Lymphoproliferative Disorders

The effect of IgVH mutational status on the induction of apoptosis by rituximab in patients with heavily pretreated B-cell chronic lymphocytic leukemia: evidence from a clinical phase I/II trial

We investigated tumor cell apoptosis *in vivo* in 14 heavily pretreated patients with B-cell chronic lymphocytic leukemia undergoing rituximab monotherapy. Apoptosis induction was more pronounced in patients with mutated IgVH genes than in those with unmutated IgVH genes, independently of the levels of expression of CD20, CD38, and ZAP-70 and of the presence of 17p deletion. Our results suggest an association between IgVH gene mutational status and rituximab-induced apoptosis.

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Rituximab induces apoptosis in B-cell chronic lymphocytic leukemia (B-CLL) cells *in vitro*.[1] However, the contribution of apoptosis induction to this drug's clinical activity in B-CLL remains unclear. Furthermore, novel prognostic markers such as CD38, ZAP-70, p53 deletion and IgVH mutational status have been shown to confer a poor prognosis.[2-5] The underlying pathophysiologic mechanisms are unknown and may include deregulation of apoptosis. Thus, the aim of our study was to investigate the safety and efficacy of rituximab in patients with advanced refractory B-CLL, focusing on the *in vivo* induction of apoptosis in relation to IgVH gene mutational status, p53 function and expression of CD38 and ZAP-70.

We conducted a phase I study at the Innsbruck Medical University Hospital between May 1999 and November 2002. The inclusion criteria were: (i) confirmed diagnosis of B-CLL as defined by the modified National Cancer Institute (NCI) criteria,[6] verified by immunophenotyping; (ii) expression of CD20 on tumor cells; (iii) an immediate indication for treatment according to NCI criteria,[6] and (iv) failure of at least two treatment regimens including fludarabine and/or alkylators. The study was approved by the local ethics committee and written informed consent was obtained from all participants. Rituximab was administered with appropriate pre-medication with a step-up dosing design, dividing the standard cumulative dose (1500 mg/m^2) into six weekly fractions.

Peripheral blood samples were obtained 1, 4 and 24 h after each rituximab infusion and up to 6 months after the end of rituximab treatment. Apoptotic B-CLL cells were detected by flow cytometry using fluorochrome-labeled anti-CD19 and anti-CD5 antibodies (Becton Dickinson, Vienna, Austria) together with fluoroscein isothiocyanate (FITC)-labeled annexin V (Alexis, Läufelfingen, Switzerland). Stained cells were analyzed immediately on a FACSCalibur (Becton Dickinson). Risk assessment according to ZAP-70 (Upstate, Lake Placid, NY, USA) and CD38 (CD38-PE, Immunotech, Marseille, France) expression was performed as described previously.[2,3,7] The accuracy of staining protocols and cut-off values used for risk stratification were confirmed in a large cohort of B-CLL patients (n=204).[8] IgVH genes were analyzed according to Fais and co-workers.[8] Sequencing was performed using forward primers unless quality control read from the sequencing process suggested incorrect sequence reactions. In this case sequencing was repeated using the reverse primer. A cut-off of ≥98% homology to germline sequences was used for the detection of unmutated IgVH genes.[5] p53 status was assessed by detecting deletion of chromosome 17p using a commercially available probe (ABBOTT/VYSIS, Vienna, Austria). The involvement of risk factors in sensitivity to rituximab was analyzed by means of appropriate descriptive summary statistics.

Fourteen heavily pretreated patients were included in this study (Table 1A). Their corresponding molecular risk profile is shown in Table 1B. Treatment was generally well tolerated with a mild cytokine release syndrome being the most common side effect. Overall clinical efficacy was limited, with only one patient achieving a partial response.

Table 1A. Patients' characteristics.

Parameters	*No. of patients (%)*
Total number	14 (100)
Sex	
Male	6 (43)
Female	8 (57)
Median age (years)	70
Range	48-79
ECOG Performance Status	
0	2 (14)
1	11 (79)
2*	1 (7)
Rai Stage	
I	0
II	2 (14)
III	5 (36)
IV	7 (50)
B symptoms	8 (57)
Fludarabine-refractory	12 (86)
	median (range)
White blood cells ($\times 10^9$/L)	94.3 (22.0-225.9)
Hemoglobin (g/dL)	10.6 (8.3-14.9)
Platelets ($\times 10^9$/L)	149 (9.0-231.0)
Number of previous chemotherapy regimens	5 (2-9)°

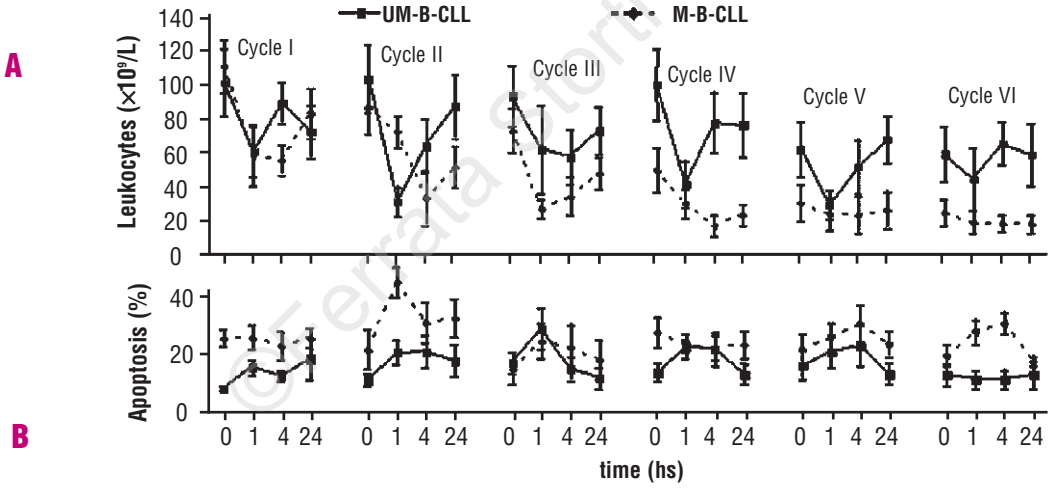
**ECOG status improved to 1 at beginning of therapy; °including one patient who had received high-dose chemotherapy and autologous stem cell transplantation.*

Table 1B. Molecular risk parameters of B-CLL patients.

PatientID	*Zap-70⁺ B-CLL [%]*	*CD38⁺ B-CLL [%]*	*Deletion of 17p*	*Homology to germline Ig VH [%]*	*Ig VH locus*
LF (#12)	**41**	25	no	90.1	VH 4-34
HMV (#13)	3	**37**	**yes**	96.3	VH 3-21 (IgG)
JP (#6)	**25**	**30**	no	95.1	VH 5-51
MZ (#14)	**68**	**97**	no	95.7	VH3-21
RM (#1)	**72**	**88**	no	97.1	VH 3-48
EM (#3)	**55**	**77**	no	97.3	VH 3-48
AP (#9)	**50**	2	no	**99.3**	VH 1-69
GP (#7)	**38**	**69**	**yes**	93.3	VH 3-66
JR (#11)	**36**	**76**	no	100.0	VH 4-34
GG (#10)	**29**	**100**	no	98.8	VH 3-49
GN (#5)	**47**	**99**	no	99.0	VH 3-23
ES (#2)	**78**	**76**	**yes**	**100.0**	VH1-69
ZH (#4)	**97**	**100**	**yes**	**99.7**	VH 1-69
HF (#8)	**86**	**97**	**yes**	**100.0**	VH 3-48

Patients were classified into high risk categories according to the following cut-off values: Zap-70 high risk was defined by ≥20% Zap-70⁺ B-CLL cells; CD38 B-CLL high risk was defined by ≥30% CD38⁺ B-CLL cells. Patients characterized as high risk in the respective category are highlighted in bold.

However, treatment with rituximab resulted in a significant ($p<0.0001$) decrease of circulating lymphocytes. All 14 patients experienced >50% reduction of circulating tumor cells (median 67.8%). Complete kinetics for circu-



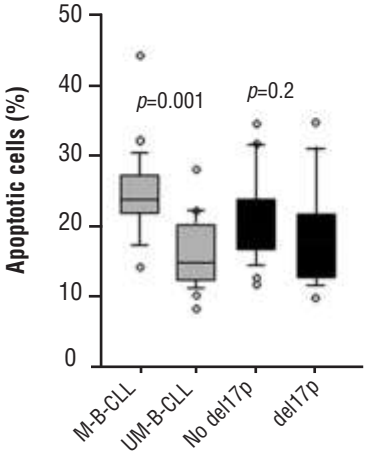


Figure 1. A. Kinetics of leukocyte numbers and apoptotic B-CLL cells in patients undergoing treatment with rituximab. Peripheral blood samples of patients with mutated (n=3) or unmutated IgVH genes (n=3) were collected before (0h) and at the indicated time points (1h, 4h and 24h) after infusion of rituximab in each cycle of the study. Leukocyte numbers (upper panel) were determined automatically with a Coulter counter at the central hematology laboratory of the University Hospital of Innsbruck. B-CLL cells undergoing apoptosis (lower panel) were determined by flow cytometry using the annexin V-FITC binding assay. **B.** Extent of apoptosis induction in B-CLL cells depends on the IgVH mutational status. Peripheral blood samples were collected before each cycle of rituximab infusion and the percentages of apoptotic B-CLL cells were determined using the annexin V-FITC binding assay. Throughout the study (four measurements per treatment cycle), a higher fraction of apoptotic cells could be detected in patients with mutated IgVH genes (n=5) than in patients with unmutated IgVH genes (n=5). In contrast, no difference in the percentage of apoptotic cells could be observed between samples from patients with (n=5) or without (n=5) p53 deletion.

lating tumor cells including apoptosis induction throughout all six cycles were available for six patients (Figure 1A). In parallel, the fraction of apoptotic cells was also increased significantly ($p<0.0001$). Of note, the maximum number of apoptotic cells detectable after rituximab infusion was significantly higher in patients with mutated IgVH genes ($p<0.0001$). The rate of spontaneously occurring apoptosis was equally distributed between both groups, which is in line with recent data from Messmer and co-workers.[10] For testing the hypothesis of an association between apoptosis induction and IgVH gene mutational status, complete data (i.e. quantification of apoptotic tumor cells immediately before and after each treatment cycle) were available for ten patients. When comparing the mean percentage of apoptotic B-CLL cells calculated from all individual apoptosis measurements throughout the study (the median number of measurements per patient was 23), we observed a significantly higher portion of apoptotic cells in the subgroup with IgVH mutations ($p=0.001$, Figure 1B). In contrast, sensitivity to apoptosis was independent of the presence of 17p deletions ($p=0.2$, Figure 1B). In summary, we were able to demonstrate that treatment with single-agent rituximab applied in a step-up dosing protocol is a safe approach even in heavily pretreated B-CLL patients with a high tumor burden. Despite only limited overall clinical efficacy, circulating tumor cells were decreased significantly, even in patients bearing 17p deletions. In particular, reduction of circulating tumor cells and apoptosis induction were significantly more pronounced in patients with mutated *IgVH* genes than in those with an unmutated *IgVH* locus. Thus, resistance to apoptosis, resulting from *IgVH* mutational status might contribute to different clinical outcomes.

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