Mutation analysis of hepcidin and ferroportin genes in Italian prospective blood donors with iron overload

Lorena Duca, Paola Delbini, Isabella Nava, Valentina Vaja, Gemino Fiorelli, and Maria Domenica Cappellini*

Maintenance of iron balance is essential for humans and requires the coordinate regulation of iron transport into plasma from dietary sources in the duodenum, from recycled senescent red cells in macrophages, and from storage in hepatocytes. Hepcidin, a recently identified antimicrobial peptide produced in the liver, has been shown to play a central role in the homeostatic regulation of iron absorption and distribution [1]. It is a negative regulator of iron absorption in the small intestine and of iron release from macrophages engaged in the recycling of iron senescent erythrocytes [2]. The human hepcidin gene contains three exons that encode a 72-aa precursor (pro-hepcidin) with a characteristic furin cleavage site immediately N-terminal to the 25-aa major hepcidin species found in plasma and urine [3]. Recently, hepcidin has been shown to regulate iron homeostasis by interaction with ferroportin, an iron cellular exporter highly expressed in absorptive enterocytes, macrophages, hepatocytes, and placental cells [4].

Hepcidin binds ferroportin, inducing its internalization and lysosomal degradation, and therefore decreases export of cellular iron to the plasma [5]. Hepcidin and ferroportin dysregulation, resulting from mutations in the genes encoding these proteins, is implicated in the pathogenesis of several iron disorders [6]. This study analyzed hepcidin and ferroportin genes in blood donors with increased iron load hereditary hemochromatosis gene (HFE) negative to clarify their possible role as primary genes responsible for iron load in individuals with a normal HFE genotype. Two-hundred voluntary blood donors originating from different areas of Italy were previously tested for hematological and iron parameters [7]. Twenty subjects (17 males and 3 females, 18–57 years) with increased transferrin saturation (%TS $>$ 50%; normal values $<$45%) or serum ferritin (SF $>$ 300 ng/mL; normal values $<$250 ng/mL) were submitted to genetic screening for hepcidin and ferroportin genes. Factors possibly responsible for the abnormalities in iron status such as HFE and TfR2 mutations, inflammation, or liver disease were excluded in all the 20 subjects. Moreover, none of them had unusual dietary habits or abnormal ethanol consumption that could explain their iron overload. The control cohort (n = 50; 30 males and 20 females, 20–60 years) included individuals with the same geographical origin, normal hematological and iron parameters, and negative results from viral and autoimmune screening studies.

Serum iron (SI), %TS, SF, aspartate transaminase (AST), and alanine transaminase (ALT) were obtained from routine clinical laboratory procedures. Red blood cells (RBC), hematocrit (Ht), hemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet count, and leukocytes were determined by standard methods. The genes of hepcidin and ferroportin were analyzed by PCR amplifications of single exons followed by mutations screening with direct sequencing and restriction analysis with endonucleases.

Allele and genotype frequencies were computed by the gene-counting method. Linkage disequilibrium (D') was calculated by performing pairwise comparisons for all SNP loci using the Haploview software. Hardy-Weinberg equilibrium (HWE) in the observed genotype distribution was assessed with the χ^2 test [8].

Twenty of 200 subjects had altered iron parameters (Table I). None of these subjects had mutations in the hepcidin gene. A polymorphism in intron 2 (IVS2 + 7G$>$A), already reported [9], has been identified in one individual. This subject carrying the mutant polymorphic allele without other mutations had a serum ferritin of 478 ng/mL and transferrin saturation of 54%. No hepcidin mutations were detected in the 100 chromosomes of healthy controls screened by enzyme digestion.

Complete sequencing of the promoter region and the eight exons of the ferroportin gene identified five common polymorphisms and one microsatellite in the promoter region of the gene [10]. The polymorphisms of the ferroportin gene were the following: 5′-UTR(−98)G$>$C, 5′-UTR(−8)C$>$G, $(CGG)_n$ 5′-UTR microsatellites, IVS1-24C$>$G, and 977 T$>$C (V221V).

The results obtained, reported as genotypes of the different polymorphism/mutations, are summarized in Table II. A comparison of the allelic frequencies between the groups was performed using the χ^2 test.

The 5′-UTR (−8G) and the −98 polymorphisms occurred at a higher frequency in the iron-loaded individuals (0.35 and 0.43, respectively) than in the control group (0.05 and 0.06, respectively). Moreover, the −8G and −98C alleles were in marked linkage disequilibrium. The frequency of the promoter microsatellite $(CGG)_7$ and $(CGG)_9$ genotypes was higher in the iron-overload group (0.30 and 0.23, respectively) than in the control group (0.28 and 0.04, respectively). The $(CGG)_9$ allele was in partial linkage disequilibrium with the −8G and the −98C allele.

The intronic polymorphisms IVS1(−24) and the T$>$C substitution in exon 6 at nt 663 (V221V) were common polymorphisms that exhibited allelic frequencies significantly higher in the subjects with altered iron parameters (0.50 and 0.65, respectively) when compared with the control group (0.30).

One known mutation [9], an A$>$G substitution at nt 1681 in exon 8 resulting in an amino acid change (R561G), was found in two subjects. One subject homozygous for this substitution showed a severe iron overload (SF = 2750 ng/mL and TS = 84%); the other carrying R561G in a heterozygous state had SF = 700 ng/mL and TS within the normal range. No other subjects were found to have this mutation. The amino acid affected by A$>$G substitution is not conserved within the species. The arginine in humans is replaced with a threonine in mice and a proline in zebrafish, suggesting that the substitution of this amino acid does not appear to be detrimental to the activity of ferroportin. It remains to be determined if the presence of R561G, in the homozygous or heterozygous state, is associated with iron burden as documented by the increase in transferrin saturation or ferritin levels.

A 744 G$>$T (Q248H) substitution, already described [9] in exon 7, was detected in subjects affected by iron overload at polymorphic frequencies (0.15). Heterozygosity was found in two individuals with moderate iron load, whereas homozygosity was present in 2 of 20 iron-loaded subjects and was associated with ferritin levels $>$600 ng/mL and transferrin saturation $>$60%.

TABLE I. Hematological and Iron Parameters in 20 Iron-Overloaded Blood Donors

Subjects	Hb (g/dL)	MCV (fL)	MCH (pg)	MCHC (g/dL)	AST (U/L)	ALT (U/L)	GGT (U/L)	SF (ng/mL)	SI (μg/dL)	TS (%)
Blood donors (n = 20)	14.7 ± 1.6	87.1 ± 1.9	29.7 ± 3.0	32.4 ± 2.2	27.5 ± 8.8	34.0 ± 21.5	27.8 ± 14.3	631 ± 526	130 ± 51	45 ± 21
Controls (n = 50)	14.0 ± 2.0	88.0 ± 1.7	32.0 ± 1.5	33.5 ± 1.5	22.5 ± 2.0	26.0 ± 1.6	22.0 ± 8.0	150 ± 70	110 ± 10	20 ± 5

TABLE II. Genotype and Allelic Frequency of Ferroportin in Blood Donors

	Genotype						Allelic frequency		
	$N_{8/8}$ (%)	$N_{8/7}$ (%)	$N_{7/7}$ (%)	$N_{8/9}$ (%)	$N_{7/9}$ (%)	$N_{9/9}$ (%)	7 (%)	8 (%)	9 (%)
Microsatellite repeats									
Cases ($n = 20$)	6 (30)	6 (30)	3 (15)	1 (5)	0 (0)	4 (20)	12 (30)	19 (47)	9 (23)
Controls ($n = 50$)	32 (64)	3 (6)	12 (24)	1 (2)	1 (2)	1 (2)	28 (28)	68 (68)	4 (4)

	Genotype			Allelic frequency		
	N_{AA} (%)	N_{Aa} (%)	N_{aa} (%)	*A* (%)	*a* (%)	*P* value
5′UTR(−8)						
Cases ($n = 20$)	9 (45)	8 (40)	3 (15)	26 (65)	14 (35)	<0.0001
Controls ($n = 50$)	46 (92)	3 (6)	1 (2)	95 (95)	5 (5)	
5′UTR(−98)						
Cases ($n = 20$)	8 (40)	7 (35)	5 (25)	23 (57)	17 (43)	<0.0001
Controls ($n = 50$)	45 (90)	4 (8)	1 (2)	94 (94)	6 (6)	
IVS1(-24)						
Cases ($n = 20$)	8 (40)	4 (20)	8 (40)	20 (50)	20 (50)	<0.05
Controls ($n = 50$)	27 (54)	16 (32)	7 (14)	70 (70)	30 (30)	
V221V						
Cases ($n = 20$)	4 (20)	6 (30)	10 (50)	14 (35)	26 (65)	<0.001
Controls ($n = 50$)	30 (60)	10 (20)	10 (20)	70 (70)	30 (30)	
Q248H						
Cases ($n = 20$)	15 (75)	4 (20)	1 (5)	34 (85)	6 (15)	<0.01
Controls ($n = 50$)	49 (98)	1 (2)	0 (0)	99 (99)	1 (1)	
L348M						
Cases ($n = 20$)	18 (90)	2 (10)	0 (0)	38 (95)	2 (5)	>0.05
Controls ($n = 50$)	50 (100)	0 (0)	0 (0)	100 (100)	0 (0)	
L348V						
Cases ($n = 20$)	19 (95)	1 (5)	0 (0)	39 (97)	1 (3)	>0.05
Controls ($n = 50$)	50 (100)	0 (0)	0 (0)	100 (100)	0 (0)	
R561G						
Cases ($n = 20$)	17 (85)	3 (15)	0 (0)	37 (92)	3 (8)	<0.05
Controls ($n = 50$)	50 (100)	0 (0)	0 (0)	100 (100)	0 (0)	

$N_{8/8}$: common homozygotes; $N_{8/7}$, $N_{8/9}$, $N_{7/9}$: heterozygotes; $n_{7/7}$, $n_{9/9}$: rare homozygotes; N_{AA}: common homozygotes; n_{Aa}: heterozygotes; n_{AA}: rare homozygotes; p allelic: Fisher's exact test on allele frequencies.

Interestingly, one of these Q248H carriers was also homozygous for V221V and IVS1(−24) polymorphisms and heterozygous for L384M common mutation in exon 7. The subject had serum ferritin and transferrin saturation values of 670 ng/mL and 70%, respectively. Q248H mutation has been hypothesized to cause hyperferritinemia in the homozygous state and lead to disease in the presence of other modifying factors [11].

Further studies are advisable to establish the possible involvement of iron-related genes in mild to moderated iron load. Although they are preliminary, our data support the hypothesis that polymorphisms of hepcidin and ferroportin genes are important contributors to iron storage and iron load.

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The role of antiphospholipid antibodies toward the protein C/protein S system in venous thromboembolic disease

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The association between venous thromboembolism (VTE) and antibodies anti-Protein C (PC)/Protein S (PS) is still uncertain. We performed a case-control study to determine the risk of VTE related to the presence of these auto-antibodies considered independently of the presence of lupus anticoagulant (LAC) or anti-cardiolipin antibodies (ACA). One hundred thirty-five patients with idiopathic VTE and 164 healthy subjects were enrolled. Anti-PC and anti-PS antibodies (both IgG and IgM) were assessed using commercially available ELISA kits. Among cases there was a higher prevalence of elevated anti-PC IgM antibodies than in controls (OR 2.44, 95%CI 1.00–5.94). The presence of anti-PC IgG and anti-PS IgG and IgM antibodies was also higher in cases than in controls, but the difference was not statistically significant. Only five patients had both anti-PC or anti-PS antibodies and LAC or ACA. We performed a stepwise multivariate logistic regression analysis showing that anti-PC IgM>95° percentile was a significant predictor of VTE after adjustment for LAC or ACA (OR 2.52, 95%CI 1.01–6.24)). Larger prospective studies are needed to confirm this finding.

Anti-phospholipid antibodies (APLA) are a heterogeneous group of immunoglobulins directed toward many targets constituted by phospholipids binding proteins. [1,2] The association among anticardiolipin-antibodies (ACA), anti-$\beta_2$microglobulin-antibodies, and LAC with venous thromboembolism (VTE) is widely accepted [3–8] but since 1990 many other targets of APLAs have been described, the most common being prothrombin and thrombin [9–12], coagulation protein C (PC) and S (PS) [13–15], annexin [16,17], thrombomodulin [18–20] and heparan-sulfate proteoglycans [13,14,21]. Some authors reported an association between anti-PC and anti-PS autoantibodies and VTE [15,22–26] but their role in the development of thrombotic disease is still a matter of debate.

We performed a case control study to evaluate the association between anti-PC/PS antibodies and VTE using as a cut-off for auto-antibodies positivity, the 95° percentile of antibodies levels calculated in controls.

A group of 179 cases and 173 healthy controls were enrolled. Forty-three (24%) of 179 cases were excluded because they were carriers of inherited thrombophilia and/or presented with other predisposing or risk factors for thrombosis. Nine (5.2%) of 173 controls were excluded because they exhibited inherited thrombophilia. Thus, 135 cases and 164 controls were included. No statistically significant difference between the two groups was observed as for age (mean age 49.57 years in cases and 49.27 years in controls; $P = 0.933$ Kruskal-Wallis test). Moreover, no statistically significant difference was seen in VTE risk according to gender (M/F 86/49 in cases and 88/76 in controls; OR 0.66, 95%CI 0.41–1.05). Considering anti-PC antibodies, the 95° percentile value in controls for IgG and IgM class was 7.1 AU/ml and 7.7 AU/ml, respectively. Twelve (8.9%) cases and 8 (4.9%) controls presented with an anti-PC IgG titre above the 95° percentile value. Fifteen (11.1%) cases and 8 (4.9%) controls showed an anti-PC IgM titre above the 95° percentile value. In bivariate analysis, cases with IgM anti-PC antibodies >95° percentile presented with a statistically significant higher risk of VTE disease than controls (OR 2.44, 95%CI 1.00–5.94) while anti-PC IgG antibodies >95° percentile did not show a significant association with VTE (O.R. 1.90, 95%CI 0.75–4.80). Considering anti-PS antibodies, the >95° percentile value in controls for IgG and IgM class was 10.3 AU/ml and 13.0 AU/ml, respectively. Eleven (8.1%) cases and 8 (4.9%) controls showed an anti-PS IgG titre above the 95° percentile value. Ten (7.4%) cases and 8 (4.9%) controls presented with an anti-PS IgM titre above the 95° percentile titre. In bivariate analysis, cases with anti-PS IgG and IgM antibodies >95° percentile presented a higher, albeit not statistically significant, risk of VTE disease than controls (OR 1.73, 95%CI 0.68–4.43 and 1.56, 95%CI 0.60–4.07, respectively) [Table I, Fig. 1]. In cases we observed a higher prevalence of LAC and/or ACA than in controls (14.8% vs 2.4%) and their association with VTE was statistically significant (OR 6.96, 95%CI 2.32–20.90). Among carriers of LAC, two subjects presented also anti-PC IgG and one had also anti-PS IgG antibodies. Among patients with ACA, one had also anti-PC IgG and one had both anti-PC IgM and anti-PS IgG antibodies. All of them were included as cases. A stepwise logistic regression analysis was used to identify, among age, gender, LAC and/or ACA and the presence of anti-PC and anti-PS IgG and IgM antibodies above the 95° percentile, significant covariates that predicted VTE. According to this method, only gender, anti-PC IgM>95° percentile and LAC or ACA were included in the multivariate analysis. Anti-PC IgM and the presence of LAC or ACA confirmed to be a risk factor for VTE (OR 2.52, 95%CI 1.01–6.24 and 7.59, 95%CI 2.50–23.03, respectively). Male gender was not shown to increase the risk of VTE (O.R. 0.65, 95%CI 0.40–1.06). [Table II]. By performing a stratification after the exclusion of patients with LAC and ACA, the same results were obtained.

Moreover, we performed the same analysis using as cut-off the 99° percentile in controls but, possibly because of the limited size of our study population, only seven subjects (6 cases and 1 control) had a titre of anti-PC IgM higher than 99° percentile found in controls. Thus, further analysis was not performed at this cut-off level.

In agreement with previous reports in the literature [27], in our study the presence of LAC or ACA was strongly associated with VTE with an estimated increased risk of about 7 times in affected patients. Our results seem to suggest a possible association between anti-PC and VTE but there are several methodological limitations which need to be taken into account. The association between IgM (but not IgG anti-PC antibodies) and VTE found in our study, deserves some comments. In fact, given that an association might exist, it has to be noted that usually the increased titre of IgG isotype is more involved in thrombotic complications than IgM. The retrospective design of our study does not allow to exclude that the presence of anti PC/PS antibodies is an epiphenomenon and not the cause of VTE and that a higher IgM titre could be a consequence other that a cause of a thrombotic event. Secondly, we can not evaluate the variation of antibodies levels during time and correlate them with the onset of VTE. Thus, VTE risk might be variable in time due to titre fluctuations in the same patient. Finally, we have not assessed the presence of antiβ_2-GPI antibodies in cases and controls and therefore we do not know the effect of the interaction, if any, between these autoantibodies and those who are directed against the protein C/protein S system.

In our study, selection bias was prevented by enrolling all consecutive patients referred to our Institution with objectively proven DVT or PE. Observation bias was prevented by having laboratory and instrumental tests for the diagnosis of VTE disease performed and interpreted by independent operators. Potentially confounding factors were avoided by excluding from the analysis carriers of inherited thrombophilia as well as patients with active cancer, pregnancy, hormonal treatment, recent surgery, trauma or prolonged bed resting. Other larger and prospective studies aimed to evaluate the VTE risk in patients with anti-PC/PS antibodies alone or in combination with other APLAs are needed. It will also be interesting to evaluate whether the presence of anti-PC and anti-PS antibodies can be associated with other clinical events such as a higher incidence of VTE recurrences or arterial thrombosis or pregnancy complications as it has been shown for other antiphospholipid antibodies.

TABLE I. Anti-PC and Anti-PS Antibodies in Cases and Controls and VTE Disease Risk in Bivariate Analysis

		Cases (n = 135)	Controls (n = 164)	OR; (95% CI) Univariate analysis
Anti-PC IgG	Positive	12 (8.9%)	8 (4.9%)	1.90; (0.75–4.80)
	Negative	123 (91.1%)	156 (95.1%)	
Anti-PC IgM	Positive	15 (11.1%)	8 (4.9%)	2.44; (1.00–5.94)
	Negative	120 (88.9%)	156 (95.1%)	
Anti-PS IgG	Positive	11 (8.1%)	8 (4.9%)	1.73; (0.680–4.43)
	Negative	124 (91.9%)	156 (95.1%)	
Anti-PS IgM	Positive	10 (7.4%)	8 (4.9%)	1.56; (0.60–4.07)
	Negative	125 (92.6%)	156 (95.1%)	

Positive: antibodies title >95° percentile calculated on controls. Negative: antibodies title ≤95° percentile calculated on controls.

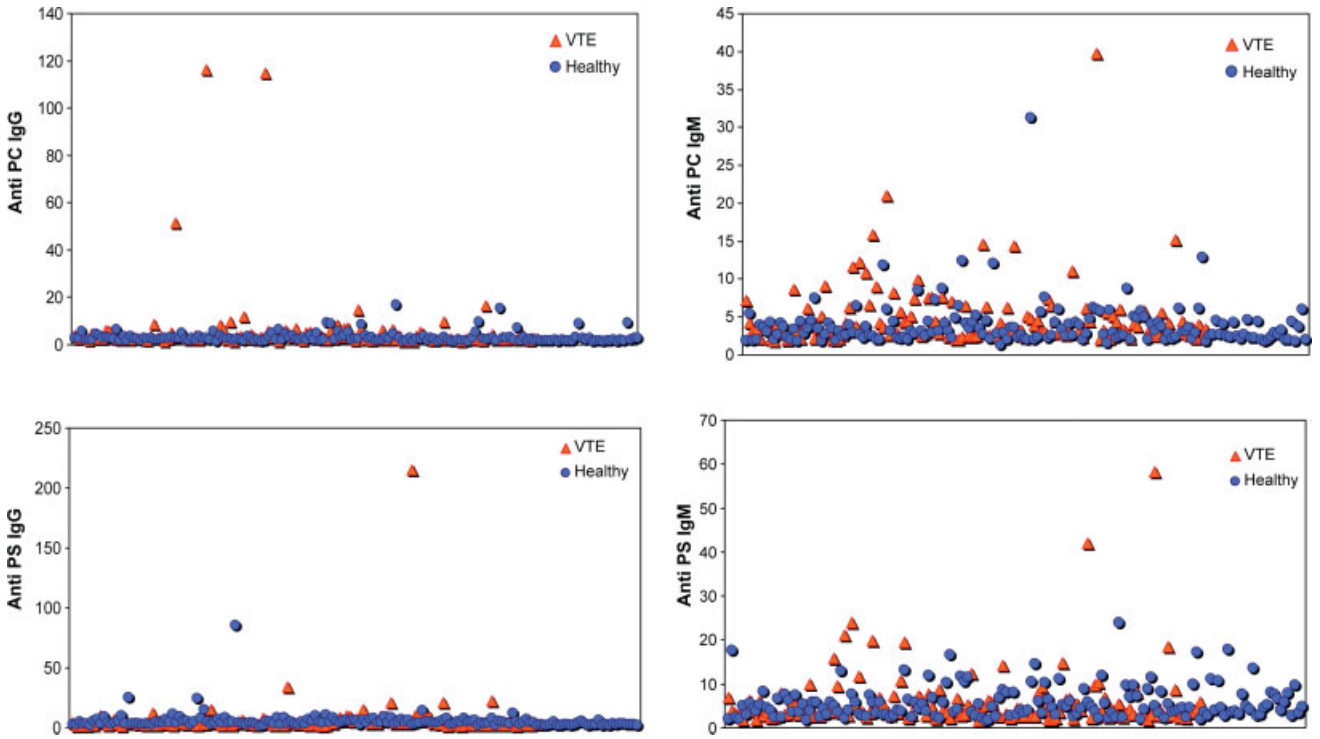

Figure 1. Individual antibodies values in cases and controls. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE II. Logistic Regression Model of Association of VTE with Variables Selected with Stepwise Method

Variables	OR; (95% Wald CI)
Male	0.65; (0.40–1.06)
Anti PC IgM>95°perc.	2.52; (1.01–6.24)
LAC or ACA	7.59; (2.50–23.03)

Area under Roc curve 0.627; Cut off 0.5: Sensibility 25.2%, Specificity 92.7% Corrected 62.2%.

Finally, according to our data and those of the literature we conclude that there is still no indication to perform routine screenings for anti-PC and anti-PS antibodies in patients with VTE.

Patients and Methods

All consecutive subjects referred from 2001 to 2003 to the Thrombosis Centre at the University of Padua with acute symptoms and objectively proven proximal deep venous thrombosis of the leg (DVT) and/or pulmonary embolism (PE) were enrolled after informed consent had been given.

DVT was documented by compression ultrasonography (CUS), PE by ventilation-perfusion lung scan or spiral CT-scan, according to the standard guidelines. Controls were healthy subjects unrelated with cases, age and gender matched, with no history of VTE, without known inherited thrombophilic defects (Factor V [FV] Leiden and G20210A prothrombin mutations, and PC, PS or antithrombin [AT] deficiency) and/or active cancer.

From an antecubital vein of all enrolled subjects, 9 ml of blood was drawn in a syringe prefilled with 1 ml of Na-Citrate 105 mmol in order to detect LAC, anticardiolipin antibodies, anti-PC and anti-PS antibodies, AT, PC, and PS plasma levels as well as FV Leiden and PT G20210A mutations.

In particular, anti-PC and anti-PS antibodies were detected by ELISA assays using kits containing highly purified human PC and PS coated to microplates (Bouty, Milano, Italy). Antibodies titre was evaluated in patients' plasma at a standard dilution of 1:100 and was expressed in arbitrary unit (AU/ml). IgG and IgM ACA were assessed by ELISA assays. In particular, microplates were coated with bovine purified cardiolipin and saturated with β2-GPI. (Bouty, Milano, Italy). IgG (GPL) and IgM (MPL) concentrations were expressed as U/ml. For LAC detection, both activated partial thromboplastin time (aPTT) and diluted Russel viper venom time (dRVVT) were used using the commercially available BCT method (Dade Behring, Germany). The inhibitory activity of patient's plasma on normal plasma was evaluated by performing aPTT and/or dRVVT in a 1:1 mixture of patient's plasma and platelet-poor pooled normal plasma after incubation at 37°C at 0′, 30′, 60′, 90′, 120′. If correction was observed, the presence of LAC was ruled out. If correction of the clotting time did not occur then confirmatory

tests were performed;. Platelets neutralization procedure was used as confirmatory test using a commercially available method (Dade Behring; Germany) using the BCT coagulation analyser (Dade Behring, Germany). The method consists of the neutralization of LAC in patient's plasma by the addition of platelets lysate which resulted in a shortening of the clotting time. The test was considered consistent with the presence of LAC when there was a correction or a shortening of the clotting time greater than 8 sec. The ratio of dRVVT measured on a mix 1:1 patient's plasma-control plasma and dRVVT of a control plasma was also determined. LAC was considered positive if one of these conditions were satisfied: presence of an aPTT-LA higher than 43 sec without correction of clotting time after the mixing test as described earlier and a positive confirmatory test or a dRVVT higher than 45 sec and a dRVVT ratio higher than 1.3. According to the Guidelines recommended by the Subcommittee for Standardization of the International Society on Thrombosis and Haemostasis patients with LAC positivity confirmed in a consecutive test performed 6 months after the first, were considered affected by LAC. Thrombophilia screening including AT, PC (antigen and activity) and PS (total and free antigen, and activity) evaluation was performed as previously described [28]. DNA analysis for factor V Leiden and G20210A prothrombin mutations was also performed as previously reported [29,30].

Subjects were interviewed for the presence of other predisposing factors for VTE including traumas, surgery, pregnancy, bed resting, active cancer. Those with inherited thrombophilia or the presence of known predisposing or risk factors for VTE were excluded from our analysis.

Statistical analysis

Statistical analysis was performed using commercially available statistics software (SAS Institute Inc., Cary, NC, USA). Comparisons between cases and controls were made using non-parametric test (Kruskal-Wallis) because continuous variables were not normally distributed. $P < 0.05$ was considered to be statistically significant. We classified autoantibodies values in two categories: values higher and lower than the 95° percentile calculated in controls, respectively. To estimate the strength of the association between the presence of autoantibodies and VTE, Odds Ratio (OR) and 95% confidence intervals (CI) were determined. After the descriptive analysis, a stepwise logistic regression analysis was used to identify significant covariates that predicted VTE, using a *P*-value to enter the model of 0.15 and to keep into the model of 0.20 this variables were considered for a multivariate analysis.

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Identification of *Chlamydia trachomatis* in a patient with ocular lymphoma

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Accumulating evidence suggests that infectious agents may play a role in ocular adnexa lymphomas (OALs) of MALT-type [1–4]. In particular, *Chlamydia psittaci*, the causative agent of psittacosis, has been detected by PCR in most patients from Italy or isolated eastern Asiatic countries with OALs in absence of other *Chlamydia species* [4–8]. These patients have also been shown to have a complete or partial response to doxycycline, recognized to be a cheap and safe treatment in these patients [5,6]. In contrast, OAL patients from other geographic areas and with different genetic background were found to be negative for *C. psittaci* DNA or had a quite variable response to antibiotic treatment, assuming that this pathogen might not play a ubiquitous role in OALs and that bacterial infection is not associated with OAL [8–12].

Here, we show the molecular detection and characterization of *C. trachomatis* [13] but not of other Chlamydiae in PBMCs and lymphoma lesions from an Italian patient with OAL. *C. trachomatis* 16S rRNA, ompA, and different HsP-60 encoding genes were demonstrated using high-sensitive nested PCR and reverse transcriptase PCR (RT-PCR) in specimens cocultured in optimized Hep-2 cell lines [14–16]. The patient successfully responded to 1-month doxycycline therapy with regression of the ocular lesion and the disappearance of bacterial DNA but not of mRNA from PBMCs. This experience expands the knowledge that other doxycylline-sensitive organisms are involved in the pathogenesis of OALs. Moreover, the continual detection of HsP-60 mRNA transcripts after clinical recovery indicates a persistent antigenic condition that can favor the onset of OAL [14,17,18]. Molecular methods combined with cell cultures would be useful for monitoring the chlamydia persistence related genes and to assess the effectiveness of therapy in these patients.

Methods Section

A 53-year-old Italian woman was referred in September 2007 to the ophthalmologist because of asymptomatic right ocular conjunctival lymphoma of MALT-type with microscopic evidence of typical salmon masses (Fig. 1a). In 2001, the patient had developed an analogous tumor in the left eye, which was successfully treated with chemotherapy (6 COP courses, cyclophosphamide, vincristine, prednisone). The treatment was generally well tolerated, and induced a complete remission state of the disease. A total body CT scan showed no lymphonode enlargement and tissue involvement while microscopic and immunocytochemical analysis of osteomedullary biopsy and the flow cytometry analysis of bone marrow cells obtained from a bone marrow aspirate documented 12% neoplastic cells of clonal origin. The multicolor flow cytometry analysis excluded the presence of tumor cells in the peripheral blood. Following chemotherapy, a complete disappearance of clonal lymphoid cells was detected. Since the end of 2001, the patient is suffering from chronic hepatitis B for which she was first treated with standard IFN monotherapy followed by antiviral nucleos(t)ide analogues, achieving suppression of HBV DNA viremia.

Because of the possible link between OALs and Chlamydia infection, the patient was referred to the laboratory of Section of Infectious Diseases to investigate Chlamydia. Fresh PBMC samples collected simultaneously with ocular biopsy after written informed consent were isolated by Fycoll-paque plus (GE Healthcare Europe GmbH, Milan, Italy) according to the manufacturer's protocol. Bioptic ocular tissue was broken up into small pieces with sterile scalp, and DNA extraction was performed according a previous described method. After aliquoting, PBMCs as well as lymphoma specimens were tested by molecular and cell culture assay (Hep-2 cell lines) based on additional centrifugation and extension of culture time. Primer sets targeting 16S rRNA, outer membrane protein (ompA/MOMP), and HsP-60 genomic regions of *C. psittaci, C. pneumoniae,* and *C. trachomatis* were amplified by nested PCR and reverse transcriptase PCR (RT-PCR) according to Contini et al. as described [11,17]. For RT-PCR, cDNA synthesis was performed using kit SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Briefly, 7 μl of RNA were added to a mixture containing: 50 mM oligo (dT)20, 10 nM dNTP, and diethyl pyrocarbonate water (DEPC-treated) until a final volume of 10 μl, incubated to 65°C for 5 min and then placed in ice at least for 1 min. cDNA synthesis mix (10 μl) was added to each RNA/primer mixture and incubated at 50°C for 50 min and at 85°C for 5 min. cDNA synthesized was employed for PCR reactions using the same protocols performed for DNA. Fresh PBMC and lymphoma specimens previously centrifuged and resuspended in RPMI 1640 medium (Gibco, Invitrogen, Carlsbad, CA) were inoculated in 24-well microtiter plates seeded with Hep-2 cell line (ATCC CCL-23). Each well was then overlaid with fresh DMEM medium (Gibco, Invitrogen, Carlsbad, CA) and incubated, as previously described, with additional centrifugations to increase the number of bacterial inclusions. For RT-PCR, cDNA synthesis was performed using a SuperScript III First-Strand Synthesis System kit (Invitrogen, Carlsbad, CA). PCR and RT-PCR were performed in supernatants and cell cultures after extraction of DNA and RNA.

Positive products of 412 bp and 527 bp corresponding to *C. trachomatis* 16S rRNA and ompA gene, respectively, and three HsP-60-encoding genes (Ct110, Ct604, Ct755) corresponding to 114 to 161 bp were detected by PCR and RT-PCR in either ocular or PBMC specimens cocultured in Hep-2 cell lines (ATCC CCL-23). Sequencing of PCR products (ABI PRISM® DNA Sequencing (Applied Biosystems, The Netherlands) did detect a strict homology (*E* value:$<$ 0.01) with *C. trachomatis*, as shown by BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST).

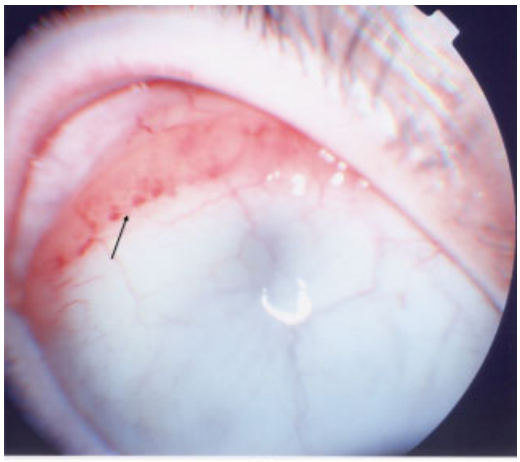
The patient was given doxycycline (100-mg tablets, Bassado; Pharmacia, Rome, Italy) 100 mg, twice a day, for 4 weeks without receiving any concomitant antiblastic or corticosteroid therapy in the period from the start of antibiotic therapy which was safe and well tolerated. *C. trachomatis* PCR DNA and mRNA were negative in PBMCs after the conclusion of antibiotic treatment and a significant reduction of the ocular lesions was observed (Fig. 1b). *C. trachomatis* eradication was monitored at molecular level by assessing patient's PBMCs 6 months later and after 9 months of follow-up. An ultrasonography performed at 6 months from therapy, showed a complete disappearance of the lesion described. At 12 months from doxycycline assumption, Ct was no longer detectable in the patient's PBMCs.

At the end of September 2008, the ophthalmologist valuated the patient again and he noted an indolent lesion with salmon red patch appearance in the right eye, which affected the bulbar conjunctiva. Histological diagnosis was again a marginal zone B-cell lymphoma of MALT-type. Ocular bioptic portions and PBMCs were reanalyzed as earlier. *C. trachomatis* 16 S rRNA and ompA gene were not detected with both molecular assays. PCR identified again all three genes products associated to HsP-60 gene, whereas RT-PCR detected an amplification signal corresponding only to the single Ct 604 portion of HsP-60 cDNA (Fig. 2).

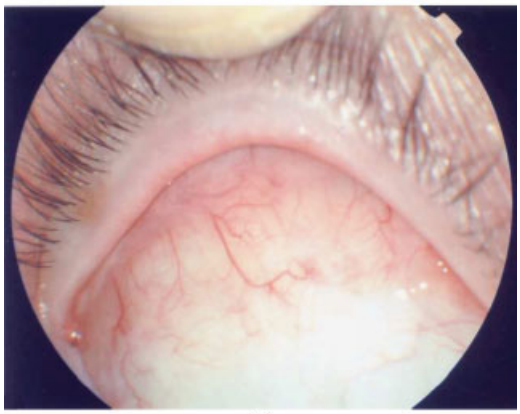
Doxycycline was readministered at the same dosage. Patient's PBMCs were revaluated 1 month after starting therapy and did detect again a molecular profile as earlier. However, a marked reduction of the eye lesion was observed.

Approximately 1 year after the suspension of antibiotic treatment, the ocular lymphoma is completely regressed as shown by ultrasonography, the PCR is negative, but the PBMC RT-PCR continues to be positive for Ct 604 of HsP-60 gene.

In the case described, *C. trachomatis* and not other chlamydiaceae was detected and identified in the PBMCs and eye from a patient with OAL with high-sensitive molecular tools. *C. trachomatis* was also capable to grow and be isolated in cell cultures. The combined use of optimized culture and molecular tools has shown to increase detection rates improving the overall sensitivity, suggesting their potential use in detecting *C. trachomatis*.



(a)



(b)

Figure 1. Right conjunctival lymphoma (a) with typical salmon color with diffuse margins (arrow) and its (b) complete regression after doxycycline treatment. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

So far, there is no molecular and epidemiological evidence of OAL of mucosa-associated lymphoid tissue (MALT)-type *C. trachomatis* associated. MALT lymphomas are common low-grade B-cell lymphomas arising from a background of chronic antigenic stimulation at a number of mucosal sites. Those originating in the eye are called OALs and may involve any part of the eye socket.

A pathogenic link between infectious agents and OALs has been well demonstrated. Infectious agents may play a role by acting through mechanisms, which include chronic antigenic stimulation and the action of infectious oncogenes. In this context, the etiological role of *H. pylori* in gastric MALT lymphomas sharing clinic pathologic features with OALs of MALT-type has been established. Similarly, other bacteria including Chlamydia may play a role in oncogenesis for their tendency to cause persistent infections.

Although *C. psittaci* is the most detected pathogen in OALs, the association between *C. psittaci* and OAL is not consistent around the world. Moreover, the question remains whether all patients with OALs of MALT-type should be treated for a possible infectious etiology.

C. trachomatis is an obligate intracellular gram negative bacterium sexually transmitted with a unique biphasic developmental stage, which has been linked with blindness (trachoma) and infertility, and it is the major causative agent in the development of pelvic inflammatory disease (PID) in women. *C. trachomatis* has also become the most frequent identifiable cause of neonatal conjunctivitis in many countries.

C. trachomatis as other *Chlamydia species* may evade the host immune response and can cause persistent infection characterized by an atypical intracellular and metabolically less active state that is difficult to resolve not only by the host-defence system but also by antibiotic therapy.

Unlike Ct 110 and Ct 755 genes which seem to predominate during active infection, the finding of dominant transcript Ct 604 of HsP-60 gene, indicates that *C. trachomatis* enters in a persistent infection state with an unusual

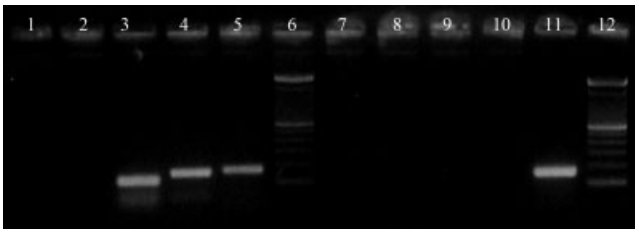


Figure 2. Gel electrophoresis of PCR and RT-PCR for detection of *C. trachomatis* specific genes and their expression in PBMC samples. Lanes 1–5; Lane 1: 16s rRNA gene (412 bp); Lane 2: ompA/MOMP gene (527 bp); Lane 3: Hsp60 (ct110, 114 bp); Lane 4: Hsp60 (ct755, 155 bp); Lane 5: Hsp60 (ct604, 161 bp); Lanes 7–11; RT-PCR. Lane 7: 16s rRNA gene (412 bp); Lane 8: ompA/MOMP gene (527 bp); Lane 9: Hsp60 (ct110, 114 bp); Lane 10: Hsp60 (ct755, 155 bp); Lane 11: Hsp60 (ct604, 161 bp); Lanes 6 and 12: 100 bp Ladder.

transcript pattern characterized by the attenuation of many Chlamydia genes including omp1 and others, as previously reported. Therefore, *C. trachomatis* HsP-60 transcripts and in particular Ct 604 indicate a persistent antigenic condition that may elicit chronic disease triggering both humoral and cell-mediated immune responses that can ultimately favor the onset or relapse of OAL. In this setting, the use of molecular methods including RT-PCR may be useful for monitoring the persistence and to assess the effectiveness of therapy.

Further investigations are clearly needed to define the significance of detection of the Chlamydia persistence related genes also in view to consider the use of discontinuous administration of oral doxycycline as prophylaxis for management of OALs.

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Decitabine combined with fractionated gemtuzumab ozogamicin therapy in patients with relapsed or refractory acute myeloid leukemia

Saeeda Chowdhury, Stuart Seropian and Peter W. Marks*

Salvage chemotherapy for patients with relapsed or refractory acute myeloid leukemia (AML) is generally associated with a low-response rate and significant nonhematologic toxicity. Both decitabine and gemtuzumab ozogamicin have activity in AML as single agents and can be administered sequentially with potential synergy due to their toxicity profiles. Twelve patients with AML, who had received a median of three prior regimens (range 1–6), were treated with decitabine 20 mg/m^2 on days 1 through 5 followed by gemtuzumab ozogamicin 3 mg/m^2 on days 6, 9, and 12. Five patients achieved a complete response (42%) and subsequently underwent hematopoietic stem cell transplantation. Three patients are in complete remission and four are still alive 7 to 16 months after treatment. The regimen was well tolerated with the primary nonhematologic toxicity of Grade 1 or 2 transaminitis observed in four patients. These results indicate that decitabine in combination with gemtuzumab is a regimen of promising efficacy worthy of further investigation in controlled trials.

Relapsed or refractory acute myeloid leukemia (AML) has a poor overall prognosis. Patients with intermediate or poor risk cytogenetics who have relapsed more than once generally have a median survival of less than a year without hematopoietic stem cell transplantation (HSCT) [1]. In addition, the survival of AML patients who relapse after treatment with HSCT is generally short. A number of salvage chemotherapy regimens have been employed in these settings with the ultimate goal of proceeding toward allogeneic HSCT. Salvage regimens include high-dose cytarabine (ara-C), mitoxantrone/etoposide, high dose ara-C/mitoxantrone, and clofarabine/cytarabine [2]. Many traditional salvage regimens are associated with significant nonhematologic toxicities, potentially limiting their use in heavily pretreated patients. The hypomethylating agents, azacitidine and decitabine, are generally well tolerated and have been demonstrated to produce responses in a small percentage of patients with relapsed or refractory AML [3]. Although gemtuzumab ozogamicin has previously been associated with significant hepatic toxicity, recent data indicate that splitting the dose can minimize this without loss of efficacy [4]. In an effort to combine agents with relatively mild and nonoverlapping nonhematologic toxicities, hypomethylating agents have been combined with the administration of gemtuzumab. For example, the administration of azacitidine in combination with low-dose gemtuzumab and hydroxyurea to an elderly population of patients with previously untreated AML was associated with a 70% response rate [5]. Following such reports, at our institution, a number of patients with relapsed or refractory AML have been treated with decitabine followed by fractionated gemtuzumab in an effort to provide salvage chemotherapy with minimal nonhematologic toxicity as a bridge to allogeneic HSCT.

Institutional review board approval was obtained for this retrospective analysis. Twelve consecutive patients with relapsed or refractory AML treated between September 1, 2006 and January 31, 2009 were included. Individuals ranged from 29 to 66 years of age. The group of patients had received a median number of three prior regimens (range 1–6). Six had received previous allogeneic HSCT and one had received an autologous

TABLE I. Demographics and Treatment Characteristics of Patients

No	Age/Sex	Cytogenetics	Prior regimens	Response	OS (mo)
1	51/F	>3 abnl	Ida/ara-C × 2, MUD HSCT	NR	1
2	66/F	t(9;11)	Ida/ara-C, Aust ICE, MRD HSCT	CR	13[a]
3	29/F	Normal	Ida/ara-C, FLAG × 2, HiDAC, Mito/Etop, CLARA	NR	<1
4	44/M	Normal	Ida/ara-C, Mito/Etop, CLARA	CR	16[a]
5	46/M	20q-	MRD HSCT, Ida/ara-C	NR	12
6	48/F	Normal	Ida/ara-C/HiDAC, CLARA	CR	7
7	62/F	Normal	Ida/ara-C/HIDAC, Mito/Etop	NR	2
8	64/F	Trisomy 8	Ida/ara-C/HiDAC, RIC MRD HSCT	NR	3
9	44/F	Trisomy 8	Ida/ara-C, MRD HSCT, Ida-FLAG, MRD HSCT	CR	11[a]
10	38/F	Normal	Ida/ara-C, MRD HSCT, HiDAC, Mito/Etop	NR	5
11	61/F	>3 abnl	Ida/ara-C	NR	<1
12	41/M	Normal	Ida/ara-C/HiDAC, Auto HSCT, CLARA	CR	7[a]

abnl, abnormalities; Aust ICE, idarubicin, cytarabine, etoposide; CLARA, clofarabine, cytarabine; CR, complete response; FLAG, fludarabine, cytarabine, G-CSF; HiDAC, high dose cytarabine, Ida/ara, idarubicin/cytarabine; Mito/etop, mitoxantrone, etoposide; MRD HSCT, matched related donor hematopoietic stem cell transplant; MUD, matched unrelated donor; NR, no response, RIC reduced intensity conditioning. [a]Indicates that the patient is still alive as of the cut-off date of this report.

HSCT. All patients were documented by flow cytometry on peripheral blood or bone marrow aspirate to meet the criteria for CD33-positive acute myeloid leukemia and had intermediate or poor-risk cytogenetics. The cut-off date for follow-up was June 1, 2009.

Decitabine was administered at 20 mg/m^2 IV on days 1 through 5 over 1 hr each day. Gemtuzumab ozogamicin was given at 3 mg/m^2 IV on days 6, 9, and 12 over 2 hr with acetaminophen and diphenhydramine premedication each day. CBCs, electrolytes, and liver function tests were monitored closely. Standard supportive care was provided including antibiotic prophylaxis and transfusion of packed red blood cells and platelets according to the needs of the individual patients. Episodes of febrile neutropenia were treated according to standard guidelines.

Complete remission was achieved in five of 12 patients (42%, Table I). Subsequent therapy included allogeneic HSCT in all five of these individuals. One patient relapsed 2 months after HSCT and another 15 months after HSCT. Three patients are in complete remission and four patients are still alive as of the cut-off date of this report (median 12 months, range 7–16). The administration of decitabine and fractionated gemtuzumab chemotherapy was generally well tolerated. The most common toxicity of this regimen was CTCAE v3.0 Grade 1 (n = 4) or Grade 2 (n = 1) transaminitis. Hematologic recovery in patients achieving CR occurred by day 31.

In summary, in an effort to provide salvage therapy of manageable toxicity to heavily pretreated individuals with AML, patients have been treated at our institution with decitabine in combination with fractionated gemtuzumab. This therapy facilitated allogeneic HSCT as further therapy in responding patients. Complete responses in five of 12 individuals (42%) who had received a median of three prior treatment regimens are encouraging. The nonhematologic toxicities of this regimen were modest. The combination of decitabine and gemtuzumab has produced complete responses and facilitated HSCT in a sufficient number of individuals to potentially merit further investigation in controlled trials.

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Progressive transition of Epstein–Barr virus associated lymphoproliferative disease subtypes with the development of lung cancer

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Epstein–Barr virus (EBV) associated lymphoproliferative disease (LPD) comprises a wide spectrum of clinical and pathological features [1]. This variety is most systematically categorized in post-transplant lymphoproliferative disorders (PTLD) [2], in which subtypes are ordered according to disease progression from reactive polyclonal proliferation to large cell lymphoma. However, whether this categorization is applicable to LPDs other than PTLD is not well explored. Here, we present a nontransplant case of EBV-LPD that initially presented as a polyclonal self-limiting proliferation and later transited to large cell lymphoma. This transition was associated with the progression of lung cancer and its therapy. Our case demonstrates that stepwise progression of LPD is a feature that can be observed in non-PTLD cases of EBV-LPD.

Case Presentation

In November 2006, a 66-year-old male patient had fever and swelling of bilateral inguinal lymph nodes and consulted a local hospital. However, these symptoms regressed spontaneously without any treatments. In the course of medical work-ups, computed tomography (CT) revealed nodular shadow with 10-mm diameter in the right upper lobe of the lung, and this nodule was considered inactive because fluoro-deoxy-glucose positron emission tomography (FDG-PET) and tumor markers for lung cancers were all negative. Since April 2007, he had recurrent episodes of fever and multiple lympadenopathy that occurred and resolved synchronously. Titers for EBV antibodies in August 2007 were compatible with reactivation of past EBV infection as follows: EBV-VCA-IgG (1:1280), EBV-VCA-IgM (1:10), EBV-EA-IgG (1:10), and EBV-EBNA (1:20). Left inguinal lymph node was biopsied and the diagnosis of reactive lymphadenopathy was made. As consciousness disturbance (Glasgow Coma Scale E4, V3, M5 total 11) and hallucination coexisted with the fifth episode of fever and lymphadenopathy, he was transferred to our hospital in December 2007. Physical examination was as follows: body temperature 38.5°C, blood pressure 119/66 mmHg, and pulse rate 100/min. His body weight had decreased 4 kg in the previous 4 months. He had moderate hepatosplenomegaly and marked systemic lymphadenopathy. The lymph nodes were elastic hard and had tenderness. Pathological examination of his left axillary lymph node revealed EBV positive diffuse large B cell lymphoma (DLBCL). Peripheral white blood cell count was 6100/μL, of which atypical lymphocytes constituted 14.5%. Biochemical laboratory data were as follows: LDH 814 IU/l, GOT 53 IU/l, GPT 28 IU/l, BUN 31.8 mg/dl, Cre 0.86 mg/dl, CRP 2.04 mg/ml, and s-IL2R 8656 U/ml. Reanalysis of EBV-antibody titers revealed almost the same profile as August 2007: EBV-VCA-IgG (1:2560), EBV-VCA-IgM (1:10), EBV-EA-IgG (1:10), and EBV-EBNA (1:20). EBV viral load in peripheral blood was elevated (6.4×10^4/μg DNA), also suggesting reactivation of EBV infection. Despite thorough examinations including CT, electroencephalogram, and assays of cerebrospinal fluid, the etiology for consciousness disturbance was not elucidated and he recovered normal consciousness spontaneously. Enhanced CT and FDG-PET were compatible with marked exacerbation of systemic lymphadenopathy. The abnormal nodule on the right upper lung expanded to 22 mm in

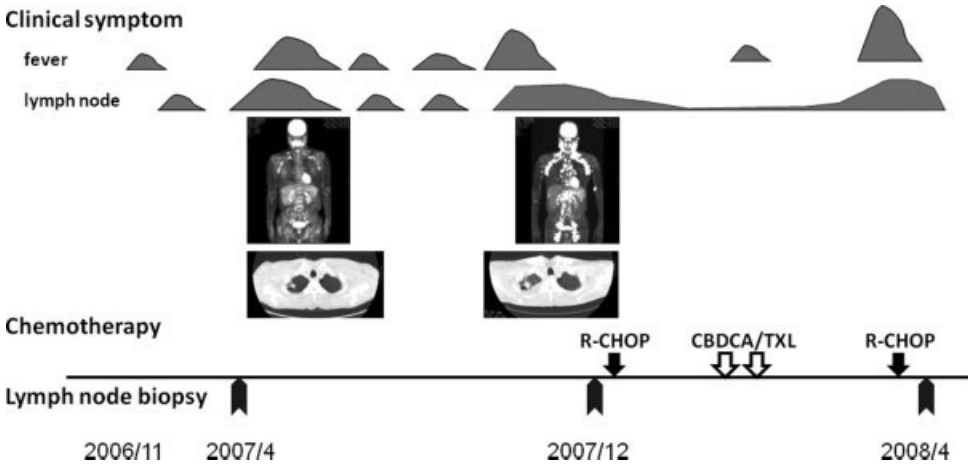

Figure 1. Illustrative presentation of the clinical course of the patient. The clinical course of the patient is presented including clinical symptoms, CT scan, FDG-PET findings, and the therapies.

diameter and FDG uptake was enhanced (SUV max 6.1). Additionally, tumor marker was elevated (CEA 234 ng/ml) and lung cancer was strongly suspected. However, therapy for malignant lymphoma was prioritized considering aggressive progression of lymphoma. After one course of R-CHOP therapy, his general condition improved dramatically and lymphadenopathy disappeared promptly. In turn, pleural effusion increased and thoracentesis revealed bloody exudative effusion containing malignant cells. The diagnosis of adenocarcinoma of the lung, stage IIIb was made. After pleurosclerosis, the patient received two courses of CBDCA (330 mg) and TXL (660 mg) therapy.

In March 2008, fever and systemic lymphadenopathy exacerbated. The patient had tremendous fluid leakage from vessels that induced diminished intravascular volume and impaired circulation dynamics. R-CHOP therapy could not stop the deterioration of organ failures and the patient died on the fifth day of chemotherapy and autopsy was performed. The clinical course of this patient is illustrated in Fig. 1.

Histopathological Findings

To examine the longitudinal transition of pathophysiological condition of EBV-LPD, we reviewed the histological findings of this patient (Fig. 2).

In April 2007, the left inguinal lymph node was first subjected to pathological examination in the local hospital and we reviewed the specimens. The nodal architecture was mostly reserved, and small clusters of medium-sized cleaved lymphocytes with apparent nucleoli were observed. The infiltrating cells were positive for CD 20, EBER-ISH, and light chain was restricted to lambda. However, IgH analysis was a polyclonal pattern. From these findings, we made the diagnosis of EBV-LPD.

In December 2007, second biopsy from left axillary lymph node revealed different features. The lymph follicle architecture was completely effaced. Medium- to large-sized atypical lymphocytes with irregular nuclei and distinct nucleoli were seen. These cells were CD 20 (+), EBER (+), LMP1 (−), EBNA2 (−), and MIB-1 index was 50–70%. This time, stain for kappa light chain was almost exclusively positive and IgH analysis showed a monoclonal pattern. From these characteristics, DLBCL, EBV-positive was diagnosed. Type I latency was suggested as the mode of EBV persistent infection.

In April 2008, the patient died and the autopsied specimens were also examined. The lymph nodes findings were the same as those of December 2007. EBV latency protein analysis was LMP1 (+) and EBNA2 (−), compatible with latency II infection.

Discussion

To better characterize the pathological features in this case, we determined the clonality of LPD lesions with two methods: immunoglobulin light chain analysis with immunohistochemistry and IgH rearrangement analysis with polymerase chain reaction (Fig. 3). In April 2007, IgH analysis was polyclonal, and light chain was significantly deviated to lambda. Considering that he developed self-limiting lymphadenopathy and the underlying structure of lymph nodes was preserved, this lesion was reactive proliferation superimposed by localized monoclonal expansion of EBV-positive B lymphocytes. This demonstrates the polyclonal nature of LPD with sporadic expansion of a subclone in the first specimen. After 8 months when he presented with exacerbated lymphadenopathy, the lymph node specimen was compatible with DLBCL in terms of morphological atypia and effacement of background structure. This time, IgH clonality was monoclonal, and light chain restriction turned to be exclusively positive for kappa. The clinical course of this case can be summarized to the progression from polyclonal to monoclonal LPD.

EBV is a gamma herpes virus that is widely known as a causative agent of infectious mononucleosis (IM). Although IM manifests as a primary infection of EBV and most of the infected B-cells are eradicated by EBV-specific CD8 cytotoxic T lymphocytes (CTL), EBV establishes a lifelong dormant infection in some of the host immune cells. Modes of latent infection in B cells are classified in one of three patterns and denominated as Type I, II, and III latencies. In Type III latency, which is seen in opportunistic LPDs, infected B cells are constantly surveyed by CTL because Type III latency specific proteins, EBNA3s, are the targets of CTLs, and development of LPD is suppressed in immune-competent status. However, when host-

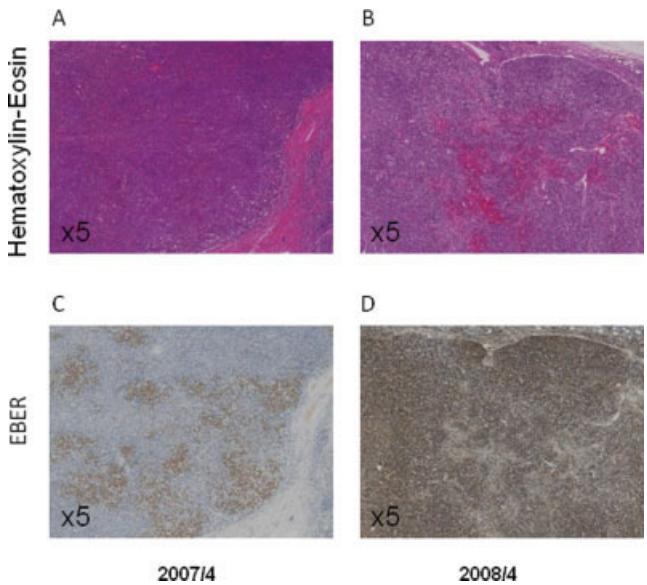

Figure 2. Histopathology of the lymph nodes. Biopsied lymph nodes were stained with hematoxylin-eosin (A, B) and EB virus-encoded RNA (EBER) (C, D). With the specimen of April 2007 (A, C), the nodal architecture was reserved and EBER was partially positive. In April 2008 (B, D), the lymph follicle architecture was completely effaced and EBER was 100% positive.

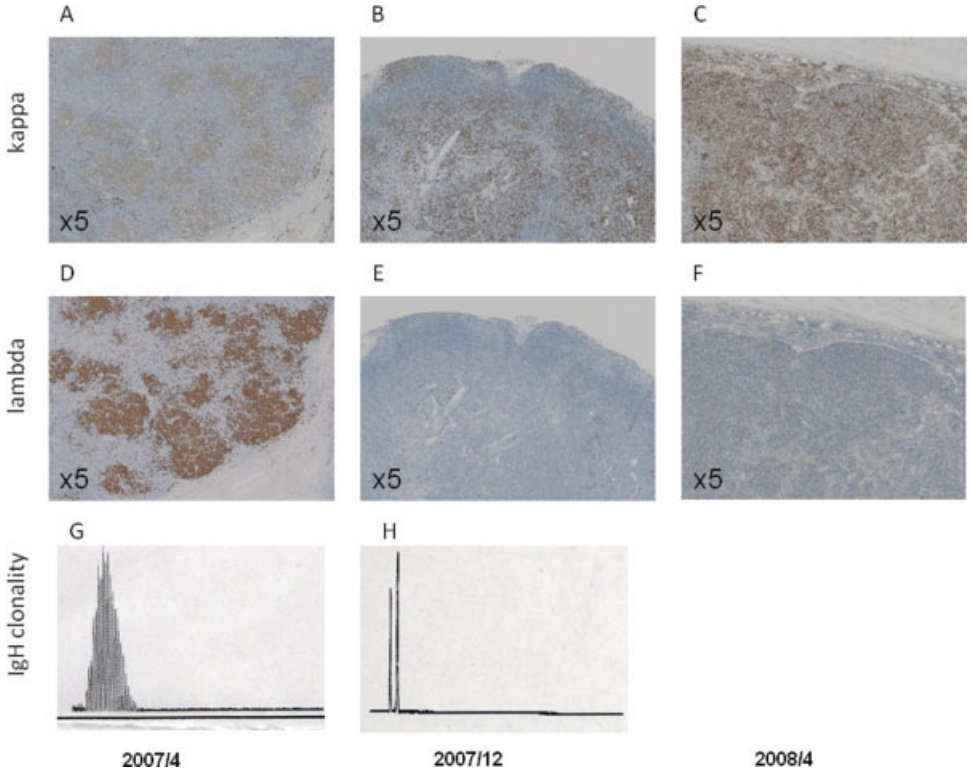

Figure 3. Clonal examinations of LPD. Clonality of LPD lesions were examined by immunohistochemistry for light chain kappa (A–C) and lambda (D–F), and by multiprimer PCR for immunoglobulin heavy (IgH) chain (G, H). The clonality was examined in three different time points. In April 2007, light chain was deviated to lambda (D), however, kappa-positive cells are also seen (A). IgH PCR showed a polyclonal pattern (G). In December 2007, light chain restriction to kappa was more prominent (B, E) and IgH PCR was a monoclonal pattern (H). In April 2008, light chain was exclusively restricted to kappa (C), leaving almost no cells with lambda chain (F).

immunity is impaired, EBV-infected B cells begin uncontrolled proliferation, which leads to the development of EBV-LPD. A number of clinical conditions have been shown to confer immunodeficiency that causes B-LPD: congenital immunodeficiency [3], acquired immunodeficiency syndrome (AIDS-related lymphoma) [4], aging (age-related EBV-associated B-LPD) [5], immunosuppressive agents for autoimmune diseases [6], and organ or hematopoietic stem cell transplantation (PTLD) [2]. The pathological categorization of LPD is most profoundly categorized in PTLD [2] and referenced as a model of progressive disease statuses. In early lesions, the underlying architecture is preserved and clinical symptoms occasionally resemble IM, and spontaneous regression is observed. Analysis of immunoglobulin heavy chain (IgH) reveals polyclonal proliferation. In polymorphic PTLD, architecture of lymph nodes is effaced to some degree and IgH analysis usually shows a monoclonal pattern. In monomorphic PTLD, architectural and cytological atypia is sufficient to be diagnosed as lymphoma. This entity is subdivided into B or T/NK lineage and majority of B cell type resemble DLBCL.

A distinct clinicopathological entity that covers EBV positive DLBCL in patients older than 50 years and without any known immunodeficiency is proposed by Oyama et al. [5] and referred to as "age-related EBV-associated B-LPD." This entity is characterized by association with poor prognostic components of international prognosis index, poorer response to conventional chemotherapy, and aggressive clinical course [5]. This case fulfills the criteria of this entity because he had neither of underlying immunodeficiency including primary immunodeficiency disorders, HIV-infection, solid organ or bone marrow allograft, or iatrogenic immunosuppression for autoimmune disorders. Although he had lung cancer and received treatment for it, concurrent cancer does not preclude the diagnosis and he developed monoclonal LPD before he received the chemotherapy for the cancer. This entity formerly comprised a spectrum ranging from polymorphic proliferation, which sometimes included reactive process, to large-cell lymphomas mostly consisting of transformed cells [5]. However, recent analysis of a large series of age-related EBV-associated B-LPDs failed to show any statistical differences in clinical features between these subtypes and they are now assumed to constitute continuing spectrum [7]. However, whether transition between these subtypes can occur within a patient has not been elucidated in this entity. In the case of PTLD, polymorphic and monomorphic lesions have distinct features in the pathogenesis. For example, early germinal center (GC) cells correspond to monomorphic and not to polymorphic lesions, whereas late GC and post GC cells correspond to both monomorphic and polymorphic lesions [8,9]. These differences of fundamental pathogenesis raise the possibility that the subtype of PTLD is determined in onset, although this issue remains controversial because occasional cases of PTLD span the spectrum of polymorphic and monomorphic subtypes synchronously or metachronously in a single patient. In contrast, such in-depth investigation has not been performed at all in age-related EBV-associated LPD. So, our case is valuable because this demonstrated the progression from polyclonal proliferation of EBV positive cells to monoclonal large cell lymphoma in a single patient and showed that subtype transition is a feature not specific to PTLD.

The role of lung cancer in the immunological deterioration in this case is a matter of debate because cancer can be both a cause and consequence of immune impairment. In addition, specific link between EBV-infection and cancer immunity is presumed because EBV-derived IL-10 (formerly called BCRF-1) strongly reduces antigen-specific T cell proliferation [10] and its immunosuppressive effect induces anergy to tumors [11].

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Conflict of interest: Nothing to report.

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Sickle cell disease caused by heterozygosity for Hb S and novel LCR deletion: Report of two patients

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The β-globin gene LCR is located ~6 kb upstream of the embryonic ε-globin gene, and is made up of five DNase I hypersensitive sites (HSs), HS 1–5. LCR plays a pivotal role in regulating the expression of downstream ε-, ^{G}γ-, ^{A}γ-, δ-, and β-globin genes in cis [1]. Deletions removing the LCR and parts of the downstream β-globin gene cluster in patients have been described [2]. These individuals present with a $(\gamma\delta\beta)^0$-thalassemia carrier phenotype. We now report two patients with severe sickle cell disease who were compound heterozygous for Hb S mutation and novel LCR deletion. In one case, HS 1–3 were deleted; in the other, HS 1–5 were deleted. In both cases, the β-like globin genes in cis to the LCR deletions were intact. Genotypically, both patients appeared to have sickle cell trait. Coinherited with either LCR deletion, these individuals presented as sickle cell disease patients. The breakpoints of these LCR deletions were defined. These results affirm that HS 2 and 3 are primarily responsible for conferring erythroid specific high-level expression of cis-linked β-like globin genes. Furthermore, LCR deletions might cause hemolytic disease of newborns.

Patient 1

A 6-year-old African-American boy presented with sickle cell disease. He had a grade III/VI systolic ejection heart murmur at 6 months, possibly a manifestation of moderate anemia at the time. At 1-year of age, he presented with acute sickle painful episode and required transfusion. He had splenic sequestrations and underwent splenectomy when he was 3½ years old. He had acute chest syndrome, painful vaso-occlusive events, and received transfusions.

At 5 years of age, his Hb was 7.3 g/dL, MCV 68 fL, reticulocyte count 6%, and serum ferritin 280 ng/mL. Hemoglobin analysis by high-performance liquid chromatography (HPLC) revealed Hb S 80.1%, Hb F 9.5%, Hb A_2 3.8%, and possibly Hb A 5.0% eluting at 2.53 min. This HPLC technique yields higher Hb A_2 level in the presence of Hb S. Beta-globin gene nucleotide sequencing showed that he was heterozygous for Hb S mutation, and no β-thalassemia mutation was detected. There was SNP heterozygosity at four sites indicative that both his β-globin gene alleles were present. He was heterozygous for the C > T polymorphism at nt –158 5′ to the ^{G}γ-globin gene (the *Xmn I* polymorphism) which is sometimes associated with elevated Hb F [3]. He had a single α-globin gene deletion of the rightward type ($-\alpha^{3.7}/\alpha\alpha$).

He was treated with hydroxyurea. While his Hb changed little (7.6 g/dL), his Hb F rose to 19% and his MCV increased to 78 fL. He improved clinically, and did not have painful vaso-occlusive episodes in the 6 months after hydroxyurea treatment.

Patient 2

An 18-year-old Puerto Rican woman presented with sickle cell disease. She was born prematurely at 32 weeks of gestation, was anemic at birth requiring transfusions and had cardiac anomalies. At 7 months of age, she was started on regular transfusion program. She had repeated vaso-occlusive events and developed aseptic necrosis of the hip.

Her Hb was 7.0 g/dL, MCV 68 fL, reticulocyte count 13%, Hb S 86.5%, Hb F 5.2%, Hb A_2 4.6%, and possibly Hb A 2.2% eluting at 2.46 min. Beta-globin gene nucleotide sequencing was performed on the patient and both her parents confirming that the proband and her father were heterozygous for the Hb S mutation (data not shown). Beta-thalassemia mutation was not found in the proband and her mother. A SNP heterozygosity was present in the β-globin gene sequences of the mother and the proband, consistent with both having two β-globin gene alleles.

These two patients had microcytic anemia (Hb 7.3 and 7.0 g/dL, MCV 68 fL in both), each with 80–90% Hb S, and presented with severe sickle cell disease. To account for the silencing of the normal β-globin gene allele, we hypothesized that a deletion of part or all of the β LCR was present. In Patient 1, MLPA revealed a deletion removing HS 1–3 (Fig. 1A). A gap-PCR test was designed for detection of this deletion (Fig. 1B). Nucleotide sequencing across the breakpoints revealed a deletion of ~12 kb spanning from nt 10,054–21,932, based on NCBI NG_000007.3 (Fig. 1C). We name this the Tennessean LCR deletion.

In Patient 2, we took advantage of her β codon 6 A/T heterozygosity and used the strategy of designing a series of PCR reactions as had been previously described to map the 3′ breakpoint of the deletion between HS 1 and the ε-globin gene (data not shown) [4]. Subsequently, MPLA confirmed that

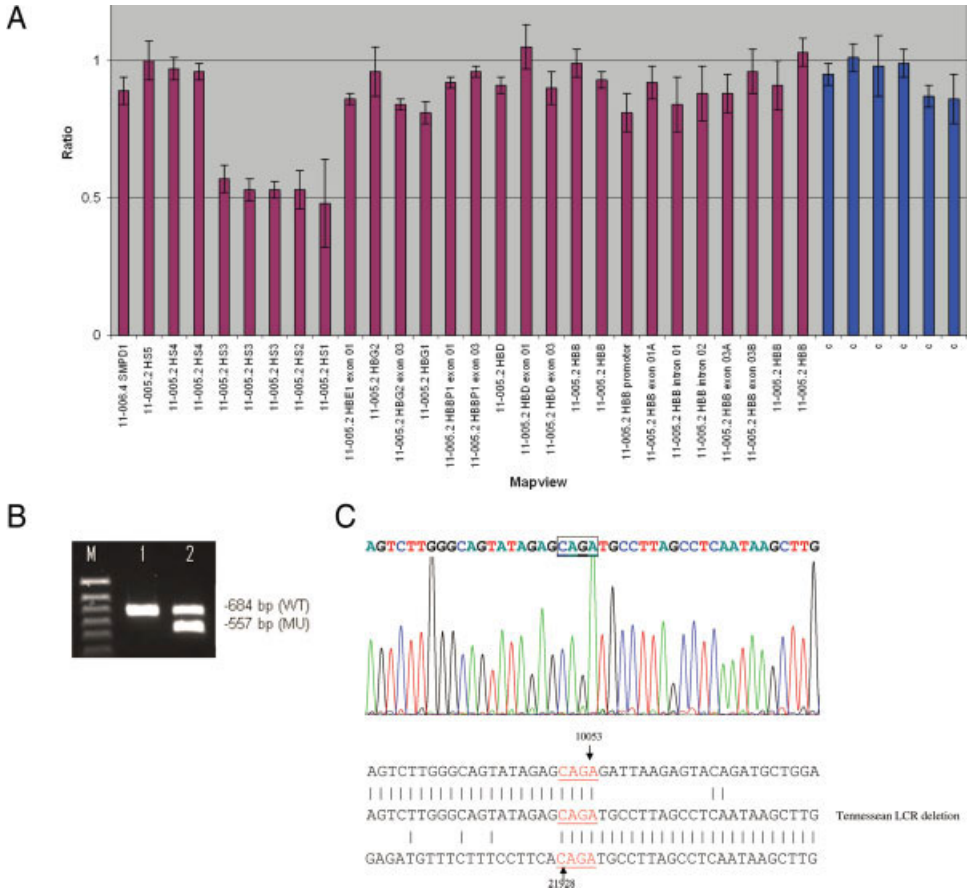

Figure 1. Tennessean LCR deletion. A: MLPA of the 6-year-old African-American boy who is heterozygous for the Tennessean deletion, showing the signals of probes on HS 1–3 equal to half of all other probes in the β-globin gene cluster. The blue-colored bars (C) represent probes for genes on other chromosomes which serve as control. B: Gap-PCR test designed to detect the Tennessean LCR deletion. Lane M, molecular size markers; Lane 1, normal individual without the deletion, showing the control band of 684 bp, using primers TF1 (GCTCACTGCACATACACTAGACAGA, nt 9574–9598 according to NG_000007.3) and TR (CCATCAATAATTCTA GCCCCA CAGGA, nt 22,009–21,984); and Lane 2, proband showing the 684 bp control band, and 557 of the mutant band, using primers TF2 (CTAGGACCTGCAAGTT ATCTGGTCA, nt 21,326–21,350) and TR. C: Alignment between the nucleotide sequencing bridging the Tennessean LCR deletion, and sequences 5′ to the upstream breakpoint and 3′ to the downstream breakpoint. The nucleotide numbering is based on NCBI NG_000007.3.

she had a deletion removing HS 1–5 but not the ϵ-globin gene. Gap-PCR primers were designed to span the breakpoints (Fig. 2A). Nucleotide sequencing and alignment revealed a deletion of ∼22.5 kb from nt 2904–25,433, based on NCBI NG_000007.3 (Fig. 2B). The same deletion was found in her mother. We name this the Puerto Rican LCR deletion.

The human β-globin gene cluster LCR is located ∼6–22 kb upstream from embryonic ε-globin gene on chromosome 11p15 [5]. It is composed of five individual DNase I HSs, each 200–400 bp in length and separated from each other by 2–4 kb. The DNase 1 hypersensitivity in HS 1–4 is formed only in erythroid cells, but that in HS 5 is present in other cell lineages. It is generally agreed that LCR acts by recruiting transcription related complexes to the β-globin gene promoters in cis and inducing the spatial reorganization to an "open" chromatin conformation to promote transcription [1,5–7]. It is yet to be conclusively determined if the HS sites function by coming together to form a holocomplex or if individual HS site alone can exert its effects.

There are 10 known LCR deletions that remove all or parts of the LCR, including the two reported here. Of these, four and possibly five do not remove any of the downstream β-like globin genes; the other five remove some or all of the downstream β-like globin genes but not the β-globin gene itself (Fig. 3). At least 10 other large deletions remove both the LCR and the entire downstream β-globin gene cluster [2].

To detect novel deletions or deletions whose breakpoints have yet to be identified can be a challenge. MPLA has recently emerged as useful to quickly detect large deletions in either β- or α-globin gene clusters, as illustrated by the present report [10]. Definitive identification of these deletions is needed for proper genetic counseling and antenatal diagnosis when it is indicated.

Kulozik et al. reported a 3 kb deletion that removed HS 1 in an Italian family [9]. The affected individual did not seem to have hematological changes that could be ascribed to the deletion suggesting that HS 1 by itself has no or minimal effect on β-globin gene expression.

Driscoll et al. [8] reported an 11-year-old Hispanic girl. Her Hb was 11.6 g/dL, MCV 59 fL, Hb A_2 3.5%, and the remainder was Hb A. No Hb S was found. This patient was found to have Hb S mutation in one of her β-globin gene alleles. Additionally, she had a de novo deletion that removed HS 2–5, but left HS 1 and the remainder of the downstream β-like globin genes unaffected (Fig. 3). This deletion must be in cis to the β-globin gene allele with the Hb S mutation, effectively silencing its expression. This is the opposite of our two cases in which the normal β-globin gene is silenced by the LCR deletion.

Both the Tennessean LCR deletion removing HS 1–3 and the Puerto Rican LCR deletion removing HS 1–5 seem to be the result of nonhomologous recombination (Figs. 2C and 3B). These deletions effectively silence the downstream normal β-globin gene expression. Interestingly, the mother of Patient 2 had a β-thalassemia trait phenotype (Hb 10.3 g/dL, MCV 61 fL) but with a normal Hb A_2 (3.1%), suggesting reduced expression of the δ-globin gene in cis to the LCR deletion.

Taken together, these results indicate that HS 2 and 3 are most responsible for enhanced expression of downstream β-like globin genes, and that the absence of HS 4 and 5 does not cause more severe phenotype. These observations are consistent with experimental data which have shown a prominent role for HS 3 and to a lesser extent, HS 2 [17,18].

In both our patients, a very small hemoglobin fraction (2–5%) was observed within the "window," in which Hb A normally elutes from the HPLC. Whether these minute fractions represent genuine Hb A requires further

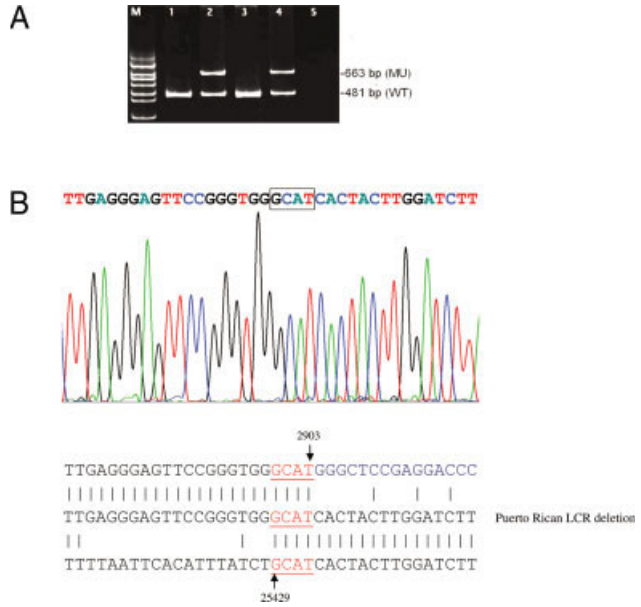

Figure 2. Puerto Rican LCR deletion. A: Gap-PCR test designed to detect the Puerto Rican LCR deletion. Lane M, molecular size markers; Lane 1, normal individual without the deletion, showing the control band of 481 bp, using primers PF1 (ACTAGAATGACATCATCAGTACCTATTGC, nt 25,007–25,035) and PR (ATGAAAGCATTAGATCTAGGATTGGGTCTAC, nt 25,488–25,457); Lane 2, proband showing the 481 bp control band, and 663 bp mutant band, using primers PF2 (TGAGGCACAGGGCCCTAAAGTAAT, nt 2295–2318) and PR; Lane 3, proband's father; Lane 4, proband's mother; and Lane 5, no DNA control. B: Alignment between the nucleotide sequencing bridging the Puerto Rican LCR deletion, and sequences 5′ to the upstream breakpoint and 3′ to the downstream breakpoint. The nucleotide numbering is based on NCBI NG_000007.3.

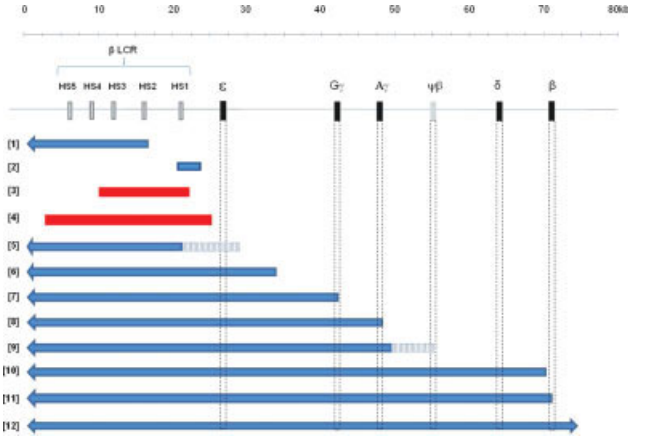

Figure 3. Schematic representation of known deletions of the β-globin gene LCR. The top line represents the approximate kilobases in nucleotide distances. HS sites of the β LCR and the globin genes are represented by boxes on the second row. The region encompassing undetermined 3′ breakpoint is represented by hatched lines. Breakpoints outside the illustrated region are indicated by arrows. [1] Hispanic LCR deletion of ~30 kb in length (Ref. 8); [2] Italian LCR deletion of ~3 kb in length (Ref. 9); [3] Tennessean LCR deletion of ~12 kb in length (this report); [4] Puerto Rican LCR deletion of ~22.5 kb in length (this report); [5] Dutch V LCR deletion of ~130 kb in length (Ref. 10); [6] English II LCR deletion of ~98 kb in length (Ref. 11); [7] English I LCR deletion of ~100 kb (Ref. 12); [8] Dutch III LCR deletion of ~112 kb in length (Ref. 13); [9] Dutch IV LCR deletion of ~99.4 kb in length (Refs. 10,14); [10] Dutch VI LCR deletion of ~160 kb in length (Ref. 10); [11] Anglo-Saxon LCR deletion of ~95.9 kb in length (Refs. 15,16); and [12] Other large deletions removing the complete β-globin gene cluster (Ref. 2).

confirmatory testing. We have reviewed the HPLC chromatograms of 15 patients with Hb S/HPFH2 or HPFH1, and none showed hemoglobin fraction eluted in the same Hb A "window" as observed in the two patients reported here (data not shown). Perhaps even with LCR deletions, a minimal residual expression of the β-globin gene in cis to the LCR deletion remains, as has been shown in transgenic mouse model [19].

The child with the Tennessean deletion had a baseline Hb F close to 10%. He was heterozygous for the C > T polymorphism at nt −158 5′ to the $^{G}\gamma$-globin gene (*Xmn I* polymorphism) that is sometimes associated with elevated Hb F [3]. It is uncertain if this polymorphism is in cis to the LCR deletion. When given hydroxyurea, his Hb F increased to 19% accompanied by significant clinical improvement. It should be of interest to investigate whether the Hb F enhancing effect of hydroxyurea requires the presence of a functional LCR in cis to the γ-globin genes.

During the neonatal period, infants heterozygous for $(\gamma\delta\beta)^0$-thalassemia due to large deletions removing the β-like globin genes have microcytosis and hemolytic anemia [13–15,20–24]. There is at least one exception to this observation [12]. The two patients described here have deletions of their LCR leading to absence of β-like globin gene expression in cis, phenotypically not distinguishable from the $(\gamma\delta\beta)^0$-thalassemia deletions. The differential diagnosis of hemolytic anemia of newborn ought to include $(\gamma\delta\beta)^0$-thalassemia heterozygosity, due to deletion removing either the entire beta-globin gene cluster or its LCR.

Methods

Blood counts were done by automated cell counters. Hemoglobin analysis was done by BioRad Variant II cation HPLC (BioRad Laboratories, Hercules, CA). Genomic DNA was extracted from peripheral blood leukocytes. The β-globin genes, and also the promoter region of $^{G}\gamma$-globin gene were amplified by PCR separately, the amplicons were purified, and direct nucleotide sequencing was carried out using BigDye Terminator v3.1 Cycle Sequencing Kit and an ABI PRISM 3100X Genetic Analyzer (Applied Biosystems, Foster City, CA) [4]. The single α-globin gene deletion of the rightward type $(-\alpha^{3.7})$ was diagnosed by multiplex gap-PCR procedures.

Long range PCR and detecting loss of heterozygosity by sequencing were used initially to search for the 3′ breakpoint of the putative deletions in one of the patients [4]. Later, large deletions in the β-globin gene cluster were searched for by multiplex ligation-dependent probe amplification (MLPA) using the SALSA MLPA kit P102-B1 HBB, lot # 0508 (MCR-Holland, Amsterdam, The Netherlands) according to manufacturer's procedures. Probes spanned the region from ~1 mb 5′ upstream of the LCR to 9 kb 3′ downstream from the 3′ β-globin gene enhancer [12]. Reference probes for 15 additional chromosomal positions were used. Fluorescent labels were then added to achieve probe separation and definition on a capillary sequencing system. The height of each LCR HS specific probe peak was then compared with those of reference probes. Results were expressed in a ratio to determine allelic loss. The breakpoints of the deletions were further defined by designing appropriate gap-PCR amplification and nucleotide sequencing [4].

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The clonality of CD3+ CD10+ T cells in angioimmunoblastic T cell lymphoma, B cell lymphoma, and reactive lymphoid hyperplasia

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T cells coexpressing CD3 and CD10 are a characteristic feature of angioimmunoblastic T-cell lymphoma (AITL) [1]. However, they are not unique to AITL, as these cells are also present in B cell lymphoma and reactive lymphoid hyperplasia [2]. To determine the significance of CD3+ CD10+ T cells, we used flow cytometry with cell sorting and molecular biology techniques for T cell gene rearrangement to study T cells from patients with AITL, B cell lymphoma, and reactive lymph node hyperplasia. We found that CD3+ CD10+ T cells in B cell lymphoma and reactive lymphoid hyperplasia were polyclonal. In early stage of AITL, they were oligoclonal, and became monoclonal as AITL progressed. These findings illustrate the differences between early and late lymphoma and could be important for the diagnosis of AITL.

AITL, a disease originally named angioimmunoblastic lymphadenopathy (AILD) was first described as an atypical lymphoid hyperplasia and/or premalignant condition in the early 1970s [3]. Subsequently, it became evident that AILD is a malignant lymphoma, and in the WHO Classification of Tumors of Hematopoietic and Lymphoid Tissues, AITL has been classified under mature T cell and NK cell neoplasms and is considered to be a subcategory of peripheral T-cell lymphoma. Currently, it is viewed as a monoclonal proliferation of T cells that express CD3, CD4, and aberrantly express CD10 [4].

The clonality of CD3+ CD10+ T cells has not been systematically studied and it is not known whether these cell are monoclonal in different stages of AITL. Thus we decided to compare early and late AITL. Because early AITL has some feature of reactive lymphadenopathy, we included in the study some cases of benign lymphoid hyperplasia and B cell lymphoma.

We analyzed a total of 84 cases collected over a 12 month period. Tissue samples included: 50 lymph nodes, 11 tonsils, 8 body fluids (pleural, abdominal, and bronchoalveolar lavage), 5 bone marrows, 4 peripheral blood specimens, and 6 others (thyroid, lung, liver, mediastinum, stomach, and hip mass). The age of the patients ranged from 2 to 81 years-old. The results are summarized in Table I. The mean number of CD3+ CD10+ T cells (as a percentage of total lymphocytes) in lymph nodes involved by reactive lymphoid hyperplasia was 2.8%. These cells were 7.0-fold lower in normal blood (mean 0.4%, $P = 0.05$), 2.7-fold higher in AITL (mean 7.6%, $P < 0.001$), and 3.5-fold higher in high-grade follicular lymphoma (mean 9.8%, $P < 0.001$).

In one case of AITL, the first biopsy showed 4% CD3+ CD10+ T cells and the second biopsy 34 days later from a different location showed an increase to 8.5%.

T cell gene rearrangement studies on the selected cases showed polyclonality of CD3+ CD10+ T cells in reactive lymphoid hyperplasia (tonsil), follicular lymphoma, mantle cell lymphoma, and small lymphocytic lymphoma/chronic lymphocytic leukemia. An early biopsy from an AITL patient showed oligoclonal CD3+ CD10+ T cells and a biopsy from the same patient 34 days later showed monoclonal CD3+ CD10+ T cells. The results are summarized in Table II.

In this study, CD3+ CD10+ T cells were present in a variety of reactive and malignant conditions. In the reactive conditions, the frequency of these cells ranged from less than 1–7.2%; however, in AITL, they were more frequent, ranging from 4.0 to 9.2% of the total lymphocyte population. T cell receptor gene rearrangement studies on the sorted CD3+ CD10+ T cells from six different cases demonstrated that these cells were polyclonal in reactive lymphoid hyperplasia and B cell lymphoma, but they were oligoclonal in early stage AITL and became monoclonal in the later stage of AITL. In this study, we demonstrated that CD3+ CD10+ T cells were higher in AITL than in reactive lymphoid hyperplasia. However, there was a high degree of overlap, making it difficult to use this feature as a diagnostic criterion. By studying subsequent biopsies of a single AITL patient, we were able to show that CD3+ CD10+ increased in number and transitioned from oligo-

TABLE I. Percentage of CD3+ CD10+ T Cells in Various Diseases and Tissues

Diagnosis[a]	Tissue[b]	*N*	CD3+ CD10+ T cells (%) Mean ± 1 SD[c]	CD3+ CD10+ T cells (%) Range[d]	*P* Value[e]
1. Normal	Blood	2	0.4 ± 0.2	0.3–0.6	0.052
	Bone marrow	1	2.5	NA	NA
	Lymph node	1	2.1	NA	NA
2. Reactive lymphoid hyperplasia					
Dermatitis	Blood	1	1.3	NA	NA
Hashimoto thyroiditis	Thyroid	2	4.4 ± 1.6	3.3–5.6	0.154
HIV	Lymph Node	2	4.0 ± 1.3	3.0–4.9	0.306
Nodular hyperplasia	Lung	1	2.6	NA	NA
Unspecified cause	Blood	1	1.5	NA	NA
	BAL	1	4.5	NA	NA
	Lymph node	25	2.8 ± 1.6	0.3–7.2	NA
	Pleural fluid	4	2.6 ± 0.4	2.1–2.9	0.826
	Tonsil	10	2.8 ± 1.2	1.3–4.7	0.931
3. B cell neoplasms					
Burkitt Lymphoma	Abdominal Fluid	1	2.3	NA	NA
	Stomach	1	<1	NA	NA
Diffuse large B cell lymphoma					
Primary mediastinal variant	Mediastinum	1	2.8	NA	NA
T cell rich variant	Lymph node	1	5.6	NA	NA
Unspecified variant	Liver	1	3.9	NA	NA
	Lymph node	1	1.1	NA	NA
	Pleural fluid	1	0.9	NA	NA
	Tonsil	1	55.4	NA	NA
Follicular lymphoma					
Grade 1	Lymph node	3	2.5 ± 0.5	2.0–3.0	0.815
Grade 2	Lymph node	2	4.2 ± 1.3	3.3–5.1	0.218
	Pleural fluid	1	4.2	NA	NA
Grade 3a	Lymph node	2	9.8 ± 3.6	7.3–12.4	<0.001
Unspecified grade	Bone marrow	1	1.8	NA	NA
MALT lymphoma	Lymph node	1	4.2	NA	NA
Mantle cell lymphoma	Lymph node	1	5.1	NA	NA
Plasma cell neoplasm	Hip	1	1.9	NA	NA
SLL/CLL	Lymph node	3	3.2 ± 1.8	1.5–5.1	0.648
All B cell neoplasms	Lymph node	14	4.4 ± 2.9	1.1–12.4	0.028
4. T cell neoplasms					
AITL	Lymph node	4	7.6 ± 2.5	4.0–9.7	<0.001
PTCL with AITL	Lymph node	1	5.5	NA	NA
5. Hodgkin lymphoma	Lymph node	2	2.8 ± 2.5	1.0–4.6	0.968
6. Miscellaneous					
CHF	Bone marrow	1	0.9	NA	NA
History of B cell lymphoma[f]	Bone marrow	1	2.1	NA	NA
History of CLL[f]	Bone marrow	1	0.2	NA	NA

[a] AITL, angioimmunoblastic T cell lymphoma; CHF, congestive heart failure; HIV, human immunodeficiency virus; MALT, mucosa associated lymphoid tissue; PTCL, peripheral T cell lymphoma; SLL/CLL, small lymphocytic lymphoma/chronic lymphocytic leukemia.
[b] BAL, bronchoalveolar lavage.
[c] SD, standard deviation.
[d] NA, not applicable due to $N = 1$.
[e] *P* values were calculated using the two tailed *t*-test. All mean values were compared to the mean of the reactive lymphoid hyperplasia (unspecified cause, lymph node) subgroup; NA, not applicable due to $N = 1$.
[f] Bone marrow was negative for tumor.

TABLE II. T Cell Beta Receptor Gene Rearrangement in Sorted CD3+ CD10+ T Cells

Case number	Age (years)	Gender[a]	Diagnosis[b]	Tissue	CD3+ CD10+ T cells (%)	T cell receptor clonality
1	7	F	Reactive lymphoid hyperplasia	Tonsil	3.0	Polyclonal
2	50	F	Follicular lymphoma, Grade 1	Lymph node	2.6	Polyclonal
3	72	M	Mantle cell lymphoma	Lymph node	5.1	Polyclonal
4	79	F	SLL/CLL	Lymph node	3.0	Polyclonal
5	62	M	AITL (early biopsy)	Lymph node	4.0	Oligoclonal
6	62	M	AITL (late biopsy)	Lymph node	8.5	Monoclonal

[a] F, female; M, male.
[b] AITL, angioimmunoblastic T cell lymphoma; SLL/CLL, small lymphocytic lymphoma/chronic lymphocytic leukemia.

clonal to monoclonal. Therefore, by utilizing flow cytometry and T cell gene rearrangement studies on sorted CD3+ CD10+ T cells, we can increase the sensitivity for detecting early phase AITL and also monitor the disease progression.

CD10+ T cells are not unique for AITL, nor can they be used as a marker to distinguish AITL from PTLC or other lymphoid proliferative disorders. There are different views as to the relationship between AILD and AITL. According to one study, the authors demonstrated that all lesions with the histologic features of AILD are neoplastic [5]. In another study, the authors considered AILD and AITL as part of a spectrum of diseases which represented a heterogenous group with similar histology [6]. Some cases of AILD have been shown to undergo spontaneous remission and do not show monoclonality, but most cases of AILD contain a monoclonal T-cell population [5,7].

Some authors view AITL as a spectrum ranging from atypical reactive lymphoid hyperplasia to frank lymphoma, however, most authors believe that AITL arises de novo as a subtype of PTCL [8,9]. During the initial phase,

AITL seems to be a reactive condition since the T cell receptor gene is usually not rearranged in the early stage [5,7–9,10]. At which point these cells become monoclonal neoplastic cells is still unclear. In one of our cases, a single lymph node showed a spectrum of disease ranging from atypical lymphoid hyperplasia, to typical AITL (with open sinuses, residual follicles, and proliferation of follicular dendritic cells), to typical monomorphic PTLC. AITL can represent a spectrum of disease, from early stage atypical hyperplasia at one end and PTCL at the other end.

Case selection: We obtained lymphoid tissue and bone marrow specimens with informed consent at the University of Kansas Medical Center by following an institutional review board approved protocol. Eighty-four cases representing a variety of tissue specimens were collected from a 12 month period, and submitted to our flow cytometry laboratory for evaluation of lymphoma. All diagnoses were rendered according to WHO criteria using histologic, immunohistochemical, flow cytometric, and cytogenetic studies.

Flow cytometry: The specimen were analyzed on a Coulter-Epics XL-MCL© flow cytometer (Fullerton, CA) using two-color flow cytometry. Lymphocytes were gated based on forward light scatter and side scatter characteristics. The CD3+ CD10+ T cells were quantified as a percentage of the total gated lymphocytes. Selected cases of CD3+ CD10+ T cells were sorted for at least 5,000 cells for the T cell gene rearrangement study.

Statistical analysis: Differences between two independent mean values were tested for statistical significance using the two-tailed *t*-test. Values of $P < 0.05$ were considered significant.

T cell gene rearrangement study: DNA was isolated from sorted cells using a home-brew salting-out extraction procedure. TCR-γ gene rearrangement studies were performed using a commercially available PCR assay (Invivoscribe, San Diego, CA). Two primer sets, spanning the Vγ-Jγ region were used in a reduced reaction volume (15 μl of each Master Mix), but otherwise according to the manufacturer's instructions. The two forward primers were differentially labeled with a yellow or green dye to distinguish the respective products, with valid size ranges of 55–85 and 155–185 base pairs for the yellow dye, and 200–235 and 235–270 base pairs for the green dye. Capillary electrophoresis using an ABI 3130 analyzer (Applied Biosystems, Foster City, CA) was used to resolve the sizes of products amplified by PCR.

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Impact of the basal metabolic ratio in predicting early deaths after allogeneic stem cell transplantation

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Early deaths after allogeneic stem cell transplantation (allo-SCT) are of major concern. On the assumption that both decreased and increased basal metabolism might relate to early deaths, we analyzed the risk factors for overall survival to days 30 (OS30) and 60 (OS60). The Harris-Benedict equation was used to calculate basal metabolism. Comparing a patient's basal metabolism (PBM) calculated from pretransplant body weight with the standard basal metabolism (SBM) calculated from standard body weight (body mass index (BMI) = 22), we defined the basal metabolic ratio (BMR) as a parameter (BMR = PBM/SBM). We retrospectively analyzed 360 adult patients transplanted between 1997 and 2006 at a single center in Japan. A multivariate analysis of OS30 showed risk factors to be: BMR ≤ 0.95 (low BMR; LBR) ($P = 0.01$), BMR > 1.05 (high BMR; HBR) ($P = 0.005$) and non-complete remission (non-CR) ($P = 0.001$), whereas a multivariate analysis of OS60 showed those risk factors to be: LBR ($P = 0.02$), HBR ($P = 0.04$), non-CR ($P = 0.002$), and performance status ≤ 1 ($P = 0.01$). OS30 and OS60 were found to be favorable in 0.95 < BMR ≤ 1.05 (average BMR; ABR) (96.8 and 90.3% for ABR, 87.1 and 76.2% for LBR, and 87.8 and 81.1% for HBR). In conclusion, BMR could prove to be a predictor of early death after allo-SCT.

Allogeneic stem cell transplantation (Allo-SCT) is a curative treatment strategy for patients with hematological disorders. However, early deaths after allo-SCT are of major concern, and must be dramatically reduced. Although a hematopoietic cell transplantation-specific comorbidity index (HCT-CI) has recently been used to identify risk assessments before allo-SCT [1], it fails to provide data on early deaths. Together with established factors such as the disease status [2], performance status (PS) [3], and HLA compatibility [4], the factor of advanced age has been recognized as a significant risk of transplant-related mortality (TRM) after allo-SCT [5,6].

It is said that age is not necessarily synonymous with aging [7]. There may be a difference between biological and chronological age, with visual estimations being used as a measure of the former [8,9]. In fact, a reduction in basal metabolism with advancing age has been observed in a number of studies [10–13], and an age-related decline in basal metabolism is partially explained by a reduction in the metabolic activity of tissue components [10,14,15]. The organ age of those patients who appear older than their chronological age may also be older than their actual age. This finding may be related to reductions in basal metabolism and organ reserve, and could eventually lead to early death after allo-SCT [16]. At the same time, it has

also been reported that basal metabolism is elevated in some cancer patients, which leads to weight loss and cachexia [16,17], though the causes of increased energy consumption in cancer patients remain unclear [18].

Organs were found to be major contributors to basal metabolism, especially the brain, liver, heart, and kidneys, and accounted for 58% ± 4.8% of the total resting energy expenditure [19]. These are vital organs and changes in their metabolism may cause functional impairment. Considering these facts, we speculated that both decreased and increased basal metabolism levels may be related to early death after allo-SCT, and we analyzed the effect of basal metabolism on overall survival to days 30 (OS30) and 60 (OS60).

We retrospectively examined 360 adult patients suffering from hematological disorders (Table I). There were 185 of 360 patients (51.4%) within 0.95 $<$ basal metabolic ratio (BMR) $\leq$ 1.05 (average BMR; ABR), with 101 (28.0%) in BMR $\leq$ 0.95 (low BMR; LBR), and 74 (20.6%) in BMR $>$ 1.05 (high BMR; HBR). Association analyses revealed that BMR might be independent of disease status ($P = 0.92$), PS ($P = 0.26$), HCT-CI ($P = 0.38$), HLA disparity ($P = 0.77$), donor source ($P = 0.54$), graft source ($P = 0.22$), conditioning ($P = 0.30$), and the period of allo-SCT ($P = 0.59$).

At day 30, 332 of 360 patients (92.2%) were still alive, and all but one was free of disease. As for the 28 deceased patients, their causes of death were: infection ($n = 8$), heart failure ($n = 4$), multiple organ failure ($n = 3$), graft failure ($n = 3$), cerebrovascular disorder ($n = 2$), noninfectious pulmonary dysfunction ($n = 2$), renal failure ($n = 2$), thrombotic thrombocytopenic purpura ($n = 2$), graft-versus-host disease (GVHD) ($n = 1$), and relapse ($n = 1$). In a multivariate analysis, LBR, HBR, and non-CR were identified as significantly unfavorable prognostic factors for OS30 (Table II). The Kaplan–Meier estimate for OS30 was 92.2% and OS30 of ABR was better than those of LBR and HBR (Fig. 1A). TRM at 30 days was lower in ABR than that in HBR and LBR (Fig. 1B).

At day 60, 304 of 360 patients (84.4%) were alive, and all but three were free of disease. Among the 56 deceased, their causes of death were: infection ($n = 16$), graft failure ($n = 6$), heart failure ($n = 5$), multiple organ failure ($n = 4$), noninfectious pulmonary dysfunction ($n = 4$), GVHD ($n = 4$), intestinal transplanted-associated microangiopathy ($n = 4$), relapse ($n = 3$), renal failure ($n = 3$), cerebrovascular disorder ($n = 2$), tacrolimus encephalopathy ($n = 2$), thrombotic thrombocytopenic purpura ($n = 2$), and liver failure ($n = 1$). In a multivariate analysis, LBR, HBR, non-CR, and PS $\geq$ 1 were identified as significantly unfavorable prognostic factors for OS60 (Table II). The Kaplan–Meier estimate for OS60 was 84.4% and OS60 of ABR was better than those of LBR and HBR (Fig. 1A). TRM at 60 days was lower in ABR than in HBR and LBR (Fig. 1B).

In this study, we demonstrated that LBR and HBR were significant risk factors for early death after allo-SCT. The causes of death within 1 or 2 months after allo-SCT may vary widely, therefore making them usually difficult to predict. Our data suggesting that HBR might be a significant risk factor for early deaths might explain that higher levels of basal metabolism were found in infected or critically ill patients. As for lower basal metabolism, we hypothesized that it might reflect a diminished organ reserve indicating older biological age, thus, causing early deaths after allo-SCT due to organ failure or other reasons.

The death rate of ABR would increase after 30 days following allo-SCT, and approached that of LBR and HBR. The respective odds ratio of death by time from transplantation in the LBR and HBR (vs. ABR) were 4.41 and 4.13 at day 30, 2.89 and 2.16 at day 60, 1.89 and 1.54 at day 100, and 1.70 and 1.45 at day 120. The reason could have been that the reserve capacity of principal organs would have exerted a relatively large impact on deaths at the beginning of allo-SCT, whereas other factors such as GVHD, infection, and relapse would have an even stronger relationship to deaths after a certain period of time from allo-SCT.

Our result suggested that special attention might be needed for patients with LBR and HBR. We assumed that the organ age of patients who looked older than their actual age might also actually be older, and that this might be related to a decrease in basal metabolism and organ reserve, which could eventually lead to early death. Therefore, careful attention would be needed, such as selecting reduced-intensity conditioning regimens (RIST) frequently used for elderly patients [20,21], thus enhancing the chances of reducing TRM [22].

BMR might be an independent prognostic factor, and thus be able to predict a prognosis that existing parameters could not. Considering that most patients in this study were in relatively good condition ($>$90% were PS 0 or 1), we assumed that in evaluating the potential risk derived from organ reserve capacity, we might discover a disparity between PS and BMR. We can calculate BMR relatively easily using a patient's age, height, and body weight, so that it could become a parameter helpful in discussions about allo-SCT indications and in predictions of clinical courses after allo-SCT.

It was reported that survival among patients at 85–95% or $<$85% of ideal body weight was significantly worse than for those in 95–145% weight category [23]. In that report, those in the former category were only about 10% of all study patients, suggesting that some of them were in extremely poor nutritional and general health. In our report, we were able to obtain a better stratification of patients by focusing on basal metabolism, which suggests that correlations among not only patients' height and weight but also their age could put them at risk of early death after allo-SCT.

There were several limitations in this study. It was a retrospective cohort study at a single center in Japan with no validation set and no actual data of basal metabolism, and with patients' conditions such as underlying diseases,

TABLE I. Patient and Donor Characteristics

No. patients	360
Sex	
Male	235
Female	125
Underlying disorders	
AML	100
CML	72
MDS	68
ALL	60
Malignant lymphoma	16
Multiple myeloma	11
ATL	4
Aplastic anemia	22
PNH	4
Others	3
Disease status	
CR	195
Non-CR	165
Age, median (range)	40.5 ± 12.5 (16–68)
HLA	
Match	261
Mismatch	99
Donor	
Related	162
Unrelated	198
Graft	
Bone marrow	269
Peripheral blood	60
Cord blood	29
Conditioning	
Myeloablative	236
BU+L-PAM+TBI10-12Gy	43
CA+CY+TBI10-12Gy	62
BU+CY+TBI10Gy	36
L-PAM+TBI10-12Gy	37
CY+TBI10-12Gy	33
BU+CY	24
BU+L-PAM	1
RIST	124
Flu+L-PAM	88
Flu+others	15
CY+TLI7.5Gy	9
CY+TBI5Gy+TLI5Gy	12
GVHD priphyraxis	
Cyclosporine+sMTX	195
Tacrolimus+sMTX	157
Others	8
BMR median (range)	0.983 (0.790–1.241)
HCT-CI median (range)	2 (0–10)
Performance status (%)	
0	66.4
1	28.0
2	4.4
3	1.2

AML, acute myeloid leukemia; CML, chronic myeloid leukemia; MDS, myelodysplastic syndrome; ALL, acute lymphocytic leukemia; ATL, adult T-cell leukemia/lymphoma; PNH, paroxysmal nocturnal hemoglobinuria; CR, complete remission; non-CR, non-complete remission; RIST, reduced intensity conditioning regimens; BU, buslfan; L-PAM, melphalan; TBI, total body irradiation; CA, cytarabine; CY, cyclophosphamide; Flu, fludarabin; TLI, total lymphoid irradiation; sMTX, short-term methotrexate; BMR, basal metabolic ratio; BMI, body mass index; HCT-CI, hematopoietic cell transplantation-specific comorbidity.

TABLE II. Risk Factors for Overall Survival

	Univariate			Multivariate		
Risk factor	HR	95% CI	*P* value	HR	95% CI	*P* value
Overall Survival 30 Days After Allogeneic Stem Cell Transplantation						
BMR						
LBR	4.41	(1.62–12.0)	0.004	3.89	(1.33–11.4)	0.01
HBR	4.13	(1.41–12.0)	0.01	5.24	(1.63–16.7)	0.005
Status non-CR	11.40	(3.38–38.5)	<0.0001	8.20	(2.32–23.4)	0.001
PS $\geq$ 1	4.52	(1.96–10.5)	0.0004	2.57	(0.99–6.67)	0.052
HCR-CI $\geq$ 3	3.14	(1.40–7.02)	0.005	2.14	(0.86–5.35)	0.10
HLA mismatch	2.18	(0.99–4.78)	0.052			
Conditioning						
Myeloablative	1.06	(0.47–2.38)	0.88			
Donor UR	1.29	(0.59–2.84)	0.53			
Graft (vs. BM)						
PB	1.96	(0.77–4.95)	0.16			
CB	1.71	(0.47–6.21)	0.83			
Age > 50 y.o.	1.51	(0.56–4.10)	0.42			
Year 1997–2001	1.15	(0.53–2.48)	0.73			
Overall Survival 60 Days After Allogeneic Stem Cell Transplantation						
BMR						
LBR	2.89	(1.48–5.65)	0.002	2.53	(1.18–5.41)	0.02
HBR	2.16	(1.01–4.63)	0.04	2.35	(1.00–5.52)	0.04
Status Non CR	4.41	(2.31–8.40)	<0.0001	3.47	(1.60–7.52)	0.002
PS $\geq$ 1	3.89	(2.09–7.25)	<0.0001	2.49	(1.21–5.10)	0.01
HCR–CI $\geq$ 3	2.25	(1.26–4.01)	0.006	1.47	(0.74–2.93)	0.27
HLA mismatch	5.92	(3.04–11.5)	<0.0001	1.93	(0.97–3.86)	0.06
Conditioning						
Myeloablative	1.82	(1.02–3.25)	0.04	1.04	(0.52–2.09)	0.91
Donor UR	1.73	(0.95–3.14)	0.07			
Graft (vs. BM)						
PB	1.61	(0.76–3.39)	0.22			
CB	4.37	(1.90–10.1)	0.005			
Age > 50 y.o.	0.76	(0.40–1.44)	0.76			
Year 1997–2001	0.69	(0.38–1.24)	0.21			

Abbreviations are explained in Table I. We included factors that were significant in the univariate analyses (i.e. $P < 0.05$) in the multivariate analyses. By way of exception, because donor source, especially cord blood, was strongly associated with HLA mismatch as expected, we excluded donor source from multivariate analysis for OS60.

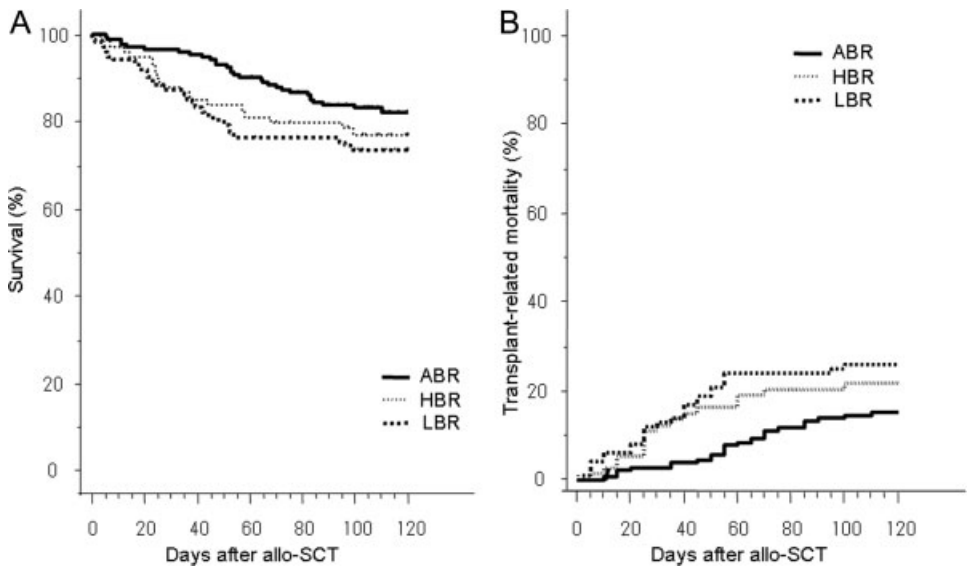

Figure 1. Overall survival and cumulative incidence of TRM according to basal metabolic ratio (BMR). A: OS30 proved better in an average BMR (ABR) (n = 185) than in a low BMR (LBR) (n = 101) or high BMR (HBR) (n = 74) (96.8% for ABR, 87.1% for LBR, and 87.8% for HBR). OS60 was better in an ABR than in LBR or HBR (90.3% for ABR, 76.2% for LBR, and 81.1% for HBR). B: Transplant-related mortality was lower in an ABR than in a LBR or HBR (2.7% at day 30 and 8.2% at day 60 for ABR, 12.2% at day 30 and 18.9% at day 60 for HBR, and 12.9% at day 30 and 23.8% at day 60 for LBR, respectively).

pretransplant therapies, donors, conditioning regimens varying widely. This new parameter and its prognostic significance require validation from other cohorts of patients. Although we simply intended to establish a reference value, we encountered some potential problems in using US-based equations (the Harris-Benedict equation and BMI).

In conclusion, both decreased and increased basal metabolism may be related to early death after allo-SCT. BMR could prove useful as a new predictive parameter for early death, especially, in cases where it would be difficult to predict outcomes by relying on existing parameters. Efforts at further validation are warranted, and it would be of considerable value to develop a more sensitive predictive system in combination with other parameters to avoid early death after allo-SCT.

Methods

The study population (excluding rescue allo-SCTs for engraftment failure) consisted of 360 adult Japanese patients who underwent allo-SCTs between January 1997 and December 2006 at the Japanese Red Cross Nagoya First

Hospital. Diagnosis was based on cytology, karyotype, and immunophenotyping of marrow cells. Since treatment before the introduction of allo-SCT was based on the therapeutic strategy current at that time, the indications of allo-SCT were judged by our transplant team. Protocols were approved by the hospital's institutional review boards, and all patients provided informed consent.

All the data were retrieved retrospectively from the medical records by experienced clinicians in our transplant team. Medical records were documented in a predetermined manner by the team's experienced nurses and clinicians, and all records were approved by the chief of our transplant center.

Donors were selected based on a combination of serotyping and genotyping performed for HLA-A and -B and -DRB1 according to the standard procedures. Donor and recipient pairs were considered matched when displaying identical HLA-A, -B, and -DRB1 loci. Mismatches included at least one disparity at one of these loci. As for GVHD prophylaxis, cyclosporine and short-term methotrexate were used for allo-SCT in malignant diseases from HLA-matched related donors and in non-CR patients from unrelated UR donors. Tacrolimus and short-term methotrexate were used for allo-SCT in non-malignant diseases from related donors, from HLA-mismatched related donors, and in CR patients from unrelated donors. Supportive care measures were taken using our local protocols, mainly focusing on the prevention of infection, such as oral care, maintaining a clean pubic area, early administration of antibiotics to treat fever, and on pain control, such as cooling of the oral mucosa, and the administration of NSAIDs or narcotics, especially for pain due to mucosal damage.

To assess the potential impact of each patient's overall status, we focused on basal metabolism. The Harris-Benedict equation, which is one of those most commonly employed, was used to estimate basal metabolism, calculating it from a person's weight, height, and age [24]. Comparing a PBM calculated from a patient's pretransplant body weight with SBM calculated from standard body weight (BMI = 22), we defined the BMR as a parameter (BMR = PBM/SBM) to show the comparison between patient data and calculated reference values, similar to the procedure used in electrocardiograms (QT/QTc) [25] and pulmonary function tests [26]. BMI is a global marker of body fat, a condition also widely prevalent in Japan. Standard body weight calculated from BMI = 22 has been accepted as a reference value [27]. We used this standard body weight as a benchmark to calculate SBM. The patient's height was measured on admission by an experimental nurse. The patient's body weight was calculated under the following conditions: wearing light clothing, at the time of awakening, between 1 and 2 weeks before allo-SCT, before conditioning and without fluid administration or the median of three points in the case of patients requiring continuous fluid administration.

We divided BMR into three groups; LBR, ABR, and HBR, because the standard deviation of the metabolic rate was reported as 5.5% of the average value [28].

The end points of this study included OS30 and OS60. OS were measured from the date of transplantation to that of death from any cause. Kaplan–Meier product-limit estimates were conducted to determine OS30 and OS60. The cumulative-incidence function was used for estimates of TRM [29]. Univariate and multivariate analyses to determine risk factors used logistic regression analyses. Chi-square test was used for association analyses.

As for parameters, we analyzed BMR (LBR vs. ABR vs. HBR), disease status (CR vs. non-CR), ECOG PS [30] (0 vs. $\geq$1), HCT-CI ($\leq$ 2 vs. $\geq$3), HLA disparity (match vs. mismatch), donor source (related vs. unrelated), graft source (BM vs. PB vs. CB), age at allo-SCT ($\leq$50 years vs. $>$50 years), conditioning regimen (myeloablative regimens vs. RIST), and the time of allo-SCT administration (between 1997 and 2001 vs. between 2002 and 2006). A significance level of $P < 0.05$ was used for all analyses. The StatView programs (SAS Institute, Cary, NC) and the "cmprsk" package available in R (R Development Core Team, 2005) were used for the analyses, which were based on all data available as of August 2007.

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