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.

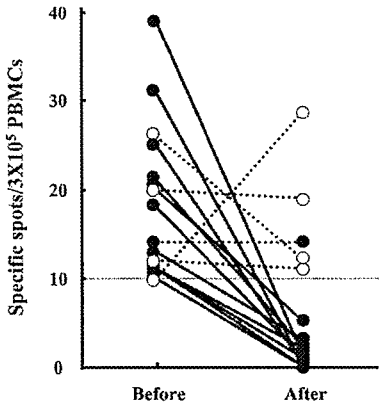

Fig. 8. Kinetics of hTERT-specific T cell responses before and after curative treatments. PBMCs were obtained before and 6 months after treatments and analyzed. Open circles show the patients without tumor recurrence within 1 year after treatment. Closed circles show the patients with tumor recurrence 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.

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 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.

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

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ABSTRACT

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 ($CD3^+$, $CD4^+$, $CD8^+$, $CD45RO^+$, $TIA\text{-}1^+$, 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.

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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

[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		NS2		MC		*P**
	No.	%	No.	%	No.	%	
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.

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

	B-Cell Group		T/CM Group		FDC Group		Null-Cell Group	
Characteristic	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.

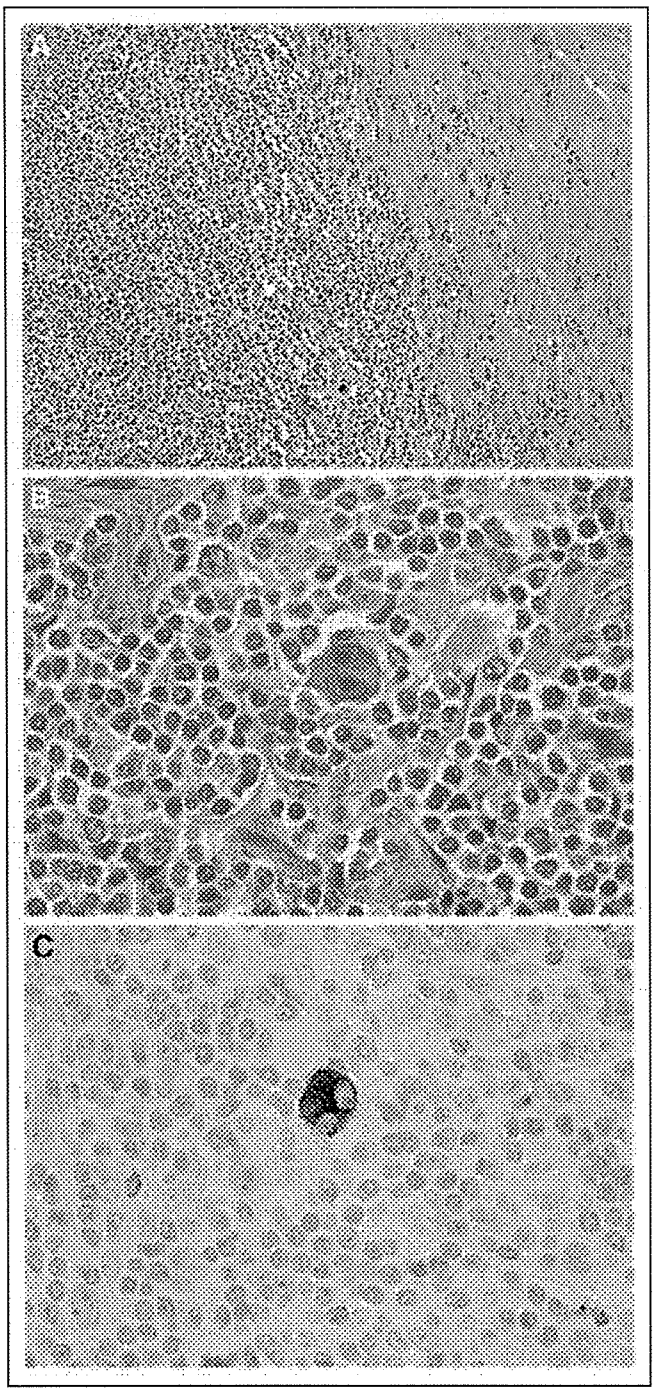

Fig 1. Classical Hodgkin's lymphoma (CHL) with T-cell and/or cytotoxic molecule expression. (A) T-cell and/or cytotoxic molecule–positive CHL patient sample shows fibrous collagen bands dividing the lymph node into nodules and is categorized as nodular sclerosis (original magnification ×40). (B) Reed-Sternberg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

Table 3. Clinical Characteristics According to Phenotype

Characteristic	B-Cell Group (n = 63) No.	%	T/CM Group (n = 27) No.	%	FDC Group (n = 22) No.	%	Null-Cell Group (n = 212) No.	%	P*
Sex									.35
Male	42		15		17		149		
Female	21		12		5		63		
Ratio	2.0		1.25		3.4		2.37		
Age, years									.048
Median	57		44		55		46		
Range	9-89		13-84		16-82		9-88		
> 50	38	60	11	41	14	64	88	42	.019
PS > 1	7	11	8	30	5	23	33	16	.14
Clinical stage III/IV	21	33	12	44	12	55	90	43	.33
B symptoms	16	31	10	40	10	53	67	37	.43
Bulky mass	5	10	4	15	5	25	31	17	.43
Extranodal > 1 site	6	13	6	24	1	5	24	14	.34
WBC > 15,000/μL	1	3	4	19	4	25	20	14	.11
Hb < 10.5 g/dL	5	14	6	29	5	33	39	28	.33
Serum albumin < 4.0 g/dL	13	39	13	65	6	55	63	55	.28
LDH > normal	9	32	6	29	4	40	61	52	.094
Treatment									
Type of chemotherapy									.15
ABVD	23	66	9	41	9	64	77	58	
ABVD/C-MOPP	3	8	3	14	5	36	17	13	
C-MOPP	1	3	3	14	0	0	11	8	
BEACOPP	0	0	1	5	0	0	5	4	
CHOP	6	17	3	13	0	0	7	5	
Other	2	6	3	13	0	0	16	12	
Chemotherapy only	22	61	11	48	9	56	74	51	
Chemotherapy and RT	13	36	11	48	5	31	59	41	
RT only	0	0	0	0	0	0	6	4	
Observation	1	3	1	4	2	13	5	4	
Response to combination chemotherapy†									.22
CR	26	81	11	58	11	85	86	78	
PR	6	19	5	26	2	15	17	16	
NR	0	0	3	16	0	0	7	6	
Relapse/progressive disease	8	23	13	59	5	38	54	40	.054
Survival, months									.0041
Median	21.9		15.4		56.0		28.3		
Range	1.2-142+		4.5-145		7.5-163+		2.0-254+		

Abbreviations: T/CM, T-cell and/or cytotoxic molecules; FDC, follicular dendritic cell; PS, performance status; Hb, hemoglobin; LDH, lactate dehydrogenase; ABVD, doxorubicin, bleomycin, vinblastine, and dacarbazine; C-MOPP, cyclophosphamide, vincristine, procarbazine, and prednisone; BEACOPP, bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine, and prednisone; CHOP, cyclophosphamide, doxorubicin, vincristine, and prednisone; RT, radiation therapy; CR, complete response; PR, partial response; NR, no response.

*χ^2 test for independence, or Fisher's exact probability test, B *v* T/CM *v* FDC *v* null.

†ABVD, ABVD/C-MOPP, C-MOPP, BEACOPP, or CHOP.

DISCUSSION

Our study in 324 consecutive patients with Hodgkin's lymphoma had three major findings. First, among the four phenotypic subclassifications (B-cell, T/CM, FDC, and null-cell groups), the T/CM group had a significantly poorer prognosis in uni- and multivariate analyses. To our knowledge, this is the first study to report the prognostic significance of this factor. Second, among the histopathologic groups (NS, NS2, and MC) of CHL, no significant differences were found in clinical features, except age at onset and sex ratio. Finally, EBV positivity was more prevalent in MC, occurred mostly in older men, and was not identified as an independent prognostic factor.

T-cell marker and/or CM expression has been demonstrated immunohistochemically on H-RS cells in approximately 5% to 20% of CHL patients, although there is little information in the literature regarding the clinicopathologic significance of their expression. In our series, T/CM marker expression was detected in 27 (8%) of 324 CHL patients, and was significantly associated with an adverse prognosis.

Genotypic evidence from several groups has indicated that the expression of T-cell phenotype on H-RS cells is aberrant.[10,17] Consistent findings regarding T-cell marker positivity and its prognostic significance have been reported.[17] In one report, however, the proportion of T-cell marker expression was low.[10] Conversely, CM positivity was reported in 10% to 18% of CHL patients.[18,19] Our relatively lower percentage (6%) of cytotoxic phenotype in CHL patients might have been influenced by the exclusion of borderline cases, which posed a problem in differential diagnosis from Hodgkin's-like ALCL under the Revised European-American Lymphoma classification.[13]

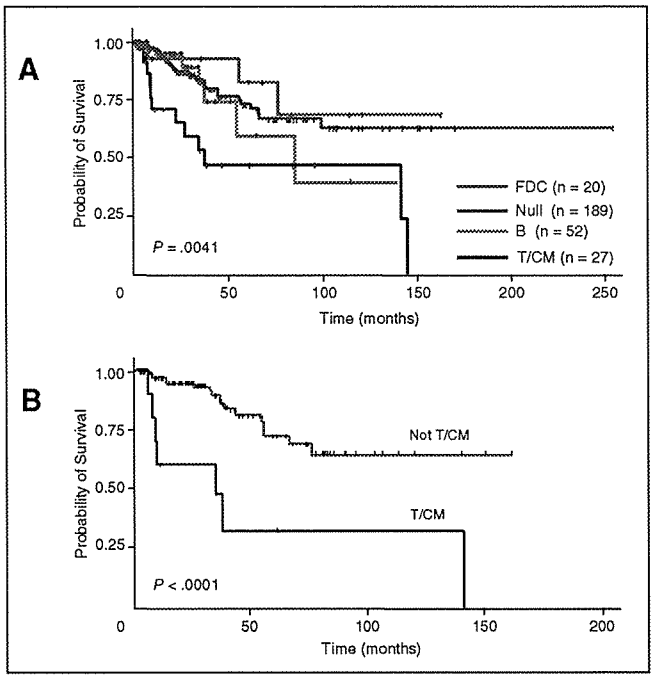

Fig 2. Survival data for four subgroups by phenotypic differentiation (B cell, T cell and/or cytotoxic molecules [T/CM], null cell, follicular dendritic cells [FDC]) in classical Hodgkin's lymphoma. (A) Disease-specific survival according to four phenotypic groups. (B) Prognosis of patients with the T/CM phenotype (———) is significantly poorer than that of those without this phenotype (– – – –) in classical Hodgkin's lymphoma patients who received chemotherapy with doxorubicin, bleomycin, vinblastine, and dacarbazine.

We reported previously that CM expression has an independent prognostic impact associated with unfavorable survival in nodal peripheral T-cell lymphoma, unspecified.[15] Moreover, TIA-1 and/or granzyme B expression on Hodgkin's-like ALCL was significantly associated with an adverse prognosis (Asano et al, submitted for publication). These data suggest that the expression of CMs may be predictive of the overall survival of CHL patients. The case of a CHL patient with evidence of clonal T-cell receptor γ (TCR-γ) gene rearrangement who had considerably shorter disease-specific survival has been reported.[17] Studies of TCR-γ rearrangement in H-RS cells have been technically challenging. A clonal TCR-γ chain gene was undetected in any of the patients with successful amplification of DNA by polymerase chain reaction analysis. This finding indicates that few patients with the T/CM phenotype have CHL of possible T-cell origin, although problems may have existed in the sensitivity of TCR-γ gene detection. The biologic significance of T/CM expression in CHL without genetic evidence of T-cell origin remains to be elucidated. These issues warrant additional investigation.

According to the WHO classification, histopathologic grouping in CHL is made in consideration of background inflammatory cells, including lymphocytes, plasmacytes, histiocytes, and eosinophils. In this study, we compared these morphologic groups (NS, NS2, and MC) in terms of clinical characteristics and survival, but found no significant differences among them, except for a younger age at onset and higher ratio of females in NS. As reported previously,[14] the present MC group was characterized by a higher ratio of positivity for EBV compared with the NS group.

The clinicopathologic significance of EBV as a prognosticator in CHL patients is still controversial.[20-26] Several recent studies have documented a marked survival disadvantage in older EBV-positive CHL patients compared with EBV-negative patients.[21,22] In our study, however, no significant survival difference was seen between EBV-positive and -negative patients. These results conflict with those reported by others, but the clinical features of our EBV-positive patients were compatible with those reported previously.[20,23,24]

The prognostic significance of B-cell or FDC marker in CHL is also controversial.[27] In this study, the expression of B-cell and FDC markers was detected in 20% and 7% of CHL cases, respectively. The B-cell group showed a relatively unfavorable clinical course compared with the null-cell group, whereas that of the FDC group was relatively favorable. These results may be in keeping with a recent report which identified the FDC marker as an independent favorable prognostic factor for overall survival in patients with diffuse large B-cell lymphoma.[28]

Clinical prognostic factors for CHL have been studied by Hasenclever et al.[29] They showed that the IPFP score is useful in

Table 4. Cox Proportional Hazards Model HR and 95% CI Estimates for Death As a Result of Lymphoma-Related Causes in Patients With CHL

		Univariate			Multivariate Total CHL			Multivariate ABVD Therapy Group			Multivariate Advanced CHL		
Variables	Unfavorable Factors	HR	95% CI	*P*	HR	95% CI	*P*	HR	95% CI	*P*	HR	95% CI	*P*
Phenotype	T/CM	3.07	1.61 to 5.86	.001	3.97	1.85 to 8.48	< .0001	9.23	3.17 to 20.9	< .0001	2.62	1.05 to 6.50	.038
Serum albumin	< 4.0 g/dL	3.83	1.69 to 8.68	.001	2.32	0.95 to 5.70	.066	2.31	0.73 to 7.26	.15		—	
Performance status	> 1	2.64	1.46 to 4.76	.001	1.57	0.76 to 3.27	.22	2.31	0.88 to 6.08	.09		—	
Stage	III/IV	1.94	1.10 to 3.41	.021	1.37	0.64 to 2.94	.42	1.84	0.68 to 4.97	.23		—	
Hemoglobin	< 10.5 g/dL	1.79	0.99 to 3.21	.052	1.25	0.60 to 2.61	.56	1.08	0.40 to 2.88	.88		—	
Age	> 45 years	1.71	0.98 to 2.96	.058	2.55	1.23 to 5.29	.012	1.72	0.65 to 4.55	.28		—	
Lymphocyte count	< 600/μL	2.24	0.94 to 5.32	.068	1.45	0.58 to 3.60	.43	1.25	0.27 to 5.93	.78		—	
EBV	Positive	1.59	0.90 to 2.78	.11		—			—			—	
WBC	> 15,000/μL	1.76	0.69 to 4.47	.23		—			—			—	
Histology	NS2	1.49	0.73 to 3.06	.27		—			—			—	
CD15	Negative	1.38	0.78 to 2.45	.28		—			—			—	
Sex	Male	1.11	0.61 to 2.03	.72		—			—			—	
IPFP score	5 or more	3.18	1.48 to 6.85	.003		—			—		2.73	1.19 to 6.24	.018

Abbreviations: HR, hazard ratio; CI, confidence interval; CHL, classical Hodgkin's lymphoma; ABVD, doxorubicin, bleomycin, vinblastine, and dacarbazine; T/CM, T-cell and/or cytotoxic molecules; EBV, Epstein-Barr virus; NS2, nodular sclerosis grade 2; IPFP, International Prognostic Factor Project.

determining the prognosis of advanced CHL, and in clinical decision making for individual patients. In the present study, and consistent with other findings,[30] the IPFP score was found to have prognostic significance in CHL. Moreover, among patients with early-stage (I/II) CHL, those with an IPFP score of 3/4 showed a poorer prognosis than those with low-risk score (< 3), although there were no patients with a high IPFP score (5 or more) in the stage I/II patients (data not shown). One notable consideration is that T-cell or cytotoxic phenotype remained a significant prognostic factor even after adjustment for IPFP score.

Compared with Western CHL reports, the patients in this study were characterized by a low NS rate, low CD15 positivity, and poor prognosis.[14,27,31] According to these findings, the patients may have included far fewer NS cases with a favorable prognosis and $CD15^+$ $CD30^+$ phenotype than in these Western studies. However, the T/CM phenotypic appearance of H-RS cells is present in Western as well as Japanese patients,[10,17-19] possibly indicating that the T/CM phenotype in CHL carries a poor prognosis in both Western and Asian patients.

In conclusion, we demonstrated that patients with CHL with the T/CM phenotype have a significantly poorer prognosis than those with the other phenotypic groups. Examination of T-cell markers in CHL patients is recommended as a routine pathologic practice.

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Appendix

The Appendix is included in the full-text version of this article, available online at www.jco.org. It is not included in the PDF version (via Adobe® Reader®).

Authors' Disclosures of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

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KIR Ligands and Prediction of Relapse after Unrelated Donor Hematopoietic Cell Transplantation for Hematologic Malignancy

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ABSTRACT

Recurrent malignancy remains a significant complication after allogeneic hematopoietic cell transplantation (HCT). Efforts to decrease relapse have included donor lymphocyte infusion to stimulate donor anti-recipient T-cell allorecognition of major and minor histocompatibility differences. Recently, alloreactive effects of donor natural killer cell-mediated inhibitory killer immunoglobulin-like receptor (KIR) recognition of recipient HLA-C and -B ligands have been described. We examined KIR ligand effects on risk of relapse in 1770 patients undergoing myeloablative T-replete HCT from HLA-matched or -mismatched unrelated donors for the treatment of myeloid and lymphoid leukemias. KIR ligands defined by HLA-B and -C genotypes were used to determine donor-recipient ligand incompatibility or recipient lack of KIR ligand. Among HLA-mismatched transplantations, recipient homozygosity for HLA-B or -C KIR epitopes predicted lack of KIR ligand and was associated with a decreased hazard of relapse (hazard ratio, 0.61; 95% confidence interval, .043-0.85; $P = .004$). Absence of HLA-C group 2 or HLA-Bw4 KIR ligands was associated with lower hazards of relapse (hazard ratio, 0.47; 95% confidence interval, 0.28-0.79, $P = .004$; hazard ratio, 0.56; 95% confidence interval, 0.33-0.97; $P = .04$, respectively). The decrease in hazard of relapse in patients with acute myelogenous leukemia was similar to that in patients with chronic myelogenous leukemia and acute lymphoblastic leukemia ($P = .95$). Recipient homozygosity for HLA-B or -C epitopes that define KIR ligands is likely to be a predictive factor for leukemia relapse after myeloablative HCT from HLA-mismatched unrelated donors. This effect was not observed in HLA-identical unrelated transplants.

KEY WORDS

KIR ligand • Relapse • Unrelated hematopoietic cell transplantation

INTRODUCTION

Malignant hematologic disorders can be cured by hematopoietic cell transplantation (HCT), and the availability of unrelated volunteer hematopoietic cell donors has broadened the treatment options for many patients who otherwise lack related donors [1-3]. Disease relapse after transplantation remains a significant challenge [4-6], underscoring the importance of identifying genetic and nongenetic factors that can affect relapse rates.

Recognition of recipient HLA ligand by donor natural killer (NK) cell inhibitory killer immunoglobulin-like receptors (KIRs) has been proposed as the basis for alloreactivity leading to a decrease in post-transplantation relapse and improved survival after HLA-mismatched transplantation for acute myelogenous leukemia (AML) [7,8]. Initial studies predicted donor NK alloreactivity based on a model of KIR ligand incompatibility in which donors with HLA genotypes comprised of class I ligands for inhibitory KIR are paired with recipients with HLA genotypes lacking one or more of the class I ligands present in the donor. Upon transfer to a recipient lacking the cognate class I ligand, donor NK cells expressing the relevant inhibitory KIR are released from inhibition and allow NK activation in a graft-versus-host (GVH) direction. Cytotoxicity against residual host leukemic cells leads to lower relapse. The KIR ligand incompatibility effect in other HLA-mismatched transplantation populations, however, has not been consistently observed [9-13].

Because the HLA and KIR gene complexes are encoded on chromosomes 6p21 and 19q13, respectively, individuals may have inhibitory KIR for which they have no HLA ligand, and, conversely, individuals may have HLA ligands for which they have no KIR. The independent segregation of HLA and KIR genes presents the situation wherein donor NK activation can occur when host target cells lack HLA ligands for inhibitory KIR-expressing NK cells, regardless of the HLA genotype of the NK cell itself; in other words, a recipient who is "missing HLA ligand" can trigger cytotoxicity of donor NK cells. Population frequency data demonstrate that, for the inhibitory KIR2DL2/3, -2DL1, and -3DL1 genes, nearly all individuals have a complete complement of inhibitory KIR [14-24]. In contrast, frequencies of the corresponding HLA class I ligands (HLA-C group 1, -C group 2, or -Bw4), deviate greatly from 100%. Therefore, recipient homozygosity for HLA-C group 1, HLA-C group 2, or HLA-Bw6 and therefore lack of KIR ligand for respective donor inhibitory KIRs can be predicted to occur not only between HLA-matched recipients and donors but also between HLA-mismatched pairs. Even within individuals, the "missing ligand" model has been shown to play a role in autoimmune disease, viral infection, and pre-eclampsia [25-27].

Recent studies have examined the effect of "missing HLA ligand" in allogeneic HCT. Compared with donor-recipient KIR ligand incompatibility, lack of recipient HLA ligand for donor inhibitory KIR was found to be a better predictor of outcome after HLA-haploidentical HCT [28]. Another study found that lack of ligand in the recipient occurred with 63% frequency in HLA-identical T-cell depleted sibling HCT and that missing ligand predicted higher overall survival and lower risk for acute leukemia relapse [29]. Most studies have examined the effect of either KIR ligand incompatibility or lack of KIR ligand on transplantation outcome in study populations with limited size. We report findings from a large dataset of the International Histocompatibility Working Group (IHWG) in HCT, a consortium of international transplantation centers and histocompatibility laboratories (http://www.ihwg.org). Results from this study group indicate that lack of KIR ligand in patients receiving HLA-mismatched transplants may be a predictor for protection from leukemia relapse.

METHODS

Patient Population

In total, 1770 patients receiving myeloablative conditioning followed by an unrelated HLA-matched or -mismatched bone marrow or peripheral blood stem cell HCT for treatment of AML, myelodysplastic syndrome (MDS), chronic myelogenous leukemia (CML), and acute lymphoblastic leukemia (ALL) were evaluated in this study. All had complete donor-recipient HLA-A, -B, -C, DRB1, and DQB1 allele typing data and clinical data (date of transplantation, diagnosis and stage of disease at time of transplantation, patient age at transplantation, survival status, ie, date of last follow-up or death, and date of relapse). Patient and transplant characteristics are listed in Table 1. The use of patient information and samples for this study was approved by the institutional review boards of each of the 22 participating institutions within the IHWG HCT-KIR Component.

Transplantation Procedure and Clinical Definitions

All patients received myeloablative conditioning followed by infusion of T-replete donor stem cells. Disease stage was defined as low risk (CML in first chronic phase), intermediate risk (CML in second chronic phase or accelerated phase CML; AML or ALL in remission; MDS refractory anemia subtype), and high risk (blastic CML; AML or ALL in relapse; MDS refractory anemia with excess blasts and refractory anemia with excess blasts in transformation). Relapse was defined by morphologic or cytogenetic

Table 1. *Patient and Donor Characteristics**

	Recipient with all KIR Ligands Present (n = 380)		Recipient with KIR Ligands Absent (n = 822)	
	n	%	n	%
ALL				
Remission	54	14	91	11
Relapse	6	2	29	4
AML				
Remission	51	13	105	13
Relapse	30	8	59	7
CML				
Chronic phase 1	167	44	402	49
Chronic phase 2	14	4	25	3
Accelerated phase	41	11	76	9
Blast crisis	9	2	16	2
MDS				
RA	1	<1	8	1
RAEB	2	<1	5	<1
RAEBT	5	1	6	<1
Disease severity				
Low	167	44	402	49
Intermediate	161	42	305	37
High	52	14	115	14
Patient/donor gender				
M/M	139	37	289	35
M/F	89	23	146	18
F/M	85	22	198	24
F/F	62	16	174	21
Unknown	5	1	15	2
Patient/donor ethnicity				
Caucasian	328	86	716	87
Asian	4	1	10	1
Other	23	6	34	4
Unknown	25	7	62	8
Patient/donor CMV status				
Positive/positive	70	18	171	21
Positive/negative	97	26	214	26
Negative/positive	63	16	114	14
Negative/negative	131	34	280	34
Unknown	19	5	42	5
Median patient age	34.5		35.0	
Number of HLA mismatches				
0	172	45	408	50
1	118	31	237	29
2	53	14	107	13
3	22	6	39	5
4	6	2	22	3
>4	9	2	9	1

*Non-JMDP patients and donors. ALL indicates acute lymphoblastic leukemia; AML, acute myelogenous leukemia; CML chronic myelogenous leukemia; MDS myelodysplastic syndrome; RA, refractory anemia; RAEB, refractory anemia with excess blasts; RAEBT, refractory anemia with excess blasts in transformation; M, male; F, female; CMV, cytomegalovirus.

evidence of disease in the peripheral blood or bone marrow. Overall survival was calculated for the interval between date of transplantation and death or date of last follow-up. For survival analyses, reinduction of remission was not considered.

KIR Ligand Assignment

High-resolution typing of recipient HLA-B and HLA-C alleles was used to segregate patients into the following KIR ligand groups: HLA-C group 1 alleles (Ser77 and Asn80) recognized by KIR2DL2 and 2DL3; HLA-C group 2 alleles (Asn77 and Lys80) recognized by KIR2DL1; HLA-Bw4–positive alleles (Arg79, Ile80, Arg83 or Arg79, Thr80, Arg83) recognized by KIR3DL1; and Bw6-positive alleles (Arg79, Asn80, Gly83), which are not known ligands for any inhibitory KIR. Patients were grouped according to homozygosity for HLA-C group 1, HLA-C group 2, or HLA-Bw6. Under the assumption that an individual has a full complement of inhibitory KIRs, patient homozygosity for HLA-C group 2 indicates lack of ligand for donor inhibitory KIR2DL2 or 2DL3; patient homozygosity for HLA-C group 1 implies lack of ligand for donor inhibitory KIR2DL1; and patient homozygosity for HLA-Bw6 implies lack of ligand for donor inhibitory KIR3DL1.

Statistical Analysis

Cox regression models were fit to examine the association of homozygosity for HLA-C group 1, HLA-C group 2, or HLA-Bw6 with hazards of mortality and relapse. All models were adjusted for severity of disease (categorized as low, intermediate, and high), patient age at transplantation, cytomegalovirus serostatus, number of mismatched HLA alleles where appropriate (modeled as a continuous linear variable capable of taking on a value from 0 to 10), and transplantation center where appropriate (categorized as a center contributing to the Japanese Marrow Donor Program [JMDP] vs a center not contributing to JMDP). All reported 2-sided P values were estimated from the Wald test, and no adjustments were made for multiple comparisons. Therefore, P values between .01 and .05 should be considered as suggestive rather than conclusive evidence of a difference.

RESULTS

Effect of Donor-Recipient Ethnicity

In total, 1350 patients (76%) were homozygous for KIR epitopes HLA-C group 1, group 2, or HLA-Bw6 and therefore could be considered to be lacking ≥1 ligand for donor inhibitory KIR. Of these patients, 264 (20%) relapsed. Among the 420 patients who were heterozygous for the HLA-C KIR epitopes and HLA-Bw4–positive, and therefore not lacking KIR ligand, 93 relapsed (22%). The adjusted hazard of relapse among patients lacking a ligand was not statistically significantly different from that among patients in whom all ligands were present (hazard ratio [HR], 0.89; 95% confidence interval [CI], 0.70-1.14;

Table 2. *Distribution of KIR Epitope Homozygosity by Transplantation Center*

HLA Genotype	KIR Ligand Absence	Non-JMDP		JMDP	
		n (%)	Relapse (%)	n (%)	Relapse (%)
HLA-C group 1, HLA-C group 2, and HLA-Bw4	None	380 (32)	89/380 (23)	40 (7)	4/40 (10)
HLA-C group 2 and HLA-Bw4	HLA-C group 1	140 (12)	34/140 (24)	2 (<1)	0/2 (0)
HLA-C group 1 and HLA-Bw4	HLA-C group 2	203 (17)	42/203 (21)	319 (56)	63/319 (20)
HLA-Bw6	HLA-Bw4	167 (14)	34/167 (20)	33 (6)	5/33 (15)
HLA-C group 2 and HLA-Bw6	HLA-C group 1 and HLA-Bw4	16 (1)	3/16 (19)	1 (<1)	0/1 (0)
HLA-C group 1 and HLA-Bw6	HLA-C group 2 and HLA-Bw4	296 (25)	53/296 (18)	173 (30)	30/173 (17)

P = .37). Because analysis of KIR epitopes is based on HLA genotypes and frequencies of HLA alleles can vary dramatically between ethnically different populations, we examined the dependence of the KIR ligand effect on ethnicity, where ethnicity was modeled based on transplantation center. The distribution of KIR epitope homozygosity and the percentage of patients who relapsed in each of the categories differed between transplant pairs contributed by the JMDP and other centers and registries (non-JMDP; Table 2). These disparities in KIR epitope distribution could largely be attributed to the high prevalence of HLA-C group 1 allele homozygosity in the Japanese population, leading to an over-representation of patients who lacked HLA-C group 2 ligand for donor KIR2DL1. The small number of JMDP patients who were not homozygous for HLA-C group 1 precluded an informative analysis of the effect of a missing KIR ligand in this group, and therefore all subsequent analyses of KIR ligand effects were restricted to the non-JMDP population.

Effects of Degree of HLA Mismatch and KIR Ligand Homozygosity in Non-JMDP Transplants

The adjusted hazard of relapse among patients missing a KIR ligand was not statistically significantly different from that among patients not missing a KIR ligand among patients in the non-JMDP group (HR, 0.84; 95% CI, 0.65-1.09; P = .19). However, there was evidence to suggest that the effect of a missing ligand differed among patients matched for all 10 HLA-A, -B, -C, DRB1, and DQB1 alleles compared with those mismatched for ≥1 allele (P = .009). In particular, the adjusted hazard of relapse was slightly higher in the matched group among patients missing a KIR ligand than among those who were not (89 of 409 [22%] relapses among patients missing a ligand vs 31 of 172 [18%] relapses among patients not missing a ligand; Figure 1A; HR, 1.26; 95% CI, 0.83-1.90, P = .27). In contrast, among patients mismatched for ≥1 HLA-A, -B, -C, DRB1, or DQB1 allele, the adjusted hazard of relapse was statistically significantly lower among those missing a ligand than among those not missing a ligand (77 of 415 [19%] relapses among patients missing a ligand vs 58 of 208 [28%] relapses among patients not missing a ligand; Figure 1B; HR, 0.61; 95% CI, 0.43-0.85; P = .004). Although the decrease in the hazard of relapse was greatest among HLA-C group 1 homozygous patients, other groups also experienced less relapse than the group not missing a ligand (Table 3), and the difference between patients homozygous for HLA-C group 1 and patients

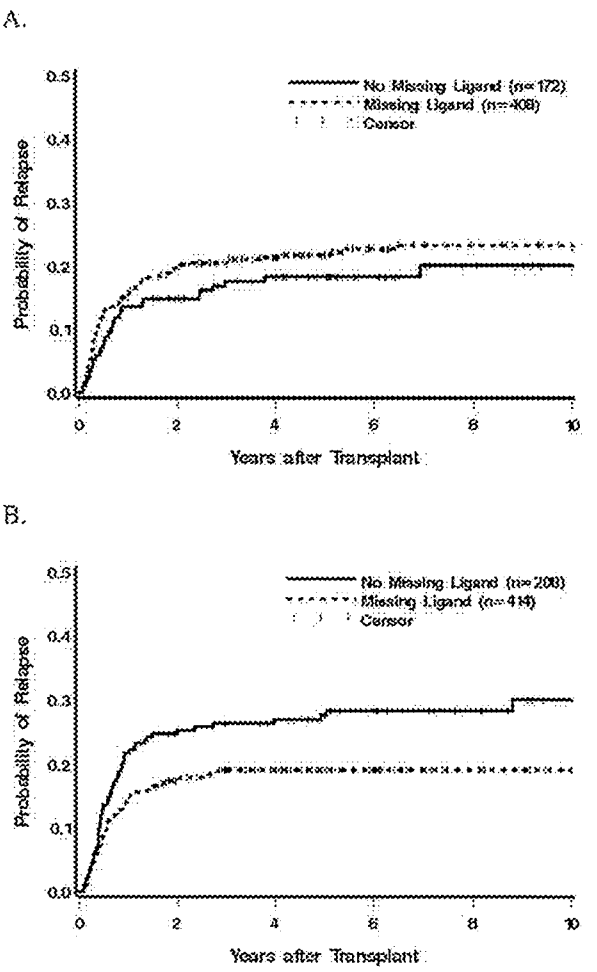

Figure 1. Probability of relapse in HLA-matched (A) or HLA-mismatched (B) patients lacking KIR ligand (broken line) or not lacking KIR ligand (solid line). Lack of recipient KIR ligand was defined as HLA-C group 1, group 2, or Bw6 homozygosity.

Table 3. *Effect of Missing KIR Ligand on Relapse in HLA-Mismatched Patients*

KIR Ligand Absence	HR	95% CI	P
None (n = 208)	1	—	—
HLA-C group 1 (n = 70)	0.85	0.49–1.48	.56
HLA-C group 2 (n = 122)	0.47	0.28–0.79	.004
HLA-Bw4 (n = 90)	0.56	0.33–0.97	.04
HLA-Bw4 and HLA-C group 1 (n = 13)	0.89	0.28–2.86	.85
HLA-Bw4 and HLA-C group 2 (n = 119)	0.64	0.39–1.06	.08

in the other missing ligand groups was not statistically significant (P = .33).

Although lack of ligand was associated with a statistically significantly lower hazard of relapse among patients who received transplants from an HLA-mismatched donor, there was no effect seen on overall survival (HR, 0.91; 95% CI, 0.73-1.12; P = .36). In the HLA-matched group, there was no effect of lack of KIR ligand on relapse or survival even when analyzed within specific epitope groups (data not shown).

Effect of Missing KIR Ligand within Disease Groups of the Non-JMDP Dataset

Recent studies have demonstrated a missing KIR ligand effect in AML, but not in CML or ALL in T-cell–depleted HLA-matched transplants [29]. We therefore compared the effect of missing KIR ligand on relapse in patients between these disease groups. Among HLA-mismatched transplants in the present study, the effect of missing a KIR ligand on relapse was similar among patients with AML (Figure 2A; HR, 0.62; 95% CI, 0.34-1.10; P = .10) and those with CML (HR, 0.66; 95% CI, 0.39-1.12; P = .12) and ALL (HR, 0.66; 95% CI, 0.39-1.02; P = .06; Figure 2B; combined HR, 0.59; 95% CI, 0.38-0.91; P = .02). A formal test of interaction between disease (AML vs CML or ALL) and missing ligand yielded P = .95.

Contribution of Specificity and Degree of HLA Mismatch to Missing KIR Ligand Effect in Non-JMDP Transplants

Among the 622 donor-recipient pairs mismatched for ≥1 HLA-A, -B, -C, DRB1, or DQB1 allele, 355 (57.1%) were single-allele mismatched, 160 (25.7%) were 2-allele mismatched, and 107 (17.2%) were mismatched for ≥3 alleles. There was no evidence that the effect of missing ligand was dependent on the number of mismatched alleles (P = .26, test for interaction between number of mismatches and presence of missing ligand). The effect of missing ligand on the hazard of relapse was also similar across the various degrees of HLA allele mismatching. When the study was restricted to single-allele mismatches only, a similar conclusion was obtained (data not shown).

Comparison of Missing KIR Ligand and KIR Ligand Incompatibility in Non-JMDP Transplants

In 428 HLA-B and/or HLA-C mismatched pairs, the KIR ligand incompatibility model and the missing KIR ligand model could be compared directly. There was no statistically significant difference in relapse between the 189 patients who could be characterized by KIR ligand incompatibility in the GVH direction and the 239 KIR ligand compatible patients (HR, 1.16; 95% CI, 0.76-1.78; P = .48). In the same group of HLA-B and/or -C mismatched patients, the 276 patients who were missing a KIR ligand had a lower hazard of relapse compared with the 152 patients who were not missing a ligand, although it did not reach

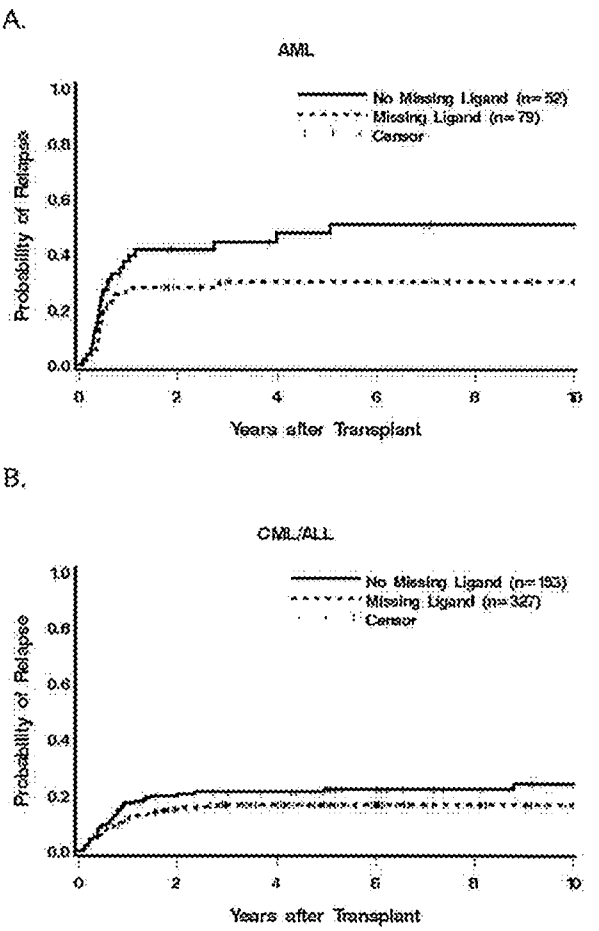

Figure 2. Probability of relapse in leukemia patients lacking (broken line) or not lacking (solid line) KIR ligand. A. Effect of missing ligand in AML. B. Effect of missing ligand in CML and ALL. The relative difference in the probability of relapse between the missing and non-missing groups was comparable in the AML and non-AML groups, even though the absolute difference in probabilities was larger in the AML group due to a higher overall probability of relapse.

statistical significance (HR, 0.70; 95% CI, 0.46-1.06; $P = .09$). KIR ligand incompatible donor-recipient pairs who also lacked KIR ligands demonstrated a decreased hazard for relapse compared with KIR ligand incompatible pairs who did not lack ligands (HR, 0.59; 95% CI, 0.33-1.05; $P = .07$). Neither KIR ligand incompatibility nor missing ligand conferred a survival benefit (data not shown).

DISCUSSION

Clinical experience demonstrates that the stage of disease at the time of allogeneic transplantation is a strong predictor of disease recurrence [4-6]. The availability of other measurements for predicting the risk of post-transplantation relapse could enable transplantation clinicians to tailor the transplantation procedure to individual patient risk and more effectively plan post-transplantation preventive measures and monitoring strategies. By using recipient homozygosity for HLA-C group 1, group 2, and Bw6 as markers for KIR ligand absence, we found a beneficial effect of missing recipient ligand on the risk of post-transplantation leukemia relapse in patients receiving HLA-mismatched unrelated donor transplants.

Although the beneficial effect of KIR ligand absence was seen in recipients of HLA-mismatched allografts, there was no apparent association between the locus specificity of HLA mismatch and the effect of lack of KIR ligand in the recipient. This was somewhat surprising, because it might be expected that specific mismatches at the HLA-B and HLA-C loci would potentiate KIR-driven effects through a cell-surface *cis*-mediated KIR-HLA mechanism [30] or through lack of engagement of licensed KIR [31,32]. Larger sample sizes may be needed to elucidate the HLA locus mismatches most relevant to KIR-mediated alloreactivity. A previous report found that recipients expressing an HLA-C group 1 allele had a higher overall survival compared with recipients homozygous for HLA-C group 2 in HLA-identical sibling HCT for various myeloid malignancies, but the survival advantage could not specifically be demonstrated to be due to lower relapse [33]. A more recent study of 111 patients who received an unrelated allograft for the treatment of various hematologic malignancies also demonstrated a deleterious effect of HLA-C group 2 homozygosity on survival [34]. The present IHWG dataset provides a large number of patients with complete 5-locus HLA allele typing information with which to analyze ligand effects in the matched and mismatched settings. In this dataset, we demonstrate that recipient homozygosity for HLA-C group 1 and HLA-Bw6 each confers a statistically significant decreased risk for relapse and that homozygosity for HLA-C group 2 does not have a deleterious effect on outcome. The magnitude of the protective effect on relapse appears largest in the group homozygous for HLA-C group 1 (and therefore lacking the HLA-C group 2 ligand). Differences in binding affinity between the inhibitory KIRs and their HLA ligands are known, with KIR2DL1 exhibiting the strongest affinity for its ligand HLA-C group 2 when compared with the other inhibitory receptors and their ligands [35,36]. It is therefore possible that absence of HLA-C group 2 ligand for its high-affinity inhibitory KIR leads to the most robust release from NK inhibition and therefore the highest potential for NK activation.

NK effects against AML have previously been demonstrated; however, effects against CML and ALL have been less clear [7-9,29,33]. In this analysis, there was a nearly identical association between lack of KIR ligand and relapse seen among patients with AML relative to patients with CML or ALL, even after adjusting for severity of disease. Although the total patient material demonstrated a statistically significant effect on relapse, segregation into disease categories resulted in patient numbers insufficient to reach statistical significance. Definitive conclusions regarding the effect of KIR ligands in disease-specific groups will require even larger numbers of patients in future studies.

This study identified a beneficial effect of recipient KIR epitope homozygosity in lowering risk of relapse, but a corresponding effect on survival was not observed. This may be due to the increased morbidity and mortality associated with mismatched unrelated HCT in which GVH disease is more prevalent. In addition, the missing ligand effect was seen in HLA-mismatched transplants and not in HLA-matched transplants. The favorable effect of lack of KIR ligand previously reported in HLA-identical transplants was apparent in the setting of ex vivo allograft T-cell depletion [29], a manipulation that may enhance NK function [8]. A recent study examining KIR reconstitution after unrelated donor HCT demonstrated a correlation between the presence of donor T cells in the allograft and lower NK KIR expression, which was associated with inferior survival [37]. Although our study population was restricted to T-cell–replete transplants, insufficient data existed to adjust the analysis for the degree of post-transplantation immunosuppression, the increased use of which in HLA-mismatched transplants may promote NK alloreactivity through T-cell suppression. An alternative explanation for the missing KIR ligand effect in HLA-disparate HCT is the possibility that donor-derived cytotoxic T-cell clones expressing inhibitory KIR are contributing to the observed antileukemic effect, and their expansion may be amplified in the HLA-disparate setting [13,38,39]. In minimally T-cell–depleted HLA-nonidentical transplants, T-cell alloreactivity dominates NK reactivity [13]. In this analysis, rates of

GVH disease were comparable between the HLA-matched and -mismatched groups (73% vs 75% for grades 2-4 and 35% vs 37% for grades 3-4). Even if the T- or NK-lymphocyte subsets contributed to the lower relapse rates observed in this study, the antileukemic effect appears to be related specifically to lack of recognition of KIR ligands and not to T-cell allorecognition of mismatched non-KIR epitope HLA alleles. It is therefore unlikely that T-cell allorecognition and GVH disease are responsible for the missing KIR ligand effect on relapse seen in the HLA-mismatched group.

This analysis supports the use of recipient HLA genotyping for the prediction of inhibitory KIR-mediated NK effects, with lack of KIR ligand being statistically associated with lower rates of relapse, whereas KIR ligand incompatibility is not. Therefore, the analysis does not support the deliberate selection of HLA-C or -B mismatched donors to capture KIR ligand incompatibility effects. The importance of considering the known ethnic differences in HLA class I and KIR gene and allele frequencies was demonstrated in this study. Due to these differences, the effect of missing KIR ligand could be tested only in the non-JMDP population, because the available JMDP data resulted in some ligand groups too small for meaningful comparisons. A larger JMDP dataset with sufficient numbers of study group members is currently being analyzed (Y. Morishima, personal communication). Correlation of donor KIR genotyping with HCT outcome will certainly be necessary to achieve a more comprehensive understanding of NK effects in HCT. The activating KIRs, whose ligands remain unclear, also likely contribute to transplantation outcome: donor activating KIRs have been associated with decreased relapse [40] and decreased cytomegalovirus activation [41]. Until more information is known about the clinical significance of donor KIR receptor allele diversity and the role of activating KIRs and their ligands, recipient KIR epitope homozygosity may serve as an important prognostic tool in aiding the selection of preventive and treatment options for patients with leukemia.

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