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IDENTIFYING THE STAGE OF NEW CLL PATIENTS USING TK, ZAP-70, CD38 LEVELS

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Serum thymidine kinase (TK), zeta-associated protein of 70 kDa (ZAP-70) and CD38 levels have been shown to be correlated with survival in chronic lymphocytic leukaemia (CLL). ***Aim*****: To investigate the possible correlations between TK, ZAP-70 and CD38 levels as prognostic markers in new diagnosed Rai stages of CLL patients.** ***Methods*****: 120 CLL patients were enrolled. ELISA was used to measure serum TK level, flow cytomerty — to determine ZAP-70 and CD38 expression applying ZAP-70 Kit and monoclonal antibody to CD38, respectively.** ***Results:*** **Significantly higher levels of TK were found in the high progression group of CLL patients that corresponded to stage II (Rai classification). An elevated level of TK, CD38 and ZAP-70 together was also found in the II stage. The coefficient of correlation between CD38 and ZAP-70 is reliable ($p < 0.001$). There is also a correlation between the level of TK and the disease stage ($p < 0.05$). Other parameters do not show this correlation.** ***Conclusion*****: The determination of TK, ZAP-70 and CD38 together allows patients susceptible to a possible stage of the disease, to be identified. Estimation of the factors at an early stage of the disease may allow an earlier commencement of treatment.**
Key Words: **TK, ZAP-70, CD38, chronic lymphocytic leukaemia, flow cytometry, ELISA.**

Chronic lymphocytic leukaemia (CLL) follows a remarkably heterogeneous course. While some patients survive for sustained periods of time without ever requiring treatment, others succumb rapidly to the aggressive and drug resistant disease [1]. The Rai and Binet [2, 21] clinical staging systems are valuable in classifying CLL patients into broad prognostic subgroups. Clinical stages, however, have some limitations and this has led to a search for novel parameters with improved predictive power. The determination of thymidine kinase (TK), CD38 and zeta-associated protein of 70 kDa (ZAP-70) are increasingly utilized as prognostic factors for CLL [3–7]. ZAP-70 is a molecule normally expressed in T cells that helps them respond to antigen through their receptor. ZAP-70 protein was detected inside CLL cells by flow cytometry. The expression of ZAP-70 becomes discordant with increasing clinical stage [4–7, 10–12]. The CD38 expression is an independent prognostic marker [8, 9], also detected by flow cytometry. TK is a pyrimidine metabolic pathway enzyme and has a key role in the complementary alternative salvage pathway of deoxyribonucleic acid (DNA) synthesis. This enzyme is responsible for the catalytic conversion of deoxythymidine to deoxythymidine monophosphate [15]. To date, studies have shown that high TK levels are associated with an advanced clinical stage.

The aim of this study was to test the hypothesis that elevated serum TK, CD38 and ZAP-70 levels depend on the stage of new CLL patients.

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Abbreviations used: CLL – chronic lymphocytic leukaemia; TK – serum thymidine kinase; ZAP-70 – zeta-associated protein of 70 kDa.

MATERIALS AND METHODS

The study group consisted of 120 patients who were diagnosed with CLL for the first time. We have used the Rai classification for the estimation of clinical stages. The selection of patients was carried out at the National Centre of Haematology in the period from July, 2007 to July, 2008. In our clinic the level of TK, ZAP-70 and CD38 were determined for all new CLL patients. Five millilitres of clotted peripheral blood were collected for the study, and serum samples were aliquoted, following centrifugation of whole blood at 600g for 10 min at room temperature, and stored at — 70 °C until testing. The most appropriate thresholds to define ZAP-70 and CD38-positivity were controversial, the proposed cut-off levels being different [20, 22, 24]. Therefore, our study group was divided into three groups based on the degree of activity of the disease, i.e. low, middle and high degree of activity. The research design, patients' information and consent forms were approved by the Central Ethics commission.

Thymidine kinase assay. TK is a cellular enzyme involved in a salvage pathway for DNA synthesis. As long as the level of TK in serum or plasma is low, it is better to base the measurements on the enzymatic activity [15, 16]. The TK levels are not routinely determined since this requires a time consuming immune assay. The new ELISA technique, (Divi Tum kit, Biovica, Sweden), was used for TK level detection. As substrate for TK the DiviTum assay uses Bromo-deoxyuridine which is phosphorylated to its monophosphate. In order to immobilize and remove the monophosphate produced from the solution it is further phosphorylated to the tri-phosphate by kinases present in the reaction solution. The tri-phosphate is immobilized by DNA synthesis. After the TK activity incubation

is completed, the plate is washed and incubated with an anti-bromodeoxyuridine-antibody conjugated to alkaline phosphatase followed by a second wash. The alkaline phosphatase bound thus corresponds to the TK activity in the sample. The assay measures the activity of the enzyme, defined by the change of the absorbance. The minimal level for detection of TK (sensitivity) was 100 ng/L in this method. The permitted TK levels variations were in the interval 0 to 50 ng/L. We created a scale involving three ranges of TK as follows: TK 100–500 ng/L defines a low degree of activity of the disease. The middle degree of activity of the disease was defined when the level of TK was from 501 to 1000 ng/L. The high degree of activity of the disease was taken to be TK > 1000 ng/L.

ZAP-70 assay. One of the most differentially expressed genes is the gene encoding the zeta-associated protein of 70 kDa (ZAP-70), which is normally expressed in T cells and NK cells. In practice flow cytometry turned out to be the preferred technique for assaying ZAP-70 expression in CLL cells. Crespo *et al.* [5] were the first to describe such a flow cytometric method, and they confirmed the value of ZAP-70 as a surrogate marker for the IgVH mutation status. We determined the level of ZAP-70 using the PN 772587 kit from Beckman Coulter, Inc. We had defined three levels of change in the parameter: The ZAP-70 result corresponds to the norm in interval 0-10 %. This interval implies a low degree of activity of the disease. The result in interval 10–20% corresponds to a boundary region for ZAP-70, this region reflects a medium degree of disease. An increase to more than 20% for ZAP-70 corresponds to the high degree of disease [6, 7, 11]. Table 1 contains information about the distribution of the ZAP-70 levels and the degree of activity of the disease. ZAP-70 expression was determined by flow cytometry using heparinized samples of peripheral blood stored at room temperature for up to 24 h. After establishing the presence of a $CD19^+/CD5^+$ population and diagnosis of CLL, analysis of ZAP-70 expression was performed. The cells were incubated with CD19 (PC5; Beckman-Coulter, Miami, FL USA) and CD5 (FITC; Beckman-Coulter, Miami, FL USA) for 15 min. Intraprep reagent 1 (Beckman Coulter Miami, FL USA) was added and incubated for 15 min. After centrifugation and removal of the supernatant, Intraprep Reagent 2 (Beckman Coulter Miami, FL USA) was added. After gentle vortexing, antibody to ZAP-70 (PE, Beckman-Coulter, Miami, FL USA) was added and the sample was incubated for an additional 15 min. A parallel patient sample was processed identically with fluorochrome labelled isotype control antibodies and used to determine the background. After centrifugation, removal of the supernatant and re-suspension, the cells were analyzed by flow cytometry. The small mononuclear cells were gated using forward and right angle scatter. Within this population, the $CD19^+/CD5^+$ population was selected for further analysis. The percentage of cells in this population positive for ZAP-70 was determined [14].

Table 1. Level of ZAP-70 and degree of CLL activity

Level of ZAP-70 (%)	Degree of activity
0–10	low
10–20	middle
20 and more	high

CD38 assay. CD38 expression on leukemic lymphocytes was the first marker to be correlated with IgVH mutations [23]. Eventually, however, it was found that the relationship is not absolute and that, according to some studies, CD38 expression may vary over time [22]. Recently, it has been found that CD38 is expressed on CLL cells [24]. We defined three levels of the parameter: the results in the interval from 0 to 10 % equivalent norm for CD38 parameter or low degree of activity of the disease, the interval 10–20 % is a boundary region for CD38 and this region corresponds to the middle degree of disease. Increase of the norm to more than 20% for CD38 corresponds to the high degree of disease [3, 22]. Table 2 contains information on CD38 expression level and CLL activity. We used two reagents, CD23 FITC (Beckman-Coulter, Miami, FL USA) and CD38 PE (Beckman-Coulter, Miami, FL USA), incubation time 15 min. A parallel patient sample was processed identically with fluorochrome labelled isotype control antibodies and used to determine the background. After incubation the Immunoprep reagent system was added, and the test tubes were processed on a work station, the cells were analyzed by flow cytometry [18]. The CD38 was determined by the Crespo method. CD38 and ZAP-70 were determined in the Hematopathology laboratory in the Riga Centre of Haematology. Reagents and equipment EPICS XL from Beckman Coulter were used for this method.

Table 2. Level of CD38 expression and CLL activity

Level of CD38 (%)	Degree of activity
0–10	low
10–20	middle
20 and more	high

RESULTS

The 120 patients enrolled in this study (60 male, 60 female) had an age range of 34-86 years (median 67 years). The median age of males and females was 65.6 and 69.3 years, respectively. The Rai stages were as follows (Table 3). Seven patients from the cohort of the 120 patients were newly diagnosed with the Rai stage 0; 38 were Rai stage I; 40 were Rai stage II; 19 were Rai stage III; 16 were Rai stage IV. The largest subgroup studied therefore was the Rai stage II (see Table 3).The Fig. 1 shows the number of patients in all CLL stages.

Table 3. Characteristics of the 120 cases studied

Nr	Parameter	Number
1	Number of cases	120
2	Males/females	60/60
3	Age median/range (years)	67/34-86
4	Rai 0 stage	7
5	Rai I stage	38
6	Rai II stage	40
7	Rai III stage	19
8	Rai IV stage	16

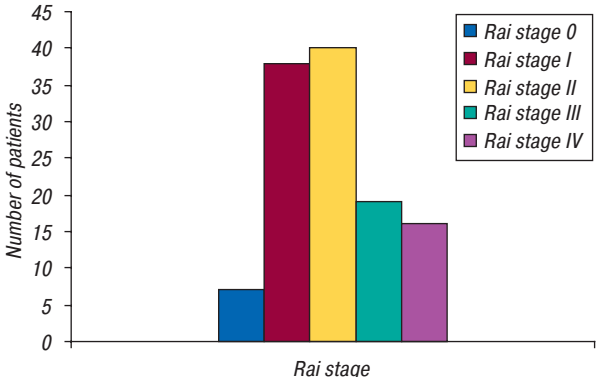

Fig. 1. Distribution of CLL patients by Rai stages

For the statistical analysis we divided all of the patients into three groups: a low risk group, containing the patients with the Rai stages 0 and I; a middle risk group with patients' Rai stage II; a high risk group with patients from Rai stages III and IV. Table 4 contains information about the numbers of patients and their median ages in each group. The information in Table 5 and Fig. 2 shows the number of patients in each stage within the corresponding TK parameters group. The interval from 0 to 50ng/L had only one patient, belonging to the low risk group. There were 30 patients in the interval from 100 to 500ng/L (Rai stages 0–I containing 17 patients; Rai stage II — 8; Rai stages III and IV — 5). There were 34 patients in the interval from 501 to 1000 ng/L (Rai stages 0-I containing 12 patients; Rai stage II — 10; Rai stages III and IV — 12). There were 55 patients in the interval > 1000ng/L (Rai stages 0-I contain 15 patients; Rai stage II — 22; Rai stages III and IV — 18). In the low risk group the highest number of patients was in the interval from 100 to 500 ng/L. In the middle risk group the highest count of patients were in the interval > 1000 ng/L. The same situation was seen in the high risk group. Table 6 and Fig. 3 contain information on the parameter ZAP-70. The low risk group (corresponding to Rai stages 0 and I) consisted of 45 patients. The group of 29 patients belonged to the interval from 0 to 10%. The 10 patients were in the interval from 10 to 20% in the same group. The interval with level of ZAP-70 > 20% in the low risk group contained 6 patients. There were 40 patients in the middle risk group (corresponding to Rai stage II). The interval from 0 to 10% consisted of 22 patients. There were 7 patients in the interval from 10 to 20 % in the same group. The group of 11 patients belonged to the interval with a level of ZAP-70 more than 20%. The 35 patients were in the high risk group (corresponding to Rai stages III and IV). The interval from 0 to 10% contained 20 patients. The 8 patients were in the interval from 10 until 20% in the same subgroup, and 7 patients — in the interval more than 20%. Table 7 and Fig. 4 contain information about the CD38 distribution between the cohorts of CLL patients divided in the groups. There were 29 patients in the interval from 0 to 10% in the low risk group (that corresponds to Rai stages 0—I). The interval from 10 to 20% contained 12 patients in the same group. The same low risk group with the level of CD38 more than 20% consists of 4 patients. The intermediate group (that corresponds to Rai stage II) contained 23 patients in the interval from 0 to 10%. Only 7 patients were in a group with the interval from 10 to 20%. The group from 10 patients were in the interval > 20%. In the high risk group (that corresponds to Rai stages III–IV) consisted from 25 patients. In the interval from 0 to 10% were 25 patients. Only 3 patients were in the interval from 10 to 20% within the same group. The high risk group with the interval more than 20% included 7 patients. However, each marker considered individually did not contain important prognostic information, the significant correlations between the stages and the level of TK ($p < 0.05$), and between the level of CD38 and ZAP-70 ($p < 0.001$) were observed (Table 8).

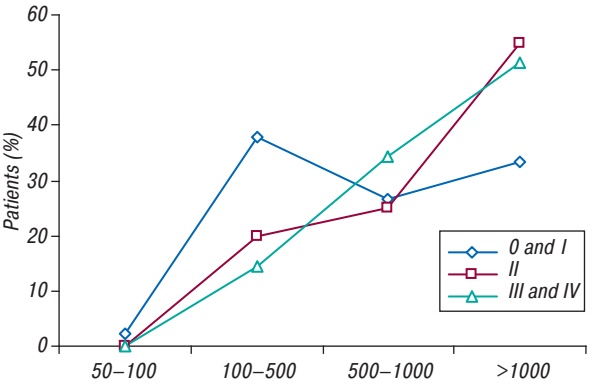

Fig. 2. The levels of TK in all CLL groups (ng/L)

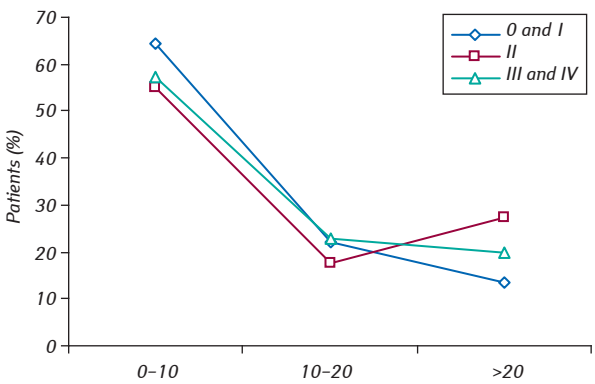

Fig. 3. The levels of ZAP-70 in all CLL groups (%)

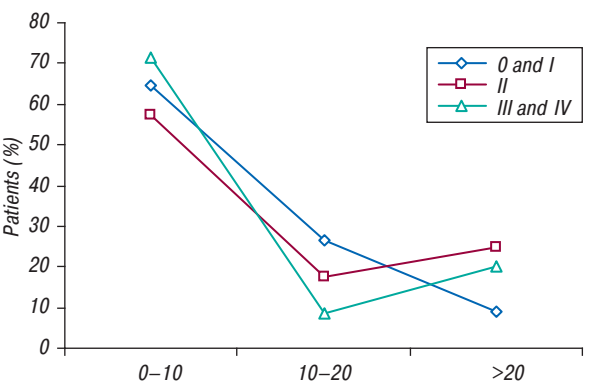

Fig. 4. The levels of CD38 in all CLL groups (%)

Table 4. Distribution of patients between the progression's groups and median ages for each group

Group of progression	stage	number of patients	mean age+(years)
low	0 and I	45	67.56
middle	II	40	67.95
high	III and IV	35	66.71

Group of progression	stage	number of patients	mean age+(years)
	total	120	67.44

Table 5. Number and percentage of patients with various levels of TK in all stages of CLL

			Stage			Total
			0 and I	II	III and IV	
TK (ng/L)	0–50	Number	1	0	0	1
		% within stage	2.2	0	0	8
	100–500	Number	17	8	5	30
		% within stage	37.8	20.0	14.3	25.0
	501-1000	Number	12	10	12	34
		% within stage	26.7	25.0	34.3	28.3
	>1000	Number	15	22	18	55
		% within stage	33.3	55.0	51.4	45.8
Total		Number	45	40	35	120
		% within stage	100.0	100.0	100.0	100.0

Table 6. Number and percentage of patients with the various levels of ZAP-70 in all stages of CLL

			Stage			Total
			0 and I	II	III and IV	
ZAP-70	0–10%	Number	29	22	20	71
		% within stage	64.4	55.0	57.1	59.2
	10–20%	Number	10	7	8	25
		% within stage	22.2	17.5	22.9	20.8
	>20%	Number	6	11	7	24
		% within stage	13.3	27.5	20.0	20.0
Total		Number	45	40	35	120
		% within stage	100.0	100.0	100.0	100.0

Table 7. Absolute number and percentage of patients with the different levels of CD38 in all stages of CLL

			Stage			Total
			0 and I	II	III and IV	
CD38	0–10%	Number	29	23	25	77
		% within stage	64.4	57.5	71.4	64.2
	10–20%	Number	12	7	3	22
		% within stage	26.7	17.5	8.6	18.3
	>20%	Number	4	10	7	21
		% within stage	8.9	25.0	20.0	17.5
Total		Number	45	40	35	120
		% within stage	100.0	100.0	100.0	100.0

Table 8. Correlation between all parameters

		Age	Gender	ZAP-70	CD38	TK	Stage
Age	p	1	0.1	0.5	-0.1	0.5	-0.5
	N	120	120	120	120	120	120
Gender	p	0.1	1	-0.5	-0.5	0.5	-0.1
	N	120	120	120	120	120	120
ZAP-70	p	0.5	-0.5	1	0.001	0.1	0.1
	N	120	120	120	120	120	120
CD38	p	-0.1	-0.5	0.001	1	0.5	0.5
	N	120	120	120	120	120	120
TK	p	0.5	0.5	0.1	0.5	1	0.05
	N	120	120	120	120	120	120
Stage	p	-0.5	-0.1	0.1	0.5	0.05	1
	N	120	120	120	120	120	120

Note: p — Pearson's coefficient; N — total number of patients.

DISCUSSION

Previous reports have recognized the role of serum TK in predicting progression free survival in CLL patients. In our report we try to show the relationship between the levels of TK and stage of disease for new cases of CLL [16, 17, 19]. The aim of this study includes investigation of associations between elevated TK levels and other markers — ZAP-70 and CD38 expression, and to ascertain if any parameters were associated with elevated TK. Table 8 contains information on correlation between all these parameters. We have based our interpretation on earlier published data [26, 27]. As reported previously, some difficulties arise on choosing a limit for CD38. In our work, we have selected the limits for CD38 and ZAP-70 in the intervals from 0 to 10%, from 10% to 20% and > 20%. The level of TK from 0 to 50ng/L was accepted as a norm (ELISA technique, Divi Tum kit, Biovica, Sweden). The positive results for TK all are > 100 ng/L. All cases with positive results are divided into three groups: from 100ng/L to 500ng/L, from 501ng/L to 1000 ng/L and > 1000 ng/L. All new patients are analyzed and divided into groups. As reported previously [23], these limits are chosen empirically to compare positive and negative cases. This empirical choice is confirmed by Hamblin *et al.* [8]. In the papers [14, 28] information is presented claiming that the elevation of ZAP-70 level reflects the progression of illness.

As reported by Knauf *et al*. [29] the increase of TK activity is conducive to progress of the disease in the low progression group of patients. Our results show that there is a correlation between the levels of TK and stages of the disease ($p < 0.05$). Another work [25] indicates that a high level of TK induces further progress of the disease in the advanced Rai stage. As our report contains only information on new patients, we can not offer a prognosis regarding the future progress of the disease. Similar findings are presented in some other papers [19, 20, 30]. However, in these papers the level of TK in the different groups of patients is not taken into account. In our case, three parameters are analyzed thus providing more information.

We find that the highest levels of all three markers are in the middle progression group of patients. This group of patients should be analyzed more carefully. We also have identified the correlation between CD38 and ZAP-70 ($p < 0.001$).

There are the same number of patients with the expression levels of CD38 and ZAP-70 > 20% in the high progression group. Also, there is a correlation between the stages and the TK level ($p < 0.05$), however, there is no correlation between the stages and ZAP-70 and CD38 parameters. When we examine all the parameters, the highest number of patients corresponds to the high progression group. The separation of patients into prognostically significant groups make possible to evaluate the results. In agreement with previous reports, the TK levels are found to increase advancement and thus do not lose a predictive potential. This is in contrast with the ZAP-70 expression which has recently been suggested to loose its discriminating ability in advanced clinical stages (Rai) [11, 13].

Our study demonstrates that the value of serum TK levels determination along with other markers allow to improve risk stratification of patients with CLL at diagnosis. However, each marker individually does not reveal the complete information. All markers need to be assessed in complex.

In conclusion, the estimation of TK, ZAP-70 and CD38 parameters together shows that there are correlations between the stages of CLL and the TK level ($p < 0.05$), and between the levels of CD38 and ZAP-70 ($p < 0.001$). The other parameters show no significant correlations. Separating the patients into three subgroups according to the stages facilitates the

statistical analysis. The analysis of TK, ZAP-70 and CD38 parameters at diagnosis could identify patients for future, more intensive study of these subgroups.

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