doi:10.4149/neo_2011_02_140

Quantification of ZAP-70 expression in chronic lymphocytic leukemia: T/B-cell ratio of mean fluorescence intensity provides stronger prognostic value than percentage of positive cells

L. SMOLEJ[1], V. VROBLOVA[2], M. MOTYCKOVA[1], K. JANKOVICOVA[2], D.SCHMITZOVA[2], J. KREJSEK[2], J. MALY[1]

[1]Charles University in Prague, School of Medicine and University Hospital in Hradec Kralove Department of Medicine, Division of Hematology, e-mail: smolej@fnhk.cz; [2]Institute of Clinical Immunology and Allergology, Hradec Králové, Czech Republic

Received September 7, 2010

Expression of ZAP-70 measured by flow cytometry belongs to the most powerful prognostic parameters in chronic lymphocytic leukemia (CLL). However, many technical factors such as setting of the positivity threshold may significantly influence results.. Quantification using mean fluorescent intensity (MFI) may eliminate the subjective error which is inevitable in the isotype control method. The aim of the present project was therefore to assess the prognostic significance of ZAP-70 using three different methods. Between 2005 and 2010 we measured ZAP-70 expression in 157 patients with CLL (108 males, 49 females, median age 60 years [range, 31-82]; low/intermediate/high Rai risk in 41/48/11%). Expression of ZAP-70 was determined by flow cytometry using phycoerythrin (PE)-conjugated monoclonal antibody, clone 1E7.2.

Evaluation was performed by 1) percentage of positive cells compared to isotype control (cut-off 20%), 2) MFI ratio of T-cells/CLL cells (cut-off 3.0); 3) MFI ratio of ZAP-70/isotype control on CLL cells (cut-off 2.5). MFI method with T-cells/CLL cells ratio was the best in the identification of patients with unfavourable outcome: ZAP-70 positive patients had significantly shorter time to treatment (TTT, median 24 vs. 55 months, p=0.0001) and overall survival (OS, median 97 vs 174 months, p=0.0074). The differences in TTT a OS were not significant with the use of isotype percentage and MFI isotype methods. Combined analysis of ZAP-70 with CD38 expression or IgVH mutation status lead to identification of a subgroup with the longest TTT and OS (ZAP-70 and CD38 negative, p<0.0001 and p=0.012; ZAP-70 negative and mutated IgVH genes, p<0.0001 and p=0.0019).

In conclusion, our results suggest that measurement of ZAP-70 expression in CLL by MFI using T-cells/CLL cells ratio might be the optimal method for accurate prediction of clinical course. Combined analysis of ZAP-70 with CD38 or IgVH mutation status further refined individual patient´s prognosis.

Key words: chronic lymphocytic leukemia, ZAP-70 expression, mean fluorescence intensity, isotype control, prognosis

The hallmark of chronic lymphocytic leukemia (CLL) is extraordinary heterogeneity of the clinical course. While patients with aggressive disease need early treatment and have significantly short overall survival, many others have indolent disease without need of therapy and have similar survival to CLL-free age- and gender-matched counterparts [1]. Classical Rai and Binet staging systems are not able to identify many patients with dismal outcome because most patients are nowadays diagnosed in early stages [2]. Thus, modern prognostic parameters such us mutation status of immunoglobulin heavy chain genes (IgVH), genetic aberrations, Zeta-Associated Protein of 70 kilodaltons (ZAP-70) and others have been used in order to improve assessment of individual patient´s prognosis [1,3]. Expression of ZAP-70 is one of the most powerful prognostic parameters in CLL with negative prognostic impact on time to treatment as well as overall survival; the predominant method for quantification being flow cytometry [4-9]. However, there are many technical reasons which may significantly influence the results and lead to problems with the standardization of the method, e.g. clone of the monoclonal antibody, type of conjugate, and most importantly, setting of the threshold for positivity [10,11]. Usage of mean fluorescence intensity (MFI) for quantification of ZAP-70 may eliminate the operator error present when placing the cursor for cut-off in the traditional isotype control method [12-15]. Therefore, the aim of the present study was to determine the prognostic significance of ZAP-70 in CLL using the isotype method as well as MFI.

Patients and methods

Between May 2005 and June 2010, we measured ZAP-70 in 157 patients (pts) with CLL (108 males, 49 females, median age 60 years [range, 31-82]; low/intermediate/high Rai risk in 41/48/11%). Results of IgVH mutation status and fluorescence in situ hybridization (FISH) cytogenetics were available in 130 and 131 patients, respectively. The median follow-up of living patients was 48 months (mo)(range, 2-451). Treatment was initiated for progressive disease in 98 pts and 31 pts have died during the follow-up. One hundred and nine pts (69%) were previously untreated at the time of sampling; 48 (31%) have undergone treatment in the past. Descriptive statistics are summarized in Table 1. Diagnosis of CLL was based on standard National Cancer Institute-sponsored Working Group 1996 criteria. The study was approved by local ethics committee, performed according to the Helsinki declaration and patients provided signed written consent.

Analysis of ZAP-70. ZAP-70 expression was quantified by flow cytometry (Epics XL, Beckman Coulter) using fresh heparinized whole blood without cell selection. Volume of 25µl of peripheral blood (1x106 cells/ml) was incubated (15 minutes, 18-25°C) with 5µl membrane monoclonal antibodies (CD3-FITC, CD19-PE, Beckman Coulter, USA). Cells were subsequently fixed (15 minutes, 18-25°C) with reagent 1 (IntraPrep Permeabilization Reagent, Beckman Coulter, USA). After washing, permeabilization was performed (15 minutes, 18-25°C) using reagent 2 (IntraPrep Permeabilization Reagent, Beckman Coulter) and cells were incubated (15 minutes, 18-25°C in dark) with phycoerythrin-conjugated ZAP-70 (clone 1E7.2, Caltag Laboratories, USA) or isotype IgG1 (Caltag Laboratories, USA) monoclonal antibodies. Samples were analyzed directly after washing. Quantification of ZAP-70 expression was performed by three methods: 1. percentage of positive cells compared to isotype control (cut-off 20%), 2. MFI ratio of T-cells/CLL cells (cut-off 3.0); 3. MFI ratio of ZAP-70/isotype control on CLL cells (cut-off 2.5). Value of the percentage ZAP-70 / IgG1 positivity and MFI of CLL cells was determined from CD19-positive gate; minimum of 50,000 cells was collected. The T-cell MFI was determined from the gate of CD3-positive cells (minimum collection of 1000 cells). The T-cell/CLL cell MFI ratio of 3.0 and ZAP/IgG1 MFI ratio of 2.5 were used as the threshold for positivity. Results were analyzed using software Flow Jo (Tree Star, Inc; USA). All measurements were performed on fresh cells and within 6 hours from blood collection.

Table 1. Descriptive statistics of CLL patients.

	n	%
Total number of patients	157	
Males	108	69
Median age (range)	60 (31-82)	
Rai risk (n=156)		
Low	64	41
Intermediate	75	48
High	17	11
IgVH mutation status (n=130)		
unmutated	81	62
mutated	49	38
FISH (n=131)		
negative	32	24
del 13q	41	31
trisomy 12	18	14
del 11q	33	25
del 17p	7	5
CD38		
positive	43	27
negative	114	73

Statistical evaluation. Software Analyse-It (Analyse-It Software Ltd., UK) and MedCalc (Mariakerke, Belgium) were used for statistical analysis. Differences in time to treatment and overall survival were assessed using log-rank test and survival curves were constructed according to Kaplan-Meier method. P-values lower than 0.05 were considered significant. Optimal cut-off values for the MFI methods of ZAP-70 quantification were determined by receiver operator curve (ROC) analysis.

Results

MFI method with T-cells/CLL cells ratio was the best for identification of patients with unfavourable outcome: ZAP-70 positive patients had significantly shorter time to treatment (TTT, median 24 vs. 55 months, p=0.0001) (Fig. 1) and overall survival (OS, median 97 vs 174 months, p=0.0074)(Fig. 2). The differences in TTT a OS were not significant with the use of ZAP-70 percentage method (TTT, median 24 vs. 55 mo, p=0.0001, OS, median 97 vs 174 mo, p=0.0074)(Figs. 3-4) or MFI ZAP/IgG1 method (TTT, median 24 vs. 55 mo, p=0.0001, OS, median 97 vs 174 mo, p=0.0074)(Figs. 5-6, Table 2). There-

Table 2. Comparison of different methods for ZAP-70 assessment. Abbreviations: TTT, time to treatment; OS, overall survival; MFI, mean fluorescence intensity. Survival is given in months.

	Isotype percentage				MFI isotype				MFI T-cell/CLL cell		
	positive	negative	p		positive	negative	p		positive	negative	p
n	40%	60%		n	53%	47%		n	36%	64%	
TTT	31	44	0.123	TTT	32	39	0.543	TTT	24	55	0.0001
OS	128	174	0.241	OS	128	162	0.456	OS	97	174	0.0074

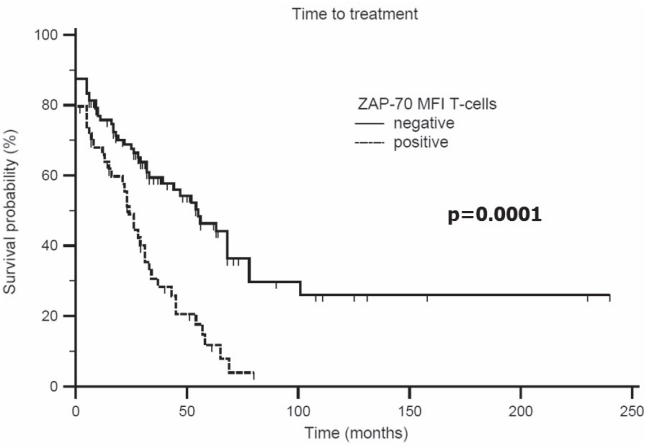

Figure 1. Time to treatment with MFI T-cell/CLL cell ratio method.

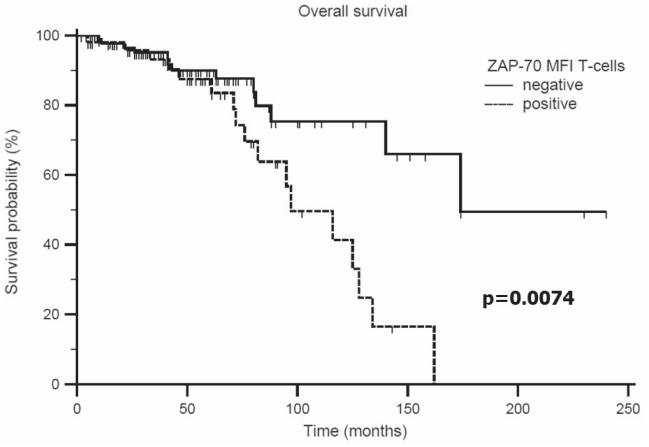

Figure 2. Overall survival with MFI T-cell/CLL cell ratio method.

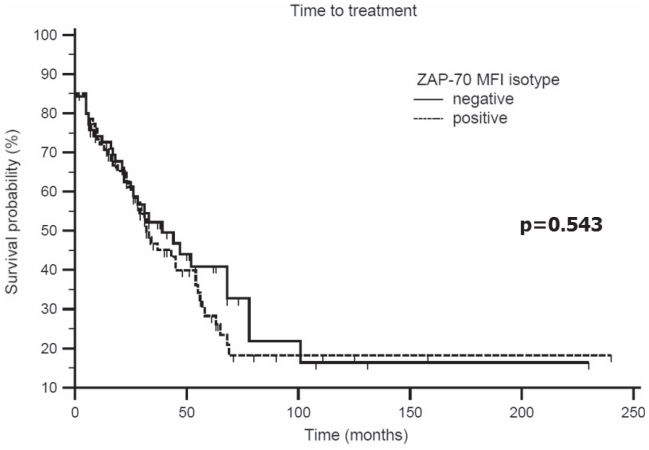

Figure 3. Time to treatment with MFI isotype method.

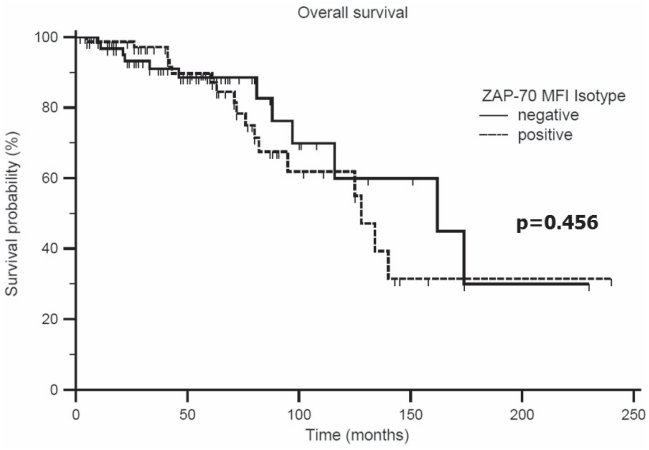

Figure 4. Overall survival with MFI isotype method.

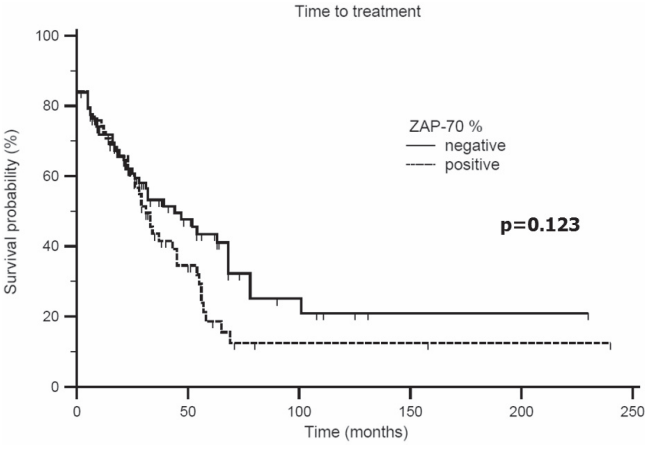

Figure 5. Time to treatment with isotype percentage method.

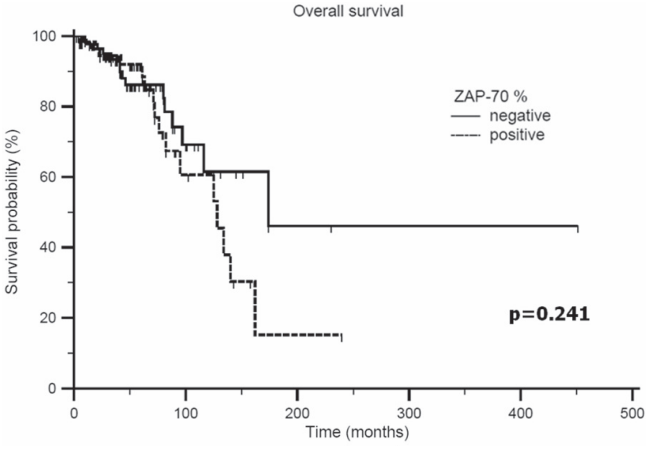

Figure 6. Overall survival with isotype percentage method.

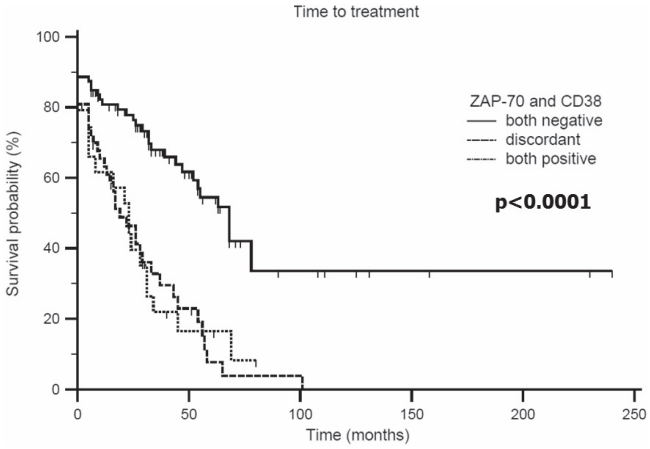

Figure 7. Time to treatment with combination of ZAP-70 and CD38

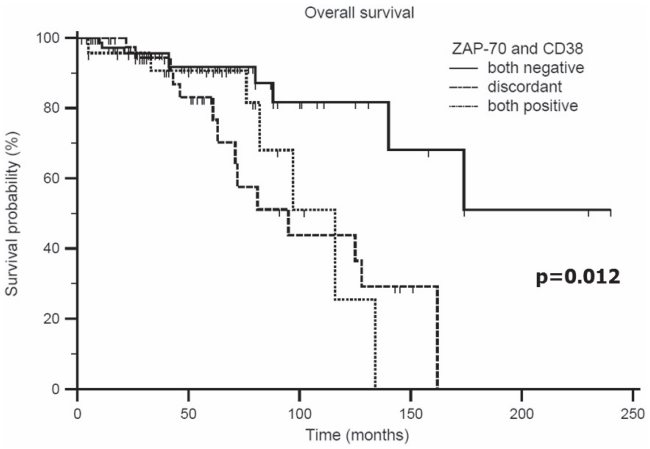

Figure 8. Overall survival with combination of ZAP-70 and CD38

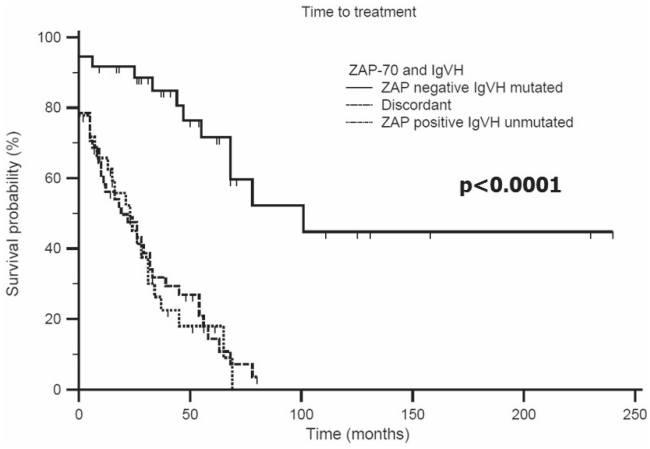

Figure 9. Time to treatment with combination of ZAP-70 and IgVH mutation status

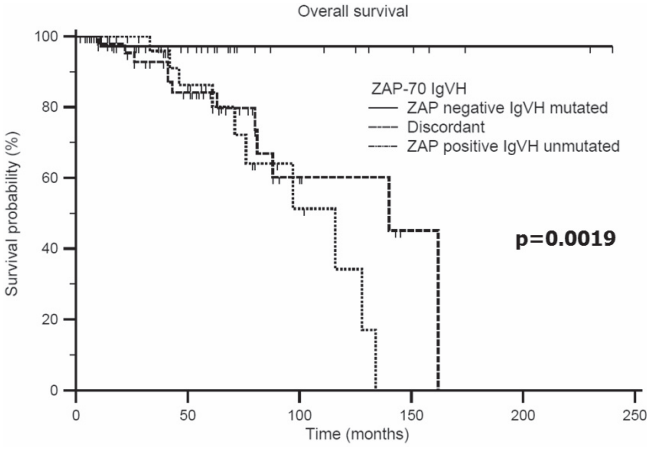

Figure 10. Overall survival with combination of ZAP-70 and IgVH mutation status

fore, we used the MFI T-cell/CLL cell ratio for further analyses. Combination of ZAP-70 with CD38 expression or IgVH mutation status lead to identification of a subgroup with excellent prognosis in term of longest TTT and OS (ZAP-70 and CD38 negative, $p<0.0001$ and $p=0.012$; ZAP-70 negative and mutated IgVH genes, $p<0.0001$ and $p=0.0019$).

Discussion

Assessment of ZAP-70 by flow cytometry is associated with several considerable technical issues which have so far precluded a worldwide standardization of this method [11]. They include: variability in the use of antibody clones and conjugates, different permeabilization methods and especially different approaches in setting the threshold of positivity. This is because ZAP-70 expression on CLL population displays continous spectrum rather than forming a separate population. Historically, the most common methods have been setting the threshhold using the isotype (negative) control [6,7] or T-cells/NK-cells (positive control) [4,5,9]. In the present study, we assessed ZAP-70 using three methods – the traditional isotype percentage method, MFI T-cell/CLL cell ratio and MFI ZAP/isotype ratio. By far best separation of ZAP-70 positive and negative cases on the basis of TTT and OS were obtained with the method using MFI ratio of T-cells/CLL cells. The use of other two methods did not result in significant differences in TTT or OS. Measurement of values of ZAP-70 change by mean fluorescence intensity (MFI) ratio has been recently reported as being more reliable, less subjective and therefore superior over assessment of percentage of ZAP-70 positive cells [11,13,14,18]. Measurement using MFI ration reduces the subjective error potentially present when assessing ZAP-70 expression by percentage of positive cells. In a large international standardization project, the best parameter for

reporting the results of ZAP-70 measurement was indeed MFI ratio of T-cells/CLL cells [11]. These results have been confirmed by Rossi et al. and Kern et al. [14,18]. Our results are in concordance with these studies including the prognostic influence on survival parameters. Other authors suggested the use the MFI ratio of CLL cells/normal B-cells [19,20] or molecules of equivalent soluble fluorochrome (MESF) [21]. The potential drawbacks of our study include presence of previously treated CLL patient in our cohort and also the fact that time between diagnosis and ZAP-70 measurement was longer in some patients. These two factors may have caused some bias because the values of ZAP-70 may change during the course of CLL with or without therapy [15,16]. From the technical point of view, the limit of MFI T-cell/CLL cell ratio could also lie in relatively low numbers of T-cells in advanced CLL. In addition, variability in the expression of ZAP-70 in T-cells of CLL patients has been reported [22]. Therefore, validation of the results on a larger cohort would be desirable. Importantly, we demonstrated that combined analysis of ZAP-70 and CD38 or ZAP-70 and IgVH mutation status is useful as it improves the prognostic stratification. Patients negative for ZAP-70 and CD38 or those who were ZAP-70 negative and had mutated IgVH had markedly better prognosis than the remaining subgroups. These results are in concordance with studies by Schroers, Hus and Del Principe [8,9,12]. From practical point of view, combined analysis of ZAP-70 and CD38 could be appropriate in elderly patients in whom costly test such as IgVH mutation status or FISH are questionable and often not performed for financial reasons.

In conclusion, our results suggest that use of ZAP-70 measurement by T-cell/CLL cell method is associated with improvement of CLL prognostication and prediction of clinical course. Furthemore, we believe that this newer approach might be more useful and less operator dependent than isotype percentage method which could also enable large interlaboratory comparisons of results. Combined analysis of ZAP-70 and CD38 or IgVH mutation status offers a possibility for refining the individual patient´s prognosis.

Acknowledgements. We thank Dr. Soňa Peková for analysis of IgVH mutation status and Dr. Lenka Kučerová for analysis of FISH. Supported by research project MZO 00179906 from Ministry of Health, Czech Republic.

References

[1] SHANAFELT TD. Predicting clinical outcome in CLL: how and why. Hematology Am Soc Hematol Educ Program. 2009: 421–9.

[2] PANOVSKÁ A, DOUBEK M, BRYCHTOVÁ Y, MAYER J. Chronic lymphocytic leukemia and focusing on epidemiology and management in everyday hematologic practice: recent data from the Czech Leukemia Study Group for Life (CELL). Clin Lymphoma Myeloma Leuk. 2010; 10: 297–300. doi:10.3816/CLML.2010.n.061

[3] ZENZ T, FRÖHLING S, MERTENS D, DÖHNER H, STILGENBAUER S. Moving from prognostic to predictive factors in chronic lymphocytic leukaemia (CLL). Best Pract Res Clin Haematol. 2010; 23: 71–84. doi:10.1016/j.beha.2009.12.003

[4] CRESPO M, BOSCH F, VILLAMOR N, BELLOSILLO B, COLOMER D et al. ZAP-70 expression as a surrogate for immunoglobulin-variable-region mutations in chronic lymphocytic leukemia. N Engl J Med 2003; 348: 1764–1775. doi:10.1056/NEJMoa023143

[5] DURIG J, NUCKEL H, CREMER M, FUHRER A, HALFMEYER K et al. ZAP-70 expression is a prognostic factor in chronic lymphocytic leukemia. Leukemia 2003; 17: 2426–2434. doi:10.1038/sj.leu.2403147

[6] ORCHARD JA, IBBOTSON RE, DAVIS Z, WIESTNER A, ROSENWALD A et al. ZAP-70 expression and prognosis in chronic lymphocytic leukaemia. Lancet 2004; 363: 105–111. doi:10.1016/S0140-6736(03)15260-9

[7] RASSENTI LZ, HUYNH L, TOY TL, CHEN L, KEATING MJ 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. doi:10.1056/NEJMoa040857

[8] SCHROERS R, GRIESINGER F, TRUMPER L, HAASE D, KULLE B et al. Combined analysis of ZAP-70 and CD38 expression as a predictor of disease progression in B-cell chronic lymphocytic leukemia. Leukemia 2005; 19: 750–758. doi:10.1038/sj.leu.2403707

[9] DEL PRINCIPE MI, DEL PG, BUCCISANO F, MAURILLO L, VENDITTI A et al. Clinical significance of ZAP-70 protein expression in B-cell chronic lymphocytic leukemia. Blood 2006; 108: 853–861. doi:10.1182/blood-2005-12-4986

[10] BOJARSKA-JUNAK A, GIANNOPOULOS K, KOWAL M, DMOSZYŃSKA A, ROLIŃSKI J. Comparison of methods for determining zeta-chain associated protein – 70 (ZAP-70) expression in patients with B-cell chronic lymphocytic leukemia (B-CLL). Cytometry B Clin Cytom. 2006; 70: 293–301. doi:10.1002/cyto.b.20133

[11] LETESTU R, RAWSTRON A, GHIA P, VILLAMOR N, BOECKX N et al. Evaluation of ZAP-70 expression by flow cytometry in chronic lymphocytic leukemia: A multicentric international harmonization process. Cytometry B Clin Cytom. 2006; 70: 309–14. doi:10.1002/cyto.b.20132

[12] HUS I, PODHORECKA M, BOJARSKA-JUNAK A, ROLINSKI J, SCHMITT M et al. The clinical significance of ZAP-70 and CD38 expression in B-cell chronic lymphocytic leukaemia. Ann Oncol 2006; 17: 683–90. doi:10.1093/annonc/mdj120

[13] LE GARFF-TAVERNIER M, TICCHIONI M, BRISSARD M, SALMON C, RAYNAUD S et al. National standardization of ZAP-70 determination by flow cytometry: the French experience. Cytometry B Clin Cytom. 2007; 72: 103–8. doi:10.1002/cyto.b.20350

[14] ROSSI FM, DEL PRINCIPE MI, ROSSI D, IRNO CONSALVO M, LUCIANO F et al. Prognostic impact of ZAP-70 expression in, Villamor N, Boeckx N chronic lymphocytic leukemia: mean fluorescence intensity T/B ratio versus percentage of positive cells. J Transl Med. 2010; 8: 23. doi:10.1186/1479-5876-8-23

[15] POULAIN S, BENARD C, DAUDIGNON A, LE BARON F, MOREL P et al. Is ZAP-70 expression stable over time in B chronic lymphocytic leukaemia? Leuk Lymphoma. 2007; 48: 1219–21. doi:10.1080/10428190701286488

[16] VROBLOVA V, VRBACKY F, HRUDKOVA M, JANKOVICOVA K, SCHMITZOVA D et al. Significant change in ZAP-70 expression during the course of chronic lymphocytic leukemia. Eur J Haematol. 2010; 84: 513–7. doi:10.1111/j.1600-0609.2010.01425.x

[17] HUS I, BOJARSKA-JUNAK A, DMOSZYNSKA A, WASIK-SZCZEPANEK E, SIEKLUCKA M et al. ZAP-70 and CD38 expression are independent prognostic factors in patients with B-cell chronic lymphocytic leukaemia and combined analysis improves their predictive value. Folia Histochem Cytobiol 2008; 46: 147–52. doi:10.2478/v10042-008-0022-7

[18] KERN W, DICKER F, SCHNITTGER S, HAFERLACH C, HAFERLACH T. Correlation of flow cytometrically determined expression of ZAP-70 using the SBZAP antibody with IgVH mutation status and cytogenetics in 1,229 patients with chronic lymphocytic leukemia. Cytometry B Clin Cytom. 2009; 76: 385–93. doi:10.1002/cyto.b.20483

[19] GACHARD N, SALVIAT A, BOUTET C, ARNOULET C, DURRIEU F et al. Multicenter study of ZAP-70 expression in patients with B-cell chronic lymphocytic leukemia using an optimized flow cytometry method. Haematologica. 2008; 93: 215–23. doi:10.3324/haematol.11622

[20] SHANKEY TV, FORMAN M, SCIBELLI P, COBB J, SMITH CM et al. An optimized whole blood method for flow cytometric measurement of ZAP-70 protein expression in chronic lymphocytic leukemia. Cytometry B Clin Cytom. 2006, 70: 259–269.

[21] KAY S, HERISHANU Y, PICK M, ROGOWSKI O, BARON S et al. Quantitative flow cytometry of ZAP-70 levels in chronic lymphocytic leukemia using molecules of equivalent soluble fluorochrome. Cytometry B Clin Cytom 2006; 70: 218–26. doi:10.1002/cyto.b.20078

[22] ZUCCHETTO A, BOMBEN R, BO MD, NANNI P, BULIAN P et al. ZAP-70 expression in B-cell chronic lymphocytic leukemia: evaluation by external (isotypic) or internal (T/NK cells) controls and correlation with IgV(H) mutations. Cytometry B Clin Cytom 2006; 70: 284–92. doi:10.1002/cyto.b.20127