

POLYMORPHISM OF ADHESION MOLECULE CD31 AND ITS ROLE IN ACUTE GRAFT-VERSUS-HOST DISEASE

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Abstract Background. Graft-versus-host disease (GVHD) caused by poorly defined minor (i.e., other than HLA) histocompatibility antigens remains a serious problem in recipients of bone marrow transplants. We sought to determine whether the CD31 adhesion molecule is a minor alloantigen.

Methods. We directly sequenced samples of complementary DNA (cDNA) encoding CD31 molecules from 21 unrelated normal subjects. Sequence-specific primers were then designed to amplify alleles by the polymerase chain reaction, thereby permitting CD31 typing of genomic DNA from additional normal subjects. To assess the relevance of CD31 matching to bone marrow transplantation, we performed CD31 typing of 46 recipients of bone marrow (32 without GVHD and 14 with severe [grade III or IV] acute GVHD) and their HLA-identical sibling donors. The immunoreactivity of CD31 phenotypes with anti-CD31 monoclonal antibodies was compared by flow cytometry.

Results. Direct sequencing of cDNA for CD31 from

the 21 normal subjects identified a single polymorphism, CTG→GTG (Leu→Val), at codon 125; we designated the resulting alleles CD31.L and CD31.V, respectively. The CD31 genotypes of these and 142 other unrelated subjects were of the expected frequencies. Among the transplant recipients, 71 percent of those with acute GVHD had CD31 genotypes that were not identical to the donor's genotype, as compared with 22 percent of the recipients without GVHD ($P = 0.004$). The binding of anti-CD31 monoclonal antibodies as measured by fluorescence-activated cell sorting correlated with the CD31 types of homozygous cell lines.

Conclusions. The adhesion molecule CD31 is polymorphic. When donor and recipient genotypes are not identical, the risk of GVHD increases. Prospective CD31 typing may reduce the risk of acute GVHD. (N Engl J Med 1996;334:286-91.)

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GRAFT-VERSUS-HOST disease (GVHD) is a frequent and often fatal complication of allogeneic bone marrow transplantation. Chemoprophylaxis of the recipient, reduction of the number of T cells in the donor marrow, or both have improved the outcome, but acute GVHD still occurs in 10 to 40 percent of recipients, even when the donor is a sibling who is genotypically identical at the major histocompatibility (HLA) locus.¹⁻⁴ GVHD in recipients of HLA-identical marrow has been attributed to incompatibility between minor histocompatibility antigens. Currently, differences at such minor loci are detectable in a limited way and only by complex tests of cytotoxic T lymphocytes; moreover, the molecular polymorphisms corresponding to minor-locus alloantigens in humans have not yet been identified.⁵⁻¹¹

We postulated that a likely site for minor-locus alloantigens would be the cell surface of vascular endothelial cells, a point of first contact between transplanted tissue and its host. However, alloantigens on vascular endothelial cells have not been well characterized by biochemical techniques or the use of alloantibodies.¹²⁻¹⁹ We therefore sought polymorphisms in genes encoding molecules on the surface of such cells, as a way of identifying new alloantigenic systems. The molecule selected for study was the platelet-endothelial-cell adhesion molecule 1 (PECAM-1, or CD31), a 120-to-130-kd membrane glycoprotein that is constitutively expressed on vascular endothelial cells, bone marrow stem cells, plate-

lets, and most circulating leukocytes.^{20,21} A member of the immunoglobulin superfamily, the CD31 molecule has eight protein domains — six extracellular, one transmembrane, and one cytoplasmic. Four CD31 sequences, obtained from independently cloned samples of complementary DNA (cDNA), have been published.^{20,22-24} They show eight nonrecurring discrepancies in amino acids scattered throughout the extracellular domains, suggesting polymorphism of CD31. To test this possibility, we studied the two parts of the extracellular domains of CD31 that have the most numerous reported discrepancies in the sequence (codons 97 through 158 and 347 through 416). We found a polymorphism of CD31 that correlated with the results of phenotyping using anti-CD31 monoclonal antibodies and with the occurrence of acute GVHD in patients who had received bone marrow transplants.

METHODS

Molecular Biologic Techniques

CD31 polymorphism was first identified by direct automated sequencing of the targeted regions of cDNA from randomly selected, unrelated subjects. This method avoids artifacts of cloning and permits the identification of heterozygous bases.²⁵ We first extracted total RNA using Trizol (GIBCO BRL, Gaithersburg, Md.) from cultured umbilical-vein endothelial cells from 12 normal infants and peripheral-blood mononuclear cells from 9 randomly selected normal blood donors. These RNA samples were reverse-transcribed, and the cDNA was amplified by the polymerase chain reaction (PCR) with Gene Amp RNA PCR kits (Perkin-Elmer Cetus, Branchburg, N.J.) and primer pairs 1, 2, and 3 (Table 1), designed to amplify the regions of interest in the CD31 molecule. One member of each pair of primers was biotinylated. The other member of the pair was used as the sequencing primer and was fluoresceinated directly, or the sequencing reaction was labeled with fluorescein-labeled deoxyadenosine triphosphate. Using as template the single-strand biotinylated PCR product, which was immobilized on streptavidin-coated magnetic beads (Dy-

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nal, Oslo, Norway), we sequenced cDNA directly. The reactions were performed with T7 sequencing kits (Pharmacia, Uppsala, Sweden), and the results were interpreted with an Automated Laser Fluorescent DNA sequencer (Pharmacia). The positions of the codons in the cDNA are cited according to the nucleotide-sequence data base of the European Molecular Biology Laboratory (accession number, M 28526), as submitted by Newman et al.,²⁰ and the positions of the introns are given as in the sequence described by Kirschbaum et al.²⁶ The codons we expected to be polymorphic corresponded to discrepancies identified by comparing published sequences of CD31.^{20,22-24,26}

Sequence-specific primers (primer pairs 4 and 5, Table 1) were then designed for allele-selective amplification by PCR of the genomic sequences from codons 97 through 158. These primers were used to test 142 randomly selected samples of DNA from unrelated subjects that were available in our tissue-typing laboratory. Genomic DNA was extracted (Biosynthesis, Lewisville, Tex.) and amplified by PCR with the sequence-specific primers for 35 cycles (MJ Research, Watertown, Mass.) of 94°C for one minute, 63°C for one minute, and 72°C for one minute in PCR reagents (Perkin-Elmer Cetus). The PCR products were electrophoresed on agarose gel to determine the presence or absence of bands representing each targeted allele.

Fluorescence-Activated Cell Sorting

We used three murine antihuman CD31 monoclonal antibodies: L133 (Becton Dickinson, Mountain View, Calif.), 7E8,²⁷ and EF70 (provided by E. Engleman, Stanford University). Umbilical-vein endothelial cells were incubated at 4°C for 30 minutes with each antibody individually, washed with phosphate-buffered saline, and then incubated at 4°C for 30 minutes with fluoresceinated goat antimouse immunoglobulin (Tago, Burlingame, Calif.) before analysis by fluorescence-activated cell sorting with a flow cytometer (Epics, Coulter, Hialeah, Fla.). Values for the mean channel number associated with each peak were calculated after conversion from a log scale to a linear scale and subtraction of the background values. Differences of fewer than 30 channels were considered to be not significant.

Selection of Patients

All protocols were approved by the Institutional Review Board at Stanford University. Informed consent was obtained from each patient (and each normal subject or parent of a normal infant). Each patient had received bone marrow from an HLA-identical sibling. GVHD was diagnosed and graded as a consensus of the opinions of the attending transplant physicians, according to previously published, standard clinical criteria,^{28,29} summarized as follows. The hallmarks of grade III acute GVHD are a maculopapular eruption involving 25 to 50 percent of the body-surface area or generalized erythroderma, moderately elevated serum bilirubin concentrations (3.0 to 14.9 mg per deciliter [51 to 255 μmol per liter]), moderate diarrhea (more than 60 ml of stool per kilogram of body weight per day or more than 1500 ml per day), and marked general clinical impairment. The most important criteria for grade IV acute GVHD are generalized erythroderma with the formation of bullae and often with desquamation, very high serum bilirubin concentrations (≥15 mg per deciliter [256 μmol per liter]), severe diarrhea (more than 90 ml of stool per kilogram per day or more than 2000 ml per day, with abdominal pain), and extreme clinical impairment. To minimize uncertainty in the grading of acute GVHD, we compared only the two most clear-cut diagnostic groups: patients with no GVHD for at least 100 days after transplantation and patients with severe (i.e., grade III or IV) acute GVHD. This design excluded patients with chronic GVHD or mild acute GVHD (groups that together constitute approximately half of our patients undergoing bone marrow transplantation), but it also eliminated diagnostic ambiguity and avoided the problem of determining whether chronic and acute GVHD are different diseases.

DNA was selected for CD31 testing solely on the basis of the availability of samples obtained before transplantation that had been kept frozen in the clinical laboratory after routine pretransplantation testing for HLA loci and DNA microsatellites. There was DNA from 32 patients without GVHD and 14 patients with acute GVHD, a yield that approximated the ratio of patients in those two groups at our institution² (and unpublished data). The personnel performing

the CD31 typing were kept unaware of the classification of each patient's GVHD.

We compared the CD31 alleles in the recipients of bone marrow with the alleles in their HLA-matched sibling donors and determined whether the CD31 genotypes were identical, compatible (i.e., the recipient had no CD31 alleles that were foreign to the donor), or mismatched (i.e., the recipient had a CD31 allele that was foreign to the donor) for GVHD.

Statistical Analysis

The goodness of fit to the Hardy-Weinberg equilibrium³⁰ and the statistical significance of differences between groups were calculated by Fisher's exact test (two-tailed) for all two-by-two tables, by the rank-sum test (two-tailed) for differences in ages, and by the chi-square test for all other tables.³¹ Odds ratios and 95 percent confidence intervals were computed by standard methods.³²

RESULTS

Molecular Polymorphism

The sequencing of cDNA from cultured umbilical-vein endothelial cells from 12 infants and peripheral-blood mononuclear cells from 9 blood donors revealed a polymorphism in the targeted regions of the CD31 gene and distinguished homozygotes bearing different genotypes from each other and from heterozygotes (Fig. 1). A frequent $\text{CTG} \rightarrow \text{GTG}$ substitution was found in codon 125 of domain 1, corresponding to the presence of a Leu-125

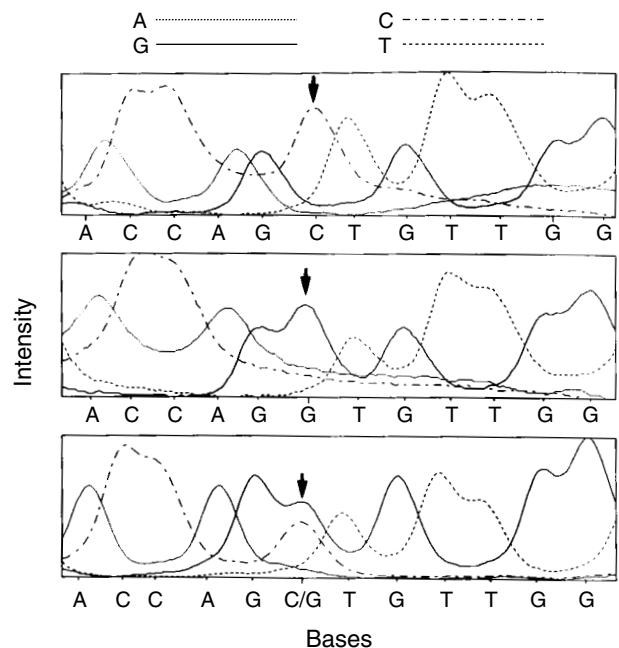


Figure 1. Results of Direct Sequencing of cDNA to Detect Polymorphism of CD31 Molecules.

The figure shows typical results obtained when primer pair 1 (as defined in Table 1) is used as described in the Methods section to amplify its target region in samples of cDNA from three subjects. In each panel, the positions of the bases are shown on the horizontal axis, and the intensity of fluorescence for each band of the sequencing gel is shown on the vertical axis. The arrows indicate the position of the first base of codon 125 — C in the top panel (showing a Leu homozygote), G in the middle panel (a Val homozygote), and C/G in the bottom panel (a Leu/Val heterozygote).

or a Val-125 allele, respectively, which we provisionally designated CD31.L and CD31.V. No other polymorphism was seen. In particular, the polymorphisms suggested by other investigators to be present at codons 115, 151, 375, and 391 were not found in any of the 21 samples (i.e., the 42 haplotypes) we sequenced (Table 1).

We assessed the genomic sequences of CD31 in DNA samples from 142 unrelated, randomly selected normal subjects, 38 of whom were white, using allele-specific PCR amplification (Table 1). With this method, a 342-base-pair band was produced only by the CD31.L alleles with primer pair 4 and only by the CD31.V alleles with primer pair 5. Among the 163 subjects studied overall, the combined frequencies of CD31.L homozygotes (0.30), CD31.V homozygotes (0.28), and CD31.L/CD31.V heterozygotes (0.42) were a good fit (chi-square, 3.04; $P=0.24$) to the Hardy-Weinberg equilibrium, as was the fit when only the smaller subgroup of whites was used.

Immunoreactivity

We sought evidence of an immunologic difference between the two CD31 alleles by flow-cytometric analysis of cultured umbilical-vein endothelial cells homozygous for CD31, using three anti-CD31 monoclonal antibodies. Figure 2 shows that antibody L133 bound equally to cells of both homozygous types, whereas antibody 7E8 bound to cells of the CD31.L type two to

four times more than to those of the CD31.V type. Antibody EF70 bound 1.6 to 1.7 times more to cells of the CD31.L type than to those of the CD31.V type. This differential immunoreactivity was also seen in pairwise comparisons among the seven other umbilical-vein endothelial cell lines homozygous for either CD31.L or CD31.V that were tested with the three monoclonal antibodies (data not shown).

Association with GVHD

We sought to determine the clinical relevance of the CD31 Leu→Val polymorphism in patients who had received bone marrow from sibling donors with HLA-identical genotypes. Because current protocols of bone marrow transplantation are rarely complicated by graft rejection, the more frequent problem of GVHD was chosen as an outcome measure of non-HLA histoincompatibility.¹⁻⁴ Patients with no GVHD were compared with those who had grade III or IV acute GVHD. These two well-defined groups had similar clinical characteristics (Table 2) before transplantation and a similar distribution of HLA and ABO antigens (data not shown). Among the characteristics generally agreed to affect the risk of acute GVHD (age, disease status, GVHD prophylaxis, and whether or not the donor and the recipient are of the same sex),^{2,4} only the median ages of the two groups differed significantly (the patients with acute GVHD were older).

The results of CD31 typing of the patients and their donors are shown in Table 3. CD31 genotypes were distributed similarly in the two groups. In contrast, the groups differed significantly ($P=0.004$) when the donor-recipient pairs were compared with regard to CD31 identity; non-identical CD31 types were more frequent (71 percent, or 10 of 14 patients) among the recipients with acute GVHD than among the recipients without GVHD (22 percent, or 7 of 32 patients). The odds ratio for acute GVHD in patients with CD31-nonidentical donors, as compared with patients who had CD31-identical donors, was 8.9 (95 percent confidence interval, 2.3 to 35.2). Incompatibility (as compared with the immunologically more stringent characteristic of nonidentity) between the recipient and the donor regarding CD31 was also a significant risk factor for acute GVHD (odds ratio, 7.2; 95 percent confidence interval, 1.6 to 32.7; $P=0.03$).

Because children have GVHD less often than adults, we analyzed the data again after excluding the

Table 1. Identification of CD31 DNA Polymorphism.

PRIMER PAIRS*	TARGET	POLYMORPHIC CODONS		PHENOTYPE AT CODON 125†		
		EXPECTED‡	OBSERVED	L	V	L/V
				<i>no. of subjects</i>		
Used in direct sequencing of cDNA (n = 21)§				4	7	10
Pair 1		115, 125, 151	125			
	5'GCACAGAGAGTTATTTTATTCCTG	Codon 97 (domain 1)				
	5'CAATTGTGAAGTGTATTGGGG	Codon 158 (domain 2)				
Pair 2		375, 391	None			
	5'ACTCAAGATTTCACCAAGATAGCC	Codon 347 (domain 4)				
	5'CTTTTATGACCTCAAAGTGGGC	Codon 408 (domain 5)				
Pair 3		375, 391	None			
	5'GTGAAAGACTGAACCTGTCCTGC	Codon 376 (domain 4)				
	5'CATAAGAAATCCTGGGCTGGG	Codon 416 (domain 5)				
Used in PCR amplification of genomic DNA (n = 142)¶				45	38	59
Pair 4		—	—			
	5'CTGCCTTCCTTCGGGTTGCA	CTG (Leu-125) allele				
	5'CAAGGACTCACCTCCACCAACAG	End of intron 2				
Pair 5		—	—			
	5'CTGCCTTCCTTCGGGTTGCA	GTG (Val-125) allele				
	5'CAAGGACTCACCTCCACCAACAC	End of intron 2				
		Codon 125				

*For each pair shown, the first primer listed is the forward primer, and the second is the reverse primer. The forward primer in pair 1 had an additional, unique 5' extension of 5'CGACGTTGTAACGACGGCCAGT matching the M13U sequencing primer in the Pharmacia sequencing kit.

†L denotes homozygosity for leucine, V homozygosity for valine, and L/V heterozygosity for leucine and valine.

‡These codons were expected to contain polymorphisms on the basis of discrepancies identified in published sequences of CD31, as described in the Methods section.

§Based on the study of cultured umbilical-vein endothelial cells from 12 normal infants and peripheral-blood mononuclear cells from 9 randomly selected normal blood donors.

¶Based on the study of 142 randomly selected DNA samples from unrelated subjects that were available in the tissue-typing laboratory.

six patients under the age of 18 years, a group in which five patients had no GVHD and one had acute GVHD. When only adults were considered (median age among those without GVHD, 31 years; among those with acute GVHD, 36 years; $P=0.04$), CD31 nonidentity became an even greater risk factor for GVHD ($P=0.001$). Nonidentity was found in 10 of the 13 patients with acute GVHD (77 percent), but only 5 of the 27 patients without GVHD (19 percent; odds ratio, 14.7; 95 percent confidence interval, 3.2 to 67.6).

DISCUSSION

The HLA system of major histocompatibility antigens is well understood in humans, but little is known about other clinically important alloantigens. We studied the CD31 adhesion molecule as a possible minor alloantigen because of its tissue distribution and suspected heterogeneity in the population. We identified an allelic Leu→Val polymorphism at codon 125 of the CD31 gene. Because we identified no other replacements at codon 125 in the 42 haplotypes we sequenced and because the frequencies of the various genotypes among the patients we tested fit the Hardy-Weinberg equilibrium, the Leu and Val alleles accounted for the great majority (approximately 98 percent) of the polymorphisms at that codon. We could not verify the amino acid replacements reported by others at codons 115, 151, 375, and 391. These apparent variations in findings may be due to errors in cloning or sequencing or to the presence of rare, as yet unvalidated alleles (with gene frequencies of 0.02 or less).

The distribution of the alleles at codon 125 correlated with the ability of homozygous cell lines to react with particular anti-CD31 monoclonal antibodies. It is known that a single Leu→Val substitution in immunoglobulin light chains gives rise to allotypes that can be distinguished with the use of alloantibodies³³ and that a difference of a single amino acid between allelic forms of cell-surface molecules can cause the production of alloantibodies. Examples are the exchange of proline for leucine at codon 33 of the platelet-specific HPA-1 antigen³⁴ and the exchange of aspartic acid for leucine at codon 156 of the HLA B44 allele. The rejection of bone marrow allografts has been traced to the difference of a single amino acid between the HLA B44 alleles.³⁵

The immunogenicity of minor histocompatibility antigens can be determined definitively *in vivo* on the basis of graft rejection or the occurrence of GVHD among recipients of HLA-identical grafts. We therefore looked for a relation between CD31 matching and GVHD in patients who had received bone marrow from siblings with the same HLA genotype. Recipients of marrow from CD31-nonidentical donors had a substantially higher risk of acute GVHD than recipients of CD31-identical marrow. As expected,^{2,4} the median age of the patients without GVHD was lower than that of those with acute GVHD.

We used CD31 identity between the graft and the

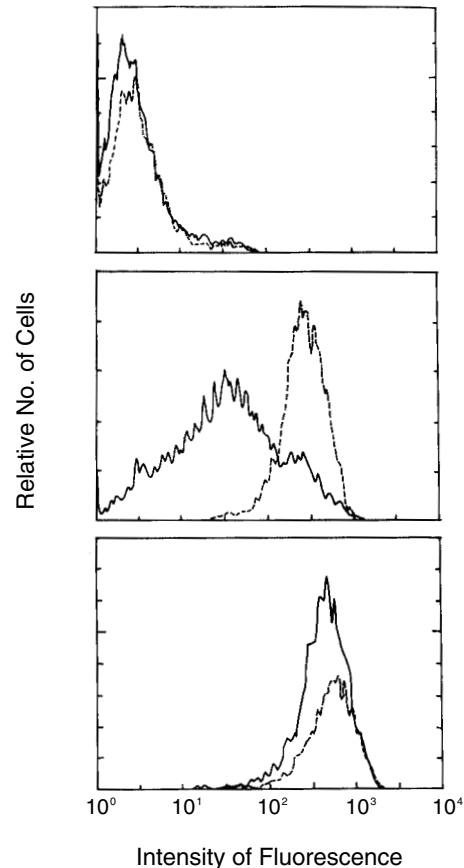


Figure 2. Fluorescence-Activated Cell-Sorter Analysis of Binding of Anti-CD31 Monoclonal Antibodies to Umbilical-Vein Endothelial Cells.

Three anti-CD31 monoclonal antibodies were incubated individually with umbilical-vein endothelial cells from CD31.L homozygote cell line 69E (dashed lines) and CD31.V homozygote cell line 63E (solid lines). Channels showing the relative intensity of fluorescence are shown on a log scale on the horizontal axis, and relative numbers of cells per channel on the vertical axis. The top panel shows a control consisting of phosphate-buffered saline, the middle panel antibody 7E8, and the bottom panel antibody L133. The values for the mean channel number associated with each peak obtained with antibody 7E8 were 208 for cell line 69E and 87 for 63E; with antibody L133, they were 277 and 276, respectively.

host rather than compatibility (in which the recipient had no CD31 alleles that were foreign to the donor) to assess the relation between CD31 and acute GVHD. This approach required no preconceived notions about mechanisms or directions of compatibility and averted the need to address poorly understood phenomena of transplantation, such as hybrid resistance.^{36,37} Nevertheless, when we analyzed the CD31 compatibility (rather than identity) of the host with the graft, the results were similar. CD31 thus appears to be a clinically important minor histocompatibility antigen in humans.

The occurrence of GVHD in some recipients of CD31-identical marrow indicates that CD31 alloantigens are not the exclusive determinants of transplanta-

tion compatibility at minor loci, a finding consistent with reports about other minor histocompatibility markers.⁵⁻¹¹ The clear involvement of the CD31 alleles in GVHD has potentially important clinical implications. Prospective matching for CD31 alleles may reduce the risk of GVHD in patients undergoing bone marrow transplantation by affording the opportunity to select among suitable related donors with HLA-identical genotypes or among unrelated, HLA-matched donors when more than one such donor is available.

Other, more complex, explanations for the association between CD31 and GVHD are possible. For example, in the CD31 segments we did not sequence there could be amino acid substitutions that contribute to alloantigenicity and are found predominantly with specific alleles at codon 125. Alternatively, CD31 alleles may serve only as markers for other minor histocompatibility loci that are linked to CD31 on chromosome 17.³⁸ However, the immunologic difference between the two CD31 phenotypes is consistent with the hypothesis that CD31 itself has alloantigenicity.

CD31 is believed to have a role in attracting leukocytes to inflammatory sites, the activation of lympho-

Table 2. Clinical Characteristics of the Transplant Recipients Studied.

CHARACTERISTIC	No GVHD (N = 32)	ACUTE GVHD (N = 14)	P VALUE
Age (yr)			0.03
Median	29	35	
Range	6-51	12-54	
	<i>no. of patients</i>		
Sex			0.86
Male	18	9	
Female	14	5	
Race or ethnic group			0.36
White	21	10	
Hispanic	5	2	
Asian	6	1	
Black	0	1	
Disease status*			0.11
Early	25	9	
Intermediate	5	1	
Advanced	2	4	
Sex			0.84
Donor and recipient same sex	13	7	
Donor female, recipient male	8	3	
Donor male, recipient female	11	4	
GVHD prophylaxis in addition to cyclosporine			0.42
Prednisone	15	9	
Methotrexate	15	5	
Both	2	0	
Diagnosis			0.68
Acute lymphocytic leukemia	9	2	
Acute myelogenous leukemia	9	6	
Chronic myelogenous leukemia	8	3	
Myelodysplastic syndrome	0	1	
Non-Hodgkin's lymphoma	4	1	
Refractory anemia with excess blast cells	1	1	
Thalassemia	1	0	

*Early denotes disease in a first chronic phase or a first complete remission; intermediate, disease in a second complete remission or a first relapse; and advanced, disease in a third or subsequent complete remission, a second or subsequent relapse, or a blast crisis.

Table 3. Relation of CD31 Polymorphism to the Occurrence of GVHD.

CD31 STATUS	No GVHD (N = 32)	ACUTE GVHD (N = 14)	P VALUE
Recipient's genotype			0.69
CD31.L homozygote	15	8	
CD31.V homozygote	5	1	
CD31.L/V heterozygote	12	5	
Genotypes of donor and recipient			
Identical	25	4	
Nonidentical			
Both homozygous	1	2	
Heterozygous donor, homozygous recipient	4	4	
Homozygous donor, heterozygous recipient	2	4	
Genotypes matched for identity			0.004
Identical	25	4	
Nonidentical	7	10	
Genotypes matched for compatibility*			0.03
Compatible	29	8	
Incompatible	3	6	

*If the recipient has no CD31 alleles that are foreign to the donor, the genotypes are considered compatible; if any CD31 alleles found in the recipient are not also found in the donor, the genotypes are considered incompatible.

cytes, interactions between leukocytes and endothelial cells, and perhaps angiogenesis.^{21,39} It would be interesting to know whether the CD31 polymorphism we found affects these physiologic functions differentially and whether the functions have a role in the relation between CD31 polymorphism and GVHD.

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