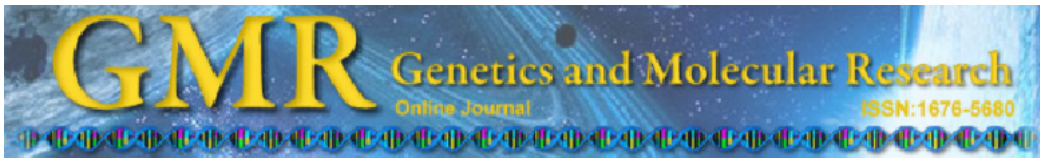


# Analysis of CD38 and ZAP70 mRNA expression among cytogenetic subgroups of Iranian chronic-lymphocytic-leukemia patients

**H. Teimori[1], M.T. Akbari[2,3], M. Hamid[4], M. Forouzandeh[5] and E. Bibordi[6]**

[1]Cellular and Molecular Research Center, School of Medicine, Shahrekord University of Medical Sciences, Shahrekord, Iran
[2]Department of Medical Genetics, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran
[3]Tehran Medical Genetics Laboratory, Tehran, Iran
[4]Molecular Medicine Division, Biotechnology Research Center, Pasteur Institute of Iran, Tehran, Iran
[5]Department of Medical Biotechnology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran
[6]Shariati Hospital, Tehran University of Medical Sciences, Tehran, Iran

Corresponding author: M.T. Akbari
E-mail: mtakbari@modares.ac.ir

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**ABSTRACT.** Chromosomal abnormalities and ZAP70 expression profile are two major independent prognostic markers in B-cell chronic lymphocytic leukemia. We investigated a possible correlation between these two markers. ZAP70 expression using real-time RT-PCR was examined in 20 B-cell chronic lymphocytic leukemia patients with del13q14, 13 patients with del11q22, 15 patients with trisomy 12, and 16 patients with no detected chromosomal abnormalities. Molecular analysis revealed that ZAP70 expression in the del13q subgroup was the same as in the control group, while it increased 2.78-fold in the del11q subgroup and 2.95-fold in the trisomy 12 subgroup, compared to the 15 cases in the control group. Comparison of the mean and standard

 

deviation of the ZAP70 expression profile within the subgroups showed it to be highly variable among the individuals of the del11q and trisomy 12 subgroups, versus tight clustering for the del13q subgroup. Therefore, there is a correlation between del13q aberration, which has good prognosis with normal levels of ZAP70 expression. Due to a high degree of variation, no conformity is seen for del11q and trisomy 12 subgroups, making this grouping poor for prognostic discrimination. As a result, neither of these markers can serve as sole discriminators to determine the course of the disease; the use of both markers improves prognostic assessment.

**Key words:** Chronic lymphocytic leukemia; ZAP70; CD38; Cytogenetic abnormalities

## INTRODUCTION

B-cell chronic lymphocytic leukemia (B-CLL), a hematopoietic neoplasm of B-lymphocytes, is the most common form of leukemia in adult life. One of the most important research issues in B-CLL is to identify prognostic factors that can predict the clinical course of the disease. Recently, several prognostic factors have emerged. Mutational status of the immunoglobulin heavy chain variable region (IgVH) is one of the most reliable prognostic markers in B-CLL. Unmutated IgVH regions are associated with poor prognosis, whereas mutated IgVH regions represent a good prognosis (Hamblin et al., 1999). However, mutational analysis of IgVH is both laborious and expensive, and it is inaccessible for many clinical genetic laboratories. Therefore, the identification of a surrogate marker for mutational status of IgVH has become an important goal.

Because measuring CD markers is rapid and convenient by flow cytometry, they became the first biological markers to be extensively studied as a surrogate marker. CD38 and CD23 were two markers that were correlated with IgVH mutational status, but their prognostic value has been the subject of many controversies (Damle et al., 1999; Hamblin et al., 2002; Krober et al., 2002; Schroers et al., 2005) and they required further investigation. Gene expression profiling with cDNA microarray revealed that several expressed genes can correlate with IgVH mutational status in B-CLL patients. ZAP70 (70-kDa zeta-chain associated protein kinase), a member of the Syk protein tyrosine kinase family, is one of the most promising surrogate markers in this regard (Crespo et al., 2003; Ferrer et al., 2004). High expression of ZAP70 was correlated with unmutated IgVH subtype, and low expression of ZAP70 was observed in mutated IgVH subtype (Orchard et al., 2004; Catherwood et al., 2006).

However, the biological role of ZAP70 in B-CLL is still unknown, and it does not completely correlate with IgVH mutational status. Therefore, it cannot be taken as the sole marker with hundred percent accuracy.

Additionally, chromosomal abnormalities have a prognostic role in B-CLL. The most common chromosomal abnormalities in B- also CLL are deletions at 13q14 (about 50%), 11q22 (7-20%) and 17p13 (about 10%) and trisomy 12 (15-25%). Deletion of 13q14 is associated with good prognosis, whereas deletion in 11q22 and 17q13 are said to have poor prognosis. Trisomy 12 is thought to confer an intermediate prognosis (Moreno and Montserrat 2008).

 

In the present study, CD markers and ZAP70 mRNA expression level were determined in the del13q14, del11q22 and trisomy 12 subgroups of Iranian B-CLL patients to investigate prognostic value of CD38 and ZAP70 and the possible relationship between ZAP70 expression and CD38 and cytogenetic subgroups.

## MATERIAL AND METHODS

### Patients

The clinical diagnosis of B-CLL was made based on criteria defined by the National Cancer Institute Working Group (NCI-WG) (Cheson et al., 1988). The Ethics Committee of Tarbiat Modartes University approved the study, and samples were taken following informed consent. Samples from 64 patients who had been newly diagnosed with B-CLL or at least were taken off treatment for the past six months were included. Patients were staged at the time of diagnosis according to the Rai staging. Six patients were diagnosed at stage 0; 9 at stage I; 30 at stage II; 14 at stage III, and 5 at stage IV. Forty-six were males and 18 cases were females. Patients were 42 to 81 years old. The median suffering time was 51.6 ± 1.3 months, ranging from 13 to 300 months. Mean and range of some hematological characteristics within the subgroups are summarized in Table 1.

**Table 1.** Some hematological characteristics of B-CLL patients.

| Subgroup | Platelet ($10^6$/mL) | | % Lymphocytes | | WBC ($10^9$/L) | | RBC ($10^9$/mL) | |
|---|---|---|---|---|---|---|---|---|
| | Mean | Range | Mean | Range | Mean | Range | Mean | Range |
| Del13q | 177 ± 10 | 89-271 | 68 ± 3.0 | 32-90 | 24 ± 2.3 | 11-47 | 4.4 ± 0.1 | 2.7-5.4 |
| Del11q | 158 ± 13 | 95-247 | 73 ± 4.8 | 38-93 | 38 ± 4.7 | 13-80 | 4.4 ± 0.2 | 3.0-5.7 |
| Trisomy 12 | 160 ± 22 | 49-401 | 70 ± 3.5 | 38-89 | 45 ± 7.5 | 9-115 | 4.4 ± 1.9 | 3.2-5.7 |
| Undetected | 160 ± 22 | 61-415 | 79 ± 2.1 | 65-95 | 39 ± 7.2 | 19-121 | 4.3 ± 0.2 | 1.2-5.7 |
| Total | 171 ± 8 | 49-415 | 73 ± 1.7 | 32-95 | 41 ± 5.9 | 9-121 | 4.3 ± 0.9 | 1.2-5.7 |

WBC = whole blood cell; RBC = red blood cell.

After clinically diagnosed with B-CLL, patients were referred for routine cytogenetic evaluation. Following conventional chromosomal analysis and I-FISH study of patients' bone marrow and/or peripheral blood with RB1, ATM and chromosome 12 centromeric probes, four groups emerged: 20 cases with del13q, 13 cases with del11q, 15 cases with trisomy 12, and 16 patients with undetected chromosomal abnormality. These four groups were investigated for ZAP70 expression status. Fifteen normal individuals (age- and gender-matched) were similarly analyzed as the control group. Peripheral blood was collected in EDTA tubes from the patients and individuals of the control group.

### Immunophenotyping

Peripheral blood mononuclear cells were separated from heparinized venous blood by density gradient centrifugation using Ficoll-Paque. The cells were washed twice with phosphate-buffered saline (PBS) and were incubated with FITC-conjugated anti-mouse antibody (IQ-Products, The Netherlands) for: CD2, CD5, CD10, CD11c, CD19, CD20, CD22, CD23,

CD25, CD38, FMC7, and HLA-DR. The stained cells were sorted using the FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) and the data were analyzed using the CellQuest software.

## RNA extraction and qualification

Two milliliters EDTA-preserved fresh whole blood was diluted with 2 mL sterile PBS and carefully transferred to 4 mL Ficoll-Paque (Biomol, The Netherlands). The new sample was centrifuged for 15 min at 2000 rpm. The buffy coat was isolated in a 1.5-mL Eppendorff microtube. After normalizing the specimens, total RNA was extracted from the buffy coat using the High Pure RNA Isolation kit (Roche, Germany) according to manufacturer instructions. The quality and concentration of the extracted RNA samples were spectrophotometrically determined by measuring optical density at 260 and 280 nm (Genova Life Science Analyser-DNA, Jenway, England).

## cDNA synthesis

First-strand cDNA was generated with the use of random hexamer primers and employing a commercially available kit (Revert Aid First-Strand cDNA Synthesis kit, Fermentas, Life sciences, Germany) following manufacturer instructions. For determining the accuracy of the cDNA synthesis reaction, cDNA was amplified by PCR with ZAP70 primers. The PCR products were then analyzed by electrophoresis on a 2% garose gel.

## Gene expression analysis

A quantitative real-time RT-PCR method was established to determine ZAP70 expression. The amplification was performed with a real-time rotary analyzer (Rotor-Gene 6000, Corbett, Australia), and data were automatically analyzed by the Rotor-Gene software according to the comparative ΔΔCt method at the end of reactions. cDNA sample from a 32-year-old healthy man was used as a calibrator. Housekeeping gene β-glucoronidase (GUSB) was used as the reference gene for normalization. One standard curve for ZAP70 and one for GUSB were obtained using 10-fold serial dilutions of the calibrator's cDNA. Primers of ZAP70 and GUSB are shown in Table 2.

**Table 2.** Characteristics of primers used for RQ-PCR in this study.

| Gene | Accession No. | Amplification length | Forward primer | Reverse primer | Ref. |
|---|---|---|---|---|---|
| ZAP70 | NM-001079 | 187 bp | 5'GTTGACTCATCCTCAGAGACGAATC | 5'AGGTTATCGCGCTTCAGGAA | (Kienle et al., 2005) |
| GUSB | NM-000181 | 100 bp | 5'GAAAATACGTGGTTGGAGAGCTCATT | 5'CCGAGTGAAGATCCCCTTTTTA | (Aerts et al., 2004) |

Real-time RT-PCR amplification was performed in a final reaction volume of 20 μL containing 2 μL target cDNA, 0.7 μL forward primer, 0.7 μL reverse primer, 4 μL master mix (LightCycler Fast Start DNA Master Sybr Green I, Roche, Germany) and 12.6 μL PCR-grade water. The thermal cycling conditions for both genes were 10 min at 95°C followed by 40 cycles of denaturation at 94°C for 12 s, annealing at 60°C for 15 s and extension at 72°C for 12 s. All reactions were in duplicate.

 

## Statistical analysis

Raw data were analyzed by means of the SPSS program version 15 software. One-way ANOVA was used for determination of statistical significance of ZAP70 expression in cytogenetic subgroups versus the control group. When significant difference was established, three *post hoc* comparison tests (Tukey, Duncan and Scheffe) were utilized to categorize differences. Statistical relationship between ZAP70 expression and Rai stages and CD38 was evaluated by a two-tailed Pearson correlation test. Differences with P less than 0.05 were considered to be statistically significant.

## RESULTS

Flow cytometry for the mentioned markers was successfully performed on all patients' blood samples. Mean and range of five distinctive B lymphocyte CD markers, i.e., CD5, CD19, CD20, CD23, and CD38, were collected from the patients' profiles and are summarized in Table 3.

**Table 3.** Mean and range of CD5, CD19, CD20, CD23, and CD38 within cytogenetic subgroups.

| Subgroup | CD5 | | CD19 | | CD20 | | CD23 | | CD38 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | Range | Mean | Range | Mean | Range | Mean | Range | Mean | Range |
| Del13q | 53 ± 4.8 | 21-99 | 70 ± 4.2 | 38-97 | 54 ± 5.4 | 17-88 | 55 ± 4.0 | 6-85 | 24 ± 3.9 | 1-60 |
| Del11q | 43 ± 5.6 | 4-77 | 62 ± 6.9 | 16-92 | 61 ± 5.1 | 32-85 | 58 ± 6.2 | 1-82 | 41 ± 6.2 | 3-73 |
| Trisomy 12 | 49 ± 7.8 | 2-94 | 73 ± 5.1 | 38-96 | 52 ± 7.3 | 11-88 | 55 ± 3.3 | 36-70 | 26 ± 4.7 | 2-59 |
| Undetected | 42 ± 6.8 | 4-93 | 65 ± 5.7 | 10-93 | 45 ± 7.4 | 2-95 | 45 ± 6.2 | 4-81 | 35 ± 6.3 | 1-84 |
| Total | 45 ± 3.2 | 2-99 | 68 ± 2.6 | 10-97 | 52 ± 3.2 | 2-95 | 52 ± 2.6 | 1-85 | 33 ± 2.7 | 1-84 |

ZAP70 expression from 20 patients with del13q14, 13 patients with del 11q22, 15 patients with trisomy 12, 16 undetected chromosomal abnormalities, and 15 control cases were performed by real-time PCR using cDNA samples. Melting curve analysis showed that all amplification products were of the expected size, without any genomic DNA contamination.

The quality of amplification performance was evaluated from the standard curves generated from 10-fold serial dilutions of the calibrator's cDNA. The efficiency value of the reactions ranged from 0.94 to 1.01 for ZAP70 and from 0.98 to 1.05 for GUSB. The correlation coefficient of each amplification was very close to 1. The expression ratio between ZAP70 and GUSB was automatically produced at the end of each reaction for each sample by the RotoGene software. Pearson's correlation test showed weak correlation between CD38 and Rai stages (coefficient of 0.3 at 0.04 level), whereas highly significant correlation between ZAP70 expression and Rai stages (coefficient of 0.418 at 0.001 level).

Pearson's correlation test between ZAP70 mRNA and CD38 markers revealed a weak correlation between ZAP70 mRNA and CD38 expression (0.236 at a 0.007 significance).

Statistical analysis of the expression ratio measured in cytogenetic subgroups produced the following results. Mean and standard deviation of the expression ratio in the control group was 1.007 and 0.320, respectively. These two parameters in the del13q subgroup were 0.8857 and 0.304, in the del11q subgroup 2.7838 and 1.23, in the trisomy 12 subgroup

2.9547 and 1.60, and in the undetected group 1.0013 and 0.339. The distribution of ZAP70 expression ratio to GUSB in cytogenetic subgroups and the control group is shown in Figure 1.

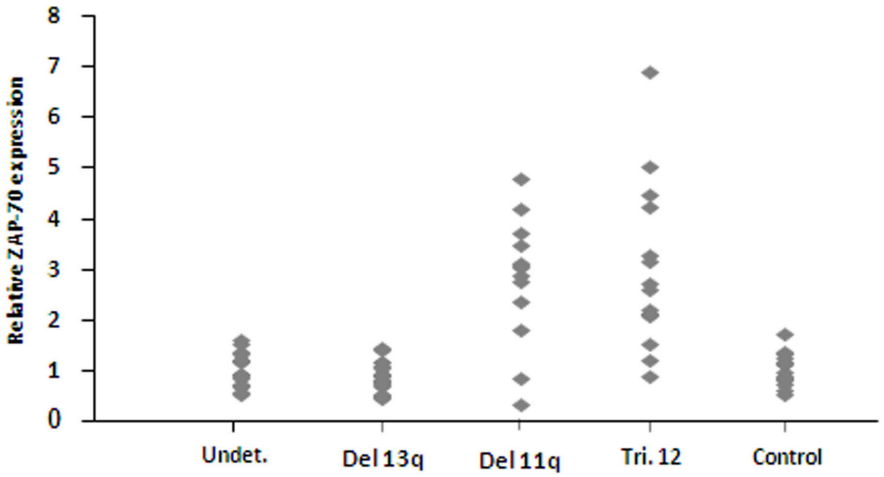


**Figure 1.** The histogram of ZAP70 expression within different study groups. Undet. = undetected group; Tri. 12 = trisomy 12.

ANOVA indicated that there was a significant difference in the distribution of ZAP70 expression ratio between the cytogenetic subgroups and the control group. All three *post hoc* tests revealed similar results. They classified 3 cytogenetic subgroups and undetected and control groups (5 study groups in total) as two statistically significant subsets. Del113q subgroup, undetected and control groups were in one subset, while trisomy 12 and del11q subgroups were grouped in another subset. Results of these three statistical analyses are summarized in Table 4.

**Table 4.** Statistical analysis of ZAP70 expression using three *post hoc* tests.

| Test | Group | Number | Subset for alpha = 0.05 | |
|---|---|---|---|---|
| | | | 1 | 2 |
| Tukey HSD[a,b] | Del13q | 20 | 0.8857 | - |
| | Undetected | 16 | 1.0013 | - |
| | Control | 15 | 1.0073 | - |
| | Del11q | 13 | - | 2.7838 |
| | Trisomy 12 | 15 | - | 2.9547 |
| Significance | | | 0.995 | 0.983 |
| Duncan[a,b] | Del13q | 20 | 0.8857 | - |
| | Undetected | 16 | 1.0013 | - |
| | Control | 15 | 1.0073 | - |
| | Del11q | 13 | - | 2.7838 |
| | Trisomy 12 | 15 | - | 2.9547 |
| Significance | | | 0.722 | 0.593 |
| Scheffe[a,b] | Del13q | 20 | 0.8857 | - |
| | Undetected | 16 | 1.0013 | - |
| | Control | 15 | 1.0073 | - |
| | Del11q | 13 | - | 2.7838 |
| | Trisomy 12 | 15 | - | 2.9547 |
| Significance | | | 0.997 | 0.990 |

Means for groups in homogeneous subsets are displayed. [a]Uses harmonic mean sample size = 15.607. [b]The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

The statistical analysis shown in Table 4 indicates that ZAP70 expression in the del13q subgroup and in the undetected group was the same as the control group, but it was overrepresented in trisomy 12 and del11q subgroups. ZAP70 expression had about 2.95- and 2.78-fold overexpression in trisomy 12 and del11q subgroups, respectively.

## DISCUSSION

The clinical staging systems proposed by Rai or Binet are the strongest and best validated predictors of clinical outcome in CLL. Both systems classify CLL patients into low, intermediate and high risk subgroups based on clinical observations and standard laboratory tests. Patients with early stage of the disease (Rai stages 0 and I or Binet stage A) have generally an indolent course with long survival or good prognosis, while advanced stage of disease (Rai stages III and IV or Binet stage C) shows an aggressive course with short survival or poor prognosis. Most potential prognostic markers have been shown to contribute to the information that correlates with clinical stages of the disease (Rai et al., 1975; Binet et al., 1981). Consequently, clinical staging systems are the mainstay for assessing prognosis in patients with CLL.

Independent prognostic value of CD38 expression was evaluated by Pearson's correlation test. There was a weak correlation between Rai stages and CD38 expression (correlation coefficient of 0.3). The importance of CD38 as a prognostic marker has been the subject of many controversies. The data of this study agree with the research that did not strongly confirm CD38 prognostic impact (Hamblin et al., 2002; Krober et al., 2002).

On the other hand, ZAP70 expression showed a good correlation with Rai stages. This correlation indicated that the increased ZAP70 expression was correlated with the Rai grading scale, representing severity of the patient's clinical condition. The Pearson coefficient value of 0.42 demonstrated that there is a direct relationship between disease progression and increased ZAP70 expression, and it may be considered a prognostic marker to assess a patient's condition. The importance of ZAP70 as an independent prognostic marker has been shown in numerous earlier studies (Wiestner et al., 2003; Kim et al., 2004; Rassenti et al., 2004; Stamatopoulos et al., 2007).

Statistical analysis of ZAP70 and CD38 expression showed a very weak correlation between these two markers. This means that CD38 value is not in concordance with ZAP70 expression value in patients. Based on the weak prognostic impact of CD38, good predictive value of ZAP70 expression and finally weak concordance of ZAP70 and CD38, it could be concluded that CD38 would not be a reliable prognostic marker in CLL and that simultaneous evaluation of CD38 and ZAP70 has no considerable clinical value in prognosis in CLL. This finding is in agreement with the earlier results of Ibrahim et al. (2001), Orchard et al. (2004) and Del Principe et al. (2006).

Next, the ZAP70 expression was analyzed in the cytogenetic subgroups and the control group using ANOVA. Except for the del11q subgroup, the other groups consisted of at least 15 individuals. This sample number was initially inferred by one-way ANOVA, for determining sample size using the MiniTab program. In three other research reports, sample size for each group was in this range between 15-20 individuals (Ibrahim et al., 2001; Kienle et al., 2005; van't Veer et al., 2006; Chen et al., 2008). Therefore, the sample size was considered to be satisfactory.

Statistical analysis of the ZAP70 expression profile within the cytogenetic subgroups indicated that expression in the del13q subgroup was similar to that of the control group, and the small difference between two mean values can be attributed to random sampling. Although the undetected subgroup showed a good clustering and similarity with the control group, its interpretation was impossible due to the fact that their chromosomal abnormalities were not known. However, expression within the two subgroups of del11q and trisomy 12 increased significantly. This observation was similar to the findings by other investigators (Kienle et al., 2005; Schroers et al., 2005; Krober et al., 2006).

Comparison of means and standard deviations for the cytogenetic subgroups and the control group illustrated that standard deviations for the del13q subgroup and the control group were much smaller than those of del11q and trisomy 12 subgroups. As can be seen in Figure 1, the distribution of ZAP70 expression value within del11q and trisomy 12 subgroups has a great range compared to del13q and undetected subgroups with compact clustering. It can be concluded that contrary to the del13q subgroup, ZAP70 expression profile in the two poor prognosis subgroups of del11q and trisomy 12 is highly variable.

If a cut-off value for ZAP70 expression level is calculated by adding 3-fold standard deviation to the mean of the control group, the value 1.97 is obtained, for which only 10 patients from the del11q subgroup (77%) and 10 patients from the trisomy 12 subgroup (66.6%) showed higher expression. If borderline values are excluded, the patient number decreases. As a result, it can be concluded that the concordance between ZAP70 expression and cytogenetic subgroups is not a hundred percent and shows their independence.

According to the well-known prognostic significance of cytogenetic abnormalities and ZAP70 expression, good correlation of ZAP70 expression within cytogenetic subgroups, and moderate concordance of ZAP70 with cytogenetic subgroups, it could be inferred that combined analysis of the two mentioned markers could prognosticate the disease outcome more favorably and serve for precise definition of prognosis if the value of one predictor factor is doubtful.

In conclusion, according to this study, CD38 could not be regarded as a reliable prognostic factor, and has a poor concordance with ZAP70 expression profile. Furthermore, simultaneous evaluation of CD38 and ZAP70 has no considerable prognostic value in B-CLL. However, the relationship between the cytogenetic subgroups and ZAP70 expression profile did show a considerable correlation: expression level in del13q subjects is similar to normal control individuals whereas its mean value is increased in del11q and trisomy 12 subgroups despite the variation between different patients. As a result, the study of these two independent prognostic factors shows significant concordance, which provides a better prognosis.

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