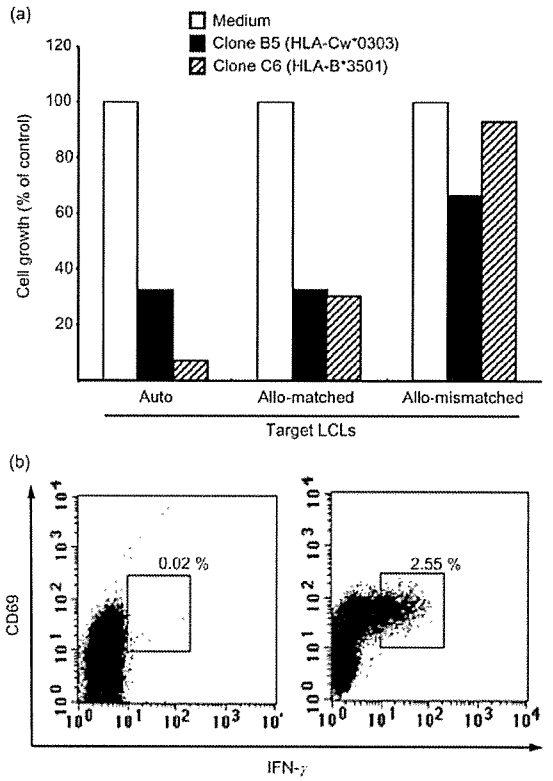

Fig. 7. Effects of EBNA1-specific CTLs on EBNA1-expressing cells. (a) EBNA1-specific CTL clones inhibit *in vitro* outgrowth of HLA-matched LCLs. Target LCLs (2×10^4) were cultured in triplicate wells of round-bottomed 96-well plates with EBNA1-specific CTL clones (1×10^4) or medium alone (control). After 4 weeks culture, the number of LCLs in the culture at each setting was counted. Cell growth (percentage of control) was calculated as [no. LCLs from the culture with CTLs (clone B5 or clone C6)]/[no. LCLs from the culture without CTLs (medium)] × 100. The B-cell (LCL) identity of the outgrowing cultures was confirmed by analysis of CD19 expression by flow cytometry. Data from one representative experiment of two are shown. (b) Detection of IFN-γ-producing anti-EBNA1 CTLs recognizing HLA-Cw*0303-positive cancer cells expressing EBNA1. B5 clones (5×10^5) were incubated with 2×10^6 MKN45-Cw0303 cells or MKN45-Cw0303-ΔGA-EBNA1 cells for 6 h, in the presence of brefeldin A for the last 5 h. After incubation, the cell suspensions were fixed with 4% paraformaldehyde in PBS and then permeabilized with IC Perm and stained with PE–cyanin-5.1-labelled anti-CD8, PE-labelled anti-CD69 or FITC-labelled anti-human IFN-γ mAbs. Stained cells were analysed by flow cytometry. Fifty thousand events were acquired for each analysis. The proportions of IFN-γ^+ CD69$^+$ CTLs among CD8$^+$ lymphocytes are indicated for one representative experiment of two performed.

analyses have shown that LMP2-specific CTLs are present in the infused CTLs used for adoptive immunotherapy and might have antiviral activity in patients with a good response to immunotherapy for HD (Bollard *et al.*, 2004). Interestingly, the CTL line from one NPC patient who attained a complete response was shown to contain a relatively large T-cell population for an EBNA1-derived CTL epitope (Straathof *et al.*, 2005). This suggests that increased attention should be focused on the contribution of EBNA1-specific CTLs to EBV cellular immunity. In this study, we showed two EBNA1-specific CTL clones to cause strong, specific inhibition of LCL outgrowth *in vitro*, which is consistent with recent observations with HLA-B8- and HLA-B*3501-restricted CTL clones (Tellam *et al.*, 2004; Voo *et al.*, 2004). C6 CTLs failed to respond to an HLA-Cw*0303-expressing gastric cancer cell line transduced with full-length EBNA1, although they produced IFN-γ when GAr-depleted EBNA1 was transduced (Fig. 7b). These data suggest differential antigen-processing machinery and presentation on class I molecules between LCLs and gastric cancer cells.

In conclusion, we have established EBNA1-specific CTL clones from PBMCs of a healthy donor by using EBNA1 mRNA-transfected DCs, and identified a novel CTL epitope of EBNA1 presented by HLA-Cw*0303 and -Cw*0304 molecules. The induction method adapted may be useful for generating EBNA1-specific CTLs and for investigating cellular immunity against EBNA1. Finally, the induced EBNA1-specific CTLs recognized EBNA1-expressing gastric carcinoma cells in the context of HLA-Cw*0303 *in vitro*, suggesting that EBNA1 is an important antigen for the further development of CTL therapy for EBV-associated malignancies.

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Identification of an HLA-A24-restricted cytotoxic T lymphocyte epitope from human papillomavirus type-16 E6: The combined effects of bortezomib and interferon-γ on the presentation of a cryptic epitope

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About 50% of cervical cancers are associated with human papillomavirus type 16 (HPV-16), and since the HPV-16 E6 and E7 oncoproteins are constitutively expressed in the tumor cells, they are attractive targets for cytotoxic T lymphocyte (CTL)-mediated immunotherapy. Nevertheless, only a limited number of HPV-16 E6 epitopes have been identified to date. Using reverse immunological methods, we have generated a CTL clone against the HPV-16 $E6_{49-57}$ epitope restricted by HLA-A*2402, which is the most common allele in Japan and relatively frequent worldwide, capable of lysing 293T cells transduced with HLA-A*2402 and HPV-16 E6. Although it was unable to recognize the SiHa cervical cancer cell line positive for HPV-16 and HLA-A*2402, the cells became susceptible to lysis when transduced with E6-E7 genes, which was unexpectedly offset by pretreatment with interferon (IFN)-γ alone. Interestingly, however, combined pretreatment with a proteasome inhibitor, bortezomib and IFN-γ fully restored CTL-mediated lysis of the original SiHa cells. Furthermore, such intervention of 2 of 4 other cervical cancer cell lines expressing HPV-16 E6 and HLA-A*2402 was found to induce IFN-γ production by specific CTLs. Tetramer analysis further revealed that induction of $E6_{49-57}$-specific T cells was possible in 5 of 7 patients with HPV-16-positive high grade cervical intraepithelial neoplasia or cervical cancer by *in vitro* stimulation with $E6_{49-57}$ peptide. Thus, these findings together indicate that $E6_{49-57}$ is a candidate epitope for immunotherapy and immunological monitoring of such patients.

Key words: cytotoxic T lymphocyte; tumor immunity; epitope, cervical neoplasm; bortezomib

Cervical cancer, the second most common cancer in women worldwide, with 250,000 new cases diagnosed each year,[1] is causally linked with human papillomavirus (HPV), the first proposed necessary cause of a human cancer as assessed by the World Health Organization.[2] High-risk HPV-16 is the most common type in all countries, with an overall prevalence of more than 50% in cervical cancers,[3] and 42.4% in Japanese patients.[4] Approximately 95% of HPV infections of the anogenital tract resolve spontaneously because of immune responses,[5] and the risk of cervical cancer is markedly increased in patients with immunodeficiency.[6–8] These findings suggest that the immune system plays a pivotal role in preventing development and progression of cervical cancer.

Two HPV oncoproteins, E6 and E7, which can inhibit the tumor suppressors, p53 and RB respectively, are constitutively expressed in cervical cancer cells and appear to be required to maintain their malignant growth.[9] These viral oncoproteins, which are not present in normal cells, have been considered to be attractive targets for specific immunotherapy against cervical cancer, and identification and characterization of cytotoxic T lymphocyte (CTL) epitopes for HPV have facilitated the development of peptide vaccines against cervical cancer. Most of the CTL epitopes identified to date for HPV-16 E6 and E7 are restricted by HLA-A*0201, the most frequent HLA allele in Caucasian populations. Identification of functional HPV-16 epitopes restricted by HLA-A*2402, which is very common in Japanese (phenotype frequency ~60%) and other Asian populations, should provide an opportunity of immunotherapy of HLA-A*2402^+ patients.

Proteasomes play a pivotal role in signal transduction, transcriptional regulation, response to stress, and control of receptor function by orderly degrading cellular proteins.[10] In addition, they make a critical contribution to the generation of antigenic peptides, displaying both exact N and C termini, or intermediates with exact C termini and N-terminal extensions with lengths ranging from 3–24 aa. $CD8^+$ T cells recognize 8–10 aa antigenic peptides presented on MHC class I molecules after transportation into endoplasmic reticulum and further N-terminus trimming. It is known that cells exposed to interferon (IFN)-γ up-regulate other catalytic components and can be equipped with "immunoproteasomes," which favor the generation of peptides suited for presentation by MHC I molecules. Thus, inhibition of proteasome functions by inhibitors can cause not only cellular apoptosis by affecting cellular regulatory proteins,[10] but also modulation of the generation of some antigenic peptides by proteasomes, leading to loss or gain of antigenicity.[11–13] Among various proteasome inhibitors, bortezomib, a boronic acid dipeptide, is the only one to have progressed to clinical trials in cancer patients, and has shown striking activity in patients with advanced multiple myeloma.[14] Bortezomib is a selective but reversible inhibitor of both standard proteasomes and immunoproteasomes.[15–18]

In the present study, using a reverse immunological approach, we identified an HLA-A*2402-restricted HPV-16 E6-derived nonameric epitope, $E6_{49-57}$. A $CD8^+$ T cell clone, specific for this epitope, initially failed to recognize untreated HPV-16^+ cervical

Abbreviations: B-LCL, B-lymphoblastoid cell line; BIMAS, bioinformatics and molecular analysis section; CD40-B, CD40-activated B; CIN, cervical intraepithelial neoplasia; CTL, cytotoxic T lymphocyte; ELISA, enzyme-linked immunosorbent assay; HPV-16, human papillomavirus type 16; IFN, interferon; PBMCs, peripheral blood mononuclear cells.

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cancer cell lines; however, recognition was evident on bortezomib treatment, particularly with simultaneous IFN-γ treatment in combination. Furthermore, induction of $E6_{49-57}$-specific T cells detectable by HLA-A24/$E6_{49-57}$ tetramers was possible in 5 of 7 patients with HPV-16^+ high grade cervical intraepithelial neoplasia (CIN) or cervical cancer by *in vitro* stimulation with the $E6_{49-57}$ peptide. These results indicate $E6_{49-57}$ to be an endogenously processed HLA-A*2402-restricted CTL epitope, and the limited supply of HPV-16 E6 peptides observed in cervical cancer cells may be, at least in part, overcome by coadministration of IFN-γ and bortezomib.

Material and methods

Blood donors and cell lines

This study was approved by the Institutional Review Board of Aichi Cancer Center. Peripheral blood samples were obtained from patients with cervical cancer or cervical intraepithelial neoplasia (CIN) and healthy volunteer donors. All samples were collected after obtaining written informed consent according to the Declaration of Helsinki. HLA typing was carried out at the HLA Laboratory (Kyoto, Japan). Peripheral blood mononuclear cells (PBMCs) were isolated by a standard method, and CD8-positive and -negative fractions were isolated using CD8 microbeads (Miltenyi Biotec, Bergisch-Gladbach, Germany) and cryopreserved until use. B-lymphoblastoid cell lines (B-LCLs) were established by infecting an aliquot of PBMCs with B95-8 supernatant and maintained in RPMI1640 supplemented with 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate and penicillin/streptomycin (referred below as complete medium).

CD40-activated B (CD40-B) cells were generated as previously described,[19] using a human CD40L-transfected NIH/3T3 cell line.[20] HPV-16-positive cervical cancer cell lines, CaSki, SiHa (ATCC, Manassa, VA), SKGIIIa and SKGIIIb, the latter 2 derived from a patient with a moderately differentiated epidermoid cervical cancer,[21] BOKU (Japanese Collection of Research Bioresources, Osaka, Japan) and 293T human embryonic kidney cells were cultured in IMDM, supplemented with 10% FCS, 2 mM L-glutamine and penicillin/streptomycin.

HPV genotyping

Cervical scrapes or tumor samples were evaluated for the presence of HPV DNA using a general primer (Gp5+/GP6+)-based PCR method as described previously.[22] Samples showing positive bands by ethidium bromide staining were further examined by PCR-based restriction fragment length polymorphism methods, using consensus primers for E6 ORF of HPV-16, -18 and -33, followed by digestion with *Rsa* I and *Sau* 3AI.[23] When the Gp5+/Gp6+ primer could not detect HPV DNA in cervical scrapes, total cellular DNA isolated from paraffin-embedded samples was explored as an alternative approach.

Construction of HPV-16 E6-E7 fusion gene expression plasmids

Plasmids encoding an in-frame HPV-16 E6-E7 fusion protein, devoid of 8 nt consisting of the E6 stop codon, the E7 start codon and 2 nt between them, were generated using a conventional overlapping PCR method. In brief, E6 and E7 genes were individually amplified using a template plasmid encoding genomic E6 and E7 (GenBank accession no. AF003015 and K02718) with the following primers: 5′ E6, 5′-ATAgtcgacTCACCATGTTTCAGGACCCACAGGA-3′; 3′ E6, 5′-TGTATCTCCATGCAGCTGGGTTTCTCTACGT-3′; 5′ E7, 5′-GAAACCCAGCTGCATGGAGATACACCTACAT-3′; 3′ E7, 5′-TATggatccTTATGGTTTCATAGAACAGATGG-3′.

The 3′ E6 and 5′ E7 primers contained overlapping sequences (underlined) for the targeted region. Restriction enzyme sites for *Sal*I and *Bam*HI are indicated with lower case letters. The 2 PCR products were conjugated by second PCR, using 5′ E6 and 3′ E7 primers and the final PCR product (E6-E7) was digested with *Sal*I and *Bam*HI and inserted into the pIRES2-EGFP vector (BD Biosciences, San Jose, CA). In addition, an E6-E7-IRES-EGFP fragment excised from pIRES2-EGFP/E6-E7 with *Xho*I and *Not*I was reinserted into the pLBPC retroviral vector and transfected into the Phoenix-GP-GALV cell line[24] (a gift from H.-P. Kiem, Fred Hutchinson Cancer Research Center, Seattle, WA, and G. Nolan, Stanford University, Stanford, CA) for retroviral gene transfer. HLA-A24-negative cell lines (BOKU and CaSki) were transduced retrovirally with HLA-A*2402 cDNA as previously described.[25] HLA-A*2402-transfected cell lines are referred to as "cell line name/A24." After 48 hr, HLA-A*2402-transduced cell lines were further infected with the retroviral vector pLBPC encoding E6-E7-IRES-EGFP. Individual transduced cells were selected with predetermined concentrations of puromycin.

Synthetic peptides and in vitro CTL induction

Peptides with the HLA-A*2402 binding motif within HPV-16 E6 and E7 were predicted by computer algorithms available at the BioInformatics & Molecular Analysis Section (BIMAS) web site (http://bimas.dcrt.nih.gov/molbio/hla_bind)[26] and synthesized using standard Fmoc chemistry.

Induction of CTLs was performed according to standard procedures as described previously.[19] Briefly, thawed $CD8^+$ cells (0.5×10^6/ml) were cocultured with irradiated (96 Gy) autologous peptide-pulsed (10 μM) CD40-B cells (0.5×10^6/ml) in 2 ml RPMI 1640, supplemented with 9% pooled human serum, 2 mM L-glutamine and penicillin/streptomycin (referred to as CTL medium) in the presence of recombinant human IL-7 (50 U/ml) and IL-12 (5 ng/ml; all from R&D Systems, Minneapolis, MN) in 48- or 24-well plates at 37°C in 5% CO_2. On days 7 and 14, the $CD8^+$ cells were restimulated as above. One and three days after each stimulation, recombinant human IL-2 (Chiron, Emeryville, CA) was added to the cultures to a final concentration of 20 IU/ml.

MHC stabilization assay

Binding of the synthesized peptides to HLA-A*2402 molecules was evaluated by an MHC stabilization assay, using T2-A24 cells, as described earlier.[27] Briefly, HLA-A24-transduced T2 cells (2×10^5) were incubated with 200 μL AIM-V medium (Invitrogen) and each of the peptides at a concentration of 10 μM at 27°C for 16 hr, followed by incubation at 37°C for 3 hr. Surface HLA-A24 molecules were then stained with the anti-HLA-A24 mAb (One Lambda, Canoga Park, CA) and FITC-conjugated anti-mouse mAb (BD Biosciences, San Jose, CA). Expression was measured with a FACS Calibur flow cytometer and CellQuest software (BD Biosciences), and mean fluorescence intensity (MFI) was recorded. Percentage MFI increase was calculated as follows: Percent MFI = (MFI with the given peptide − MFI without peptide)/(MFI without peptide) × 100.

Intracellular IFN-γ staining and T cell cloning

Intracellular IFN-γ was detected as previously described with modifications.[28] Briefly, a total of $0.3–0.5 \times 10^6$ cells from each T cell line were stimulated with autologous B-LCL (1×10^6) pulsed with each of 3 peptides (10 μM) in complete medium for 6 hr in the presence of 1 μg/ml brefeldin A and 20 IU/ml IL-2 at 37°C. After incubation, cells were stained with the aid of a Cytofix/Cytoperm Plus kit (BD Biosciences) according to the manufacturer's instructions. In brief, all cells were transferred into 5-ml round-bottomed tubes, washed and stained with PE-conjugated anti-CD3 (BD Biosciences) and PE-cyanine-5-conjugated anti-CD8 (Caltag, Burlingame, CA) for 15 min on ice. After membrane-staining, cells were washed and fixed with 250 μl of Cytofix (BD Biosciences) for 10 min on ice, stained intracellularly with FITC-conjugated anti-IFN-γ (BD Biosciences) for 30 min on ice and then analyzed as above.

T cells from CTL lines showing intracellular IFN-γ production were cloned by limiting dilution in 96-well round-bottomed plates with 5×10^4 PBMCs (33 Gy-irradiated) and 1×10^4 B-LCLs (66

TABLE 1 – CHARACTERS OF SYNTHETIC PEPTIDES AND RESULTS OF THE MHC STABILIZATION ASSAY

Peptide	Sequence	Score (BIMAS)	Score (SYFPEITHI)	% MFI increase
$E6_{49-57}$	VYDFAFRDL	240	23	54
$E6_{87-95}$	CYSVYGTTL	200	20	55
$E6_{98-106}$	QYNKPLCDL	300	21	58
CMV $pp65_{328-336}$	QYDPVAALF	168	24	325

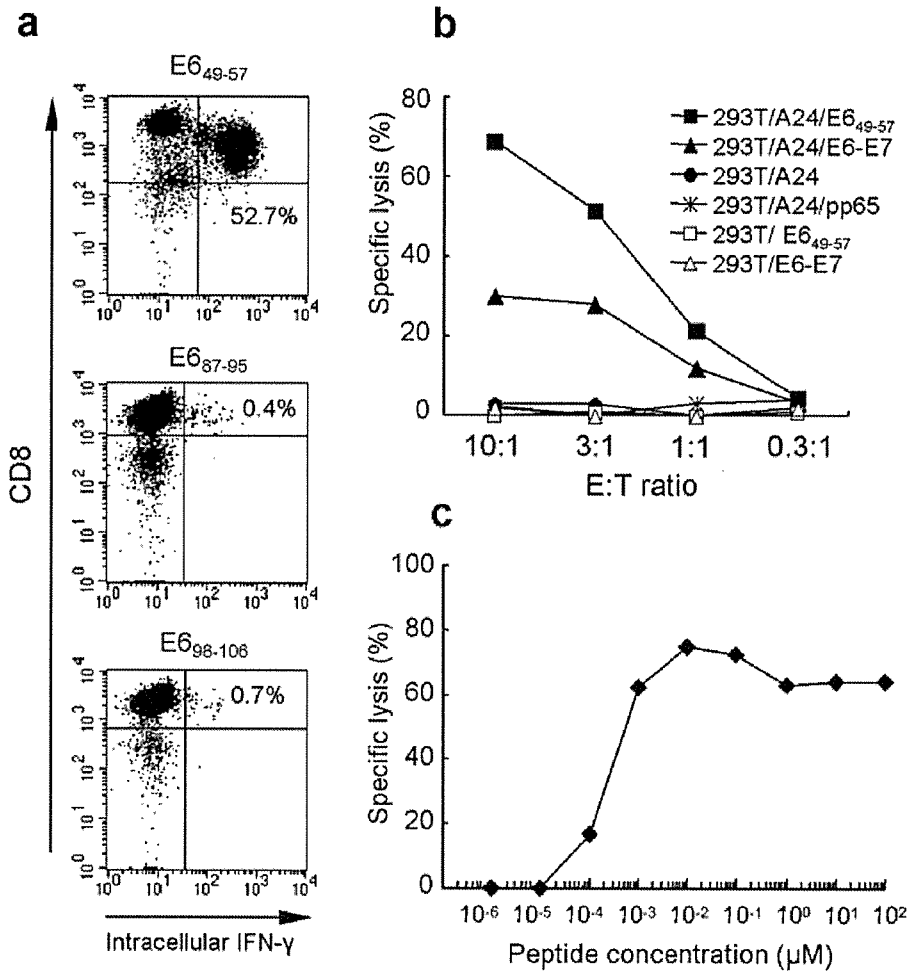

FIGURE 1 – Generation and characterization of HPV-16 E6-specific CTL lines and a clone. (*a*) Intracellular IFN-γ staining of CTL lines. $CD8^+$ cells were cocultured with irradiated autologous peptide-pulsed CD40-B cells in the presence of IL-7 and IL-12. On days 7 and 14, they were restimulated and 1 and 3 days after each stimulation, IL-2 was added to the cultures. Growing T cells were stimulated with autologous B-LCL pulsed with each of the 3 peptides indicated (Table I) for 6 hr in the presence of brefeldin A and IL-2. After incubation, cells were stained anti-CD3 and anti-CD8 followed by anti-IFN-γ, and then flow cytometric analysis was conducted. The percentage of cells gated for CD3 is shown. (*b*) Cytolytic activity of an HPV-16 $E6_{49-57}$-specific CTL clone. 2B2-CTL was isolated from the T cell line specific for the $E6_{49-57}$ peptide by limiting dilution, and cytotoxicity assays were performed by standard 4-hr ^{51}Cr release assay against various 293T target cells: 293T/A24, HLA-A*2402-transduced 293T cells; $E6_{49-57}$, $E6_{49-57}$-pulsed cells; E6-E7, E6-E7 fusion gene-transduced cells; pp65, HLA-A*2402-restricted cytomegalovirus pp65, control peptide-pulsed cells. (*c*) Evaluation of synthetic peptides for epitope reconstitution activity. Autologous B-LCLs were labeled with ^{51}Cr, then pulsed with serial dilutions of HPV-16 $E6_{49-57}$ peptide and used as targets for 2B2-CTL at an E:T ratio of 20:1.

Gy), 30 ng/ml anti-CD3 mAb (OKT3; Ortho Diagnostics, Raritan, NJ) and 50 IU/ml IL-2 in 200 μl CTL medium. Outgrowing wells were identified after 10–14 days and further expanded in 25-cm^2 flasks.

Cytotoxicity assay

Target cells were labeled with 0.1 mCi of $Na_2{}^{51}CrO_4$ overnight, and 1×10^3 target cells/well were mixed with CTL at the E:T ratios indicated in a standard 4-hr cytotoxicity assay in 96-well round-bottom plates. All assays were performed in triplicate. Peptide-pulsed target cells were incubated with peptide (1 μM) for 30 min after ^{51}Cr labeling, and washed twice before use. When indicated, target cells were treated with 100 U/ml IFN-γ (R&D Systems) for 48 hr, 10 μM epoxomicin (Peptide Institute, Osaka, Japan) for 5 hr or 10 μM bortezomib (Millennium Pharmaceuticals, Cambridge, MA) for 5 hr before cytotoxicity assay. Percent-

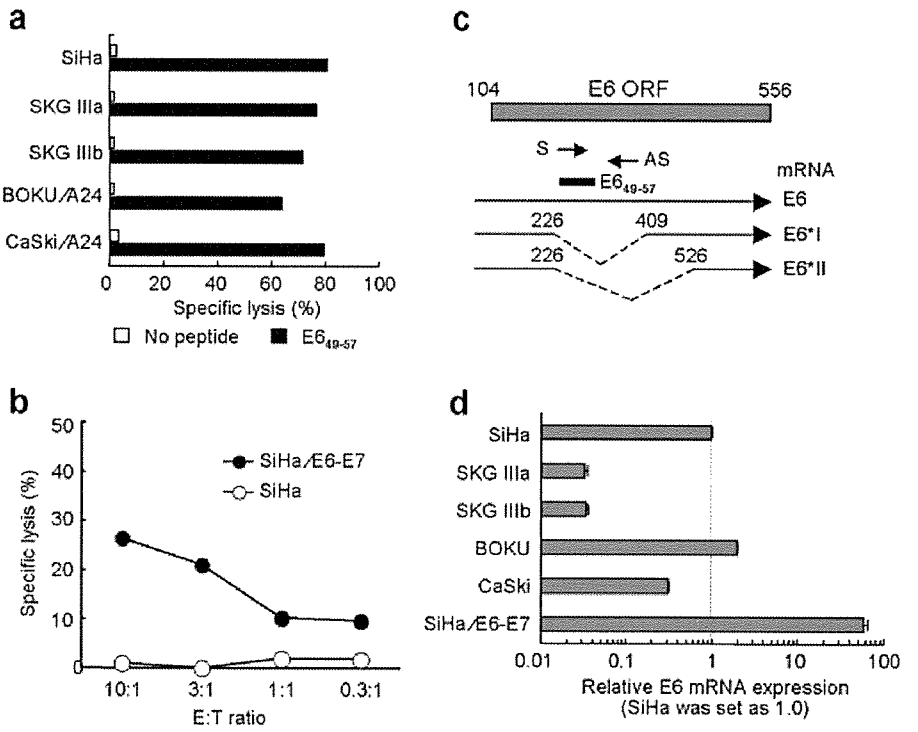

FIGURE 2 – Failure of 2B2-CTL lyse HLA-A*2402$^+$ HPV-16$^+$ cervical cancer cell lines and induction of susceptibility in SiHa cells by forced expression of the HPV-16 E6-E7 gene. (*a*) Cytolytic activity of 2B2-CTL against ^{51}Cr-labeled cervical cancer cell lines with or without the $E6_{49-57}$ peptide (1 μM) at an E:T ratio of 10:1. (*b*) Cytolytic activity of 2B2-CTL against ^{51}Cr-labeled original SiHa cells or those with E6-E7 fusion gene transduction (SiHa/E6-E7). (*c*) Schematic representation of the HPV-16 E6 ORF, mRNAs, locations of the $E6_{49-57}$ epitope and primers used to quantify HPV-16 E6 mRNA expression. (*d*) Real-time RT-PCR analysis of expression of the unspliced form of HPV-16 E6 mRNA in cervical cancer cell lines and SiHa/E6-E7. Expression of unspliced HPV-16 E6 transcripts in each sample was normalized to the internal GAPDH level, and values are expressed relative to the level found in SiHa cells as 1.0.

age specific lysis was calculated as follows: [(Experimental cpm − Spontaneous cpm)/(Maximum cpm − Spontaneous cpm)] × 100. For peptide reconstitution assays, ^{51}Cr-labeled HLA-A*2402$^+$ B-LCLs were incubated for 30 min in medium containing 10-fold serial dilutions of the peptides and then used as targets in standard cytotoxicity assays.

Enzyme-linked immunosorbent assay

Ten thousand CTLs per well were cocultured with the same number of target cells in complete medium in 96-well polypropylene plates. When necessary, target cells were pretreated with IFN-γ and individual proteasome inhibitor, (either) alone or in combination. After overnight incubation at 37°C, 50 μl of supernatant was collected and IFN-γ was measured by enzyme-linked immunosorbent assay (ELISA).

Isolation of mRNA and quantitative RT-PCR for detection of E6 transcripts

Messenger RNA was isolated from ~8 × 10^6 cells of cervical cancer cell lines using a μMACS mRNA Isolation Kit (Miltenyi Biotec). One half microgram of mRNA was converted into cDNA in 30 μl of reaction mixture using an oligo(dT)$_{15}$ primer (Roche, Indianapolis, IN), and M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA). Quantitation of the HPV-16 E6 transcripts was performed using a fluorescence-based real-time detection method (ABI PRISM 7700 Sequence Detection System (TaqMan); Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The primer and probe sequences used to detect the unspliced form of E6 (GenBank Accession No. K02718) were as follows (Fig. 2*b*): sense, 5′-TGCATAGTATATAGAGATGGGAATCCA-3′ (nt 254–280); antisense, 5′-ACGGTTTGTTGTATTGCTGTTCTAAT-3′ (nt 364–389); probe, 5′-(FAM)-TGCTGTATGTGATAAATG-(MGB)-3′ (nt 282–300).

A primer and probe set for human GAPDH was used as an internal control. PCR was performed in a 1× TaqMan Universal PCR master mix containing 10 pmol of each sense and antisense primer, and 2 pmol of probe in a total volume of 25 μl (all from Applied Biosystems). The temperature profile was as follows: 50°C for 2 min, 95°C for 10 min and then 95°C for 15 sec and 60°C for 1 min for 40 cycles. Samples were quantified using relative standard curves for each experiment. All results were normalized with respect to the internal control, and are expressed relative to the levels indicated.

Tetramer construction and flow cytometric analysis

HLA-peptide tetramers were produced as described previously.[29] For staining, cells were incubated with the tetramer at a concentration of 20 μg/ml at room temperature for 15 min followed by FITC-conjugated anti-CD3 (BD Biosciences) and Tricolor anti-CD8 mAb (Caltag) on ice for 15 min. Cervical cancer cell lines were stained with FITC-labeled anti-HLA class I (Beckman Coulter, Fullerton, CA) and analyzed as above.

Results

HPV-16 $E6_{49-57}$ peptide-specific CTLs can be generated and lyse E6-transduced 293T cells

The primary amino acid sequence of HPV-16 E6 and E7 was first analyzed for consensus motifs for novel nonameric peptides capable of binding to HLA-A*2402, the most frequent allele in the Japanese population, and the top 3 candidate peptides with scores of >50 using the BIMAS computer algorithm were chosen (Table I). All were from E6 sequences, as E7 did not contain peptide motifs suitable for HLA-A*2402-binding. The MHC stabilization assay revealed that these peptides similarly increased HLA-A24 expression on the cells, although the increase was inferior to that with a strong A24-binder peptide, $CMVpp65_{328-336}$.[27] These results indicate that the peptides indeed bound and stabilized the HLA complex on cell surfaces.

HLA-A*2402$^+$ PBMCs collected from one CIN patient and 3 healthy volunteer donors were tested for *in vitro* induction of CTLs specific for these peptides. After the third stimulation, cytolytic activity of the CTL lines was assessed. A CTL line that could

lyse peptide-pulsed autologous B-LCL was successfully generated from the CIN patient but not from healthy volunteer donors. Intracellular cytokine staining demonstrated that $E6_{49-57}$ peptide-pulsed autologous B-LCL induced IFN-γ production in 52.7% of $CD3^+$ cells from the CTL line, whereas induction with the other 2 peptides was negligible (Fig. 1*a*). Therefore, T cells specific for the $E6_{49-57}$ peptide (VYDFARDL) were subsequently cloned. One of the representative T cell clones, designated 2B2-CTL, was further expanded and tested.

Cytotoxicity assays showed 2B2-CTL to lyse $E6_{49-57}$ peptide-pulsed HLA-A*2402-transduced 293T cells (referred as 293T/A24) and HPV-16 E6-E7-transduced 293T/A24 cells (Fig. 1*b*), indicating that 2B2-CTL is restricted by HLA-A*2402 and recognizes an endogenously processed E6-derived epitope in 293T cells. The $E6_{49-57}$ peptide was found to have high affinity to HLA-A*2402 molecules because half-maximal lysis of peptide-pulsed autologous B-LCL was achieved at a concentration of 300 pM (Fig. 1*c*), which is comparable to peptide concentrations required for reconstitution of other tumor-associated epitopes reported earlier.[30]

Failure to lyse HPV-16-positive cervical cancer cell lines by 2B2-CTL can be overcome by forced expression of full-length E6 cDNA by gene transfer

To examine the immunotherapeutic potential of HPV-16 $E6_{49-57}$-specific 2B2-CTL, cytotoxic activity against various HPV-16^+ cervical cancer cell lines was tested after transduction with HLA-A*2402 cDNA when necessary. Unfortunately, none of 5 cervical cancer cell lines was originally lysed, but all became susceptible to lysis when the $E6_{49-57}$ peptide was supplied exogenously (Fig. 2*a*), suggesting that E6 proteins are not sufficiently produced or processed by the cellular machinery in these cell lines. To determine whether forced expression of HPV-16 E6 would make such cell lines susceptible to 2B2-CTL, SiHa cells were transduced with E6-E7 cDNA (referred to as SiHa/E6-E7) and tested. As shown in Figure 2*b*, SiHa/E6-E7 cells became susceptible to lysis by 2B2-CTL, indicating that the lack of lysis of original cell lines was likely caused by an insufficient supply of E6 proteins, while the intracellular machinery necessary for processing the HPV-16 $E6_{49-57}$ epitope was retained.

Next, we performed real-time RT-PCR to quantify HPV-16 E6 mRNA. Oncogenic HPVs, such as HPV-16 and HPV-18, have been reported to potentially produce distinct E6 mRNAs including E6, E6*I, E6*II by alternative splicing (Fig. 2*c*), and these transcripts of HPV-16 have been found in cervical cancers and cell lines.[31,32] Since the $E6_{49-57}$ epitope is encoded by nucleotides 226–253, which are spliced out in E6*I and E6*II mRNAs, primers and the probe were designed to detect the unspliced form of E6 mRNA. All these cell lines expressed the unspliced E6 mRNA, but the level varied significantly (Fig. 2*d*). Indeed, the expression level in SiHa/E6-E7 cells was 60-fold higher than that in untransduced SiHa cells (Fig. 2*d*), and this large difference could account for susceptibility to lysis by 2B2-CTL.

Treatment of cervical cancer cell lines with proteasome inhibitors and IFN-γ results in recognition by 2B2-CTL

Although 2B2-CTL recognized the endogenously processed $E6_{49-57}$ epitope and lysed SiHa/E6-E7 cells, forced expression of E6 cDNA was still required to lyse SiHa cells. Since presentation of certain CTL epitopes of tumor and viral antigens is reported to be enhanced by treatment of target cells with proteasome inhibitors,[11–13] we first attempted to sensitize SiHa cells with epoxomicin or bortezomib. Epoxomicin is an irreversible proteasome inhibitor, which is about 100-fold more potent than lactacystin,[12] while bortezomib is a reversible proteasome inhibitor.[15] As shown in Figure 3*a*, treatment of SiHa cells with epoxomicin or bortezomib induced IFN-γ production from 2B2-CTL but not from an irrelevant CTL clone or SiHa cells themselves. This effect was more prominent with epoxomicin than bortezomib. Of note, addition of IFN-γ pretreatment to proteasome inhibitors induced more

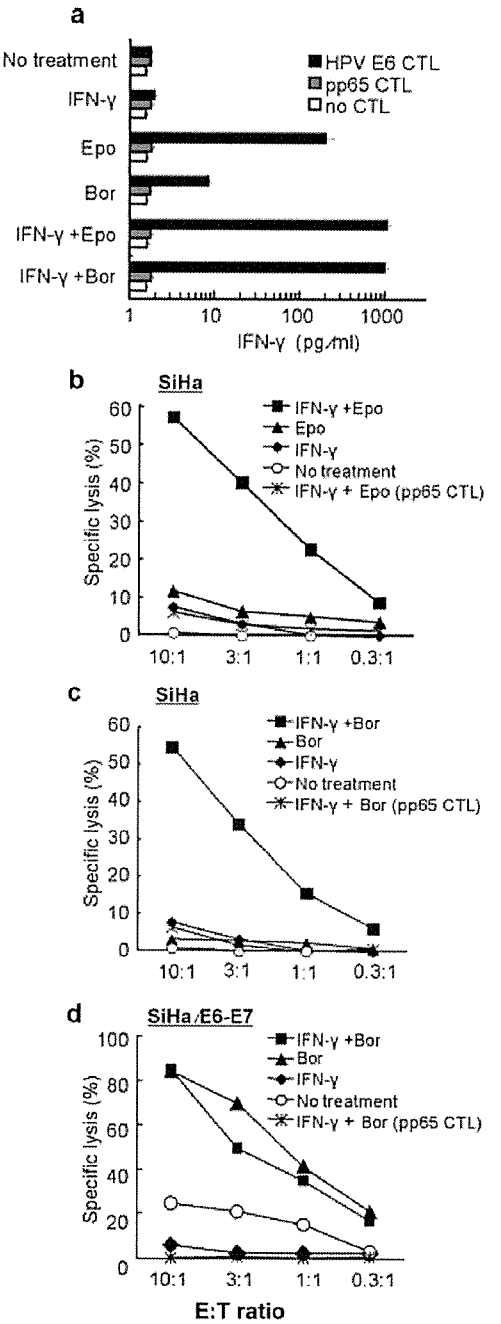

FIGURE 3 — Induction of susceptibility of SiHa cells to 2B2-CTL by treatment with proteasome inhibitors and IFN-γ. (*a*) IFN-γ production by 2B2-CTL after stimulation with pretreated SiHa cells. Supernatants were harvested and assayed by ELISA. Values are means ± SD obtained from triplicate samples in 1 of 2 independent experiments. The proteasome inhibitors used in this assay were epoxomicin (Epo) and bortezomib (Bor). pp65 CTL, with specificity for HLA-A24-restricted CMV pp65 epitope, was used as an irrelevant control CTL. (*b*) Cytolytic activity of 2B2-CTL against SiHa cells treated with epoxomicin (Epo) and IFN-γ was assessed with a standard 4-h ^{51}Cr release assay. (*c*) Cytolytic activity of 2B2-CTL against SiHa cells treated with bortezomib (Bor) and IFN-γ, assessed as in panel (*b*). (*d*) Cytolytic activity of 2B2-CTL against SiHa/E6-E7 cells treated with bortezomib (Bor) and IFN-γ assessed as in panel (*c*).

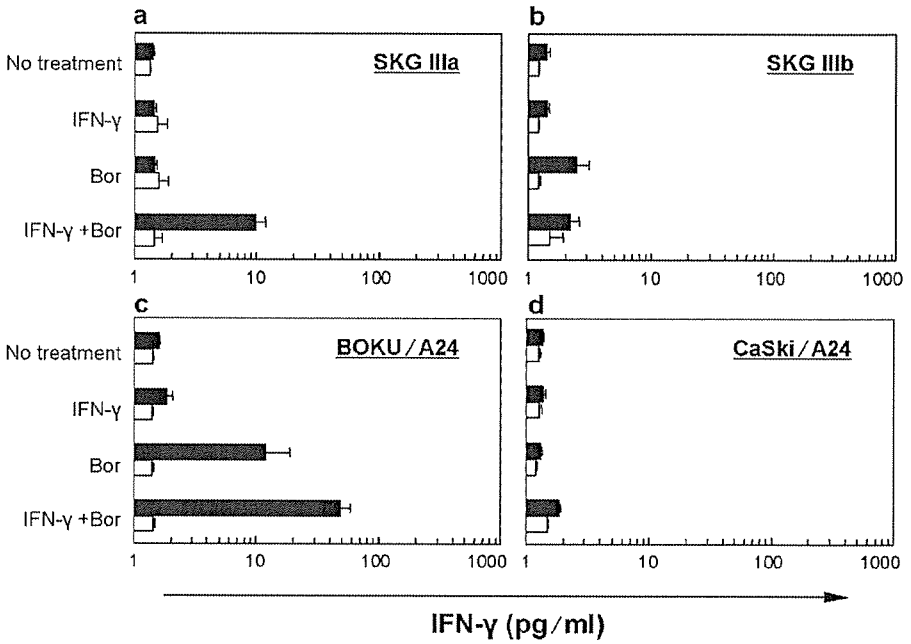

FIGURE 4 – IFN-γ production from 2B2-CTL cocultured with cervical cancer cell lines. After pretreatment of SKG IIIa (*a*), SKG IIIb (*b*), BOKU/A24 (*c*) and CaSki/A24 (*d*) cells with bortezomib (Bor) and IFN-γ, 2B2-CTL (solid bars) or no CTL (open bars) were added. Supernatants were harvested and assayed by ELISA. Values are means ± SD obtained from triplicate samples in 1 of 2 independent experiments.

IFN-γ production by 2B2-CTL, while IFN-γ pretreatment alone was not effective (Fig. 3*a*). The synergistic effect was clearly reflected by lytic activity of 2B2-CTL; SiHa cells pretreated with both IFN-γ and epoxomicin (Fig. 3*b*) or bortezomib (Fig. 3*c*) showed enhanced susceptibility to cytolysis by 2B2-CTL. Surprisingly, SiHa/E6-E7 cells, which were moderately lysed by 2B2-CTL (Fig. 2*d*), underwent no lysis when treated with IFN-γ alone (see Discussion), whereas pretreatment with bortezomib alone gave robust lysis (Fig. 3*d*).

The encouraging results of the efficacy of the combined treatment prompted us to test other cervical cancer cell lines by focusing on clinically available bortezomib.[14] Pretreatment of 4 cervical cancer cell lines (SKG IIIa, SKG IIIb, BOKU/A24 and CaSki/A24) with bortezomib and IFN-γ led to the production of IFN-γ by 2B2-CTL in BOKU/A24 and SKGIIIa, although the level of IFN-γ production was 20- and 100-fold lower, respectively (Figs. 4*a* and 4*c*), when compared with the results in SiHa cells (Fig. 3*a*). Little or no IFN-γ production by 2B2-CTL was observed upon stimulation with SKG IIIb and CaSki/A24 cells (Figs. 4*b* and 4*d*). However, when further transduced with E6-E7 cDNA, CaSki/A24 cells stimulated IFN-γ production to levels comparable to BOKU/A24 in the presence of bortezomib (data not shown), suggesting an insufficient amount of E6 protein in CaSki cells, partly accounting for the lack of recognition (Fig. 4*d*). In addition, none of these 4 cell lines pretreated with epoxomicin and IFN-γ induced significant IFN-γ production (data not shown), unlike SiHa cells. Finally, restoration of IFN-γ production by 2B2-CTL upon stimulation with the pretreated BOKU/A24 and SKGIIIa was not accompanied by efficient lysis by the CTL (data not shown).

Treatment with bortezomib reduces E6 mRNA but its effect on the cell surface expression of HLA class I is marginal

There are many intracellular steps in the generation of cell surface peptide-MHC complexes, which can be targeted in immune evasion by tumors and viruses. To investigate how bortezomib in combination with IFN-γ restored immunogenicity of the cervical cancer cell lines tested, we first examined cell surface expression of HLA class I molecules (Fig. 5*a*). As expected, expression detected by anti-pan HLA class I mAb (clone W6/32) on all the cell lines was up-regulated by IFN-γ (left panels), while 5-hr treatment with bortezomib alone exerted little effect (middle panels). The simultaneous treatment with bortezomib and IFN-γ, however, caused mild reduction of HLA class I expression of all cell lines when compared with those treated with IFN-γ alone (left *vs.* right panels). Because endogenous peptide supply is critical to stabilize cell surface HLA expression, these data indicate that at least net peptide supply to HLA molecules was only slightly affected in IFN-γ-treated cell lines by 5-hr treatment with this proteasome inhibitor.

Next, expression levels of unspliced E6 mRNA were assessed by real-time RT-PCR analysis. The value for SiHa cells was slightly up-regulated by IFN-γ, as previously reported.[33] Unexpectedly, E6 mRNA was down-regulated after treatment with bortezomib alone in all cell lines, to values ranging from 4 to 60% of the untreated cell levels (Fig. 5*b*). In SKG IIIa and SKG IIIb cells, IFN-γ slightly down-regulated E6 mRNA, with further down-regulation on addition of bortezomib. In the case of BOKU cells, bortezomib down-regulated E6 mRNA was fully restored by IFN-γ. The quantitative results of real-time RT-PCR assays were confirmed by Western blot analysis (T. Kiyono, unpublished observations). Collectively, pretreatment of the cervical cancer cell lines with bortezomib was always associated down-regulation of E6 mRNA, and addition of IFN-γ showed mixed results. These findings altogether indicated that the restoration of 2B2-CTL recognition by pretreatment with bortezomib especially in combination with IFN-γ is not due to E6 mRNA up-regulation, but rather is likely caused by modulation of proteasomal function by bortezomib, as reported previously.[11–13]

*HLA-A*2402-restricted $E6_{49-57}$-specific CD8+ T cells can be induced from CIN and cervical cancer patients despite low precursor frequencies*

Identification of the immunogenic HLA-A*2402-restricted HPV-16 E6-derived epitope enabled us to synthesize the HLA-A24/$E6_{49-57}$ tetramer, which clearly stained 2B2-CTL (Fig. 6*a*, right panel) and detected a growing population of $E6_{49-57}$-specific T cells in bulk culture after stimulation from which 2B2-CTL was generated (Fig. 6*a*, middle panel). In a prospective cohort study consisting of twenty HLA-A*2402$^+$ patients with CIN or cervical

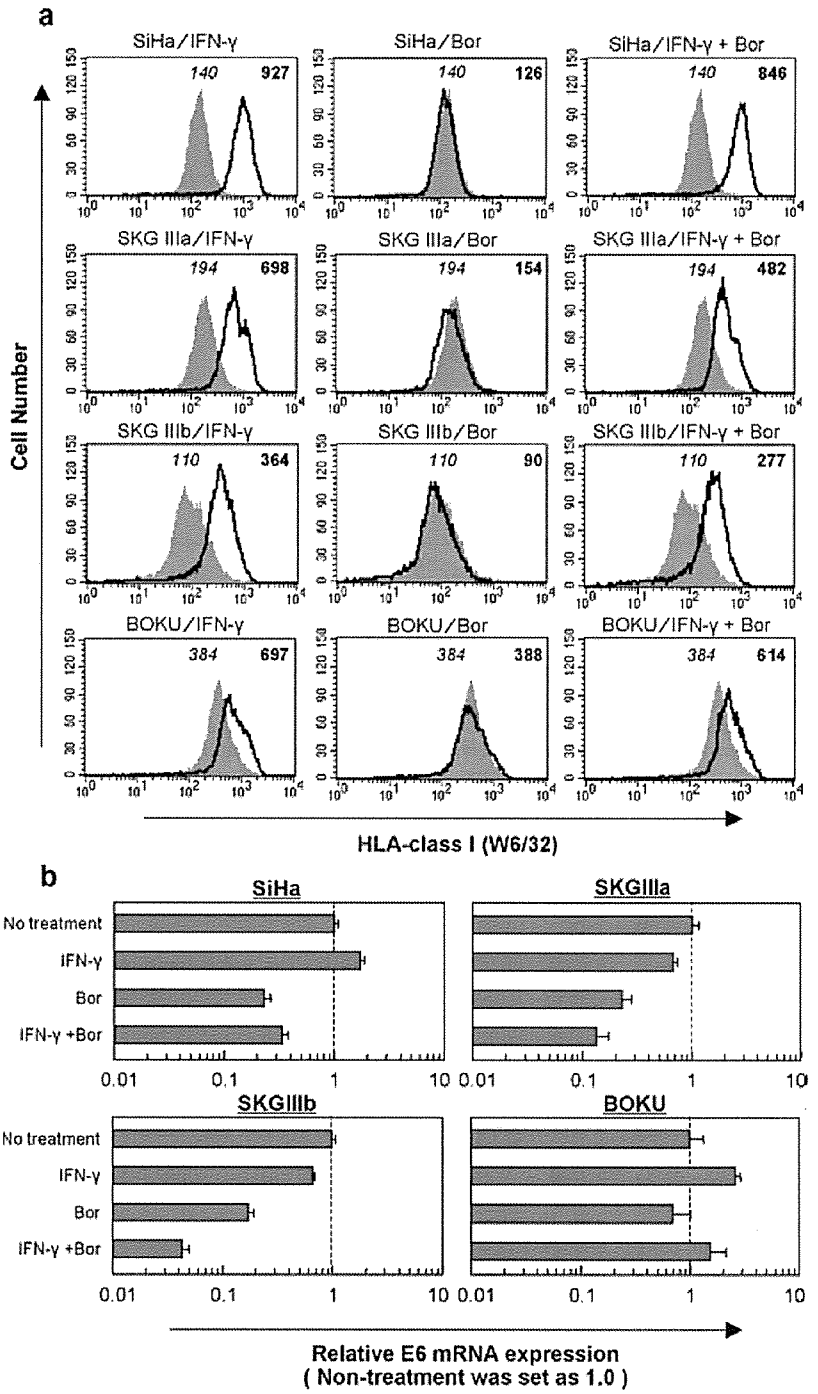

FIGURE 5 – Expression of cell surface HLA-class I molecules and unspliced form of E6 mRNA in cervical cancer cell lines after treatment with bortezomib (Bor) and IFN-γ. (*a*) Flow cytometric analysis of HLA-class I expression on SiHa, SKG IIIa, SKGIIIb and BOKU cells. Open areas represent fluorescence distributions of cervical cancer cells treated with bortezomib and IFN-γ, and filled areas represent those for untreated cervical cancer cells. The mean fluorescence intensity for each result after the indicated treatment (in bold) or no-treatment (in italics) is shown. The mean fluorescence intensity for negative controls (FITC isotype control) was always between 3 and 4. (*b*) Real-time RT-PCR analysis of unspliced HPV-16 E6 transcripts in the cell lines shown in panel (*a*), treated with bortezomib (Bor) and IFN-γ, compared with no-treatment controls. Expression of unspliced HPV-16 E6 transcripts in each sample was normalized to the internal GAPDH level, and values are expressed relative to the level found in the no-treatment control cells as 1.0.

cancer, we investigated if $E6_{49-57}$-specific $CD8^+$ T cells could be induced from their PBMCs by stimulation with peptide-pulsed autologous CD40B cells.[19] $CD8^+$ T cells were stimulated in multiple wells of 48-well plates depending on the yield from individual patients. All those undergoing conization or hysterectomy were found to be positive for certain HPV. Patient characteristics and a summary of the results are shown in Table II. Of the total, 7 were HPV-16^+, 6 were HPV-18^+ and the remaining 7 had unclassified HPV infections, which were at least not HPV-16. HLA-A24/$E6_{49-57}$ tetramer$^+$ T cells were successfully induced in 5 of 7 HPV-16^+ patients and 5 of 13 HPV-16^- patients. Distinct populations of $CD8^+$ tetramer$^+$ cells were observed in these patients after 3 rounds of stimulation, at frequencies from 0.06 to 68.1% of the $CD8^+$ T cells (Figs. 6*b*, 6*c* and Table II). However, tetramer$^+$ cells were not detectable as a distinguishable cluster in the unstimulated $CD8^+$ fraction of any of the patients tested (data not shown), implying that the precursor frequency of HLA-A*2402-restricted $E6_{49-57}$-specific T cells in unstimulated PBMCs was less

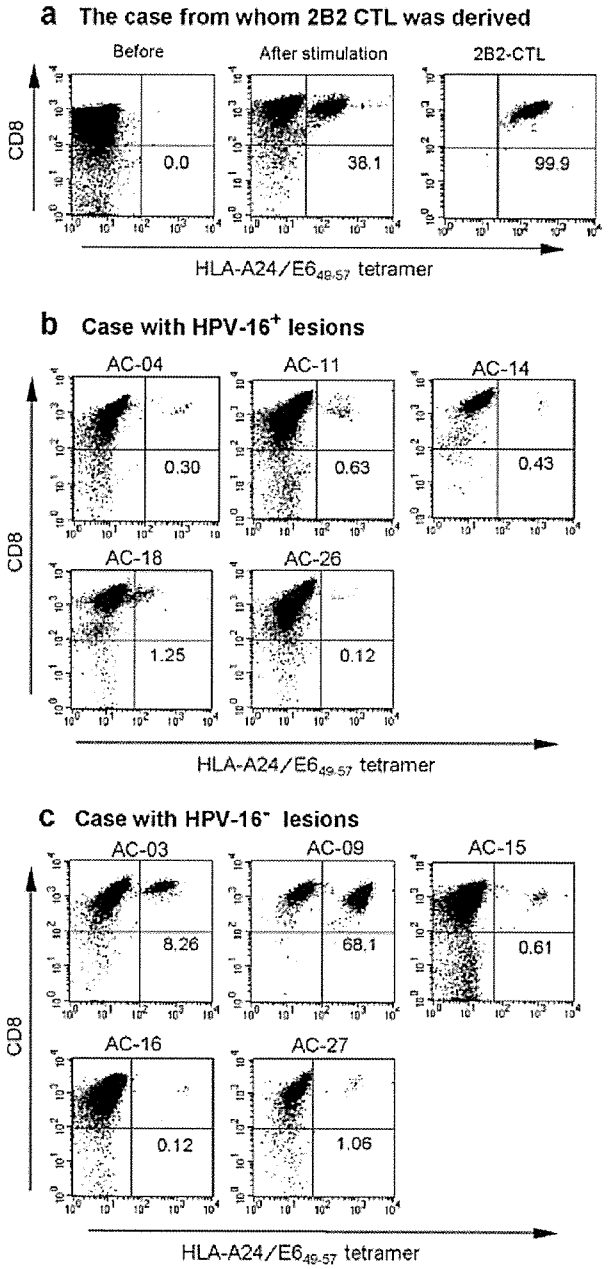

FIGURE 6 – Detection of HLA-A*2402-restricted $E6_{49-57}$-specific $CD8^+$ T cells using the HLA-A24/$E6_{49-57}$ tetramer. Unstimulated peripheral blood $CD8^+$ cells from patients with CIN or cervical cancer were stimulated with $E6_{49-57}$ peptide-pulsed CD40-B cells 3 times, as described in the Materials and Methods. The percentage of T cells binding to the tetramer among all $CD8^+$ is indicated in each panel. (*a*) Results of tetramer staining of unstimulated (left panel) and stimulated (middle panel) T cells from the patient from whom clone 2B2 was generated, and 2B2-CTL (right panel) are shown. (*b*) Representative positive staining results for T cell lines obtained from 5 of 7 patients with HPV-16-positive cervical lesions. (*c*) Representative positive staining results for T cell lines from 5 of 13 patients with HPV-16-negative cervical lesions.

than 10^{-4}. Finally, we further attempted to generate T cell lines from additional CIN patients by stimulating their PBMCs with 2 other candidate peptides (Table I), $E6_{87-95}$ and $E6_{98-106}$ in 2 and 8 patients, respectively, but no T cell lines specific for the peptides could be induced.

TABLE II – $E6_{49-57}$-SPECIFIC T CELL INDUCTION FROM PBMCS OF HLA-A*2402$^+$ PATIENTS DIAGNOSED WITH CIN OR CERVICAL CANCER

Patient group	Case	Age	Histological stage[1]	Induction efficiency[2]	% Tetramer$^+$ in $CD8^+$ cells[3]
HPV-16$^+$ patients	AC-04	24	CIN III	1/1	0.3
	AC-07	55	CIN III	0/3	—
	AC-10	58	CxCa	0/2	—
	AC-11	50	CIN III	1/4	0.63
	AC-14	40	CIN III	2/4	0.43, 0.38
	AC-18	27	CxCa	1/5	1.25
	AC-26	42	CxCa	1/2	0.12
HPV-16$^-$ patients	AC-01	58	CIN III	0/1	
	AC-03	55	CxCa	1/1	8.26
	AC-08	28	CIN III	0/2	—
	AC-09	36	CIN III	4/4	68.1, 38.9, 0.2, 0.06
	AC-15	34	CIN III	1/3	0.61
	AC-16	51	CIN III	2/3	0.12, 0.08
	AC-19	48	CIN II	0/2	—
	AC-20	52	CIN III	0/6	—
	AC-23	43	CIN III	0/5	—
	AC-24	53	CIN III	0/4	—
	AC-25	40	CIN III	0/4	—
	AC-27	36	CIN III	1/5	1.06
	AC-28	55	CIN III	0/2	—

[1]CIN, cervical intraepithelial neoplasia with histological grading; CxCa, cervical cancer.—[2]Number of wells containing A24/$E6_{49-57}$ tetramer$^+$ $CD8^+$ cells out of total wells set-up.—[3]Percentage of tetramer$^+$ cells among $CD8^+$ cells in the tetramer$^+$ wells.

Discussion

High-risk HPV E6 and E7 oncoproteins are indispensable to maintain the malignant growth of cervical cancer cells and are thus almost ubiquitously expressed in cervical lesions. It has been shown that 3 HLA-A*0201 restricted epitopes ($E7_{11-20}$, $E7_{82-90}$ and $E7_{86-93}$) induce CTL responses in HLA-A*0201 transgenic mice, and the generated CTLs lyse HLA-A*0201$^+$ HPV-16-positive CaSki cells.[34] As reported by Muderspach *et al.*,[35] increase of E7 epitope-specific reactivity in cytokine release and cytotoxicity assays was demonstrated in 10 of 16 HPV-16-positive HLA-A*0201$^+$ patients with high-grade cervical or vulvar CIN after vaccination with either $E7_{11-20}$ or $E7_{86-93}$ epitopes, and a proportion of these patients achieved partial clearance of virus infections and regression of lesions. Therefore, we sought to identify HLA-A*2402-restricted CTL epitopes from HPV-16 E6 and E7 oncoproteins by reverse immunology to facilitate immunotherapy for cervical cancer patients. The initial search for peptides carrying the HLA-A*2402 binding motif using a computer algorithm[26] gave only 3 candidate peptides within the E6 sequence. Two of these have been predicted and shown to bind to HLA-A*2402 molecules *in vitro* previously,[36] and we here demonstrated that a 2B2-CTL specific for one of these peptides, $E6_{49-57}$, could recognize an endogenously processed E6 epitope, although combined treatment of the cervical cancer cell lines with proteasome inhibitors and IFN-γ was necessary. Very recently, 2 other HLA-A*2402-restricted HPV-16 E6 CTL epitopes, $E6_{82-90}$ and $E6_{98-107}$, were reported by Hara *et al.*, whose BIMAS scores were similar to our candidate peptides (Table I), 200 and 360, respectively.[37] The reason why $E6_{82-90}$ was not included in our candidate peptides resulted from a nonsynonymous nucleotide difference corresponding to the E6 aa 82–90 region that disrupted the anchor motif for HLA-A*2402 in the HPV-16 DNA sequence we based on (GenBank accession no. AF003015). $E6_{98-107}$ was not considered because we paid little attention to decameric peptides, these being relatively rare for HLA-A*2402.[38] Comparative experiments are now underway to determine the immunogenecity among patients with CIN and cervical cancers.

The SiHa cells used in this study have been shown to express normal levels of TAP1, TAP2 and IFN-γ-inducible proteasome subunits of low molecular mass protein 2 and 7 molecules.[39] Since forced expression of E6 mRNA on transduction of E6-E7 cDNA rendered SiHa cells susceptible to lysis by 2B2-CTL and the recognition was augmented by bortezomib while it was abrogated by IFN-γ (Fig. 3*d*), and E6 mRNA was down-regulated by bortezomib while it was upregulated by IFN-γ (Fig. 5*b*, left upper panel), we speculate the presence of a complicated, but very intriguing balance among E6 protein supply, processing and destruction does exist, at least in SiHa cells. First, there seems no doubt that insufficient E6 oncoprotein supply is a major hurdle for tumor cell escape from CTL recognition. In this regard, it is of note that Evans *et al.* reported using CTL clones specific for an E6-derived epitope restricted by HLA-A*0201, $E6_{29-38}$, appropriate processing and presentation was observed in E6-transduced B-LCLs, but not in the HLA-A*0201 and HPV-16 expressing cell line, CaSki.[39] Although IFN-γ treatment upregulates immunoproteasomes and TAP proteins in CaSki cells, forced expression of E6 by a recombinant vaccinia virus encoding HPV-16 E6/E7 fusion protein was required to render them susceptible to CTL lysis.[39] In contrast, E7 has been shown to be more abundantly expressed than E6 in HPV-16-positive and HPV-18^+ cervical cancer cell lines[40,41] may explain why CTLs specific for 3 HLA-A*0201-restricted E7 epitopes ($E7_{11-20}$, $E7_{82-90}$ and $E7_{86-93}$)[36] could kill CaSki cells (HLA-A*0201^+).[34]

Second, IFN-γ gave a marginal effect on E6 mRNA expression, but its favorable effect on increased cell surface expression of HLA class I molecules was evident in most cell lines tested, which is critical for efficient recognition by $CD8^+$ T cells. Nevertheless, it is likely that IFN-γ treatment facilitated either active destruction of the $E6_{49-57}$ epitope or failure in epitope generation by immunoproteasome,[42] which is induced by IFN-γ as observed in IFN-γ-treated SiHa/E6-E7 cells. It remains to be determined in the future whether IFN-γ secreted from tumor-reactive CTLs might unexpectedly allow tumor cells to escape by further reducing the amount of epitopes possessing kinetics similar to $E6_{49-57}$. How such epitopes can sensitize cognate naive CTLs also warrants attention because professional antigen-presenting dendritic cells are equipped mainly with immunoproteasomes.

Third, bortezomib uniformly diminished mRNA expression levels probably as a result of general suppressive effects *via* NFκB inactivation, although the magnitude varied among cells (Fig. 5*b*). Nevertheless, treatment with bortezomib alone was sufficient to render SiHa/E6-E7 cells susceptible to lysis by 2B2-CTL. Because the restored IFN-γ secretion from 2B2-CTL was observed in all cervical cancer cell lines but the CaSki SKGIIIb cells, either with bortezomib alone or in combination with IFN-γ, it is of note that bortezomib functioned favorably to overcome the insufficient newly generation of HPV-16 $E6_{49-57}$ epitope for a very short time of 5 hr, probably by inhibiting proteasomal destruction of this particular epitope or another potential mechanism (see below). Immunoproteasomes are likely to have more catalytic activity against epitopes, but it is very important to note that this activity is also efficiently inhibited by bortezomib (Fig. 3*d*). Indeed, it has been reported that bortezomib can target both standard proteasomes and immunoproteasomes.[16–18] These findings encourage us to propose complementary roles of the 2 key drugs: bortezomib can facilitate cell lines to produce the $E6_{49-57}$ epitope, while IFN-γ harnesses the antigen-presenting machinery resulting in increased cell surface MHC expression. In addition, increased MHC biosynthesis due to IFN-γ may be favorable to quickly change peptide arrays presented on MHC molecules that include the $E6_{49-57}$ epitope. Finally, the possibility should be taken into consideration that target cancer cells could be prone to induction of apoptosis due to reduction of NF-κB activity by proteasome inhibitors,[43] although we did not observe increased spontaneous cell death in our ^{51}Cr-based cytotoxicity assays.

As described above, we chose proteasome inhibitors to make the limited amount of E6 protein efficiently available for antigen processing. However, several reports have demonstrated that the presentation of certain antigenic peptides is insensitive to proteasome inhibitors.[44,45] These reports provide another explanation for the enhancement of $E6_{49-57}$ epitope processing and presentation by proteasome inhibitors in our study. That is, the epitope might be generated by a nonproteasomal cytosolic protease or by a certain retained proteasome activity other than chymotrypsin-like activity, which is sensitive to epoxomicin or bortezomib treatment examined in our study. Epitopes produced in a proteasome-independent manner will have reduced competition for assembly with MHC class I molecules in the presence of proteasome inhibitors,[13] as the majority of MHC class I ligands are generated by proteasomes, resulting in rather efficient $E6_{49-57}$ peptide presentation. Finally, we surmise that enhanced CTL recognition by proteasome inhibitors is basically an epitope-specific phenomenon rather than a general one from the previous epitope-oriented reports. However, our preliminary studies showed that IFN-γ secretion from a CTL clone specific for HLA-A*0201-restricted HPV-16 $E7_{11-20}$ against SiHa/A2 (HLA-A*0201-transduced SiHa cells) was enhanced by exposure to bortezomib and IFN-γ (data not shown), suggesting that increase in epitope presentation by proteasome inhibitors may not be an exceptional phenomenon.

Results of $E6_{49-57}$ peptide-specific T cell induction from PBMCs of a prospective cohort of patients with CIN and cervical cancer demonstrated that the $E6_{49-57}$ peptide is indeed immunogenic and that most patients possess precursor T cells specific for this epitope. Youde *et al.*[46] have demonstrated HPV-16 $E7_{11-20}$ $tetramer^+$ $CD8^+$ cells in PBMCs to be rare in both CIN patients and healthy controls, but a CTL precursor population became detectable after peptide stimulation, as observed in our study. Thus, we surmise that immunity against E6 oncoprotein does exist in patients despite unsuccessful tumor eradication. There have been several conflicting results regarding CTL responses to HPV-16 E6 and E7 in cancer patients and healthy donors. Bontkes *et al.*[47] demonstrated that patients with persistent HPV-16 infection had specific memory CTL activity against E6 or E7, while no specific CTL activity was detected in HPV-16 negative patients or patients, who were cleared of HPV-16 infections. In contrast, Nakagawa *et al.*[48] demonstrated higher rates of HPV-16 E6-specific CTL responses in women, who had been cleared of HPV-16 infection compared with those who had not. In our study, HLA-A24/$E6_{49-57}$ $tetramer^+$ T cells were induced in both HPV-16^+ and HPV-16^- patients at similar rates. We speculate that some of the latter group might have been successfully cleared of HPV-16 infection but the current cervical neoplasia was caused by a different type of HPV because superinfection with multiple types of HPVs has been shown to be common.[49] In fact, among 13 HPV-16^- patients, 6 were found to have HPV-18^+ lesions, and 2 of them produced HLA-A24/$E6_{49-57}$ $tetramer^+$ T cells. In addition, the homology of E6 epitope of HPV-16 and HPV-18 is not very high (*i.e.* VYDFAFRDL *vs.* VFEFAFKDL; shared residues are underlined). These results suggest the possibility of past infection with HPV-16. The low CTL precursor frequency specific for HPV-16, possibly caused by poor natural immunogenicity of this virus as discussed above compared to more immunogenic viruses such as EBV, makes it difficult to address the question because serological tests do not detect previous infectious events with this virus.

In conclusion, our results demonstrate that $E6_{49-57}$ is a naturally-processed HPV-16 antigen, which can induce specific CTL in patients with cervical neoplasia. We also could demonstrate that the natural poor immunogenicity of HPV-associated cervical neoplasia may be in part overcome with a proteasome inhibitor, bortezomib, by facilitating generation of cryptic peptides in combination with IFN-γ. Since bortezomib and IFN-γ have been used clinically, they may provide patients suffering from advanced HPV-16 neoplasia with a new therapeutic option to be tested in future clinical studies.

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Effects of HLA Allele and Killer Immunoglobulin-Like Receptor Ligand Matching on Clinical Outcome in Leukemia Patients Undergoing Transplantation With T-cell–Replete Marrow From an Unrelated Donor

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ABSTRACT

The responsible human leukocyte antigen (HLA) locus and the role of killer immunoglobulin-like receptor (KIR) ligand matching on transplantation outcome were simultaneously identified by multivariate analysis in 1790 patients with leukemia who underwent transplantation with T-cell–replete marrow from an unrelated donor (UR-BMT) through the Japan Marrow Donor Program. The graft-versus-leukemia (GVL) effect depended on leukemia cell type. HLA-C mismatch reduced the relapse rate in acute lymphoblastic leukemia (ALL) (hazard ratio [HR] = 0.47; $P = .003$), and HLA-DPB1 mismatch reduced it in chronic myeloid leukemia (CML) (HR = 0.35; $P < .001$). In contrast, KIR2DL ligand mismatch in the graft-versus-host (GVH) direction (KIR-L-MM-G) increased in ALL (HR = 2.55; $P = .017$). An increased rejection rate was observed in KIR2DL ligand mismatch in the host-versus-graft direction (HR = 4.39; $P = .012$). Acute GVH disease (GVHD) was increased not only in the mismatch of HLA-A, -B, -C, and -DPB1, but also in KIR-L-MM-G. As a whole, the mismatch of HLA-A, -B, and -DQB1 locus and KIR-L-MM-G resulted in increased mortality. In conclusion, not only the mismatch of HLA-C and -DPB1, but also KIR-L-MM-G affected leukemia relapse, which should be considered based on leukemia cell type. Furthermore, KIR-L-MM induced adverse effects on acute GVHD (aGVHD) and rejection, and brought no survival benefits to patients with T-cell–replete UR-BMT.

KEY WORDS

KIR ligand incompatibility • HLA • Leukemia • Unrelated bone marrow transplantation

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) from a human leukocyte antigen (HLA)-matched unrelated (UR) donor has been established as one mode of curative therapy for hematologic malignancies and other hematologic or immunologic disorders [1,2]. Extensive research on genetic factors such

as HLA has produced mounting evidence of the presence of HLA alleles that drastically affect HSCT outcome through T cells. Induction of the graft-versus-leukemia (GVL) effect to reduce relapse of leukemia is considered an advantage of allogeneic HSCT [3]. There have been several large-scale analyses of UR-HSCT. The Japan Marrow Donor Program (JMDP) demonstrated the effect of matching of HLA class I alleles (HLA-A, -B, and -C) on the development of severe acute graft-versus-host disease (aGVHD) and the importance of HLA-A and -B allele matching for better survival in T-cell–replete UR-HSCT [4,5]. The Fred Hutchinson Cancer Research Center and the US National Marrow Donor Program (NMDP) reported the importance of HLA class II matching in GVHD and survival [6,7]. Updated analysis of the NMDP indicated that HLA-A allele-level mismatching, HLA-B serologic mismatching, and HLA-DRB1 mismatching are significant risk factors for severe aGVHD, and that disparity in HLA class I (HLA-A, -B, or -C) and/or HLA-DRB1 increased the mortality [8]. Furthermore, the role of HLA-DPB1 matching has been elucidated for aGVHD [9-11] and leukemia relapse [12]. However, the aforementioned reports have produced considerable conflicting results.

It has become evident that natural killer (NK) cells and the subpopulation of T cells express NK cell receptors, and that the activity of NK cells is controlled by the recognition of HLA class I molecules on the target cells by NK cell inhibitory and activating receptors [13,14]. The genotype and haplotype of the killer immunoglobulin-like receptors (KIRs) have been identified, and ligand specificities of KIRs have been characterized. C1 specificity of the HLA-C epitope (Asp80) is the ligand of inhibitory KIR2DL2/3, C2 specificity (Lys80) is the ligand of inhibitory KIR2DL1, and HLA-Bw4 is the ligand of KIR3DL1. With allogeneic HSCT, the disparities of these receptors between donor and recipient are suspected to induce transplant-related immunologic events through activation of NK cells, and evidence of the clinical outcome of HSCT in relation to KIR disparities has been accumulated [15]. However, reports of KIR ligand matching in UR-HSCT have shown contradictory results [16]. Limited patient numbers, different diseases, and various GVHD prophylaxes make it difficult to draw definite conclusions from these studies.

In the present study, the effects of HLA locus and KIR ligand matching were simultaneously analyzed in leukemia patients receiving T-cell–replete marrow from unrelated donors through the JMDP after a myeloablative conditioning regimen, focusing in particular on the influence of leukemia cell type on the GVL effect.

PATIENTS AND METHODS

Patients

A total of 1790 consecutive leukemia patients who underwent transplantation with marrow from a serologically HLA-A, -B, and -DR antigen–matched donor in Japan between January 1993 and March 2000 through the JMDP were analyzed. No patients receiving T-cell–depleted marrow and/or antithymocyte globulin (ATG) as GVHD prophylaxis were eligible for this study. Partial HLA-A and -B alleles and complete HLA-DRB1 alleles were identified as confirmatory HLA typing during the coordination process, and HLA-A, -B, -C, -DQB1, and -DPB1 alleles were retrospectively reconfirmed or identified after transplantation. The final clinical survey of these patients was completed as of June 1, 2005. Informed consent was obtained from patient and donor according to the Declaration of Helsinki, and approval was obtained from the JMDP and the Institutional Review Board of the Aichi Cancer Center.

Characteristics of patients and donors are listed in Table 1. The patients' age ranged from 0 to 59 years (median, 27 years), and donors' age ranged from 20 to 51 years (median, 35 years). There were 577 patients with acute myeloblastic leukemia (AML), of whom 186 underwent transplantation while in first complete remission (CR), 191 who did so while in second or further CR, and 200 who did so while in non-CR; 617 patients with acute lymphoblastic leukemia (ALL), of whom 236 underwent transplantation while in first CR, 207 who did so while in second or further CR, and 174 who did so while in non-CR; and 596 patients with chronic myeloid leukemia (CML), of whom 417 were in the first chronic phase (CP), 34 were in the second or further CP, 90 were in the accelerated phase, and 55 were in the blastic phase. Standard risk for leukemia relapse was defined as the status of the first CR of AML and ALL and the first CP of CML at transplantation, whereas high risk was defined as a more advanced status than standard risk in AML, ALL, and CML.

HLA Typing of Patients and Donors

Alleles at the HLA-A, -B, -C, -DRB1, -DQB1, and -DPB1 loci were identified as described previously [4,5]. HLA 6 locus alleles were typed in 1773 pairs, and HLA 5 locus alleles except HLA-DPB1 were typed in 17 pairs. HLA genotypes of HLA-A, -B, -C, -DQB1, and -DPB1 alleles of patient and donor were reconfirmed by the Luminex microbead method (100 System; Luminex, Austin, TX) adjusted for the JMDP [17] and in part by the sequencing-based typing method in 2004 and 2005. As a result, all HLA alleles that were observed with $> 0.1\%$ frequency among Japanese were identified. The numbers of

Table 1. *Patient characteristics and matching status of HLA allele and KIR2DL ligand*

	Patient Number (%) M/MM*	Patient Age Median (years) M/MM*	Patient Sex Female (%) M/MM*	Donor Age Median (years) M/MM*	Donor Sex Female (%) M/MM*	Sex Match (%) M/MM*	Stage at Transplant High (%) M/MM*	GVHD Prophylaxis Cyclosporine (%) M/MM*	Total Body Irradiation (%) M/MM*
All leukemia (n = 1790)									
HLA-A	1484/306	27/26	39/37	34/33	38/40	57/55	52/57	73/73	83/72
HLA-B	1645/145	27/26	40/34	34/35	39/36	56/63	52/51	72/76	83/84
HLA-C	1256/534	27/26	39/41	34/33	38/40	56/58	52/55	74/70	83/82
HLA-DRB1	1434/356	27/26	40/38	34/34	38/41	57/57	51/60	74/66	83/82
HLA-DQB1	1391/399	27/26	40/38	34/33	38/41	57/57	52/56	74/67	83/83
HLA-DPB1	612/1163	26/27	42/39	34/34	39/39	60/56	50/55	75/71	81/84
KIR2DL-G†	1693/97	26/27	39/35	34/34	39/43	57/74	53/63	73/64	83/84
KIR2DL-R‡	1679/111	27/25	39/40	34/32	39/60	57/51	53/59	73/67	83/84
Acute myeloblastic leukemia (n = 577)									
HLA-A	486/91	28/27	44/44	33/33	38/39	58/55	67/71	72/60	81/89
HLA-B	537/40	27/31	45/33	33/35	39/30	56/73	67/83	71/68	83/80
HLA-C	405/172	28/28	43/45	33/34	39/37	56/61	66/73	74/63	82/83
HLA-DRB1	474/103	28/27	44/43	33/33	37/47	58/55	66/77	72/63	82/86
HLA-DQB1	469/108	27/29	45/40	33/33	38/43	57/56	67/72	72/64	83/81
HLA-DPB1	206/366	27/28	48/42	34/33	40/38	58/57	65/70	71/70	81/84
KIR2DL-G†	546/31	28/28	43/55	33/33	38/39	57/65	67/71	72/52	82/83
KIR2DL-R‡	546/31	28/28	43/55	33/35	38/39	59/32	68/68	71/58	82/83
Acute lymphoblastic leukemia (n = 617)									
HLA-A	515/102	20/19	41/40	34/32	42/42	55/50	60/69	73/74	91/88
HLA-B	567/50	19/20	41/42	33/36	42/38	54/60	61/70	72/80	91/86
HLA-C	437/180	19/19	41/41	34/32	41/42	54/57	61/63	73/72	91/89
HLA-DRB1	485/132	19/19	41/42	33/33	43/36	55/52	61/64	74/70	90/90
HLA-DQB1	467/150	19/20	41/41	34/33	42/41	55/51	61/63	75/68	90/92
HLA-DPB1	190/425	19/29	43/40	34/33	38/43	61/52	61/62	77/71	89/91
KIR2DL-G†	587/30	20/17	42/20	33/35	42/40	55/53	61/73	73/73	91/83
KIR2DL-R‡	577/40	19/19	39/40	34/30	42/43	54/53	61/73	73/70	90/93
Chronic myelocytic leukemia (n = 596)									
HLA-A	483/113	32/31	33/35	34/34	35/40	59/60	29/35	73/81	76/72
HLA-B	541/55	32/29	34/27	34/37	36/38	56/60	29/36	74/78	74/85
HLA-C	414/182	32/31	33/36	35/34	35/39	60/58	30/31	74/76	75/74
HLA-DRB1	475/121	32/33	34/31	34/36	35/40	58/63	27/41	77/64	76/70
HLA-DQB1	455/141	32/31	34/33	35/33	35/39	57/65	28/35	76/69	75/74
HLA-DPB1	216/372	31/33	35/33	34/35	38/34	60/59	28/31	76/73	73/76
KIR2DL-G†	560/36	32/32	34/31	35/32	35/50	59/53	29/44	75/67	71/83
KIR2DL-R‡	556/40	32/27	34/28	35/31	36/38	59/65	29/38	75/68	75/75

Standard-first complete remission or first chronic phase; high more advanced stage than standard.
*M/MM match/mismatch in GVH direction for HLA matching.
†KIR2DL ligand mismatching in GVH direction.
‡KIR2DL ligand mismatching in HVG direction.

317

identified alleles in this study were 25 in HLA-A, 43 in HLA-B, 20 in HLA-C, 33 in HLA-DRB1, 14 in HLA-DQB1, and 21 in HLA-DPB1.

Matching of HLA Allele and KIR2DL Ligand

For the analysis of GVHD and leukemia relapse, HLA allele mismatch among the donor–recipient pair was scored when the recipient's alleles were not shared by the donor (graft-versus-host [GVH] direction). For graft rejection, HLA allele mismatch among the donor–recipient pair was scored when the donor's alleles were not shared by the patient (host-versus-graft [HVG] direction). For survival, the mismatch was defined as that of either the GVH direction or the HVG direction.

KIR2DL ligand specificity of HLA-C antigen was determined according to the HLA-C allele. The epitope of HLA-Cw3 group (C1 specificity) consists of Asn80, and that of the HLA-Cw4 group (C2 specificity) consists of Lys80.

KIR ligand mismatch in the GVH direction (KIR-L-MM-G) was scored when the donor's KIR2DL epitope of HLA-C was not shared by the patient epitope. This mismatch occurred when KIR2DL2/3- or KIR2DL1-positive effector cells were activated without the expression of corresponding HLA-C ligand (C1 or C2, respectively) on the patient's target cells. KIR ligand mismatch in HVG direction (KIR-L-MM-R) was scored when the patient's KIR2DL epitope of HLA-C was not shared by the donor. This mismatch occurred when patient KIR2DL2/3- or KIR2DL1-positive effector cells were activated without the expression of corresponding HLA-C ligand (C1 or C2, respectively) on donor cells.

Matching Status of HLA Locus in Allele Level and KIR2DL Ligand

The matching status of HLA allele matching in the GVH direction in each HLA locus and KIR ligand matching in both directions are given in Table 1. The HLA-C epitope of KIR2DL was estimated from HLA-C allele type, with 92.4% of the HLA-C allele belonging to the Cw3 group (C1 specificity) and 7.6% belonging to the Cw4 group (C2 specificity). KIR2DL ligand match in both directions occurred in 1583 pairs (88.4%). KIR-L-MM-G, which occurred in the combination of KIR2DL ligand in patient–donor pairs, was found in 97 pairs (5.4%): C1/C1 and C1/C2 in 92 pairs, C2/C2 and C1/C2 in 4 pairs, and C1/C1 and C2/C2 in 1 pair. KIR-L-MM-R, which occurred in the combination of KIR2DL ligand in patient and donor pairs, was found in 111 pairs (6.2%): C1/C2 and C1/C1 in 105 pairs, C1/C2 and C2/C2 in 5 pairs, and C1/C1 and C2/C2 in 1 pair. Mismatches in both directions were found in only 1 pair. Because all pairs were a serologic HLA-B match in this study, the combination of KIR3DL1 and its ligand of Bw4 matched in all pairs.

Definition of Transplantation-Related Events

The occurrence of aGVHD was evaluated according to grading criteria in patients who survived more than 8 days after transplantation, and chronic GVHD (cGVHD) according to the criteria in patients who survived more than 100 days after transplantation as described previously [5]. Rejection was defined as when the peripheral granulocyte count became $< 500/\mu L$ with the finding of severe hypoplastic marrow in engrafted patients. Engraftment was defined as a peripheral granulocyte count of $> 500/\mu L$ for 3 successive days in patients surviving > 21 days after transplantation.

GVHD Prophylaxis

Among the 1790 patients transplanted with T-cell–replete marrow, 1302 received a cyclosporine-based regimen and 488 received a tacrolimus-based regimen for GVHD prophylaxis. Anti-thymocyte globuline (ATG) was not given for GVHD prophylaxis.

Preconditioning Regimen

All patients were preconditioned with a myeloablative regimen, with 1480 receiving total body irradiation (TBI)-containing regimens and 310 receiving non-TBI regimens.

Statistical Analysis

All of the analyses were conducted using STATA version 8.2 (STATA Corp, College Station, TX). Overall survival rate was assessed by the Kaplan-Meier product limit method [18]. Cumulative incidences of aGVHD, cGVHD, rejection, and leukemia relapse were assessed as described previously to eliminate the effect of competing risk [19,20]. The competing events regarding aGVHD, cGVHD, rejection, and relapse were defined as death without aGVHD, cGVHD, rejection, and relapse, respectively. For each endpoint, a log-rank test was applied to assess the impact of the factor of interest.

Cox proportional hazard models [21] were applied to assess the impact of HLA allele matching (mismatch vs match [hazard risk = 1.0] as a reference group) as well as KIR ligand matching (mismatch vs match in the GVH direction and mismatch vs match in the HVG direction) including the following confounders. The confounders considered were sex (donor–recipient pairs), patient age (older: linear), donor age (older: linear), type of disease (AML, CML, or ALL), risk of leukemia relapse (high vs standard),

Table 2. *Effects of HLA and KIR ligand matching for leukemia relapse*

	All Leukemia Cell Types			Acute Myeloblastic Leukemia			Acute Lymphoblastic Leukemia			Chronic Myeloid Leukemia		
	HR*	(95% CI)	P	HR	(95% CI)	P	HR	(95% CI)	P	HR	(95% CI)	P
HLA-A	1.19	(0.89-1.59)	.251	0.92	(0.54-1.58)	.761	1.18	(0.76-1.86)	.462	1.63	(0.89-2.97)	.114
HLA-B	1.01	(0.65-1.59)	.953	1.36	(0.65-2.88)	.416	0.98	(0.48-1.98)	.952	0.62	(0.22-1.76)	.367
HLA-C	0.71	(0.53-0.96)	.025	0.8	(0.49-1.30)	.366	0.47	(0.28-0.78)	.003	1.2	(0.62-2.29)	.591
HLA-DRB1	1.05	(0.73-1.53)	.789	0.78	(0.40-1.52)	.466	0.91	(0.51-1.61)	.737	1.25	(0.55-2.85)	.59
HLA-DQB1	1.10	(0.77-1.58)	.579	1.55	(0.82-2.95)	.178	1.11	(0.63-1.95)	.71	0.86	(0.39-1.93)	.72
HLA-DPB1	0.68	(0.55-0.85)	.001	0.76	(0.52-1.09)	.137	0.92	(0.65-1.28)	.604	0.35	(0.21-0.58)	<.001
KIR2DL-G†	1.55	(0.92-2.63)	.103	1.05	(0.37-3.02)	.926	2.55	(1.18-5.52)	.017	1.23	(0.38-3.94)	.727
KIR2DL-R‡	0.73	(0.40-1.34)	.313	0.53	(0.15-1.78)	.305	1.30	(0.53-3.19)	.569	0.5	(0.14-1.80)	.292

HLA matching in GVH direction.
*Hazard ratio of mismatch with match as a reference adjusted for patient age, donor age, sex-matching disease, GVHD prophylaxis, total body irradiation, transplanted cell dose, risk status, and other matching status of HLA and KIR ligand.
†KIR2DL ligand mismatching in GVH direction.
‡KIR2DL ligand mismatching in HVG direction.

GVHD prophylaxis (tacrolimus-based vs cyclosporine-based and ATG vs cyclosporine-based), numbers of transplanted cells (linear), and preconditioning (non-TBI vs TBI). The numbers of nucleated cells before manipulation of bone marrow were replaced with the numbers of transplanted cells.

Multivariate analysis for clinical outcomes, including KIR ligand matching and HLA-C matching in all pairs (not restricted to HLA-C mismatch), made it possible to evaluate whether these factors are independent. The results of all pairs by multivariate analysis are presented in the Results section and in Tables 2, 3, and 4. HLA-C–mismatched pairs were selected for the analysis of cumulative incidence in KIR ligand matching.

RESULTS

Effects of HLA Locus Mismatch and KIR Ligand Mismatch on Leukemia Relapse

When all leukemia patients (AML, ALL, and CML) were analyzed together, HLA-C mismatch was found to be a factor reducing the relapse rate (HR = 0.71; P = .025) (Table 2). This GVL effect was remarkable in ALL patients (HR = 0.47; P = .003), especially in high risk (HR = 0.40; P = .004) but not in standard risk (HR = 0.85; P = .728). No such effect was observed in AML patients (HR = 0.80; P = .366) or CML patients (HR = 1.20; P = .591).

Cumulative incidence curves of relapse by leukemia cell type are shown in Figure 1. The relapse rate 5 years after transplantation was 16.7% (95% confidence interval [CI] = 11.6%-30.9%) for HLA-C mismatch and 29.8% (95% CI = 25.5%-34.3%) for HLA-C match in ALL patients (P = .012); 17.6% (95% CI = 12.2%-23.8%) and 25.9% (95% CI = 21.1%-30.9%), respectively, in AML patients (P = .342); and 11.7% (955 CI = 12.2%-23.8%) and 12.0% (95% CI = 9.0%-15.4%), respectively, in CML patients (P = .485).

HLA-DPB1 mismatch was shown to reduce the overall leukemia relapse rate (HR = 0.68; P = .001) (Table 2). This effect was significant in CML (HR =

Table 3. *Effects of HLA and KIR ligand matching for acute GVHD, chronic GVHD, and rejection in all leukemia cell types*

	Acute GVHD (Grade 2-4) (n = 1751)			Acute GVHD (Grade 3-4) (n = 1751)			Chronic GVHD (n = 1109)			Rejection (n = 1664)		
	HR*	95% CI	P	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P
HLA-A	1.22	(1.02-1.46)	.034	1.44	(1.11-1.86)	.006	1.41	(1.08-1.85)	.013	0.72	(0.24-2.14)	.555
HLA-B	1.43	(1.28-1.82)	.003	1.40	(1.00-1.95)	.05	1.00	(0.65-1.52)	.991	1.16	(0.32-4.16)	.82
HLA-C	1.29	(1.08-1.55)	.006	1.39	(1.06-1.83)	.017	1.38	(1.07-1.78)	.014	1.87	(0.72-4.86)	.201
HLA-DRB1	1.15	(0.90-1.47)	.254	1.09	(0.77-1.54)	.644	0.91	(0.63-1.31)	.607	0.49	(0.10-2.33)	.366
HLA-DQB1	1.02	(0.81-1.29)	.871	1.13	(0.81-1.59)	.465	1.20	(0.85-1.69)	.288	0.62	(0.07-5.16)	.536
HLA-DPB1	1.39	(1.19-1.63)	<.001	1.26	(1.00-1.60)	.053	0.86	(0.70-1.05)	.138	1.08	(0.59-2.41)	.843
KIR2DL-G†	1.70	(1.28-2.26)	<.001	2.35	(1.62-3.40)	<.001	1.13	(0.68-1.87)	.64	0.62	(0.07-5.16)	.655
KIR2DL-R‡	1.04	(0.77-1.42)	.78	1.33	(0.88-2.02)	.18	0.88	(0.55-1.42)	.603	4.39	(1.38-13.96)	.012

HLA matching in GVH direction for acute GVHD and chronic GVHD, and HLA matching in HVG direction for rejection.
*Hazard ratio of mismatch with match as a reference adjusted for patient age, donor age, sex-matching disease, GVHD prophylaxis, total body irradiation, transplanted cell dose, risk status, and other matching status of HLA and KIR ligand.
†KIR2DL ligand mismatching in GVH direction.
‡KIR2DL ligand mismatching in HVG direction.

Table 4. *Effects of HLA and KIR ligand matching for mortality*

	All Leukemia Cell Types			Acute Myeloblastic Leukemia			Acute Lymphoblastic Leukemia			Chronic Myeloid Leukemia		
	HR*	95% CI	P	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P
HLA-A	1.36	(1.16-1.59)	<.001	1	(0.75-1.34)	.978	1.46	(1.11-1.90)	.006	1.77	(1.35-2.33)	<.001
HLA-B	1.40	(1.13-1.73)	.002	1.43	(0.96-2.12)	.079	1.47	(1.03-2.09)	.036	1.18	(0.80-1.72)	.402
HLA-C	1.17	(0.99-1.37)	.067	1.18	(0.89-1.55)	.246	0.99	(0.74-1.31)	.928	1.42	(1.04-1.93)	.025
HLA-DRB1	0.92	(0.74-1.15)	.463	0.74	(0.50-1.10)	.136	1.04	(0.72-1.49)	.849	0.99	(0.65-1.50)	.951
HLA-DQB1	1.28	(1.04-1.58)	.018	1.29	(0.89-1.87)	.184	1.33	(0.93-1.90)	.108	1.18	(0.79-1.75)	.422
HLA-DPB1	1.06	(0.91-1.23)	.474	0.96	(0.75-1.24)	.772	1.33	(1.02-1.75)	.038	0.97	(0.74-1.27)	.827
KIR2DL-G†	1.80	(1.39-2.34)	<.001	1.93	(1.22-3.05)	.005	1.57	(0.96-2.56)	.069	2.23	(1.42-3.50)	<.001
KIR2DL-R‡	1.07	(0.81-1.41)	.612	1.08	(0.66-1.75)	.769	0.98	(0.59-1.61)	.934	1.07	(0.66-1.72)	.787

*Hazard ratio of mismatch with match as a reference adjusted for patient age, donor age, sex-matching disease, GVHD prophylaxis, total body irradiation, transplanted cell dose, risk status, and other matching status of HLA and KIR ligand.
†KIR2DL ligand mismatching in GVH direction.
‡KIR2DL ligand matching in HVG direction.

0.35; $P < .001$), and both high-risk and standard-risk CML had a significantly lower relapse rate of HLA-DPB1 mismatch (HR = 0.35; $P < .001$ and HR = 0.39; $P = .012$, respectively). No significant effect was observed in AML (HR = 0.76; $P = .137$) or ALL (HR = 0.92; $P = .604$).

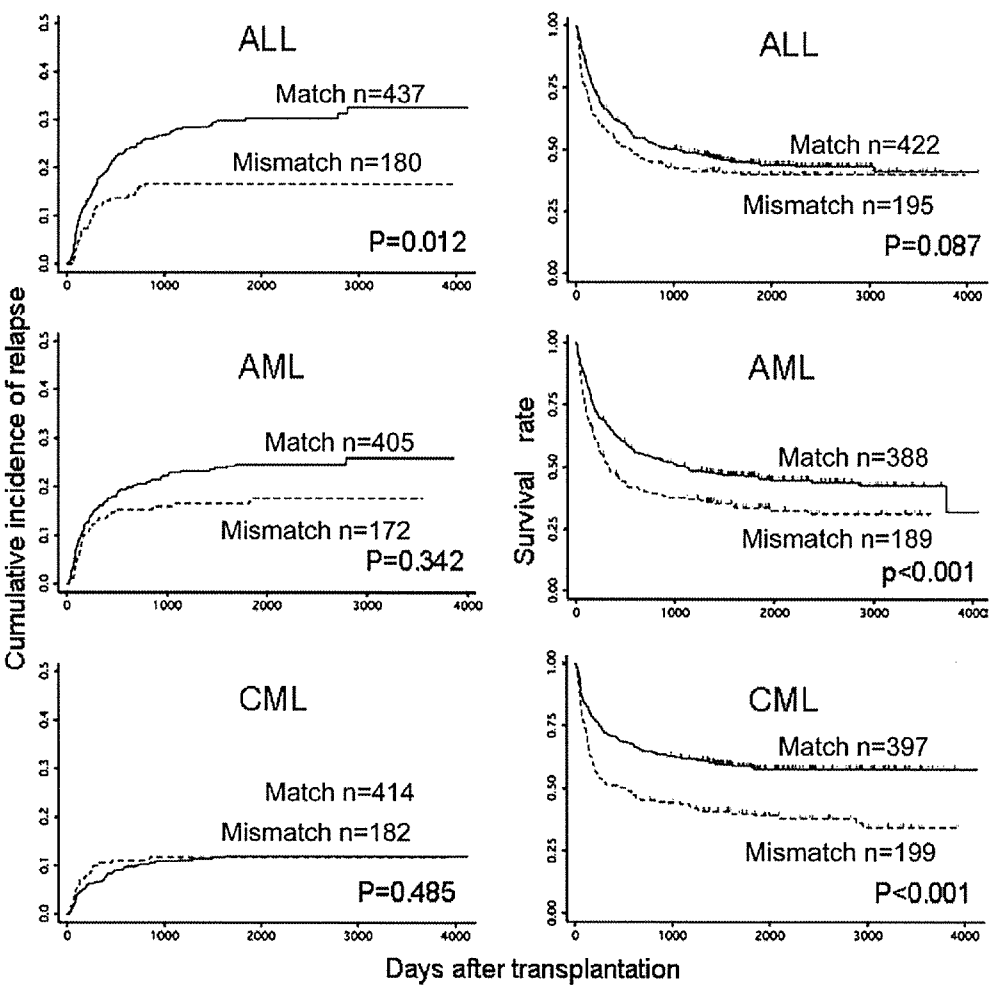

Figure 1. Cumulative incidence of relapse and survival by matching of HLA-C in patients with ALL, AML, and CML. All patients were analyzed. The direction of mismatching of HLA-C for relapse is GVH for relapse, and the direction for survival is GVH and/or HVG. The solid line represents match; the dotted line, mismatch.