have an increased risk of early disease progression and to guide treatment decision, since this technique is more amenable to application in clinical laboratories than IGHV sequencing.

*Cristina Reinoso-Martín,[1] Eloisa Jantus-Lewintre,[1] Carlos García Ballesteros,[2] Carmen Benet Campos,[2] José Ramón Mayans Ferrer,[2] and Javier García-Conde[1,3]*

*[1]Molecular Haematology Laboratory, Prince Felipe Research Centre (CIPF), [2]Haematology Service, Arnau Vilanova Hospital, [3]University of Valencia, Valencia, Spain*

*Cristina Reinoso-Martín and Eloisa Jantus-Lewintre should be considered both as first authors.*

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*Correspondence: Cristina Reinoso Martín, Molecular Hematology Laboratory, Prince Felipe Research Centre (CIPF), Avda. Autopista El Saler 16-3, 46012, Valencia, Spain, Phone: international +34.96.3289680. Fax: international +34.96.3289701. E-mail: reinosocristina@yahoo.es*

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## References

1. Hamblin T. Chronic lymphocytic leukaemia: one disease- or two? Ann Hematol 2002;81:299-303.
2. Damle RN, Wasil T, Fais F, Ghiotto F, Valetto A, Allen SL, et al. Ig V gene mutation status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukemia. Blood 1999;94:1840-7.
3. Hamblin TJ, Davis Z, Gardiner A, Oscier DG, Stevenson FK. Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukaemia. Blood 1999;94:1848-54.
4. Rosenwald A, Alizadeh AA, Widhopf G, Simon R, Davis RE, Yu X, et al. Relation of gene expression phenotype to immunoglobulin mutation genotype in B cell chronic lymphocytic leukaemia. J Exp Med 2001;194:1639-47.
5. Wiestner A, Rosenwald A, Barry TS, Wright G, Davis RE, Henrickson SE, et al. ZAP-70 expression identifies a chronic lymphocytic leukemia subtype with unmutated immunoglobulin genes, inferior clinical outcome, and distinct gene expression profile. Blood 2003;101:4944-51.
6. Crespo M, Bosch F, Villamor N, Bellosillo B, Colomer D, Rozman M, et al. ZAP-70 expression as a surrogate for immunoglobulin-variable-region mutations in chronic lymphocytic leukemia. N Engl J Med 2003;348:1764-75.
7. Del Principe MI, Del Poeta G, Buccisano F, Maurillo L, Venditti A, Zucchetto A, et al. Clinical significance of ZAP-70 protein expression in B-cell chronic lymphocytic leukemia. Blood 2006;108:853-61.
8. van Dongen JJ, Langerak AW, Bruggemann M, Evans PA, Hummel M, Lavender FL, et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. Leukemia 2003; 17:2257-317.
9. Catherwood MA, Matthews C, Niblock R, Dobbin E, Morris TC, Alexander HD. ZAP-70 mRNA quantification in B-cell chronic lymphocytic leukaemia. Eur J Haematol 2006;76:294-8.
10. Rassenti LZ, Huynh L, Toy TL, Chen L, Keating MJ, Gribben JG, et al. ZAP-70 compared with immunoglobulin heavy-chain gene mutation status as a predictor of disease progression in chronic lymphocytic leukemia. N Engl J Med 2004;351:893-901.
11. Munoz L, Lasa A, Carricondo MT, Hernandez C, Ubeda J, Nomdedeu JF. Comparative analysis of ZAP-70 expression and Ig VH mutational status in B-cell chronic lymphocytic leukaemia. Cytometry B Clin Cytom 2007;72: 96-102.
12. Van Bockstaele F, Janssens A, Piette A, Callewaert F, Pede V, Offner F, et al. Kolmogorov-Smirnov statistical test for analysis of ZAP-70 expression in B-CLL, compared with quantitative PCR and IgV(H) mutation status. Cytometry B Clin Cytom 2006;70:302-8.

## Epstein-Barr virus reactivation is a potentially severe complication in chronic lymphocytic leukemia patients with poor prognostic biological markers and fludarabine refractory disease

Chronic lymphocytic leukemia (CLL) patients who are refractory to, or have early relapse after, fludarabine and cyclophosphamide (FC) based therapy have a poor prognosis.[1] A major clinical problem in FC-refractory disease is a profound immunodeficiency, resulting in a high incidence of severe opportunistic infections.[2] The immunodeficiency may be caused either by the natural history of high-risk fludarabine-refractory CLL disease in itself and/or immunosuppressive side effects of the CLL treatment used for this group of patients. Over a two-year period, we identified 11 CLL patients with EBV-reactivation, defined as measurable EBV-DNA copies by quantitative PCR.

All 11 patients had IgG antibodies (VCA and/or EBNA) against EBV. Ten patients had negative CMV qPCR at the time of EBV-reactivation, while one (UPN 6) had concomitant CMV-reactivation. All 11 patients had biochemical signs of hypogammaglobulinemia, while 8 (UPN 1, 2, 5-6 and 8-11) patients had neutropenia. The median age at diagnosis of CLL was 58 years (range 43-75 years). The median time from CLL diagnosis to EBV-reactivation was 4.5 years (range 1-13 years). With a follow-up time of 824 days, the median overall survival from date of EBV-reactivation was 264 days, despite aggressive rituximab based chemo-immunotherapy in symptomatic cases. Eight deaths were observed, 3 as a direct result of EBV-reactivation.

All 11 patients had high-risk disease as defined by either IGVH mutational status and/or FISH analysis of recurrent cytogenetic aberrations associated with CLL (Table 1). Ten patients (UPN 1-2 and 4-11) had received fludarabine based treatment prior to EBV-reactivation, and 9 patients (UPN 1-2, 4-6 and 8-11) had fludarabine refractory disease at the time of EBV-reactivation. Whether these observations suggest that EBV-reactivation is associated with the immunosuppressive side-effects caused by treatment of advanced CLL cannot be determined from our data set. However, we do note that EBV-reactivation occurred before exposure to alemtuzumab in 4 patients (UPN 1, 7-8 and 11), and in one patient (UPN 3) prior to any CLL treatment. This last patient turned out to be fludarabine-resistant, did not receive rituximab, but responded well to standard alemtuzumab treatment on which EBV-copies in sequential blood samples disappeared. Of the 4 patients not treated with alemtuzumab, one developed proven EBV-driven CLL-related BLPD, one hemophagocytic syndrome, one a possible BLPD and one did not have any symptoms.

The clinical presentation of the patients was related to the level of EBV copies/mL plasma.

i) Low-grade EBV-reactivation. Seven patients (UPN 1-7) had low EBV-levels of up to 6,600 copies/mL. The patients were either asymptomatic (UPN 1 and 4) or presented with fever, fatigue, night sweats and/or enlarged lymph nodes, that is, symptoms identical to the development of active CLL. In retrospect, UPN 5-7 were considered to have pos-

**Table 1.** Characteristics of chronic lymphocytic leukemia patients with Epstein-Barr virus-reactivation.

| *UPN/age at CLL diagnosis/sex* | *IgV$_H$ mutational status* | *FISH/ Cytogenetics* | *CLL therapy [x]=n. of cycles* | *Interval (1)* | *Diagnosis of EBV- reactivation* | *EBV DNA copies/mL plasma* | *Symptoms at time of EBV -reactivation* | *Treatment of EBV -reactivation* | *Outcome* |
|---|---|---|---|---|---|---|---|---|---|
| 1<br>60/M | Un-mutated | Del 11q | Purine analogs [6]<br>Alemtuzumab | 20 months | Plasma | 810 | Asymptomatic | None | Died 2 months after EBV of *Aspergillus* pneumonia |
| 2<br>65/M | Un-mutated | Del 11q | Purine analogs [15] | 29 months | Plasma | Positive but no count | Fever, Richter transformation | None | Died 27 months after EBV with CMV reactivation |
| 3<br>75/M | Un-mutated | Del 11q | - | - | Plasma | 300 | Pneumonia | None | Died 8 months after EBV of *E. Coli* sepsis |
| 4<br>52/M | Un-mutated | Normal | Alkylating agents [3]<br>Purine analogs [3]<br>Alemtuzumab | 2 months | Plasma | 2300 | Asymptomatic | None | Alive 1 month after EBV |
| 5<br>60/F | Un-mutated | Del 6q<br>Trans1;9 | Alkylating agents [2]<br>Purine analogs [10]<br>Alemtuzumab | 2 months | Plasma | 1700 | Possible BLPD (2) | Rituximab, pause alemtuzumab. Later R-CHOP (3) | Died 9 months after EBV because of Richter transformation |
| 6<br>48/M | Mutated | Del 17p<br>Del 13q | Alkylating agents [7]<br>Purine analogs [1]<br>Alemtuzumab | 3 months | Plasma and BAL fluid (4) | 3900 | Possible BLPD (2) | Pause alemtuzumab | Died 2 months after EBV of pneumonia |
| 7<br>43/M | Un-mutated | Del 11q<br>Del 13q | Purine analogs [12] | 31 months | Plasma | 6600 | Possible BLPD (2) | None | Alive 4 months after EBV, searching for a bone marrow donor |
| 8<br>66/M | Un-mutated | Trisomy 12 | Purine analogs [6] | 6 months | Plasma | 45000 | Hemophagocytic syndrome | Etoposid | Died of multi-organ failure 4 days after diagnosis of hemophagocytic syndrome3 |
| 9<br>57/M | Un-mutated | Del 17p | Purine analogs [2]<br>Rituximab [2]<br>Alemtuzumab | 4 months | 1. time: plasma, LMP-1 + and EBER + in ventricular ulcer<br>2. time: plasma | 1. time: 75000<br>2. time: 46500 | 1. time: Ventricular BLPD (2)<br>2. time: Possible BLPD (2), suspected Richter transformation in BM (4) biopsy | 1. time: Pause alemtuzumab<br>2. time: R-CHOP (3) | Died during second EBV |
| 10<br>51/M | Un-mutated | Del 17p | Alkylating agents [28]<br>Purine analogs [4]<br>Alemtuzumab | 10 months | 1. time: Plasma<br>2. time: Plasma, EBER + in lymph node (2) | 1. time: 36000<br>2. time: 3.700.000 post -transplant | 1. time: None<br>2. time: BLPD (2) | 1. time: Rituximab, pause alemtuzumab<br>2. time: Rituximab | Died after RIC-HCT (4) because of BLPD (2, 5) |
| 11<br>61/F | Un-mutated | Del 11q | Purine analogs [5] | 8 months | Plasma, LMP1 + and EBER + in lymph node (2) | 770000 | BLPD (2) | R-CHOP (3) | RIC-HCT (4), alive 41 months after EBV |

*UPN 1-4: Low-grade EBV-reactivation. UPN 5-7: Intermediate-grade EBV-reactivation. UPN 8-11: High-grade EBV-reactivation. 1. Interval between first fludarabine therapy and first detected EBV-reactivation. 2. BLPD: B-cell lymphoproliferative disease. EBER: EBV encoded RNA. LMP-l: EBV latent membrane protein 1. 3. F: Fludarabine. FC: Fludarabine-Cyclophosphamide. R-FC: Rituximab-Fludarabine-Cyclophosphamide. R-CHOP: Rituximab-Adriamycin-Cyclophosphamide-Oncovine-Prednisone. 4.BAL: Bronchoalveolar lavage. RIC-HCT: Reduced intensity conditioning hemopoitic stem-cell transplantation. BM: Bone-marrow.*

sible BLPD's, in the absence of biopsy-proven EBV-driven disease. Five of these patients died.

ii) High-grade EBV-reactivation. Four patients had proven EBV-associated disease (UPN 8-11). These patients had very high EBV-levels from 45,000 to 3,700,000 copies/mL plasma. One patient (UPN 8) presented with EBV-associated hemophagocytic syndrome, and died from multi-organ failure. Three patients (UPN 9-11) developed biopsy-proven EBV-positive high-grade lymphomas. In 2 of these cases (UPN 10 and 11), we isolated and sequenced tumor-specific DNA, and analyzed the Ig sequences of the tumor cell. We found that the Ig-sequences had no homology to the Ig-sequences identified in the original CLL clones of each individual. The Ig-sequences obtained provide direct evidence that the EBV-driven BLPDs originated in B-cell clones that were not associated with the original CLL clones. Of these 3 patients, one was treated successfully with R-CHOP and subsequently proceeded to RIC-HCT, while the 2 others died despite rituximab monotherapy and R-CHOP respectively.

EBV-reactivation in CLL patients has previously been observed in small patient cohorts. The largest study describes 5 patients with solitary BLPDs, 4 with CLL.[3] All patients had received fludarabine, and 3 alemtuzumab. The lesions were clonally distinct from the original low-grade B-cell neoplasm in 3 out of 4 cases assessed. Two single case studies report on patients treated with fludarabine who develop EBV-reactivation in addition to other events.[4,5] A larger prospective study describes 4 cases of EBV-reactivation during FC therapy in 24 patients with low-grade non-Hodgkin's Lymphoma (21 patients) and CLL (3 patients).[6] Two of these EBV-reactivations occurred in CLL

patients with partial responses to FC.

The data we present suggest that EBV-reactivation in CLL may not be rare, in fact the condition could be significantly under-diagnosed. All 11 cases had signs of severe CLL associated secondary immunodeficiency. The clinical presentation of EBV reactivation was as varied as in BLPD associated with primary immunodeficiencies, and can mimic the symptoms of active CLL. Therefore, EBV-reactivation must be considered in febrile CLL patients with high-risk biological risk features and/or fludarabine-refractory disease. In the absence of clinical trials for the management of EBV reactivation in CLL, the treatment strategy should include rituximab as in the non-CLL setting, possibly in combination with chemotherapy as recommended for Richter's syndrome.[7] Further research is needed to determine a threshold for differentiation between a significant and non-significant increase in EBV copies, and to determine when rituximab therapy should be initiated.

*Julie Rath,[1] Christian Geisler,[1] Claus B. Christiansen,[2] Nina Hastrup,[3] Hans O. Madsen,[4] Mette K. Andersen,[5] Lone B. Pedersen,[1] Jesper Jurlander[1]*

*[1]Department of Hematology, The Leukemia Laboratory, Rigshospitalet; [2]Department of Microbiology, Rigshospitalet; [3]Department of Pathology, Rigshospitalet; [4]Department of Clinical Immunology, Rigshospitalet; [5]Department of Clinical Genetics, Rigshospitalet, Denmark*

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*Correspondence: Julie Rath, Department of Hematology 4041, Rigshospitalet, Blegdamsvej 9, 2100 København Ø, Denmark. Phone: international +45.35454045. E-mail: julie.christensen.rath@rh.regionh.dk*

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## References

1. Grever MR, Lucas DM, Dewald GW, Neuberg DS, Reed JC, Kitada S, et al. Comprehensive Assessment of Genetic and Molecular Features Predicting Outcome in Patients With Chronic Lymphocytic Leukemia: Results From the US Intergroup Phase III Trial E2997. J Clin Oncol 2007;25:799-804.
2. Perkins JG, Flynn JM, Howard RS, Byrd JC. Frequency and type of serious infections in fludarabine-refractory B-cell chronic lymphocytic leukemia and small lymphocytic lymphoma: implications for clinical trials in this patient population. Cancer 2002;94:2033-9.
3. Abruzzo L, Rosales C, Medeiros L, Vega F, Luthra R, Manning J, et al. Epstein-Barr virus-positive B-cell lymphoproliferative disorders arising in immunodeficient patients previously treated with fludarabine for low-grade B-cell neoplasms. Am J Surg Pathol 2002;26:630-6.
4. Mercadal S, Martinez A, Nomdedeu B, Rozman M, Gaya A, Salamero O, et al. Herpes simplex and Epstein-Barr virus lymphadenitis in a patient with chronic lymphocytic leukemia treated with fludarabine. Eur J Haematol 2006;77:442-4.
5. Compérat E, Delmer A, Le Tourneau A, Molina T, Diebold J, Audouin J. Concomitant Epstein-Barr virus-negative large B-cell lymphoma (Richter syndrome) and Epstein-Barr virus-positive B-cell lymphoproliferation after treatment with fludarabine and cyclophosphamide in a patient with B-cell chronic lymphocytic leukemia. Arch Pathol Lab Med 2006;130:1227-30.
6. Lazzarino M, Orlandi E, Baldanti F, Furione M, Pagnucco G, Astori C, et al. The immunosuppression and potential for EBV reactivation of fludarabine combined with cyclophosphamide and dexamethasone in patients with lymphoproliferative disorders. Br J Haematol 1999;107:877-82.
7. Tsimberidou AM, Keating MJ. Richter's transformation in chronic lymphocytic leukemia. Semin Oncol 2006;33:250-6.

## Rituximab in patients with hairy cell leukemia relapsing after treatment with 2-chlorodeoxyadenosine (SAKK 31/98)

We assessed the efficacy of rituximab, 375 mg/m$^2$ weekly x 4, in 26 patients with hairy cell leukemia (HCL) relapsed or progressed after prior 2-chlorodeoxyadenosine (CDA). Overall response rate (RR) was 80%, with 32% complete remission (CR). Median relapse-free-survival (RFS) was 27 months and median remission duration (RD) 33.6 months.

HCL is an indolent B-cell neoplasm. Hairy cells typically coexpress CD11c, CD25 and CD103 antigens in addition to the pan B-cell antigen CD20.[1] Patients require treatment for pancytopenia, infections or symptomatic splenomegaly and CDA has emerged as the treatment of choice.[2] A single course of CDA produces CR in up to 85% of patients and partial response (PR) in 5-25%.[3,4] With longer follow-up, relapses of HCL are common. Goodman *et al*. reported a 37% relapse rate in 209 patients treated with CDA and had at least seven years of follow-up.[5] The RR (CR and PR) after re-treatment with CDA ranges from 60 to 90% but with shorter RD. Bone marrow aplasia, prolonged cytopenias and infections increase with repetitive CDA courses.[2] Hairy cells exhibit an especially high CD20 antigen density,[6] and the anti-CD20 monoclonal antibody rituximab is considered an attractive treatment option.

We conducted a multicenter phase II trial to investigate rituximab in pre-treated HCL patients of any age who had received at least one previous course of CDA. Diagnosis and assessment of remission was established by morphology including peripheral blood smear and bone marrow examination, supported by a positive tartrate-resistant acid phosphatase (TRAP) stain and characteristic immunophenotyping. Relapse was defined as the reappearance of hairy cells in the bone marrow or as any other new disease manifestations after documented CR. Progressive disease (PD) was defined as >50% increase in the percentage of residual tumor cells, or as >50% increase of residual disease-related organomegaly after documented PR.

Between February 1998 and July 2002, 26 patients were accrued. One patient was not evaluable. Of 25 patients, 24 had classical HCL and one a prolymphocytic sub-type. Pre-treatment consisted of CDA (n=25), splenectomy (n=4), interferon-α (n=9) and alkylating agents (n=2). Nine patients had relapsed and 16 had PD. Patients' characteristics are summarized in Table 1.

Patients received rituximab 375 mg/m$^2$ weekly x 4 doses using standard infusion guidelines. Re-staging was carried out two months after treatment and then every three months during the first three years and every six months thereafter until relapse. Twenty-four of the 25 patients received all of the four planned infusions. One patient stopped treatment after the first infusion because of a dermal vasculitis. Across all follow-up visits the RR was 80% (20/25 patients; 95% CI: 64.32, 95.68) and the rate of CR was 32% (8/25 patients; 95% CI: 13.71,