] with the following modifications. Three hundred thousand unfractionated PBMCs or 100,000 TILs with 10,000 T2-A24 cells were added in duplicate cultures of RPMI 1640 medium containing 5% FCS together with the peptides at 10 μg/mL. For the mouse assay, 2×10^5 spleen cells were used for each well. The number of specific spots was determined by subtracting the number of spots in the absence of antigen from that in the presence of antigen. Responses were considered positive for the human ELISPOT assay if more than 10 specific spots were detected and if the number of spots in the presence of antigen was at least twofold greater than that in the absence of antigen.

Cytotoxicity Assay. hTERT-derived peptide-specific T cells were expanded from PBMCs in 96-well round-bottomed plates (NUNC, Naperville, IL) as previously described.[30] Briefly, 400,000 cells per well were stimulated with 10 μg/mL synthetic peptide, 10 ng/mL rIL-7, and 100 pg/mL rIL-12 (Sigma) in RPMI 1640 medium supplemented with 10% heat-inactivated human AB serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cultures were re-stimulated with 10 μg/mL peptide, 20 U/mL of rIL-2 (Sigma) and 1×10^5 mytomicin C-treated autologous PBMCs on days 7 and 14. On days 3, 10, and 17, 100 μL RPMI with 10% human AB serum and 10 U/mL rIL-2 (final concentration) were added to each well. Cytotoxicity assays were performed as previously described.[31]

Tetramer Staining and Flow Cytometry. Peptide $hTERT_{461}$-specific tetramer was purchased from Medical Biological Laboratories Co., Ltd (Nagoya, Japan). Tetramer staining was performed as previously described.[33] In brief, PBMCs and TILs were stained with CD4-FITC, CD14-FITC, CD19-FITC, CD8-PerCP (BD PharMingen, San Diego, CA), and tetramer-PE (10 μL) for 30 minutes at room temperature. Cells were washed, fixed with 0.5% paraformaldehyde/phosphate-buffered saline, and analyzed on a FACSCalibur flow cytometer. Data analysis was undertaken with CELLQuest software (Becton Dickinson, San Jose, CA).

Telomerase Assay. Telomerase activity was measured by two methods according to the manufacturer's directions. First, a polymerase chain reaction (PCR)-based telomerase repeat amplification protocol (TRAP) assay was carried out with a TRAPEZE ELISA telomerase detection kit (Intergen Co. Ltd., Auckland, New Zealand). The products of the PCR were fractionated by electrophoresis on a 10% polyacrylamide gel and then visualized by staining with SYBR-Green I (Molecular Probes, Eugene OR). Second, a TRAP enzyme-linked immunosorbent assay (ELISA) was used to quantitatively measure telomerase activity with a TRAPEZE ELISA telomerase detection kit (Intergen Co. Ltd.). Cell extracts were prepared from HepG2, HuH6, and HuH7 cells and used at 0.01 μg per assay. Telomerase activity was also measured in the tumor of 10 patients with HCC who received surgical treatment. Cell extracts were prepared from resected tumors and used at 0.1 μg per assay.

Statistical Analysis. Fisher's exact test (2-sided P-value) and the unpaired Student's t test were used to analyze the effect of variables on immune responses in HCC patients.

Results

Patient Profiles. The clinical profiles of the patients are shown in Table 1. The tumors of 37 patients were

Table 2. Peptides

Peptide	Source	Start Position	Amino Acid Sequence	HLA Restriction	Score*
$hTERT_{1088}$	hTERT	1088	TYVPLLGSL	HLA-A24	432
$hTERT_{845}$	hTERT	845	CYGDMENKL	HLA-A24	317
$hTERT_{167}$	hTERT	167	AYQVCGPPL	HLA-A24	300
$hTERT_{461}$	hTERT	461	VYGFVRACL	HLA-A24	280
$hTERT_{324}$	hTERT	324	VYAETKHFL	HLA-A24	240
$hTERT_{1009}$	hTERT	1009	AYRFHACVL	HLA-A24	200
$hTERT_{385}$	hTERT	385	RYWQMRPLF	HLA-A24	200
$hTERT_{637}$	hTERT	637	DYVVGARTF	HLA-A24	150
$hTERT_{622}$	hTERT	622	RFIPKPDGL	HLA-A24	72
$hTERT_{869}$	hTERT	869	DFLLVTPHL	HLA-A24	42
HIV env_{584}	HIV envelope	584	RYLRDQQLL	HLA-A24	720
CMV $pp65_{328}$	CMV pp65	328	QYDPVAALF	HLA-A24	168
HCV $NS3_{1031}$	HCV NS3	1031	AYSQQTRGL	HLA-A24	200
AFP_{137}	AFP	137	PLFQVPEPV	HLA-A2	3

*Estimated half-time of dissociation from the HLA-A24 or -A2 allele (min).

histologically classified as 15 well, 21 moderately, and 1 poorly differentiated HCC. Other patients were diagnosed with HCC based on typical CT findings and an elevation of AFP. The tumors were categorized as "large" (>2 cm) in 44 cases and "small" (≤2 cm) in 28 cases, and as "multiple" (≥2 nodules) in 39 cases and "solitary" (single nodule) in 33 cases. Vascular invasion of the HCC was observed in 15 cases. According to the TNM staging of the Union Internationale Contre Le Cancer (UICC) classification system (6th version),[34] 30, 26, 9, 1, 2, and 4 patients were classified as having stages I, II, IIIA, IIIB, IIIC, and IV disease, respectively.

Selection of Potential HLA-A24–Binding Peptides Within hTERT. To identify potential HLA-A24–binding peptides, the amino acid sequences of hTERT were analyzed using a computer program designed to predict HLA-binding peptides based on the estimation of the half-time dissociation of the HLA–peptide complex. Ten peptides were selected according to the half-time dissociation scores (Table 2). Two of the 10 peptides have been reported to contain HLA-A*2402–restricted epitopes (peptides $hTERT_{461}$ and $hTRERT_{324}$).[35] Next, MHC stabilization assays were performed to test the HLA-A*2402–binding capacity of these peptides using T2-A24 cells. Most peptides increased HLA-A24 expression, indicating that they bound and stabilized the HLA complex on the cell surface (Fig. 1). Peptide $CMVpp65_{328}$, which is identified as a strong binder of the HLA-A*2402 molecule,[25] also increased HLA-A24 expression. Percent MFI increase of the tested peptides except for peptides $hTERT_{1009}$, $hTERT_{385}$, and $hTERT_{622}$ was greater than that of peptide AFP_{137}, which is HLA-A2 restricted.[26]

Immunogenicity of hTERT Peptides in HLA-A*2402/K^b Transgenic Mice. To determine whether these HLA-A24–binding peptides include HLA-A*2402–restricted T cell epitopes, HLA-A*2402/K^b transgenic mice were immunized with hTERT cDNA, and the spleen cell responses were evaluated by interferon gamma (IFN-γ) ELISPOT. Six of 10 hTERT-derived peptides were recognized by the spleen cells of at least one of the primed mice (Fig. 2). Peptides $hTERT_{1009}$, $hTERT_{385}$, $hTERT_{622}$, and $hTERT_{869}$ were not recognized by any mice. These results show that peptides $hTERT_{1088}$, $hTERT_{845}$, $hTERT_{167}$, $hTERT_{461}$, $hTERT_{324}$, and $hTERT_{637}$ may be immunogenic and contain the epitopes restricted by HLA-A*2402.

T Cell Responses to hTERT-Derived Peptides Assessed by IFN-γ ELISPOT Analysis in HCC Patients. To determine whether these HLA-A24–binding peptides could be recognized by the T cells of patients with HCC, PBMC responses were evaluated by IFN-γ ELISPOT. Six of 10 hTERT-derived peptides were recognized by PBMCs of at least one patient, and 29 of 72 patients (40.3%) responded to at least one of the analyzed hTERT-derived peptides. An overview of all responses is shown in Fig. 3A. Single hTERT epitope-specific IFN-γ–producing cells were detected in 6 (8.3%), 6 (8.3%), 9 (12.5%), 5 (6.9%), 9 (12.5%), and 9 (12.5%) of 72 patients in response to the stimulation with peptides $hTERT_{1088}$, $hTERT_{845}$, $hTERT_{167}$, $hTERT_{461}$, $hTERT_{324}$, and $hTERT_{637}$, respectively. Peptides $hTERT_{1009}$, $hTERT_{385}$, $hTERT_{622}$, and $hTERT_{869}$ were not recognized by any patient.

The peptides recognized by PBMCs of the patients with HCC were comparable to those recognized by spleen cells of hTERT cDNA-immunized HLA-A*2402/K^b transgenic mice. These peptides also displayed a relatively high affinity for the HLA-A*2402 molecule compared with the negative control peptide (Fig. 1). The strength of the hTERT-specific T cell responses assessed by the frequencies of IFN-γ–producing cells in the PBMC population is between 10 and 100 specific cells per 3×10^5 PBMCs. Peptide $CMVpp65_{328}$, which includes an

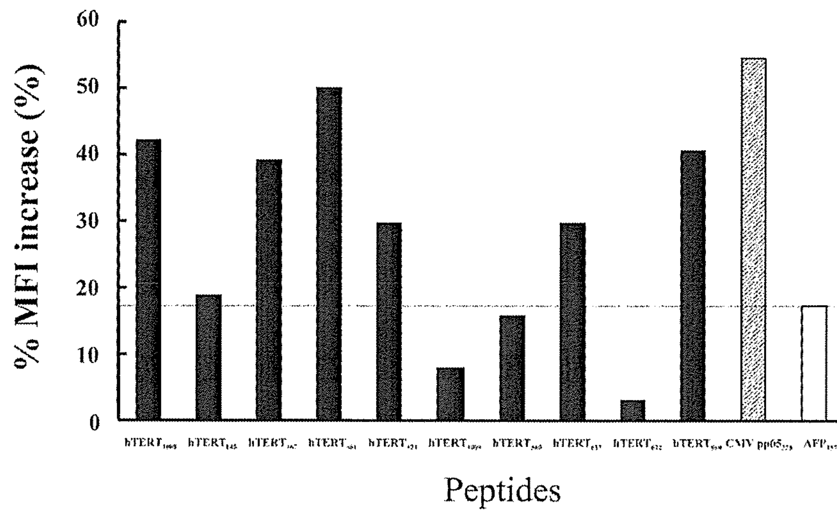


Fig. 1. MHC binding affinity. TAP-deficient T2-A24 cells were cultured for 16 hours at 26°C to enhance the expression of peptide-receptive cell surface molecules. They were incubated with individual peptides at 10 μg/mL at 37°C for 2 hours, washed, and stained with anti-HLA-A24 monoclonal antibody, anti-mouse immunoglobulin-conjugated FITC, and 1 μg propidium iodide per milliliter. The data are expressed as the percent mean fluorescence intensity (MFI) increase for live, propidium iodide-negative cells. Peptide CMVpp65$_{328}$, a previously identified CMV pp65-derived peptide known to be a strong binder to HLA-A24, was used as a positive control. Peptide AFP$_{137}$, a previously identified AFP-derived peptide known to be HLA-A2 restricted, was used as a negative control. The experiment was performed three times, and a representative result is shown. MHC, major histocompatibility complex; HLA, human leukocyte antigen; FITC, fluorescein isothiocyanate; CMV, cytomegalovirus.

epitope derived from the CMV pp65 protein, and HCVNS3$_{1031}$, which includes an epitope derived from the HCV NS3 protein, were also recognized by PBMCs of 31 of 72 (40%) and 12 of 51 (24%) patients with HCC, respectively. Conversely, no patients showed positive T cell responses against peptide HIVenv$_{584}$ derived from the HIV envelope protein, suggesting that these T cell responses were antigen-specific.

In contrast to the results for HCC patients, the ELISPOT assays for the healthy donors did not show more than 10 specific spots for all hTERT-derived peptides (Fig. 3B). The numbers of specific spots (mean ± SD) in the healthy donors were 1.4 ± 1.7, 0.6 ± 0.8, 0.8 ± 1.1, 0.7 ± 1.2, 0.5 ± 0.7, 0.6 ± 1.2, 2.0 ± 2.6, 1.7 ± 2.6, 1.6 ± 3.4, and 1.9 ± 2.9 for hTERT$_{1088}$, hTERT$_{845}$, hTERT$_{167}$, hTERT$_{461}$, hTERT$_{324}$,

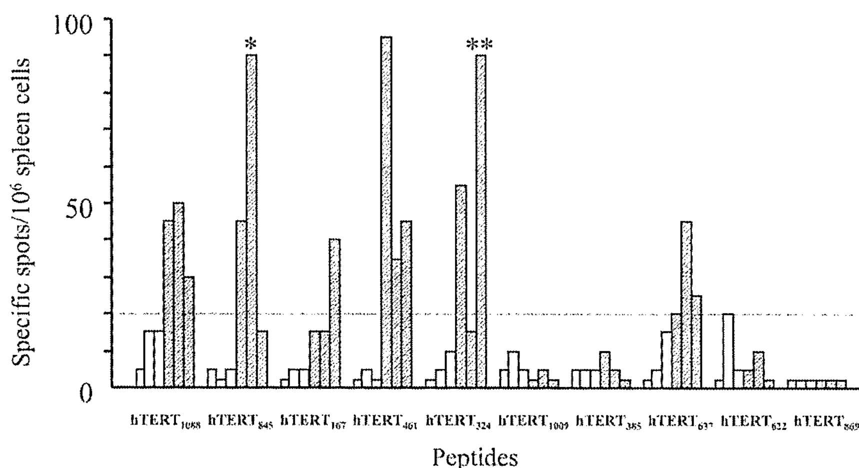


Fig. 2. Direct *ex vivo* analysis (IFN-γ ELISPOT assay) of spleen cell responses to hTERT-derived peptides in hTERT cDNA (hatched bars) or β-gal cDNA (open bars)-immunized HLA-A*2402/K^b transgenic mice. The immunization was performed in three mice for each cDNA. A positive T cell response was defined as more than 20 specific spots/1 × 10^6 spleen cells, which was the maximum response in β-gal cDNA-immunized mice. The peptide sequences are described in Table 2. * denotes 450 specific spots, ** denotes 130 specific spots. IFN-γ, interferon gamma; hTERT, human telomerase reverse transcriptase.

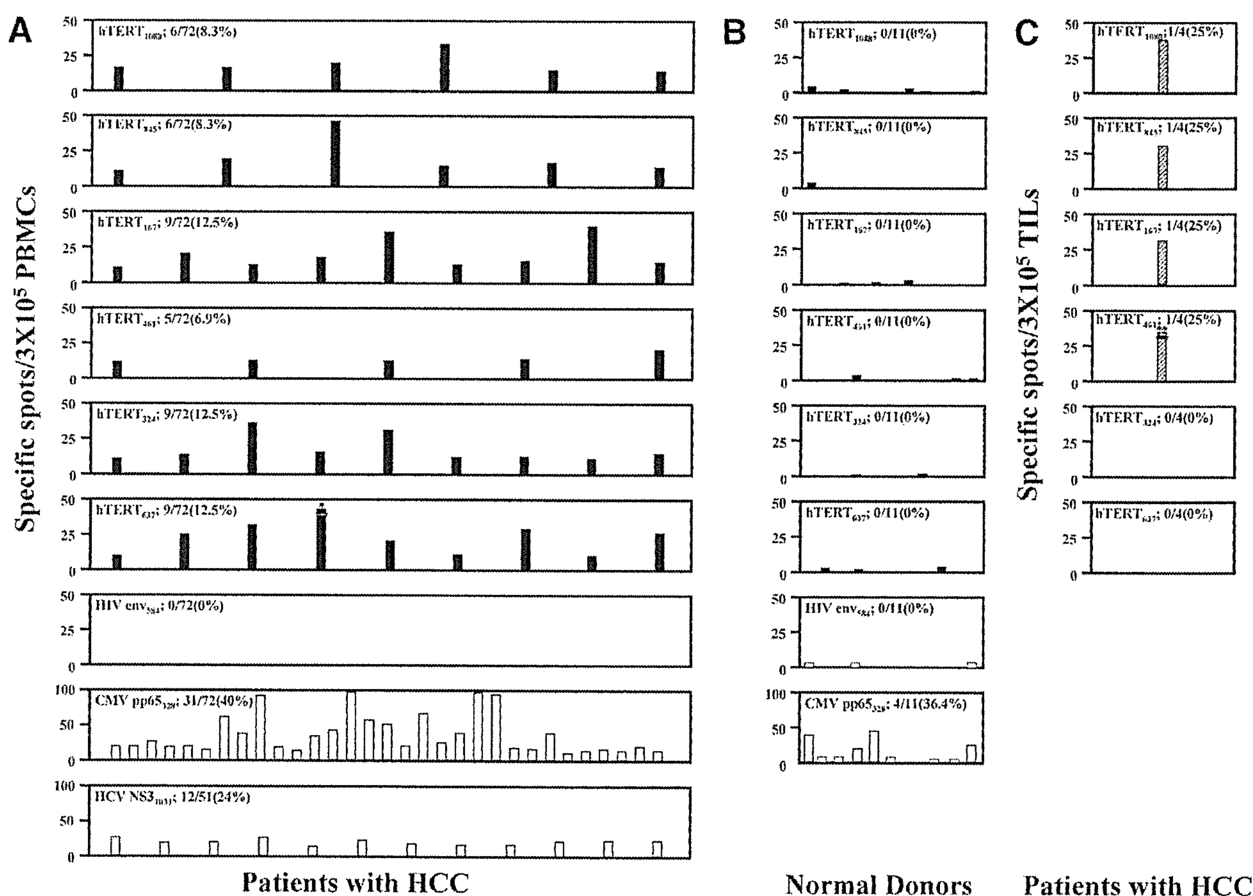


Fig. 3. Direct *ex vivo* analysis (IFN-γ ELISPOT assay) of peripheral blood T cell responses to hTERT-derived peptides (peptides $hTERT_{1088}$, $hTERT_{845}$, $hTERT_{167}$, $hTERT_{461}$, $hTERT_{324}$, and $hTERT_{637}$; solid bars) or control peptides (Peptides $HIVenv_{584}$, $CMVpp65_{328}$ and $HCVNS3_{1031}$; open bars) in HCC patients (A) and normal donors (B). Direct *ex vivo* analysis of tumor-infiltrating lymphocyte responses to hTERT-derived peptides (hatched bars) in HCC patients (C). Only significant IFN-γ responses are included in A and C. Responses were considered positive if more than 10 specific spots were detected and if the number of spots in the presence of antigen was at least twofold greater than that in the absence of antigen. The peptide sequences are described in Table 2. The data for peptides $hTERT_{1009}$, $hTERT_{385}$, $hTERT_{622}$, and $hTERT_{869}$ are excluded because there was no positive T cell response. * denotes 100 specific spots. ** denotes 243 specific spots. IFN-γ, interferon gamma; hTERT, human telomerase reverse transcriptase; HCC, hepatocellular carcinoma.

$hTERT_{1009}$, $hTERT_{385}$, $hTERT_{637}$, $hTERT_{622}$, and $hTERT_{869}$ peptides, respectively. The proportion of normal donors who showed positive T cell responses to CMV protein-derived peptides and the frequencies of the specific T cells were virtually the same as those of the HCC patients (Fig. 3B).

In ELISPOT assay using TILs, IFN-γ–producing T cells responding to peptides $hTERT_{1088}$, $hTERT_{845}$, $hTERT_{167}$, and $hTERT_{461}$ were detected as shown in Fig 3C, suggesting that hTERT-specific TILs were functional.

Cytotoxic Activity Against hTERT-Derived Peptides in HCC Patients. All hTERT-derived peptides were tested for their potential to induce HLA-A24–restricted CTLs from PBMCs of HCC patients with HLA-A24. Each peptide was tested on at least 10 patients. After three rounds of stimulation with the synthetic peptides, responder cells that had been stimulated with peptides $hTERT_{1088}$, $hTERT_{845}$, $hTERT_{167}$, $hTERT_{461}$, $hTERT_{324}$, and $hTERT_{637}$ lysed the peptide-pulsed C1R-A*2402 cells as shown in Fig. 4. Conversely, other peptides failed to induce CTLs specific for the corresponding peptides.

Cytotoxic Activity of hTERT Peptide-Specific CTLs Against Hepatoma Cell Lines. To examine whether hTERT peptide-specific CTLs induced from PBMCs of HCC patients lyse hepatoma cell lines that express hTERT, we first checked the telomerase activity in three hepatoma cells. TRAP assays showed that the three hepatoma cells expressed hTERT; however, the expression in HuH6 cells was lower than that in HepG2 or HuH7 cells (Fig. 5A). The results were confirmed in the TRAP ELISA, which is a quantitative measurement of telomerase activity. The expression levels of hTERT in HepG2 and HuH7 cells were more than twofold higher than the level in HuH6 cells (Fig. 5B).

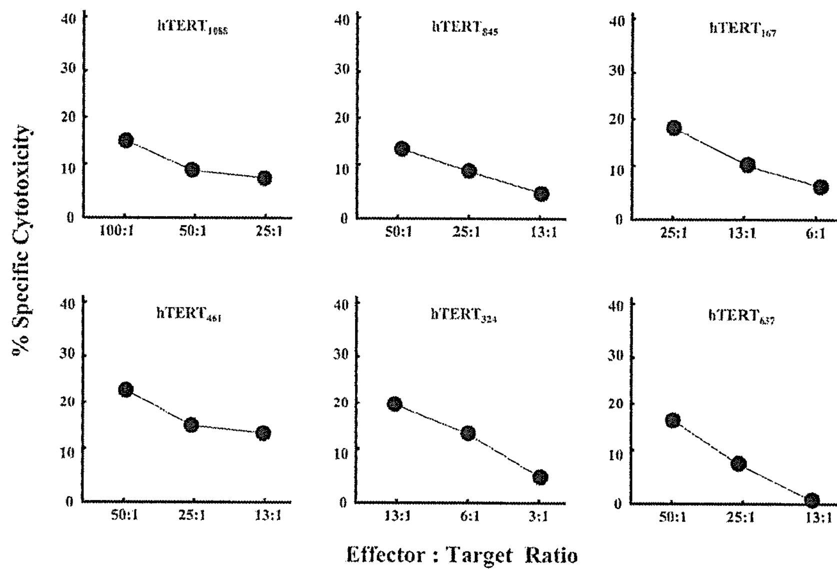


Fig. 4. Cytotoxicity of hTERT-specific T-cell lines derived with peptide in patients with HCC. The cytotoxicity of the T-cell lines was determined by a standard 6-hour cytotoxicity assay at various effector to target (E/T) ratios against C1R-A*2402 cells pulsed with one of the hTERT-derived peptides listed in Table 2. The data are indicated as the percent specific cytotoxicity, which is calculated as follows: (cytotoxicity in the presence of specific peptide) − (cytotoxicity in the absence of peptide). hTERT, human telomerase reverse transcriptase; HCC, hepatocellular carcinoma.

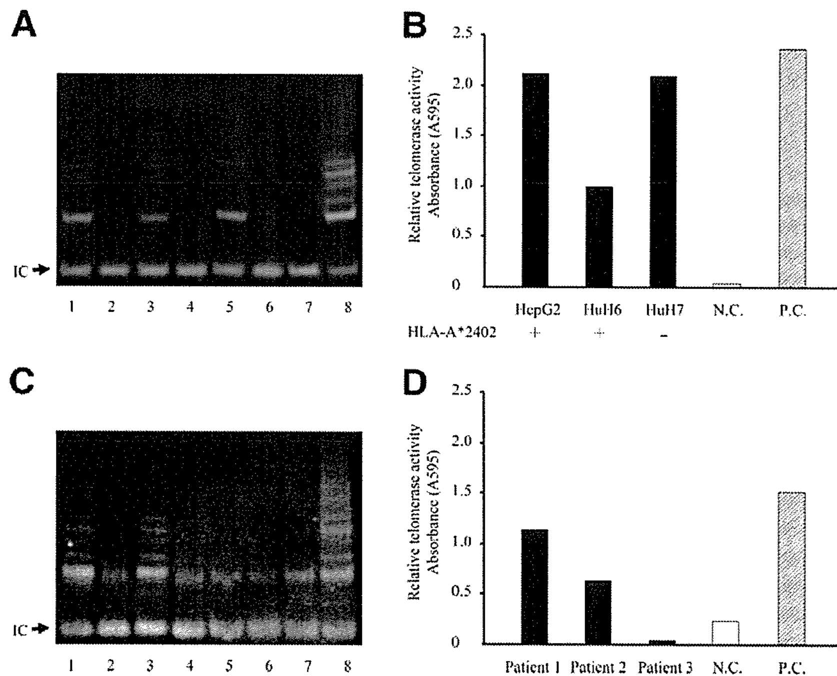


Fig. 5. Telomerase activity in hepatoma cell lines (A, B) and tumors resected by surgical treatment (C, D). A TRAP assay was carried out with 0.01 μg and 0.1 μg cell extract from hepatoma cell lines and tumors, respectively. The products of the PCR were fractionated by electrophoresis on a 10% polyacrylamide gel and then visualized by staining with SYBR-Green I. The TRAP internal control (IC) is shown for each extract. A: Lane 1; HepG2, Lane 2; HepG2 with heat, Lane 3; HuH 6, Lane 4; HuH 6 with heat, Lane 5; HuH 7, Lane 6; HuH 7 with heat, Lane 7; negative control, Lane 8; positive control. B: Lanes 1, 3, and 5, HCCs from three different patients; Lanes 2, 4, and 6, HCCs from three different patients with heat; Lane 7, negative control; Lane 8, positive control. Relative telomerase activity was measured with a TRAPEZE ELISA telomerase detection kit (TRAP ELISA) in hepatoma cell lines (C) and tumors resected by surgical treatment (D). Molecular typing of the HLA-A allele for hepatoma cell lines was performed with genomic DNA using standard site-specific oligonucleotide PCR. NC, negative control; PC, positive control; TRAP, telomerase repeat amplification protocol; PCR, polymerase chain reaction; HCC, hepatocellular carcinoma.

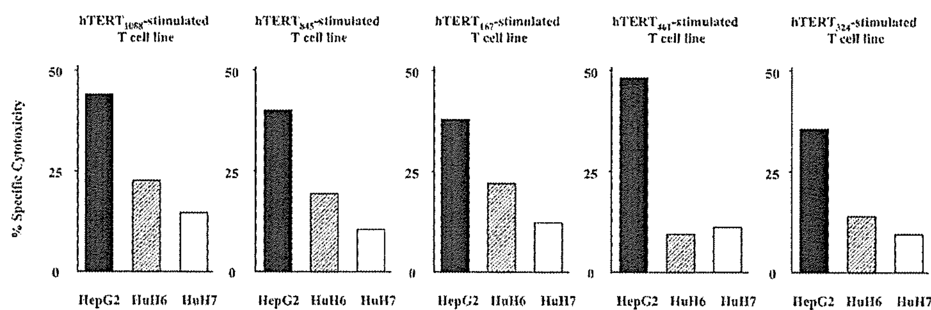


Fig. 6. Cytotoxicity of hTERT-specific T-cell lines derived with peptide against hepatoma cell lines. HepG2 (solid bar) highly expresses hTERT and has HLA-A*2402. HuH 6 (hatched bar) shows low expression of hTERT and has HLA-A*2402. HuH 7 (open bar) shows hTERT expression of the same level as HepG2 but does not have HLA-A*2402. The cytotoxicity was determined by a standard 6-hour cytotoxic assay (E/T ratio of 50:1). hTERT, human telomerase reverse transcriptase.

We next examined the cytotoxicity of hTERT peptide–specific CTLs against these hepatoma cell lines. As shown in Fig. 6, peptides $hTERT_{1088}$, $hTERT_{845}$, $hTERT_{167}$, $hTERT_{461}$, and $hTERT_{324}$-specific CTLs showed cytotoxicity against HepG2 cells, which highly express hTERT and has the HLA-A*2402 molecule. In contrast, the CTLs did not show cytotoxicity against HuH7 cells, which express hTERT at the same level as HepG2 cells but do not have HLA-A*2402. In addition, the cytotoxicity of hTERT-specific CTLs induced with peptides $hTERT_{1088}$, $hTERT_{845}$, $hTERT_{167}$, and $hTERT_{324}$ against HuH6 cells, which express HLA-A*2402 and a low level of hTERT, was weak compared with the cytotoxicity against HepG2 cells. The difference was even more marked in the cytotoxicity of CTLs induced with peptide $hTERT_{461}$, and the CTLs were not cytotoxic to HuH6 cells.

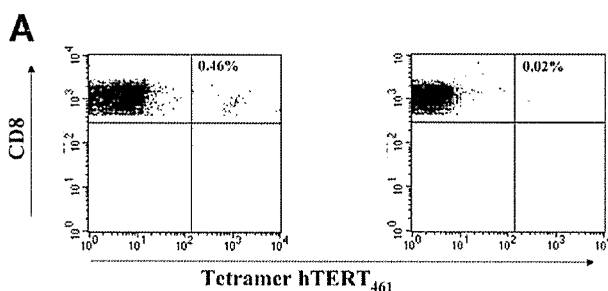
Telomerase activity was also detected in the tumor of 3 of 10 patients with HCC (Fig. 5C and D). All of the three patients showed hTERT-specific T cell responses in ELISPOT assay.

Detection of $hTERT_{461}$ Tetramer$^+$ and $CD8^+$ T Lymphocytes in PBMCs and TILs. To analyze the character of hTERT specific T cells in patients with HCC more precisely, we examined the frequencies of $hTERT_{461}$ tetramer$^+$ cells in PBMCs and TILs, and compared them with the results of ELISPOT assay. PBMCs and TILs were stained with CD4-FITC, CD14-FITC, CD19-FITC, CD8-PerCP, and tetramer-PE as described in Patients and Methods. At least 1×10^5 cells in the $CD8^+CD4^-CD14^-CD19^-$ gate were then analyzed for tetramer staining as shown in Fig. 7A.

As indicated in Fig. 7B, the frequencies of $CD8^+$ $CD4^-CD14^-CD19^-hTERT_{461}$ tetramer$^+$ cells in peripheral blood were 0.03% to 0.71% of $CD8^+$ T cells (patients 1-15). The frequencies in the patients with positive responses for ELISPOT assay were 0.06% to 0.71%. Interestingly, 7 of 10 patients without positive responses for ELISPOT assay showed 0.07% to 0.26% $CD8^+$ $CD4^-CD14^-CD19^-hTERT_{461}$tetramer$^+$ cells. These results suggest that dysfunctional hTERT-specific T cells exist in patients with HCC. Conversely, the frequency of $CD8^+CD4^-CD14^-CD19^-hTERT_{461}$ tetramer$^+$ cells in TILs was quite high (2.73%), and they were functional (patient 16).

hTERT-Specific T Cell Responses and Clinical Features of HCC Patients. To evaluate the status of hTERT-specific T cell responses in patients with HCC,

A



B

Patients	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Specific spots in ELISPOT	14	20	11	12	12	0	0	0	2	0	0	8	0	0	6	243
% of tetramer$^+$ cells	0.07	0.06	0.71	0.06	0.28	0.13	0.14	0.04	0.03	0.18	0.26	0.07	0.11	0.04	0.10	2.73

Less than 10 specific spots in ELISPOT or 0.05% of tetramer$^+$ cells

More than 10 specific spots in ELISPOT or 0.05% of tetramer$^+$ cells

More than 10 specific spots in ELISPOT and 0.05% of tetramer$^+$ cells

Fig. 7. Detection of hTERT-specific, HLA-A*2402-tetramer$^+$, $CD8^+CD4^-CD14^-$ $CD19^-$ T lymphocytes in the peripheral blood and tumor. PBMCs isolated from representative patients with HCC (A) were stained with tetrameric complexes and antibodies and analyzed on a FACSCalibur™ flow cytometer. Analysis of the association between the frequency of tetramer$^+$ cells and IFN-γ-producing cells detected in ELISPOT assay (B). Tetramer staining and ELISPOT assay were performed in 16 patients using PBMCs (patients 1-15) and TILs (patient 16). hTERT, human telomerase reverse transcriptase; PBMC, peripheral blood mononuclear cell; HCC, hepatocellular carcinoma; IFN-γ, interferon gamma; TIL, tumor-infiltrating lymphocyte.

Table 3. Univariate Analysis of the Effect of Variables on the T Cell Response Against hTERT

	Patients With Positive T Cell Response	Patients Without Positive T Cell Response	P
No. of patients	29	43	
Age (years)*	67.7 ± 9.7	66.7 ± 8.1	NS
Sex (M/F)	21/8	27/16	NS
AFP level (≦20/> 20)	13/16	14/29	NS
Diff. degree of HCC (well/ moderate or poor/ND[c])	9/6/14	6/16/21	NS
Tumor multiplicity (multiple/ solitary)	17/12	22/21	NS
Vascular invasion (+/−)	7/22	8/35	NS
TNM factor			
(T1/T2-4)	11/18	19/24	NS
(N0/N1)	28/1	43/0	NS
(M0/M1)	29/0	39/4	NS
TNM stage (I/II-IV)	11/18	19/24	NS
Histology of non-tumor liver (LC/Chronic hepatitis)	25/4	39/4	NS
Liver function (Child A/B/C)	13/14/2	30/11/2	NS
Etiology (HCV/HBV/Others)	22/3/4	37/6/0	NS

Abbreviations: NS; there was no statistical significance; ND, not determined.
*Data are expressed as mean ± SD.

we analyzed the relationship between the frequencies of peptides $hTERT_{1088}$, $hTERT_{845}$, $hTERT_{167}$, $hTERT_{461}$, $hTERT_{324}$, and $hTERT_{637}$-specific T cells detected by IFN-γ ELISPOT assay and the clinical features of patients. Table 3 shows clinical features of HCC patients who showed positive and negative T cell responses to hTERT-derived epitopes.

The clinical features of both groups were not statistically different in terms of age, sex, serum AFP levels, differentiation of HCC, tumor multiplicity, vascular invasion, TNM factors and stages, histology of the non-tumor liver, liver function, and the type of viral infection (Table 3).

Next, we examined the kinetics of hTERT-specific T cells in 16 patients who had positive T cell responses and received curative treatments by surgical resection or radiofrequent ablation, and analyzed the association between the kinetics and clinical responses. The frequencies of hTERT-specific T cells detected in ELISPOT assay decreased in most of the patients 6 months after curative treatments (Fig. 8). Only 5 of 16 patients showed positive T cell responses after treatments. Four patients whose hTERT-specific T cells were maintained had no recurrence of HCC. In contrast, 11 patients whose number of hTERT-specific T cells decreased showed HCC recurrence within 1 year after curative treatments.

Discussion

In the current study, we first attempted to identify hTERT epitopes restricted by HLA-A24, which is present

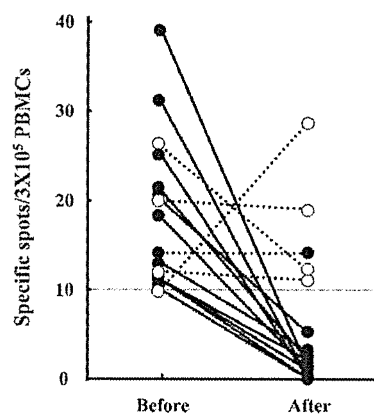


Fig. 8. Kinetics of hTERT-specific T cell responses before and after curative treatments. PBMCs were obtained before and 6 months after treatments and analyzed. Open circles show the patients without tumor recurrence within 1 year after treatment. Closed circles show the patients with tumor recurrence within 1 year after treatment. Solid and dotted lines show the patients without and with more than 10 specific spots for hTERT-derived peptides in ELISPOT assay after treatment, respectively. hTERT, human telomerase reverse transcriptase; PBMC, peripheral blood mononuclear cell.

in 60% of Japanese, 20% of whites, and 12% of Africans,[36,37] using a combined computer-based and immunological approach. Analysis of amino acid sequences of hTERT by computer showed a number of potential HLA-A24–binding peptides, and 2 of the 10 hTERT-derived peptides (Peptides $hTERT_{461}$ and $hTERT_{324}$) have been identified to contain HLA-A24–restricted CTL epitopes. Including these two peptides, six hTERT-derived peptides (peptides $hTERT_{1088}$, $hTERT_{845}$, $hTERT_{167}$, $hTERT_{461}$, $hTERT_{324}$, and $hTERT_{637}$) that showed high affinity for HLA-A*2402 induced production of IFN-γ in spleen cells and PBMCs, in hTERT cDNA-immunized HLA-A*2402/K^b transgenic mice and HCC patients, respectively. In addition, T cell lines stimulated with the peptide showed cytotoxicity against hepatoma cell lines that express HLA-A*2402 and hTERT. Taken together with the results of peptide binding, ELISPOT, and CTL assay, we concluded peptides $hTERT_{1088}$, $hTERT_{845}$, $hTERT_{167}$, $hTERT_{461}$, $hTERT_{324}$, and $hTERT_{637}$ contained HLA-A24 restricted, hTERT-specific CTL epitopes.

Interestingly, the cytotoxicity of hTERT-specific CTLs induced with peptides $hTERT_{1088}$, $hTERT_{845}$, $hTERT_{167}$, and $hTERT_{324}$ in HuH6 cells, which showed low levels of hTERT, was weak compared with the cytotoxicity in HepG2 cells with high levels of hTERT. The difference was even more marked in the cytotoxicity of CTLs induced with peptide $hTERT_{461}$, and the CTLs were not cytotoxic to HuH6. In accordance with our results, it was reported that the susceptibility of tumor cells to hTERT-specific CTLs decreased after IFN-γ

treatment because of attenuation of hTERT expression.[38] In addition, all of the patients who had telomerase activity in the tumor showed hTERT-specific T cell responses in ELISPOT assay. These results suggest that the strength of hTERT-specific cytotoxicity against hepatoma cells depends on the expression levels of the protein.

In the analysis of PBMCs in patients with HCC using $hTERT_{461}$ tetramer, the frequencies of $hTERT_{461}$ tetramer^{+} cells in PBMCs were similar to those of other tumor specific antigen-derived epitopes.[39] Furthermore, the existence of dysfunctional hTERT-specific T cells was accordant with previous reports of other tumor antigens.[39] Conversely, the frequency of $hTERT_{461}$ tetramer^{+} cells in tumors was quite high, and they produced IFN-γ. IFN-γ–producing T cells responding to other peptides $hTERT_{1088}$, $hTERT_{845}$, and $hTERT_{167}$ were also detected in tumors. These results suggest that hTERT is an attractive target for immunotherapy of HCC.

In the second part of the current study, to study the status of the host immunological response to hTERT in HCC patients, we examined the frequency of hTERT-specific T cells in the peripheral blood by ELISPOT assay with the six epitopes and analyzed the relationship between the frequency and the clinical features of the patients. ELISPOT assay showed that the frequency of reactive T cells to a single hTERT epitope was 10 to 100 per 3×10^5 PBMCs. In previous reports regarding the frequency of T cells specific for a single hTERT epitope in patients with colon or breast cancer, the number was found to be 1 to 22 per 2×10^5 PBMCs or 1 to 33 per 2×10^5 PBMCs, respectively.[18,19] In addition, single hTERT epitope–specific IFN-γ–producing cells were detected in 6.9% to 12.5% of the patients for peptides $hTERT_{1088}$, $hTERT_{845}$, $hTERT_{167}$, $hTERT_{461}$, $hTERT_{324}$, and $hTERT_{637}$. These rates are quite similar to those in previous reports.[18,19] Comparing the current results with those reports, we believe that hTERT-specific CTL responses in HCC patients are as strong as those of other cancer patients and that the newly identified hTERT epitopes are immunogenic.

From the analysis of hTERT-specific immune responses in HCC patients, we obtained evidence that clinical features, including age, sex, serum AFP levels, differentiation of HCC, tumor multiplicity, vascular invasion, TNM factors and stages, histology of the nontumor liver, liver function, and the type of viral infection, were not associated with the frequency of hTERT-specific CTLs in HCC patients (Table 3). These results suggest that hTERT-specific CTLs could be generated independently of hepatitis viral infection or serum AFP levels, which suppress the host immune response through inhibition of dendritic cells[40-42] or T cell proliferation.[43] In addition, comparing with AFP- or other tumor antigen–specific immune responses,[31,44] hTERT-specific immune responses exist and can be induced in the patients with HCC even at early stages. These results suggest the advantage of hTERT as a target for immunotherapies because the induction of tumor-specific immune responses at early stages of the tumor should be more effective for tumor growth suppression.

In the analysis of the association between kinetics of hTERT-specific T cells and clinical responses, recurrent rate of HCC was higher in the patients without maintenance of hTERT-specific T cells than in those with. This result suggests that maintenance of hTERT-specific T cells may be important to protect tumor recurrence after treatments, although there was no statistically significant difference between the two groups because of the small number of patients.

In conclusion, we identified and characterized HLA-A*2402–restricted T cell epitopes derived from hTERT. The identified epitope-specific T cells can be detected and induced by stimulating PBMCs with these peptides in HCC patients. hTERT-specific CTLs were observed even in the patients with early stages of HCC and killed hepatoma cell lines that expressed hTERT dependent on the expression level. The frequency of hTERT/tetramer^{+}CD8^{+} T cells in the tumor tissue of patients with HCC was quite high, and they were functional. These results suggest that hTERT is an important target of T-cell–based immunotherapy for HCC and that the identified epitopes could be valuable both for therapy and for analyzing the host immune responses.

Acknowledgment: The authors thank Maki Kawamura, Sanae Funaoka, and Chiharu Minami for their invaluable help with sample collection, and all patients who donated blood samples for this study.

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VOLUME 24 · NUMBER 28 · OCTOBER 1 2006

JOURNAL OF CLINICAL ONCOLOGY

ORIGINAL REPORT

Prognostic Significance of T-Cell or Cytotoxic Molecules Phenotype in Classical Hodgkin's Lymphoma: A Clinicopathologic Study

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Submitted March 9, 2006; accepted July 27, 2006; published online ahead of print at www.jco.org on September 5, 2006.

Authors' disclosures of potential conflicts of interest and author contributions are found at the end of this article.

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0732-183X/06/2428-4626/$20.00

DOI: 10.1200/JCO.2006.06.5342

A B S T R A C T

Purpose
Classical Hodgkin's lymphoma (CHL) is characterized by Hodgkin's and Reed-Sternberg (H-RS) cells, most of which are derived from germinal-center B cells. Nevertheless, one or more markers for T cells and follicular dendritic cells (FDC) may be expressed in a minority of H-RS cells in some CHL patients, although the clinical significance of this remains controversial. The aim of this study was to clarify the association between phenotypic expression and clinical outcome in CHL.

Patients and Methods
Participants were 324 consecutive CHL patients, comprising 132 patients with nodular sclerosis (NS), 35 patients with NS grade 2 (NS2), and 157 patients with mixed cellularity (MC). We evaluated the presenting features and prognosis of patients on categorization into four phenotypically defined groups: B-cell ($CD20^+$ and/or $CD79a^+$; n = 63), T-cell and/or cytotoxic molecules ($CD3^+$, $CD4^+$, $CD8^+$, $CD45RO^+$, TIA-1^+, and/or granzyme B^+; n = 27), FDC ($CD21^+$ without B-cell marker; n = 22), and null-cell types (n = 212). Other potential prognostic factors were examined.

Results
The T-cell and/or cytotoxic molecules group showed a significantly poorer prognosis than the other three groups ($P < .0001$). This finding was seen consistently in multivariate analyses. Morphologic subtyping (NS/NS2/MC) and Epstein-Barr virus positivity were not identified as independent prognostic factors.

Conclusion
The presence of T-cell and/or cytotoxic antigens in H-RS cells may represent a poor prognostic factor in CHL, even if their expression is not regarded as lineage specific. Examination of T-cell and/or cytotoxic molecules phenotype in CHL patients is recommended as a routine pathologic practice.

J Clin Oncol 24:4626-4633. © 2006 by American Society of Clinical Oncology

INTRODUCTION

The recent availability of a large number of monoclonal antibodies for leukocyte surface markers has provided further evidence for the B-cell origin of Hodgkin's and Reed-Sternberg (H-RS) cells in many but not all patients.[1,2] The application of molecular methods, single H-RS cell analysis,[3] and comparative genome expression analysis[4] has provided additional definitive evidence that H-RS cells of classical Hodgkin's lymphoma (CHL) are derived from germinal-center B cells.[5-7] Nevertheless, a small number of patients with CHL are immunoreactive for T-cell antigens,[8,9] and rare occurrences of CHL are even derived genotypically from T cells.[10,11] Adding to this complexity, we reported previously nine patients with CHL with a follicular dendritic cell (FDC) phenotype without other B-cell or T-cell markers.[12] These phenotypic analyses were interpreted variously to suggest the distinct cellular origin (B cells, T cells, or FDCs) of H-RS cells, notwithstanding that the expression of these cell-associated antigens was found to lack clear lineage specificity. Of note, the association between the expression of these markers and clinical outcome in CHL has been controversial.

In this study, we investigated comprehensively 324 patients with CHL to clarify their clinicopathologic features and survival, with special reference to phenotypic properties (four phenotypes: B cell, T cell and/or cytotoxic molecules

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[T/CM], FDC, and null cell) and positivity for Epstein-Barr virus (EBV) on H-RS cells.

PATIENTS AND METHODS

Patient Samples

A total of 324 consecutive patients with CHL diagnosed between April 1982 and March 2005 at Aichi Cancer Center Hospital (Nagoya, Japan) were selected from patient records. Approval for the study was provided by the Institutional Review Board of Aichi Cancer Center.

For the diagnosis of CHL, all patients in this study were negative for human T-cell leukemia virus type 1 antibody in sera. The tumor cells showed no sinusoidal spread and grew separately from each other in all areas of the biopsies to exclude Hodgkin's-like anaplastic large cell lymphoma (ALCL) under the Revised European-American Lymphoma classification.[13] Patients with nodular lymphocyte-predominant Hodgkin's lymphoma, which is now termed B-cell neoplasm, also were excluded.

Each patient case was reviewed independently by two pathologists (N.A. and S.N.), who used a combination of morphologic review and immunostaining to assign each patient case to one of the categories of the modified WHO classification scheme.[14] Controversial determinations were reassessed jointly by the two pathologists until a consensus was reached. Morphologically related entities, such as Hodgkin's-like ALCL and peripheral T-cell lymphoma with Reed-Sternberg-like cells, were ruled out by three external lymphoma experts (T. Yoshino, Okayama, Japan; K. Ohshima, Kurume, Japan; and Y. Matsuno, Tokyo, Japan), who were blinded to the phenotype and clinical course of the patients.

Tissue Specimens and Histology

Tissue samples were fixed in 10% formalin and embedded in paraffin, then sectioned at 5-μm intervals and stained with hematoxylin and eosin. Imprint smears of surgically rejected specimens were stained with May-Grünwald-Giemsa stain.

Immunohistochemistry

Formalin-fixed paraffin sections were subjected to immunoperoxidase studies using the avidin-biotin peroxidase complex method. Monoclonal

Table 1. Clinical and Phenotypic Characteristics According to Histology (NS *v* NS2 *v* MC)

Characteristic	NS No.	NS %	NS2 No.	NS2 %	MC No.	MC %	P*
Total No. of patients	132		35		157		
Sex							.001
Male	76		26		121		
Female	56		9		36		
Ratio	1.36		2.89		3.36		
Age, years							.0001
Median	31		50		57		
Range	12-84		5-88		4-89		
> 45	46	35	21	60	112	71	< .0001
> 60	32	24	12	34	65	41	.009
PS > 1	22	17	10	29	21	13	.089
Clinical stage III/IV	54	41	22	63	59	38	.023
Presence of "B" symptoms	42	34	16	55	45	37	.11
Bulky mass	26	21	6	20	13	10	.056
Mediastinal mass	71	58	11	39	30	24	< .0001
Extranodal > 1 site	14	12	8	29	15	12	.060
WBC > 15,000/μL	20	19	4	17	5	6	.026
Hb < 10.5 g/dL	26	25	11	48	18	21	.031
Serum albumin < 4.0 g/dL	48	53	9	69	38	51	.46
LDH > normal	36	43	14	61	30	42	.27
Survival, months							.54
Median	27.1		26.8		24.1		
Range	4.5-163+		2.0-171+		1.2-254+		
Immunophenotype†							
CD20	18 of 122	15	4 of 35	11	32 of 147	22	.19
CD21	13 of 111	12	5 of 25	20	12 of 92	13	.54
cyCD3	2 of 66	3	1 of 17	6	1 of 83	1	.47
CD4	4 of 35	11	0 of 9	0	0 of 36	0	.067
CD8	2 of 35	6	0 of 8	0	0 of 36	0	.28
CD15	90 of 131	69	28 of 34	82	84 of 154	55	.002
CD30	118 of 131	90	32 of 35	91	142 of 155	92	.90
CD45RO	5 of 104	5	1 of 29	4	1 of 113	1	.22
CD79a	3 of 34	9	1 of 8	13	8 of 43	19	.47
TIA-1	9 of 132	7	1 of 35	3	2 of 156	1	.045
Granzyme B	9 of 132	7	1 of 35	3	6 of 157	4	.42
EBV	16 of 126	13	18 of 34	53	115 of 154	75	< .0001

Abbreviations: NS, nodular sclerosis; NS2, nodular sclerosis grade 2; MC, mixed cellularity; PS, performance status; Hb, hemoglobin; LDH, lactate dehydrogenase; cyCD3, cytoplasmic CD3; EBV, Epstein Barr virus.
*χ^2 test for independence, or Fisher's exact probability test, NS *v* NS2 *v* MC.
†No. positive of No. tested patients.

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antibodies used were CD3, CD8, UCHL-1/CD45RO, L26/CD20, 1F8/CD21, Ber-H2/CD30, CD79a, and ALK1 (DAKO, Glostrup, Denmark); CD4 (Novocastra Laboratories, Newcastle, United Kingdom); LeuM1/CD15 (Becton Dickinson, Sunnyvale, CA); TIA-1 (Coulter Immunology, Hialeah, FL); and granzyme B (Monosan, Uden, the Netherlands). All antibodies were first heated in a microwave, then the antibodies were used. Reaction for the reagents was considered positive when more than 5% of the H-RS cells stained, although in practice many of the positive samples showed marking in more than 10% of cells.

In Situ Hybridization Study

The presence of EBV small RNAs was determined by in situ hybridization using EBV-encoded small nuclear early-region oligonucleotides on formalin-fixed, paraffin-embedded sections as described previously.[15]

Statistical Analysis

Differences in characteristics between the two groups were examined by the χ^2 test, Fisher's exact test, Student's t test, and Mann-Whitney U test as appropriate. Patient survival data were analyzed by the Kaplan-Meier method. Differences in survival were tested by the log-rank test. Survival for this study was evaluated in terms of disease-specific survival (DSS), measured from the date of diagnosis until the date of death as a result of a lymphoma-related cause. In DSS analysis, patients were censored at the time of death if this was from a cause unrelated to lymphoma, and deaths from treatment-related causes were classified as death from lymphoma. Univariate and multivariate analyses were performed with Cox proportional hazards regression models. Results are expressed as hazard ratios (HRs) and 95% CIs. All data were analyzed with the aid of STATA software (version 9.0, STATA Corp, College Station, TX).

RESULTS

Clinicopathologic Characteristics

Patient characteristics are summarized in Table 1. There were 223 male and 101 female patients with a median age of 48 years (range, 4 to 89). Histopathologically, they included 132 patients with nodular sclerosis (NS; median age, 31 years; range, 12 to 84 years, male-to-female ratio, 1.36), 35 with NS grade 2[16] (NS2; median age, 50 years; range, 5 to 88 years; male-to-female ratio, 2.89), and 157 with mixed cellularity (MC; median age, 57 years; range, 4 to 89 years, male-to-female ratio, 3.36). On comparison, patients with NS showed a significantly younger age at onset (P = .0001) and a higher ratio of females (P = .001). Patients with NS2 were associated significantly with several aggressive clinical parameters, namely advanced clinical stage in 22 patients (63%; P = .023) and anemia (hemoglobin < 10.5 g/dL) in 11 patients (48%; P = .031).

Immunophenotypic Characteristics

Phenotypic features are summarized in Table 1. There were significant differences in the results of positivity or negativity of H-RS cells for TIA-1, CD15, and EBV among NS, NS2, and MC patients. NS patients showed significantly higher rates for TIA-1 expression than those with NS2 or MC (P = .045), whereas MC patients showed significantly lower CD15 positivity (P = .002). Furthermore, EBV was harbored in 75% of MC patients, which is significantly higher than the ratios for NS and NS2 (13% and 53%, respectively; P < .0001).

Phenotypic Distribution of CHL

Based on the immunohistochemically recognizable features of the H-RS cell, the present series of CHL patients were delineated into four phenotypic groups, as summarized in Table 2. The first group included 63 patients with the B-cell phenotype with expression of CD20 or CD79a. The second group included 27 patients with the T/CM phenotype with expression of CD3, CD4, CD8, CD45RO, and/or CMs such as TIA-1 and granzyme B (Fig 1), but not CD20, CD79a. The third group included 22 patients with the FDC phenotype with expression of CD21, but not any of the other B- or T-cell markers. The fourth group included 212 patients with the null-cell phenotype without expression of the B-cell, T-cell, or FDC-related markers. In the T/CM group, the expression of CMs was found in 20 patients, five of whom lacked the other T-cell markers. All patients in this T/CM group were also negative for ALK1 by additional immunohistochemical staining.

Clinicopathologic characteristics of these four immunophenotypic groups are summarized in Table 3. On comparison, patients in the T/CM group had a younger onset (median age, 44 years; P = .048), higher ratio of females (male- to-female ratio, 1.25), and lower ratio of EBV on H-RS cells (35%; P = .025).

Moreover, the present series of CHL patients could be categorized into two phenotypic groups, $CD15^+$ and $CD15^-$, with CD15 expression identified in 202 (63%) of the 319 patients examined.

Table 2. Phenotypic Distribution of Classical Hodgkin's Lymphoma

Characteristic	B-Cell Group		T/CM Group		FDC Group		Null-Cell Group	
	No.	%	No.	%	No.	%	No.	%
Total No. of patients	63	20	27	8	22	7	212	65
Immunophenotype*								
CD3	0	—	4 of 21	19	0	—	0	—
CD4	0	—	4 of 16	25	0	—	0	—
CD8	0	—	2 of 16	13	0	—	0	—
CD45RO	0	—	6 of 22	27	0	—	0	—
TIA-1	0	—	12 of 26	46	0	—	0	—
Granzyme B	0	—	16 of 27	59	0	—	0	—
CD20	54 of 63	86	0	—	0	—	0	—
CD79a	12 of 20	60	0	—	0	—	0	—
CD21	0	—	0	—	22 of 22	100	0	—

Abbreviations: T/CM, T-cell and/or cytotoxic molecules; FDC, follicular dendritic cell.
*No. positive of No. tested patients.

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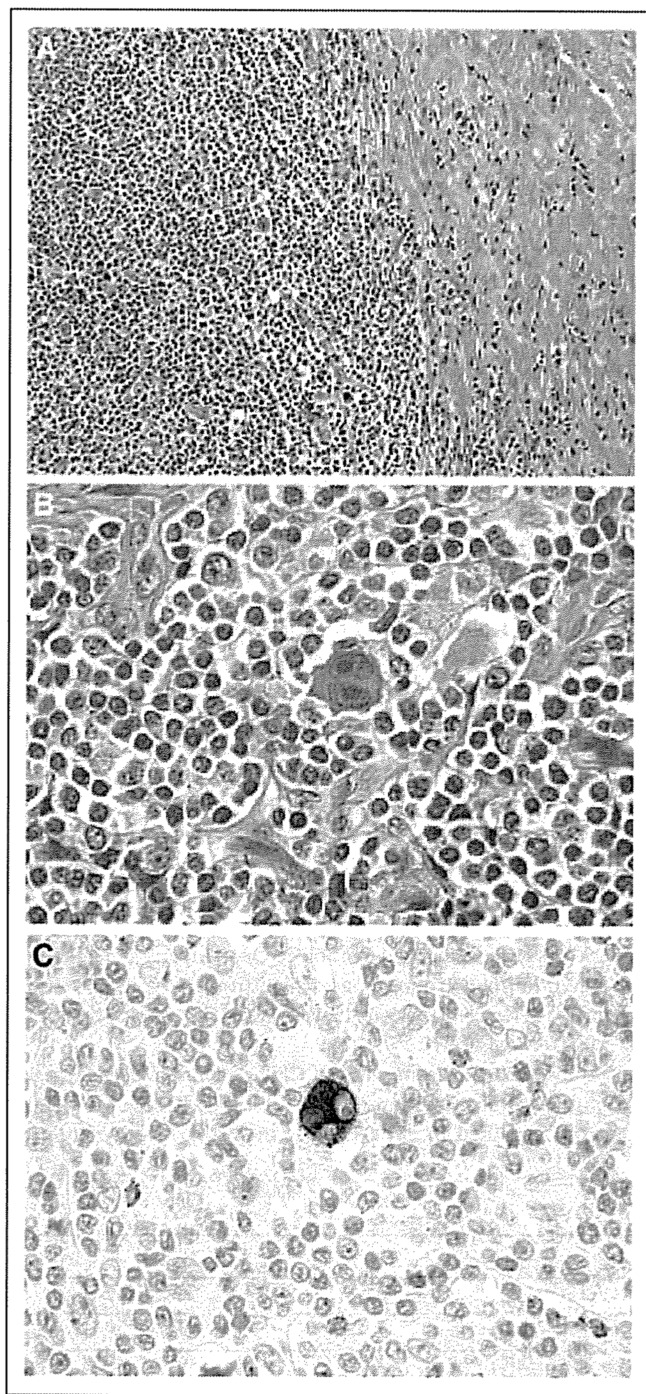

Fig 1. Classical Hodgkin's lymphoma (CHL) with T-cell and/or cytotoxic molecule expression. (A) T-cell and/or cytotoxic molecule–positive CHL patient sample shows fibrous collagen bands dividing the lymph node into nodules and is categorized as nodular sclerosis (original magnification ×40). (B) Reed-Sternberg cells are present (original magnification ×400) and (C) are immunoreactive for granzyme B (original magnification ×400).

Comparison of these patients revealed no clinical differences between them (data not shown). Seven patients showing the CD15$^-$ and CD30$^-$ phenotype were diagnosed on the basis of the morphology, and immunophenotype of the absence of B- or T-cell markers and positivity of Fascin.

EBV Distribution in CHL

EBV was detected in 149 of 314 (47%) patients, with no association seen with histopathologic group. The EBV-positive group was characterized by a higher ratio of males and an older age of onset than the EBV-negative group. CD20 expression was more frequently detected in the EBV-positive group (P = .025).

Therapeutic Response

A total of 183 patients received combination chemotherapy consisting of first-line treatment regimens as follows: doxorubicin, bleomycin, vinblastine, and dacarbazine (ABVD; 146 patients); cyclophosphamide, vincristine, procarbazine, and prednisone (15 patients); bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine, and prednisone (six patients); and cyclophosphamide, doxorubicin, vincristine, and prednisone (16 patients; Table 3). Ninety-four patients received radiation therapy, and 88 received both chemotherapy and radiation. In 106 patients with stage I/II disease, 78 patients (74%) received ABVD-based chemotherapy and six underwent radiation therapy only. No significant differences in treatment types were seen among phenotypic subgroups. In total, 77% patients (134 of 174) with CHL achieved a complete response with the initial therapy. Notably, the T/CM group showed a lower complete response rate (58%) and a higher no response rate (16%) than the other three groups.

Survival

DSS curves of the NS, NS2, and MC patients showed no significant differences among them. In Figure 2A, however, the DSS curves of the four phenotypic groups based on immunohistochemical evaluation revealed a significant difference (P = .0041). In the 139 patients who received ABVD-based chemotherapy, the survival rate of the T/CM-positive CHL patients was significantly poorer than that of the others (P < .0001; Fig 2B), and five patients showed an aggressive clinical course within 24 months of diagnosis. Median survival of stage I and II patients was 55 and 27 months, respectively. Two patients with stage I/II disease expressing the T/CM phenotype died within 12 months. Survival of the B-cell group tended to be relatively inferior to that of the null-cell group, but without statistical significance (data not shown). Finally, patients with EBV-positive CHL showed a tendency to poor prognosis compared with EBV-negative patients, but without significance by the log-rank test (P = .11).

Prognostic Factors

Univariate analysis identified 13 prognostic factors for the 288 patients of the entire series of CHL patients: phenotype (T/CM type; P = .001), serum albumin less than 4.0 g/dL (P = .001), performance status more than 1 (P = .001), and advanced clinical stage (III/IV; P = .021). The International Prognostic Factor Project (IPFP) score ($\geq$ 5) also showed prognostic significance (P = .003). Hemoglobin level less than 10.5 g/dL, age older than 45 years, and lymphocyte count less than 600/μL showed marginal significance, whereas histologic profile (NS2) was not significant (Table 4).

Multivariate analysis with individual factors showed phenotype (T/CM type: HR, 3.97; 95% CI, 1.85 to 8.48; P < .0001) and age older than 45 years (HR, 2.55; 95% CI, 1.23 to 5.29; P = .012) to be significant and independent prognostic factors in the 228 CHL patients. In the 139 patients who received ABVD-based chemotherapy, T/CM phenotype was a significant and independent prognostic factor. Moreover, T/CM phenotype also influenced survival significantly in advanced CHL patients, independent of IPFP score (Table 4).

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