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]

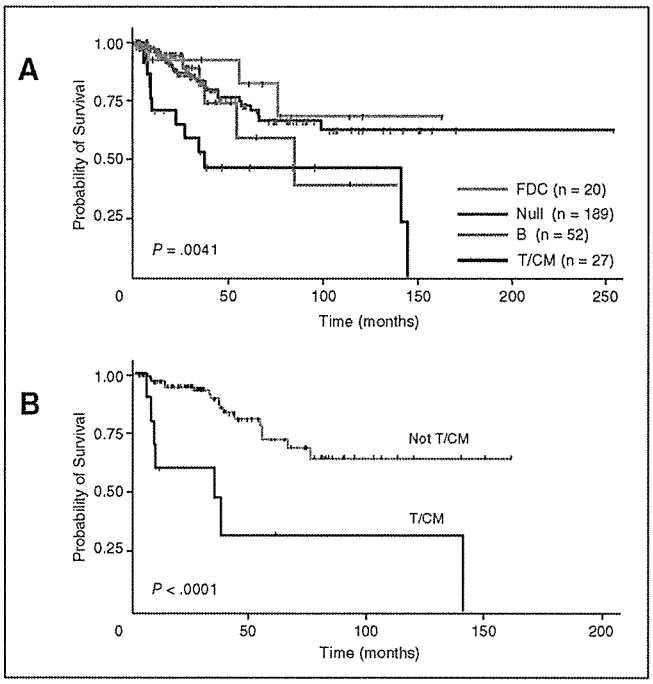

Fig 2. Survival data for four subgroups by phenotypic differentiation (B cell, T cell and/or cytotoxic molecules [T/CM], null cell, follicular dendritic cells [FDC]) in classical Hodgkin's lymphoma. (A) Disease-specific survival according to four phenotypic groups. (B) Prognosis of patients with the 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 4.78	.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

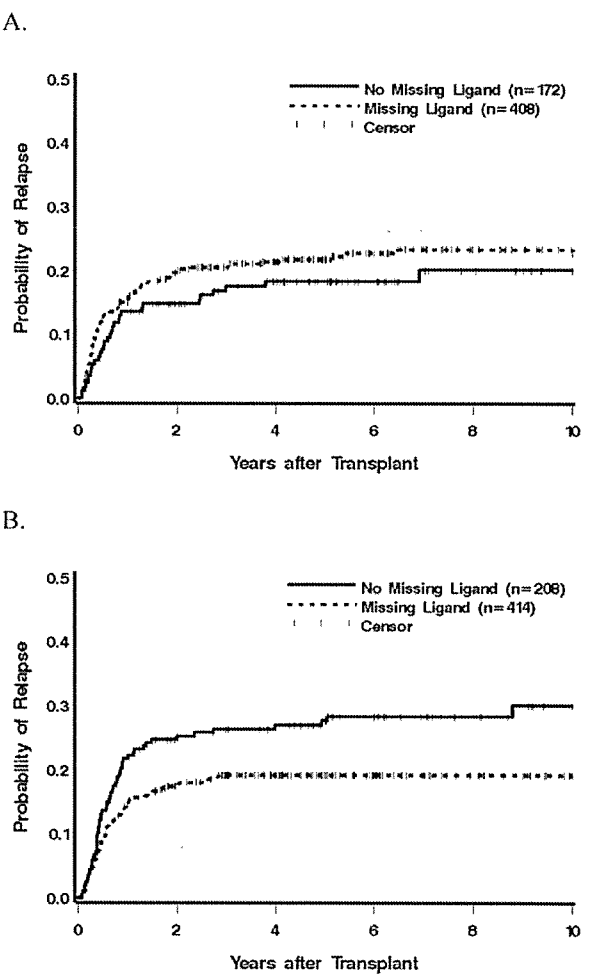

Figure 1. Probability of relapse in HLA-matched (A) or HLA-mismatched (B) patients lacking KIR ligand (broken line) or not lacking KIR ligand (solid line). Lack of recipient KIR ligand was defined as HLA-C group 1, group 2, or Bw6 homozygosity.

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

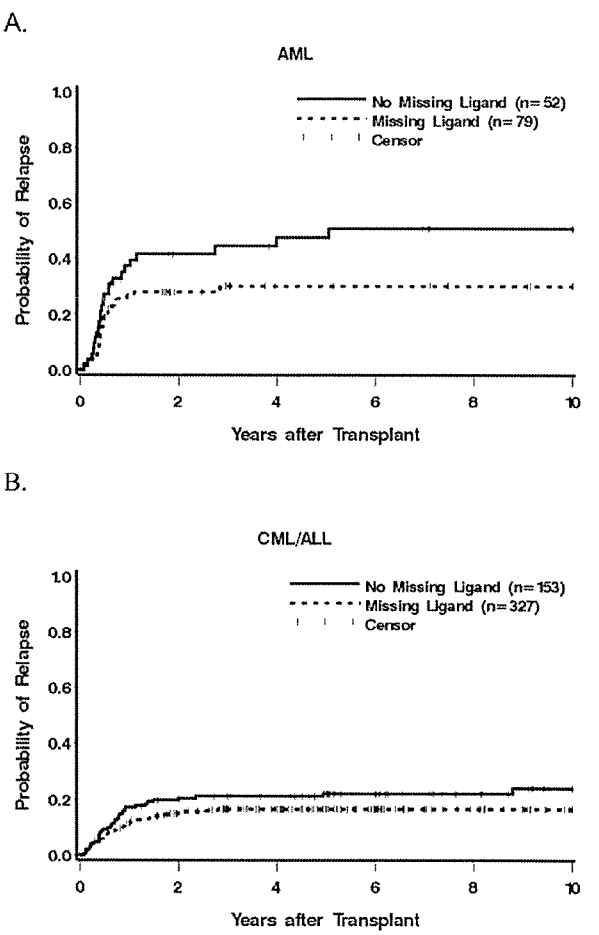

Figure 2. Probability of relapse in leukemia patients lacking (broken line) or not lacking (solid line) KIR ligand. A. Effect of missing ligand in AML. B. Effect of missing ligand in CML and ALL. The relative difference in the probability of relapse between the missing and non-missing groups was comparable 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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Expression profiling analysis of the CD5+ diffuse large B-cell lymphoma subgroup: Development of a CD5 signature

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Diffuse large B-cell lymphoma (DLBCL) accounts for 30% of non-Hodgkin's lymphomas and is known to comprise heterogeneous groups. We previously reported that CD5+ DLBCL is a clinically distinct subgroup of these tumors that is associated with poor prognosis. In our current study, we have used gene expression profiling technology in an attempt to identify new markers and to further characterize the biological features of CD5+ DLBCL. Candidate genes, which showed the greatest difference in expression between 22 CD5+ and 26 CD5- DLBCL cases, were selected from our screening and subjected to clustering analysis. This resulted in identification of a specific mRNA profile (a CD5 signature) for CD5+ DLBCL. The CD5 signature included downregulated extracellular matrix genes such as *POSTN, SPARC, COL1A1, COL3A1, CTSK, MMP9* and *LAMB3*, and comprised upregulated genes including *TRPM4*. We tested this CD5 signature for its potential use as a relevant marker for CD5+ DLBCL and found that it did indeed recognize this subgroup. The tumors identified by the CD5 signature contained most of the CD5+ DLBCL cases and some CD5- DLBCL cases. Moreover, the subgroup of cases with this CD5 signature showed a poorer prognosis. The subsequent application of the CD5 signature to the analysis of an independent series of DLBCL microarray data resulted in identification of a subgroup of DLBCL cases with a similar clinical outcome, further suggesting that the CD5 signature can be used as a clinically relevant marker of this disease. (*Cancer Sci* 2006; 97: 868–874)

Diffuse large B-cell lymphoma (DLBCL) is the most common form of non-Hodgkin's lymphoma.[1] Because it is known to include pathophysiologically and clinically heterogeneous groups, the proper identification of well-defined DLBCL subgroups has become an urgent requirement for clinicians. We previously reported that CD5+ DLBCL is a clinically distinct subgroup of DLBCL, accounting for 5–10% of all DLBCL.[2,3] Moreover, this subgroup of cases is characterized by more aggressive clinical features and poorer prognosis, compared with CD5- DLBCL.[3] Genomic aberrations that are characteristic of CD5+ DLBCL have also been identified.[4–6] CD5 positivity is essential for the detection of CD5+ DLBCL cases but is also a marker of mantle cell lymphoma (MCL), and indeed some instances of CD5+ DLBCL possess similar histological features to MCL. For such cases, the use of other markers such as cyclin D1 has become important,[1] and this indicates that the detection of CD5 alone is not sufficient to define the entire spectrum of CD5+ DLBCL tumors. Hence, CD5 can serve as an effective marker when it is used in combination with other biological indicators but it is clear that more effective markers for CD5+ DLBCL will need to be identified.

Expression profiling of mRNA has been used previously as a marker for subgroups of DLBCL.[7–10] Activated B-cell-like (ABC) and germinal-center B-cell-like (GCB) DLBCL are examples of subgroups of DLBCL that have been identified in this way.[7,8] It is thus possible that the use of expression profiling can yield novel and effective markers for defining subgroups of DLBCL, in conjunction with established markers such as CD5. With these findings in mind, we compared the expression profiles of 22 cases of CD5+ and 26 cases of CD5- DLBCL in the present study to identify better markers that may provide new insights into our understanding of the pathobiology of CD5+ DLBCL.

Materials and Methods

Patients and samples

The lymph node samples and clinical data used in our present analyses were obtained through an Institutional Review Board approved protocol from 22 patients with CD5+ DLBCL, and a further 26 individuals with CD5- DLBCL. Within these subject groups, 74% of the CD5+ DLBCL cases (14/17) and 29% of the CD5- DLBCL cases (7/24) were associated with extranodal sites (at least one site). None of these patients had a previous history of lymphoma and all 48 individuals received adequate treatment with cyclophosphamide, adriamycin, vincristine and predonine (CHOP)-like regimens. The median follow-up time was 2.4 years and the 5-year overall survival rate of the DLBCL patients was 40%. Each of the 22 CD5+ DLBCL cases was

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diagnosed for CD5 positivity by immunohistology and four of these cases were confirmed by fluorescence-activated cell sorter (FACS) analysis. All of the 26 $CD5^-$ DLBCL cases were diagnosed for CD5 negativity also by immunohistology and 23 of these were confirmed by FACS analysis. Either Leu1 (Becton Dickinson, Mountain View, CA, USA) or 4C7 (Novocastra, Newcastle, UK) was used as the monoclonal antibody for detection of CD5 antigen. The lymphomas were judged to be CD5 positive when more than 20% of the tumor cells showed positive staining.[3] Classical cytogenetics was used to confirm a negative t(11;14) in each case to exclude the diagnosis of a large cell variant of mantle cell lymphoma.

Microarray procedures

Total RNA extracts were isolated from each specimen by cesium chloride centrifugation, as described previously.[11] An oligonucleotide array, custom-made for the Cancer Institute of the Japanese Foundation for Cancer Research and on which 21 619 genes had been spotted, was used for analysis according to the manufacturer's protocol (Agilent Technologies, Palo Alto, CA, USA). The probe consisted of a mixture of an experimental Cy5-labeled cRNA and control Cy3-labeled cRNA, with the latter prepared from a pool of total RNA from 10 hyperplastic lymph node samples. Non-flagged array elements with a fluorescent intensity greater than 300 (one standard deviation below the mean of all fluorescent data) were considered well measured. Ratios of the fluorescence of the experimental Cy5-labeled samples to that of the Cy3-labeled controls were then log transformed (base 2).

Clustering analyses of microarray data

Genes that showed the greatest average difference in expression between 22 cases of $CD5^+$ and 26 cases of $CD5^-$ DLBCL after log transformation were selected from the screening. A hierarchical clustering algorithm was then applied to the DLBCL cases, according to the expression level of these genes, with the aid of Cluster and TreeView programs (http://rana.lbl.gov/EisenSoftware.htm).[12] The classification of either ABC or GCB DLBCL was based on the analysis of 100 genes identified in a previous study.[8] Our array slides contained 67 of these genes, which we used in a simple clustering method. Log-transformed ratios were centered by subtracting the median observed value of each of the genes for clustering analysis.[8]

Analysis of the published microarray data

The DLBCL gene expression profile data generated by the Cancer Genomics group were obtained from the supplemental data listed in at http://www.broad.mit.edu/cancer/pub/dlbcl.[10] None of the 176 DLBCL cases listed here had a previous history of lymphoma. The array fluorescence of these genes was log-transformed and centered by subtracting the median observed value of each of the genes for clustering analysis.

Results

Identification of CD5 signature genes for the $CD5^+$ DLBCL subgroup

Genes showing differential expression between 22 $CD5^+$ and 26 $CD5^-$ DLBCL were identified and 24 of these candidates showed an average expression difference of more than 2.5-fold between these tumor subgroups (Fig. 1a). These genes were therefore selected for clustering analysis and are referred to as CD5 signature genes. A further 70 genes showing a difference of more than 2-fold were also subjected to the same analysis.

Clustering analysis of CD5 signature genes in $CD5^+$-type and $CD5^-$-type DLBCL

Hierarchical clustering analysis with each of the identified 24 CD5 signature genes, applied to all 48 DLBCL cases, is shown in Fig. 1b. A further series of analyses using the 70 genes produced almost identical results (data not shown). It was noted that many of the genes that were found to be downregulated in $CD5^+$ DLBCL, such as *POSTN*, *SPARC*, *COL1A1*, *COL3A1*, *CTSK*, *MMP9* and *LAMB3*, are associated with the extracellular matrix (Fig. 1b). *UCHL1* and *CR2* were also found to be downregulated in $CD5^+$ DLBCL tumors and are known to be expressed in T cells, macrophages and follicular dendritic cells, which are subsets of cells that function during the immune response (Fig. 1b).

Our clustering analysis further enabled us to classify the DLBCL cases under study into two groups, one comprising 20 $CD5^+$ and 11 $CD5^-$ DLBCL cases, and the other consisting of 14 $CD5^-$ and two $CD5^+$ DLBCL samples (Fig. 1b). This indicated that the CD5 signature genes could potentially serve as markers for subgroups that are related to $CD5^+$ DLBCL. All except two cases of $CD5^+$ DLBCL could be included in the first of these two groups, which we designated as $CD5^+$-type DLBCL, in order to distinguish it from $CD5^+$ DLBCL (Table 1). The second group is referred to as $CD5^-$-type DLBCL (Table 1). One $CD5^-$ DLBCL case could not be assigned to either of these subgroups. The clinical features of $CD5^+$-type and $CD5^-$-type DLBCL are shown in Table 2. Patients with $CD5^+$-type DLBCL showed a more advanced tumor stage at diagnosis, compared with the $CD5^-$-type DLBCL cases (stage III/IV: 90% and 50%, respectively; $P = 0.0062$), and also displayed a higher international prognostic index[13] (IPI score 3–5: 66% and 27%, respectively; $P = 0.0398$). In addition, the overall survival of patients with $CD5^+$-type DLBCL after treatment with CHOP-like regimens was significantly poorer than patients with $CD5^-$-type DLBCL (Fig. 1c; $P = 0.0006$).

In the $CD5^+$-type DLBCL group, we assigned 11 $CD5^-$ DLBCL cases (Table 1), and these were further examined to explore whether in fact any similarities to $CD5^+$ DLBCL existed. Both groups showed similar clinical features except for performance status (Table 3) and no significant differences in survival were observed between the two ($P = 0.31$, log-rank test; data not shown). However, the $CD5^-$ DLBCL cases in the $CD5^+$-type DLBCL group did show a significantly poorer prognosis than their $CD5^-$ DLBCL counterparts in the $CD5^-$-type DLBCL group ($P = 0.0333$; data not shown), indicating that these 11 $CD5^-$ DLBCL cases had some clinical features that could be regarded as similar to $CD5^+$ DLBCL.

Incidence of ABC and GCB DLBCL among the 48 DLBCL subject cases

Clustering analysis using 67 of the 100 established ABC and GCB markers[8] was applied to our current 48 DLBCL cases, and it was found that these cases could be classified as either

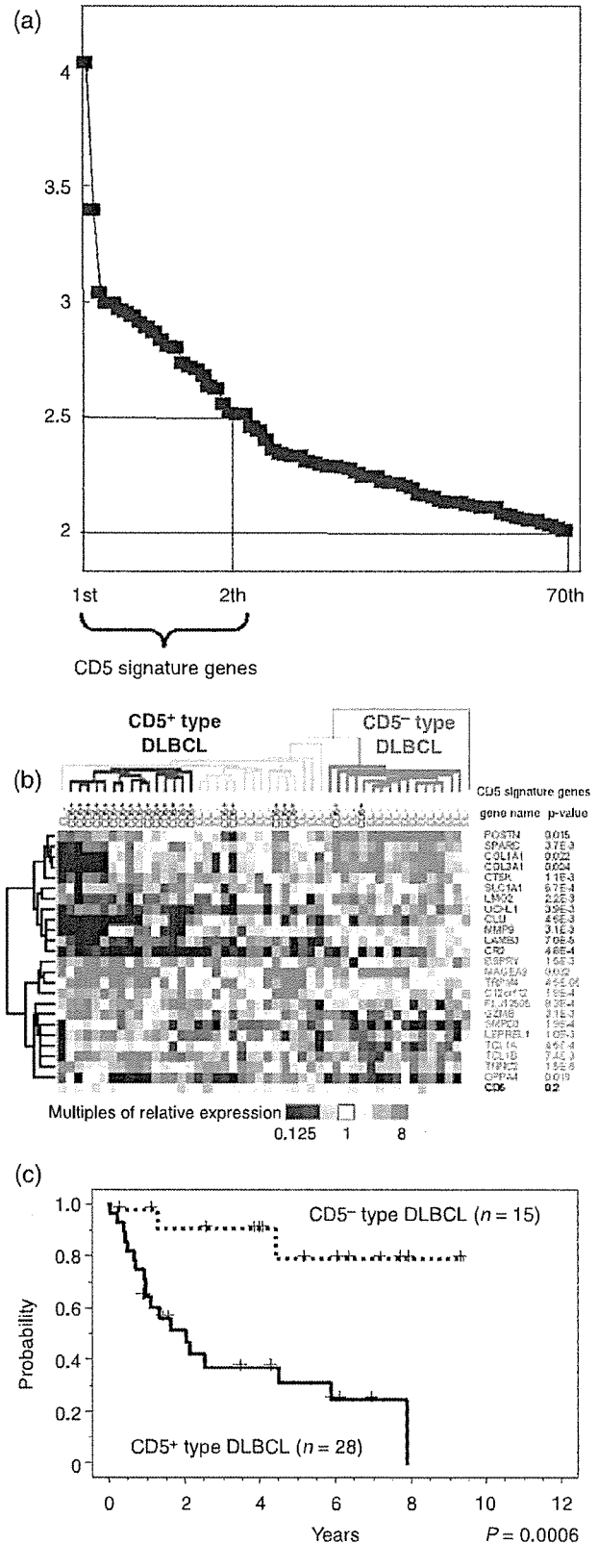

Fig. 1. CD5 signature genes define CD5⁺-type and CD5⁻-type diffuse large B-cell lymphoma (DLBCL). (a) Differences in the expression of CD5 signature genes. Genes showing the highest level of differential expression between 22 CD5⁺ and 26 CD5⁻ DLBCL cases are aligned, and multiples of the average differences in their expression levels are shown on the vertical axis. Twenty-four genes showing an average difference of more than 2.5-fold between the subgroups were designated as the CD5 signature genes. Seventy genes in total showed a difference of more than 2.0-fold. (b) Hierarchical clustering of 48 DLBCL cases via the expression levels of the 24 CD5 signature genes. Each row represents one gene. The dendrogram on the left shows the degree to which each gene is related to the others. Half of the CD5 signature genes in CD5⁺ DLBCL showed low expression levels (upper portion of the figure, shown in blue), some of which were related to the extracellular matrix (*POSTN*, *SPARC*, *COL1A1*, *COL3A1*, *CTSK*, *MMP9* and *LAMB3*). The remaining CD5 signature genes in CD5⁺ DLBCL showed high expression (lower portion of the figure, shown in red). The relative expression of *CD5* for each sample is indicated at the bottom of the figure, which also shows the *P*-values of these genes, determined using the Student's *t*-test. Each column represents one DLBCL case and the dendrogram on the top shows the degree to which each DLBCL is related to the other tumor samples in terms of gene expression. The DLBCL cases were divided into two subgroups: CD5⁺-type (left) and CD5⁻-type DLBCL (right). There was a cluster of 15 CD5⁺ DLBCL cases at the core of the CD5⁺-type DLBCL group (marked with dark blue lines). One CD5⁻ DLBCL case, located in a row on the extreme right, belonged to neither of the subgroups. (c) Kaplan–Meier analysis of the CD5⁺-type and the CD5⁻-type DLBCL cases in this study. The CD5⁺-type DLBCL patients showed a significantly poorer prognosis than their CD5⁻-type DLBCL counterparts. The *P*-values for these subgroups were analyzed using the log-rank test.

Table 1. Diffuse large B-cell lymphoma (DLBCL) subgroups defined by different markers

Surface marker	mRNA profiling marker	
	CD5⁺-type DLBCL ($n = 31$)	CD5⁻-type DLBCL ($n = 16$)
CD5⁺ DLBCL ($n = 22$)	20	2
CD5⁻ DLBCL ($n = 25$)	11	14
		$P = 0.0008^{\dagger}$

†*P*-values were calculated with Fisher's exact test.

ABC or GCB DLBCL (Fig. 2a). Kaplan–Meier analysis further revealed that the ABC DLBCL cases under analysis showed a significantly poorer prognosis, compared with the GCB DLBCL samples (Fig. 2b; $P = 0.0028$). This is in agreement with results reported previously.[7,8]

Application of the CD5 signature to published microarray data

In order to further test the validity of our CD5 signature, we applied it to published microarray data. Among the data from the Cancer Genomics group, comprising the expression level of 44 792 genes from 176 DLBCL cases, we were able to locate 22 (91%) of the 24 CD5 signature genes. Clustering analysis of these DLBCL cases, carried out with 22 of the CD5 signature genes, identified two subgroups of DLBCL, showing downregulation and upregulation of extracellular matrix genes, respectively (Fig. 3a). In addition, the mRNA profiles of these subgroups were similar to the profiles obtained from our current DLBCL cases, such that the first subgroup could be identified as CD5⁺-type and the second subgroup as CD5⁻-type. The CD5⁺-type DLBCL cases again showed a

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Table 2. Characteristics of patients with diffuse large B-cell lymphoma (DLBCL)

Characteristic	$CD5^+$-type DLBCL ($n = 31$)		$CD5^-$-type DLBCL ($n = 31$)		*P*-value†
	n	%	*n*	%	
IPI factor					
Age > 60 years	20	65	11	69	>0.99
Stage > 2	27	90	7	50	0.0062
LDH > normal	23	79	10	91	0.65
Performance status > 1	8	28	0	0	0.08
Extranodal site > 1	8	30	1	7	0.13
IPI score 3–5 (high)	19	66	3	27	0.0398

†*P*-values were calculated with Fisher's exact test. IPI, international prognostic index; LDH, lactate dehydrogenase.

Table 3. Characteristics of patients with $CD5^+$ diffuse large B-cell lymphoma (DLBCL) and $CD5^-$ cases in the $CD5^+$-type DLBCL subgroup

Characteristic	$CD5^+$ DLBCL ($n = 22$)		$CD5^-$ DLBCL cases in the DLBCL subgroup ($n = 22$)		*P*-value†
	n	%	*n*	%	
IPI factor					
Age > 60 years	13	59	8	73	0.70
Stage > 2	19	95	9	82	0.28
LDH > normal	17	85	7	70	0.37
Performance status > 1	8	40	0	0	0.0288
Extranodal site > 1	6	32	3	30	>0.99
IPI score 3–5 (high)	14	70	6	60	0.69

†*P*-values were calculated with Fisher's exact test. IPI, international prognostic index; LDH, lactate dehydrogenase.

trend toward a poorer prognosis than their $CD5^-$-type counterparts, although this difference was not statistically significant ($P = 0.0762$; Fig. 3b).

We investigated the relationship between $CD5^+$-type and $CD5^-$-type DLBCL, and also between ABC and GCB DLBCL. The 176 DLBCL cases were divided into 34 cases of ABC DLBCL, 85 cases of GCB DLBCL and 57 cases of classless leftovers on the basis of published data.[10,14] These ABC and GCB DLBCL cases could be divided into $CD5^+$-type and $CD5^-$-type DLBCL groups, so that the DLBCL cases could be classified into four subgroups by combining these two different modes of expression profiling. In the ABC DLBCL group, the $CD5^+$-type cases showed a poorer prognosis than the $CD5^-$-type cases (Fig. 4a; $P = 0.0397$). However, in the GCB DLBCL group, there was no significant difference in survival outcome between the $CD5^+$-type and the $CD5^-$-type cases (Fig. 4b; $P = 0.5073$). Thus, the clinical outcome for the $CD5^+$-type cases in the ABC DLBCL group was poorer than the outcomes in the other three subgroups.

We also attempted to apply the CD5 signature to other published microarray data. However, as only eight (33%) of the 24 CD5 signature genes could be found among the Lymphochip microarray data,[8] we considered that the results of any analysis using so few genes would not be meaningful.

Discussion

In a Japanese study, $CD5^+$ DLBCL was identified as a known subgroup of DLBCL that is associated with poor prognosis.[3] Furthermore, $CD5^+$ malignancies in Asian countries are different from those in Western countries, as evidenced by the incidence of chronic lymphocytic leukemia (CLL), which is the most frequent leukemia in Europe and the USA, but occurs at one-fifth of this rate in Japan.[1,15,16] In this regard, clinicians in Asian countries are in a better situation to investigate $CD5^+$ DLBCL due to a lower level of noise that would be caused by high incidence of CLL and CLL-related malignancies. In our current study, we attempted to elucidate novel markers and further characterize the biological features of $CD5^+$ DLBCL by means of expression profiling. Differentially expressed genes between $CD5^+$ and $CD5^-$ DLBCL samples were selected and subjected to clustering analyses, resulting in the identification of a specific mRNA profile of $CD5^+$ DLBCL, which we refer to as a CD5 signature.

The CD5 signature shows a characteristic profile featuring downregulated genes that are associated with the extracellular matrix. This profile was also found in the DLBCL samples from independent array data. The lack of an extracellular matrix may well be partially responsible for the aggressive clinical features that are characteristic of $CD5^+$ DLBCL. Our CD5 signature was compared with some reported gene sets that are concerned with the DLBCL subgroups. (1) Gascoyne *et al.* have reported 10 genes to be differentially expressed in $CD5^+$ and $CD5^-$ DLBCL.[17] *MMP9* is one of these genes and was in fact included in our CD5 signature gene set. (2) Kobayashi *et al.* have previously described an mRNA profile for $CD5^+$ DLBCL,[18] but our current findings are somewhat discordant with these previous data. The study of Kobayashi *et al.* reported

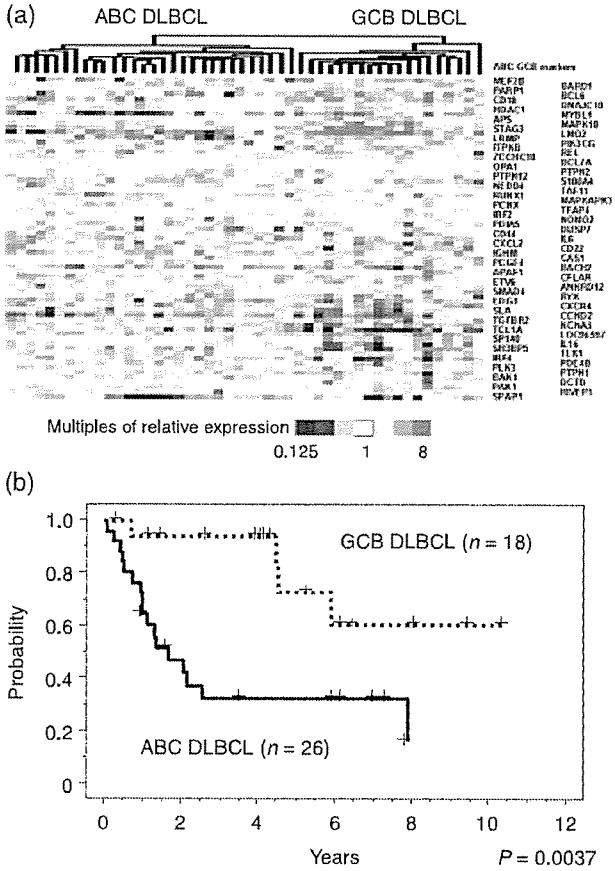

Fig. 2. Designation of activated B-cell-like (ABC) and germinal-center B-cell-like (GCB) diffuse large B-cell lymphoma (DLBCL) subtypes among the 48 DLBCL cases in this study. (a) Hierarchical clustering of the 48 DLBCL subject cases based on the expression levels of 67 known ABC and GCB marker genes that are also common to our dataset.[8] Our DLBCL samples were classified as ABC (left) and GCB (right) DLBCL in terms of the specific mRNA profiles of these marker genes. (b) Kaplan–Meier analysis of the ABC and GCB DLBCL samples among the current 48 DLBCL cases. This survival analysis showed significant differences between these subgroups.

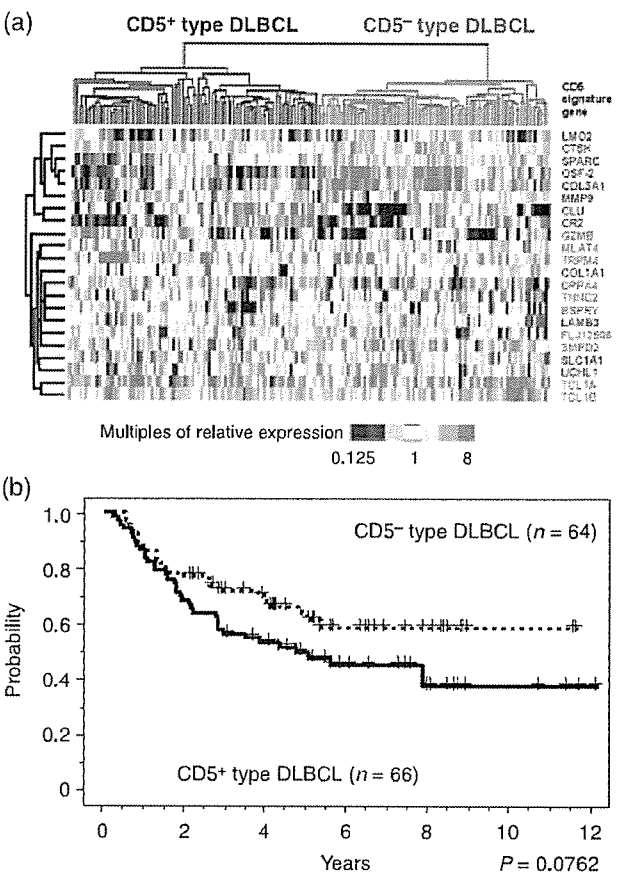

Fig. 3. Characterization of CD5⁺-type and CD5⁻-type diffuse large B-cell lymphoma (DLBCL) with existing published microarray data. (a) Clustering analysis of DLBCL samples from the Cancer Genomics group in terms of the expression levels of 22 of the CD5 signature genes. The DLBCL samples from published microarray data were classified as CD5⁺-type (left) and CD5⁻-type (right) DLBCL. The CD5⁺-type DLBCL was again characterized by downregulation of the genes that are related to the extracellular matrix, but there were some exceptions. The genes that showed a low expression level in our CD5⁺-type DLBCL cases (Fig. 1b) are indicated in blue and those with high expression (Fig. 1b) are shown in red. (b) Kaplan–Meier analysis of the Cancer Genomics group DLBCL patients that were subgrouped by means of the CD5 signature. The CD5⁺-type DLBCL patients again showed poorer prognosis than the CD5⁻-type DLBCL patients, although this difference was found not to be statistically significant. The *P*-values for these subgroups were analyzed with the log-rank test.

ITGB1 as one of the strong classifier genes showing high expression in CD5⁺ DLBCL, whereas our present analysis shows its expression in CD5⁺ DLBCL to be 1.28 times lower on average, compared with CD5⁻ DLBCL. The classifier gene *CD36* was also reported by Kobayashi *et al.* to show high expression levels in CD5⁺ DLBCL, but was found to be only 1.29 times higher in our present analysis. Significantly, none of the CD5 signature genes that we identified overlap with these previously characterized classifiers. These differences may be partly due to the fact that the study of Kobayashi *et al.* incorporated a 2400 spotted array, whereas we have used a 21 619 gene array. (3) The lymph-node signature is a profile of DLBCL reportedly related to clinical outcome and includes many extracellular matrix-associated genes.[8] Of these 375 lymph-node signature genes, 124 were included in our present data set and a further eight of our 24 CD5 signature genes can be found in their data set.[8] However, only three of these CD5 signature genes overlap, whereas the remaining five differ from the lymph-node signature genes, suggesting that our CD5 signature is likely to be different from the lymph-node signature. (4) We previously reported that ABC DLBCL was closely related to CD5⁺ DLBCL,[6] but none of the ABC and GCB markers[7] overlap with the CD5 signature genes. However, *IRF4*, which is one of the markers that shows high expression in ABC DLBCL, was found in our current analysis to be expressed at a level that was on average 1.56 times higher in CD5⁺ DLBCL than in CD5⁻ DLBCL. Other markers that have low expression in ABC DLBCL include

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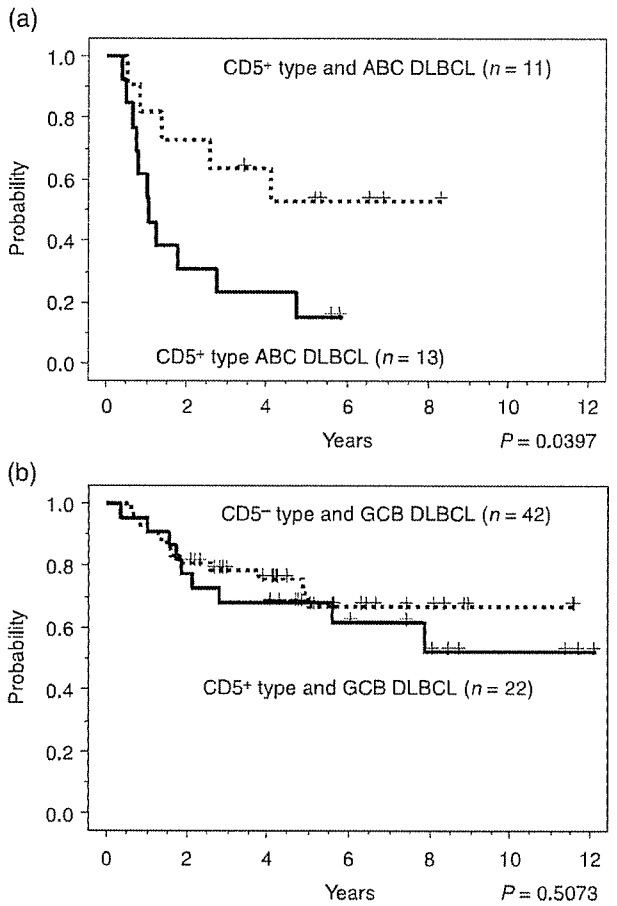

Fig. 4. Comparison of the CD5⁺-type and the CD5⁻-type diffuse large B-cell lymphoma (DLBCL) in combination with activated B-cell-like (ABC) and germinal-center B-cell-like (GCB) DLBCL. Kaplan–Meier analysis of (a) ABC DLBCL and (b) GCB DLBCL patients from the Cancer Genomics group subgrouped by means of the CD5 signature. The clinical outcomes were poorest for ABC patients with CD5⁺-type DLBCL. In contrast, there was no significant difference in survival outcome between the CD5⁺-type and the CD5⁻-type GCB subgroups. The *P*-values for these subgroups were analyzed with the log-rank test.

CD10 and *BCL6*, expressed 1.39 times and 1.88 times lower, respectively, in CD5⁺ DLBCL, and this is in agreement with results reported previously.[6] (5) Array comparative genomic hybridization (CGH) analyses have previously uncovered some genomic imbalances that characterize CD5⁺ DLBCL,[5,6] one of which is a loss of 9p21. This is consistent with our present mRNA profile analysis, which shows that the expression of *CDKN2A*, located on 9p21, is on average 1.5 times lower in CD5⁺ DLBCL, compared with CD5⁻ DLBCL. However, we did not include this gene in the CD5 signature because its differential expression was lower than the 2.5-fold cut-off threshold. This threshold for inclusion in the CD5 signature is probably one of the reasons why none of the CD5 signature genes is located on chromosomal loci that have been shown to be lost or gained in lymphoma.

Although the CD5 signature genes are statistically representative of CD5⁺ DLBCL, there were some CD5⁻ DLBCL cases found to express the CD5 signature. Eleven of 26 CD5⁻ DLBCL cases expressed the CD5 signature, according to our microarray data, and although only 5–10% of the published cases were expected to be CD5⁺ DLBCL,[3] 50% of all DLBCL cases from the Cancer Genomics group were found to express the CD5 signature. The incidence of CD5⁻ DLBCL cases expressing the CD5 signature can be partly explained by the results of the clustering analysis of our current cases (Fig. 1b). The CD5⁺-type DLBCL group that we identified is composed of two parts: a central cluster consisting of only CD5⁺ DLBCL cases and the rest comprising mainly CD5⁻ DLBCL patients. This indicates that the CD5 signature provides a rough identification of CD5⁻ DLBCL with expression profiles that are similar to those of CD5⁺ DLBCL, and that these cases may in fact resemble CD5⁺ DLBCL. Indeed, these same CD5⁻ DLBCL cases did show clinical features that resemble CD5⁺ DLBCL. We speculate that CD5⁻ DLBCL cases that show clinical features similar to CD5⁺ DLBCL will become evident also in independent array data sets.

We utilized CD5 mRNA expression levels to determine which samples could be assigned to the CD5⁺ DLBCL subgroup among the available DLBCL cases of the Cancer Genomics group. However, this turned out to be ineffective because the CD5 expression levels evaluated by microarray did not correlate with the results obtained by immunostaining or FACS analysis. This discrepancy was most likely caused by background cells such as T cells that express CD5 more strongly than CD5⁺ DLBCL. However, when the CD5 signature was applied to independent data sets, it was found to be a useful tool that could be used to assign CD5⁺ DLBCL cases.

The CD5⁺-type DLBCL tumors showed a significantly poorer clinical outcome than the CD5⁻-type DLBCL in our present analysis. The significance of the differences in survival outcome between CD5⁺-type and CD5⁻-type DLBCL was further examined by analysis of the independent data set from the Cancer Genomics group and the *P*-value for this set was calculated as 0.0762, whereas that for our current study was 0.0037. Although the independent data set did not show significance, there was a measurable trend toward a poor prognosis, which should be noted. Interestingly, however, the CD5⁺-type DLBCL of the ABC type showed the worst prognosis, whereas the CD5⁻-type DLBCL of the ABC type showed a better prognosis that was almost equivalent to the GCB DLBCL cases. The two types of DLBCL of the GCB type, however, showed no significant difference in clinical outcome. We contend therefore that the CD5 signature could serve as an effective future marker of DLBCL when used in combination with ABC/GCB profiling subtypes. Hence, both ABC/GCB and the CD5 signatures are likely to be effective in identifying the DLBCL subtype with the poorest prognosis and to thus help determine the most appropriate treatment for DLBCL patients.

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