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Original Article / *Klinik Çalışma - Araştırma*

The Effect of ZAP-70 Expression on Disease Progression in Early-Stage (Binet A) B-CLL Patients

Erken Evre (Binet A) B-KLL Hastalarında ZAP-70 Ekspresyonunun Hastalık Progresyonuna Etkisi

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Objectives: Chronic lymphocytic leukemia (CLL) is a heterogeneous disease with various clinical findings. Prognostic markers predicting the clinical outcome in early-stage CLL would play a major role in disease management. Various studies have shown that zeta-associated protein 70 (ZAP-70) provides significant prognostic information. This retrospective, single-centre study evaluates the effect of ZAP-70 on disease progression in early-stage B-CLL.

Patients and Methods: Fifty early-stage patients with a median age of 66 years (range 47-86 years) were enrolled in this study. ZAP-70 expression was measured in peripheral blood samples using flow cytometry. Cut-off value for ZAP-70 positivity was set at 20%.

Results: Treatment had to be initiated in 21 of the 50 patients within a median of 42 months. The median time to initial treatment was 24 months. Twelve of the 50 patients (24%) were ZAP-70 positive. Ten of the 12 patients (83%) in the ZAP(+) group and 11 of the 38 patients (29%) in the ZAP(-) group required treatment on follow-up. The median treatment-free interval in ZAP(+) and ZAP(-) groups were 25 and 72 months, respectively (p=0.0001).

Conclusion: ZAP-70 provides significant prognostic information and predicts treatment requirement in early-stage B-CLL patients. Ongoing studies will highlight the need for a different management policy of ZAP-70(+) early-stage CLL.

Key words: Chronic lymphocytic leukemia; ZAP-70; prognosis.

Amaç: Kronik lenfositik lösemi (KLL) değişken klinik bulgular gösterebilen heterojen bir hastalıktır. Erken evre KLL'de klinik seyri önceden gösterebilecek prognostik belirteçler tedavi planlanmasında belirleyici rol oynayabilir. Çeşitli çalışmalarda zeta ilişkili protein 70'in (ZAP-70) önemli bir prognostik gösterge olabileceğine dair veriler elde edilmiştir. Tek merkezli ve retrospektif olarak planlanmış bu çalışmada erken evre B-KLL hastalarında ZAP-70'in hastalık progresyonu üzerine etkisi araştırılmıştır.

Hastalar ve Yöntemler: Ortanca yaşı 66 (dağılım 47-86) olan 50 erken evre B-KLL hastası çalışmaya dahil edildi. ZAP-70 ekspresyon düzeyleri çevresel kanda akım sitometri yöntemi ile çalışıldı. ZAP-70 pozitifliği için eşik değer %20 olarak belirlendi.

Bulgular: Ortanca 42 aylık izlemde çalışmaya alınan 50 hastadan 21'inde tedavi gerekliliği ortaya çıktı. Tanıdan tedaviye kadar geçen ortanca süre 24 aydı. Elli hastanın 12'sinde (%24) ZAP-70 pozitif bulundu. ZAP-pozitif gruptaki 12 hastanın 10'unda (%83) ve ZAP-negatif gruptaki 38 hastanın 11'inde (%29) izlem sırasında tedavi başlanması gerekti. ZAP pozitif ve negatif gruplarda tedaviye kadar geçen ortanca süre sırasıyla 25 ve 72 aydı (p=0.0001).

Sonuç: Erken evre B-KLL hastalarında tedavi gerekliliğini belirlemede ZAP-70 düzeylerinin önemli prognostik değeri olduğu görülmektedir. Tamamlayıcı çalışmalar ZAP-70 pozitif erken evre hastalarda farklı bir tedavi politikasının gerekli olup olmadığını ortaya koyacaktır.

Anahtar sözcükler: Kronik lenfositik lösemi; ZAP-70; prognoz.

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Chronic lymphocytic leukemia (CLL) is the most common type of leukemia in developed countries. It is a heterogeneous disease characterized by a variable clinical course.[1] Some patients have a more stable and indolent disease that never needs treatment, whereas in others the disease shows an aggressive behavior requiring early treatment.[2] The two clinical staging systems developed by Rai et al.[3] and Binet et al.[4] have been widely used to predict survival outcome and treatment requirement in CLL. However, patients are usually in early stage when diagnosed and these staging systems fail to differentiate a rapidly progressive course from an indolent one in early disease. There have been various efforts to establish novel molecular, genetic and biological prognostic markers for identifying CLL patients with poor prognosis.[5-13]

Recently, mutational status of the gene encoding the variable region of the immunoglobulin heavy chain (IgV_H) was reported to be the strongest prognostic marker predicting clinical outcome in CLL. While patients with unmutated IgV_H gene have a rapid disease progression requiring early treatment, those with mutated IgV_H genes do significantly better and have a long survival.[12-17] Moreover, the prognostic value of the mutational status of IgV_H genes is not affected by disease stage. IgV_H mutation analysis with DNA sequencing is, however, difficult and expensive to perform routinely.[11] Therefore, investigators have been in search of other markers that reflect the prognosis and are easy to analyze. Of these, CD38 expression and some chromosomal aberrations such as deletions of 17p and 11q have been proposed as significant independent prognostic markers.[9,10,12,15,18]

Recent studies have found that ZAP-70, an intracellular tyrosine kinase protein normally expressed in T and NK cells, was highly expressed in IgV_H unmutated CLL patients rather than in IgV_H mutated ones.[19] ZAP-70 was shown to be the best surrogate marker for the IgV_H mutational status,[11,17,20,21] and a good indicator of patients with aggressive clinical course.[8,20-22] Hence, ZAP- 70 has the potential to be a clinically useful molecular marker of prognosis in CLL. ZAP 70 protein expression can easily be measured by flow-cytometry.[8,21,22]

In this study, we aimed to determine the prognostic impact of flow-cytometrically determined ZAP-70 expression levels on disease progression in early stage CLL patients where Binet and Rai staging systems fail to predict the outcome.

PATIENTS AND METHODS

A total of fifty early-stage (Binet A) CLL patients (31 males, 19 females; median age 66 years; range 47 to 86 years) were enrolled in this study. The median follow-up time was 42 months. Twelve of the 50 patients (24%) were on treatment when they entered the study. Clinical diagnosis of CLL was based on standard morphological and immunophenotypical criteria. NCIWG criteria were used for assessing progressive disease and to initiate treatment.[23] The time from diagnosis of CLL to initial treatment was defined as the "treatment-free interval" (TFI). Detailed clinical information and treatment histories were available for all patients included in the study. Peripheral blood samples were obtained in EDTA containing tubes from all participating patients and were analyzed for the expression of ZAP-70 as well as other typical markers of CLL and immunophenotyped within four hours of collection, using flow-cytometry, as described below. For newly diagnosed patients ZAP-70 was measured as a part of the routine diagnostic flow cytometric analysis. For those with an established diagnosis of CLL, ZAP-70 was analyzed when the patients entered the study. Patients were accepted as ZAP-70 (+) when ZAP-70 expression level was above 20%.

Analysis of ZAP-70

In brief, venous blood samples were collected into EDTA-tubes and mononuclear cells were prepared by density gradient centrifugation using Histopaque. Samples were then treated with Fix&Perm (Caltag, Burlingame, CA, USA) according to the manufacturer's instructions. Surface labeling prior to intracellular staining was done with anti-CD3 phycoerythrin-cyanin 5.1(PC5), anti-CD56-(PC5), anti-CD5-fluorescein isothiocyanate and anti-CD19-phy-

Table 1. The effect of ZAP-70 expression on treatment requirement and TFI

	All patients	ZAP-70 negative	ZAP-70 positive	*p*
Treatment requirement	21/50 (42%)	11/38 (29%)	10/12 (83%)	p=0,002*
Median treatment-free	52	72	25	0,00001**
interval (months)	SE=11.03	SE=9.79	SE=2.14	
	CI=30.39-73.61	CI=52.81-91.19	CI=20.81-29.19	

SE: Standard error; CI: Confidence interval. *chi-square test; **log-rank test.

coerythrin Texas red antibodies, as described elsewhere.[21] The antibodies were all purchased from Beckman Coulter (Immunotech, Marseille, France). For intracellular staining of ZAP-70, a phycoerythrin conjugated antibody, purchased from Caltag (Burlingame, CA, USA) was used. For each sample a negative control tube containing the relevant isotypic antibodies was studied and the working voltages were set accordingly. The minimum level of ZAP-70 positivity of the CD3+CD56+ population was determined through quadrant analysis and used as the threshold for measuring ZAP-70 in the CD19+CD5+cell gate. The gating strategies followed that of Crespo et al.;[21] first lymphocytes were gated to exclude debris monocytes and doublets, then CD3 and CD56 positive cells and CD5+19 positive cells were gated on. Analyses were performed on an EPICS XL cytometer with System II software. At least 5-10,000 events were counted for each sample.

Statistical Analysis

All statistical analysis was done using SPSS v.11 for Windows. The TFI was estimated by the Kaplan-Meier method and groups were compared utilizing the log-rank test. Data from patients who had not yet received treatment were regarded as censored. Chi-square test was performed for group-wise comparison of categorical variables. Results were considered statistically significant at $p<0.05$.

RESULTS

Of the 50 early stage B-CLL patients 12 (24%) were ZAP-70 positive and 38 (76%) were ZAP-70 negative. Median ZAP-70 expression levels were 8.3% (0.28-19.70%) and 53.1% (31.2-78.4%) in the ZAP-70 negative and positive groups, respectively. Treatment requirement in the ZAP-70 positive group was as high as 83%, whereas it was only 29% in the ZAP-70 negative group. A markedly shorter median TFI of 25 months was observed in the ZAP-70 positive group, in contrast to 72 months in the ZAP-70 negative group (p=0.00001). There was a highly significant difference between ZAP-70 positive and negative groups as far as treatment requirement and TFI were concerned. See Table 1 and Figure 1.

DISCUSSION

In CLL, up to two thirds of patients are in early stage at diagnosis and do not need treatment initially. Current management policy for those patients is "wait and watch". However, CLL is a heterogeneous disease with variable clinical course. This holds true for patients in the early stage; some of them rarely require any treatment during the entire disease course while others rapidly progress to advanced forms.[24] For years, researchers have been in search of some reliable and practical prognostic parameters to

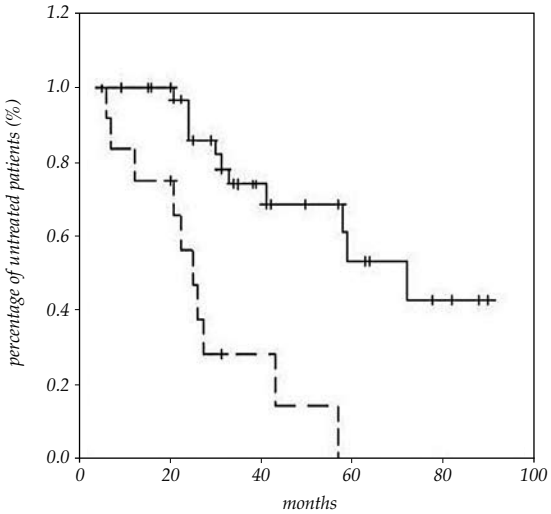

Fig. 1. *ZAP-70 expression and time to treatment-free interval.*

identify high risk patients within the early stage group.

Initially, CD38 expression as determined by flow cytometry was thought to serve as a surrogate marker for IgV_H mutational status but subsequent studies displayed conflicting results.[9,10,12,15] At present, CD38 positivity is accepted as an independent prognostic factor in CLL.[6,7] However, CD 38 expression levels are not always stable; they may alter during the course of the disease.[18]

Chromosomal aberrations have been detected up to a rate of 60-80% in CLL. Among them, deletions of 17p and 11q have been associated with unfavorable prognosis. The deletion of the p53 locus (17pl3) was described as one of the strongest independent predictors for aggressive behavior, resistance to chemotherapy and early death.

At present, IgV_H gene mutational status seems to be the most powerful predictor of clinical outcome in B-CLL.[12-14,16] CD38 has been suggested as a surrogate marker for IgV_H mutational status, but the results of CD38-related studies are conflicting.[10,15] A good correlation between IgV_H mutational status and ZAP-70 expression has recently been demonstrated by different investigators.[11,20,21] ZAP-70 expression is stable over time (during disease) and can be relatively easily measured in diagnostic laboratories where flow cytometry is available.[11,17,21] Furthermore, ZAP-70 expression was shown to be an independent prognostic predictor, irrespective of IgV_H mutational status.[8,17,20,25] In most of the trials cited above the study population was not homogeneous in terms of disease stage, i.e. they included patients at different stages of CLL. Some of the earlier studies did not even include a sub-analysis for early-stage patients. In contrast to this, we focused mainly on the clinical significance ZAP-70 expression in early-stage (Binet A) patients with regard to treatment-free interval and proportion of treatment requirement.

The results of our study showed that increased levels of ZAP-70 expression in leukemic cells was strongly associated with a higher percentage of treatment requirement and a shorter treatment-free interval in early-stage B-CLL patients. Patients with ZAP-70 (+) CLL were characterized by a more aggressive clinical course than those with ZAP-70 (-) CLL. Treatment had to be initiated in 83% of our ZAP-70 (+) patients within a median follow-up period of 42 months. On the contrary, only 29% of ZAP-70 (-) patients required treatment during follow-up. TFI in the ZAP-70 (+) and ZAP-70 (-) groups was 22 and 72 months, respectively (p=0.00001). The results of our study were similar to those of Crespo et al.[21] They reported in their study a subset of 44 early-stage CLL patients of whom 29 were ZAP-70 (+) and 18 were ZAP-70 (-). The median time to progression was 29 months in the ZAP-70 (+) patients, whereas it had not been reached in the ZAP-70 (-) group within a follow-up of 63 months. Rassenti et al.[17] reported 2.9 and 9.2 years of median time from diagnosis to initial treatment in the ZAP-70 (+) and ZAP-70 (-) patients, respectively. Disease stage was not specified in this study. Del Giudice et al.,[26] on the contrary, were not able to show any TFI difference between ZAP-70 (+) and ZAP-70 (-) groups in 92 stage A, CLL patients.

Although there is not a separate subgroup analysis for early-stage CLL patients, the study done by Dürig et al.[8] was one of the pioneering works demonstrating the prognostic function of ZAP-70 expression in CLL. They reported a heterogeneous study population with TFIs of 38 and 120 months for ZAP-70 (+) and (-) patients, respectively. The proportion of patients requiring treatment during follow-up was 59% in the ZAP-70 (+) group and 15% in the ZAP-70 (-) group.

In conclusion, our retrospective data of early stage CLL patients indicate that increased ZAP-70 expression is an adverse prognostic parameter strongly predicting a shorter treatment-free interval. Thus, ZAP-70 expression detected by flow cytometry could identify patient subsets that have distinct treatment requirements. But before integrating ZAP-70 into treatment algorithms of CLL, controlled, large-scale, prospective studies should be done to establish the true value of this new marker. Another important

issue is associated with the measurement technique of ZAP-70. The flow cytometry protocol of ZAP-70 must be reviewed and standardized prior to running larger trials.

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