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Original Article

The Effect of *B-Cell Lymphoma 2* and *BCL2-Associated X* Polymorphisms on the Survival of Acute Lymphoblastic Leukemia Patients: Application of Frailty Survival Models

Navideh Nikmohammadi [1,2], Parvin Sarbakhsh [2], Mozghan Moazami Goudarzi [3], Mehdi Talebi [4], Majid Farshdousti-Hagh [3], Jamileh Malakouti [5], *Neda Gilani [2]

1. *Student Research Committee, Tabriz University of Medical Sciences, Tabriz, Iran*
2. *Department of Statistics and Epidemiology, Faculty of Health, Tabriz University of Medical Sciences, Tabriz, Iran*
3. *Hematology and Oncology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran*
4. *Department of Applied Cell Sciences, Faculty of Advanced Medical Sciences, Tabriz University of Medical Sciences, Tabriz, Iran*
5. *Department of Midwifery, Faculty of Nursing and Midwifery, Tabriz University of Medical Sciences, Tabriz, Iran*

***Corresponding Author:** Email: gilanin@tbzmed.ac.ir

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Abstract

Background: *B-cell lymphoma 2 (BCL-2)* and *BCL-2 associated X (BAX)* polymorphisms are important in the apoptosis process, response to treatment and survival in Acute Lymphoblastic Leukemia (ALL) patients. We aimed to investigate the effect of these genes with other predictors corresponding to the survival of ALL patients with an appropriate frailty survival model.

Methods: Our study was performed in 2020 on sixty-two cases of childhood aged 3-16 (year) with ALL disease who were selected by convenience sampling from the two hospitals of Tabriz, Iran. RFLPPCR method was used for genotyping the promoter region of the *BAX* and *BCL-2* genes. We used different frailty survival models, to control heterogeneity between individuals due to unmeasured factors affecting their survival. All analyses were implemented using Stata 16.

Results: Based on the result of log-logistic model along with frailty gamma, the proportional odds (standard error) of survival for a CC allele of *BCL-2* patient compared to a AA allele patient were 6.0 (1.47); $P<0.001$ and for a AC of *BCL-2* allele patient were 0.57 (1.23); $P=0.009$. Patients with AG allele of *BAX* had 2.05 (1.26) times greater odds of surviving than a AA allele patient ($P=0.003$). The odds of survival of patients with abnormal white blood cell (WBC) were 92% less than normal WBC ($P<0.001$).

Conclusion: With controlling unmeasured factors affecting, the *BCL-2* and *BAX* genes promoter polymorphism are effective in the survival rates for ALL.

Keywords: Acute lymphoblastic leukemia; *B-Cell Lymphoma 2*; *BCL2-Associated X;* Frailty models; Survival

Introduction

Leukemia is one kind of cancer that originates in the bone marrow and the blood-forming tissues of the body that intercepts typical blood function by abnormal cell partition (1). One form of leukemia

that is very well-known in children is acute lymphocytic leukemia (ALL) (2).

This malignancy is treated in different manners such as chemotherapy, immunotherapy, and radiation. Clinical therapies firstly perform their antitumor activities by stimulating intracellular death planning (3). The apoptosis process is a physiological cell programmed to die. Detection of the key proteins involved in apoptosis exposure is an appealing way to impede the development of many diseases including cancer. Percept how these proteins affect the apoptotic pathways may lead to more efficient cancer treatments and survival of the patients. The discovery of apoptosis pathways and the development of specific molecules that include apoptosis of tumor cells display that cell death can be targeted therapeutically (4). Chemotherapeutic drugs and ionizing radiation (IR) damage DNA cells and they are involved in the apoptosis process. There is an association between increased resistance to chemotherapy and reduction apoptosis activity (5-8). Susceptibility to apoptosis is the main key to response to anti-neoplastic therapy (9).

Proteins P53, *B-cell lymphoma 2 (BCL-2)*, *BCL2 associated X (BAX)* genes are central to this process of apoptosis. Anti-apoptosis effects of *BCL-2* protein are important in multidrug resistance. More expression of *BCL-2* initiates the growth factor withdrawal, IR, glucocorticoids, and multiple chemotherapeutic agents, these process prevents cell death (10, 11). High expression of *BCL-2* was associated with an appropriate response to therapy (12, 13). *BCL-2* is a member of a family of *BCL-2* homologs. The role of *BCL-2* is important in the apoptosis process. In addition, *BAX* protein is one of the homogeneous genes versus *BCL-2*. Its activity is against the anti-apoptosis effect of *BCL-2* in the apoptosis process (14). However, the previous studies have evaluated the effect of *BCL-2* promoter (SNP -938C>A) genotyping and *BAX* (SNP G-248A) polymorphism on patient outcomes without considering time to event or frailty term (12, 15).

The notion of frailty offers a suitable way to introduce unobserved heterogeneity and associations into models for survival data. Also, longitudinal repeated measurement data can be including for survival models for accurate predictions (16, 17).

In this study, we utilized different frailty survival models, to control heterogeneity between individuals due to unmeasured factors, to know whether of *BCL-2 C-938A* (rs2279115) *SNPs* and *BAX G-248A* (rs4645878) *SNP* polymorphism with other covariates can have significant effects on survival of acute lymphoblastic leukemia patients.

Materials and Methods

Study design and participants

Our study was performed in 2020 on sixty-two cases of childhood aged 3-16 (year) with acute lymphoblastic leukemia. They had been diagnosed by bone marrow aspiration, flow cytometry, cell counts. Patients with 3-16 years old were recruited from the two Shahid Ghazi Tabatabai and Children's Hospital of Tabriz, Iran with the convenience sampling method and were seen by a pediatric oncologist. The patient sampling process took 6 months and patients were followed for one year. Among the patients who were diagnosed with acute lymphoblastic leukemia cancer, patients with unstable clinical conditions, people who received blood products within 10 days before sampling and patients with bone marrow transplantation were excluded.

All stages of the work have been carried out by following the Code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans. The written consent was obtained from parents or legal guardians of children. The ethical aspects of this study were approved by the institutional ethics committee of Tabriz University of Medical Sciences, with code IR.TBZMED.REC.1398.1203.

Assessments

Morphological characteristics of the bone marrow and flow-cytometry of all patients were prepared. Expression of CD7, CD10, CD19, HLA-DR, CD20, CD22, CD3, CD34 and CD45 based on

protocols for routine hospital practice was evaluated. Expression of CD3 marker was classified into normal (<20(ng/mL)) and non-normal (≥20 (ng/mL)) groups. The counts of WBC were ranged at two groups normal (abnormal), according to Children`s Reference Ranges for Routine Hematology Tests (18).

DNA extraction

DNA from all blood cells was extracted with a salting-out method. DNA concentration and clarity in each sample were measured with a NanoDrop 1000 Thermo scientific Spectrophotometer (Wilmington, DE, USA). DNA extracts with a visual density ratio between1.6 to 1.9 at 260/280 nm were chosen for the subsequent steps.

Amplification of the BCL-2 promoter region

BCL-2 genotype was determined with the Amplification of genomic DNA. Primers were ready by Bioneer (Daejeon, S. Korea). Forward primer: 5´TTATCCAGCTTTTCGG-3´ and the reverse primer: 5´GGCGGCAGATGAATTACAA-3´ were used. SensoQuest Thermocycler (Göttingen, Germany) was used for enzyme chain reactions (PCR), it was a final volume of twenty-five µL, containing 12.5 µL Master Mix Red (Amplicon, Odense, Denmark), 1.25 µL of each primer, six µL dH2O and four µL genomic DNA.

Amplification of the BAX promoter region

BAX genotype was such as with the consolidation of genomic DNA. Forward primer: 5'-CGGGGTTATCTCTTGGGC-3' and the reverse primer: 5'-GTGAGAGCCCCGCTGAAC-3'were used. PCR was accomplished in a final volume of twenty-five µL containing 12.5 µL Master Combine Red (Amplicon), 1.25 µL of each primer (Bioneer), six µL dH2O and four µL genomic DNA.

Restriction enzymes analysis of the BCL-2 and BAX genes

BCL-2: Aliquots of 6 µL of each PCR production were digestible with 1 unit of restriction nuclease at 37°C a nightlong with 1 µL 10× enzyme buffer. Screening of the samples for the *BCL-2* C-938A (rs2279115) *SNPs* was performed by restriction enzyme BccI (New England BioLabs, Ipswich, UK). The homozygous CC (wild-type) genotype was unreal as one major 252-base combine (bp) band. The AC heterozygous genotype showed each the undigested 252-bp band and also the digestible 154- and 98-bp bands, and also the digestible 154- and 98-bp product delineated the AA genotype.

BAX: The PCR productions (6 µL) were incubated a nightlong with one µL restriction nuclease at 37°C

Screening samples for the *BAX G-248A* (rs4645878) *SNP* was performed with the restriction enzyme ASCII (New England BioLabs). Three major bands of 352, 256, and 96 bp were been in the homozygous GG (wild-type) genotype. The 256-bp band was a lot of severe. The heterozygous AG genotype resulted in the loss of a limitation site for ASCII in one of the *BAX* promoters. The genotyping results showed the 352-bp band, 256 bp band, and 96-bp band. The 352-bp band was most intense, versus, the 96-bp band was mostly invisible. The 352-bp band was been in the homozygous AA genotype. For more detailed information and the PCR productions conditions in every step (19).

Statistical analysis

Descriptive statistics are reported as mean (±SD) for quantitative data and as frequency and percentage for qualitative data. For inferential section, parametric survival models (with and without frailty) were used to determine effective factors corresponding to the survival of acute lymphoblastic leukemia patients. The other covariates such as WBC, CD3, CD7, gender, and baseline age entered the models to assay the adjusted effect of genes. To compare the different parametric models and choose the best model, the information-theoretic criteria (such as AIC and BIC). The Hazard ratio (HR) with its standard error (SE) have reported for the exponential and Weibull models, and the results of log-logistic models represented by proportional odds (PO) with its SE. Also time

ratio reported for log-normal models (20, 21). Statistical analysis was done by Stata version 16 (College Station, TX: StataCorp LLC; 2019). *P*-values less than 0.05 were considered statistically significant.

Results

Out of 62 acute lymphoblastic leukemia patients, 41(66.1%) were male and 21(33.94%) female. The mean (± SD) of age at diagnosis patients was 7.4 (±3.37) years and the median (±SE) survival time was found 86 (±14.48) months. The mean (±SD) count of WBC was) $4.5\pm3.6)\times 10^9$ /L ranging from 1.3×10^9/L-21×10^9/L. Genotyping of the promoter region of the *BCL-2* gene (C-938A) showed the following allele frequencies (%) in the ALL patients: AA in 33 children (53.23%), AC in 18 (29.03%) and CC in 11 (17.74%). Similarly, for *BAX* gene (G-248A) the frequencies of AA, AG, GG alleles were 15 (24.2%), 24 (38.7%), 23 (37.1%), respectively. The restricted mean of survival time in patients with different genotypes were AA allele 104 months; AC allele 121 months and CC allele, 303 months; and for AA, AG, GG alleles of BAX gene were 134, 114 and 162 months, respectively. Based on the Fig. 1, for all categories of *BAX* polymorphism, CC alleles of the *BCL-2* polymorphism had a lower rate of mortality.

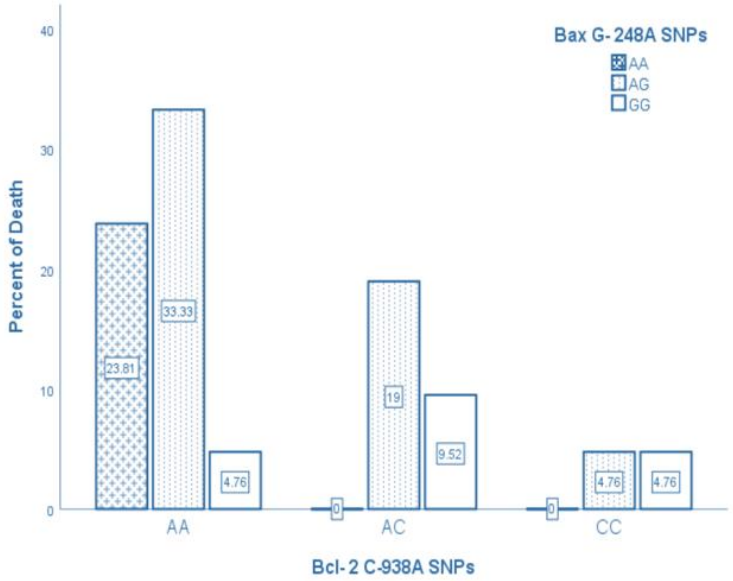

Fig. 1: Mortality rate (%) in acute lymphoblastic leukemia Patients alleles of the *BCL-2* C-938A polymorphism and *BAX G-248A SNP*

Tables 1 and 2 represent HR (SE) for univariate and multivariate exponential and Weibull and PO (SE) for log-logistic models and time ratio for log-normal with and without frailty, respectively. Frailty term was significant in most models, including the final model (results not shown). The finding of Table 1 showed that without adjusting for other variables; age is a significant factor under the exponential, log-normal, Weibull and log-logistic without frailty term. This means that older patients had a higher risk of death than others did; (for example Weibull distribution: 1.12(0.06), P=0.035). In a multivariate scenario; underlying the best model, WBC was a significant factor in survival rate. The survival of patients with abnormal WBC was less than others were; (0.52 (1.20); P<0.001). The survival difference of patients with AC allele and AA allele in *BCL-2* polymorphism was significant (0.57 (1.23); P=0.009)).

Table 1: Results of univariate parametric models with and without frailty

Variable	*Model*	*Without frailty*			*Gamma frailty*			*Inv-Gussian frailty*		
		HR¥	SE†	P	HR¥	SE†	P	HR¥	SE†	P
Age (yr)	Exponential	1.20	0.06	0.043*	1.13	0.07	0.054	1.12	0.07	0.054
	Weibull	1.12	0.06	0.035*	1.30	0.32	0.285	--	--	--
	Log-normalτ	0.89	0.04	0.049*	0.90	0.05	0.138	0.92	0.05	0.182
	Log-logistic$\pounds$	0.89	1.05	0.030*	0.90	1.06	0.107	0.91	1.06	0.197
Gender (female)	Exponential	0.56	0.27	0.244	0.56	0.27	0.549	0.56	0.27	0.248
	Weibull	0.54	0.26	0.216	0.62	0.89	0.724	--	--	--
	Log-normalτ	1.49	0.61	0.327	1.09	0.43	0.823	1.10	0.34	0.745
	Log-logistic$\pounds$	1.62	1.53	0.262	1.01	1.55	0.980	0.99	1.46	0.988
CD3 (ng/mL)	Exponential	0.94	0.58	0.921	0.91	0.62	0.895	0.91	0.61	0.900
	Weibull	0.92	0.57	0.900	0.00	0.02	0.606	--	--	--
	Log-normalτ	1.30	0.75	0.647	2.09	0.87	0.076	--	--	--
	Log-logistic$\pounds$	1.19	1.76	0.750	1.97	1.49	0.092	2.03	1.48	0.075
CD7 (ng/mL)	Exponential	1.00	0.01	0.979	0.99	0.009	1.00	1.00	0.009	0.996
	Weibull	0.99	0.01	0.996	0.94	0.08	0.514	--	--	--
	Log-normalτ	1.00	0.00	0.747	1.00	0.00	0.130	--	--	--
	Log-logistic$\pounds$	1.0	0.01	0.861	1.01	1.005	0.158	1.008	1.005	0.128
WBC (abnormal)	Exponential	1.54	0.79	0.397	1.58	0.86	0.400	1.58	0.85	0.399
	Weibull	1.55	0.79	0.387	--	--	--	--	--	--
	Log-normalτ	0.62	0.27	0.276	0.57	0.19	0.096	--	--	--
	Log-logistic$\pounds$	0.64	1.57	0.336	0.61	1.43	0.184	0.58	1.41	0.125
				BCL2 C-938A (rs2279115)						
AC	Exponential	0.78	0.38	0.625	0.77	0.39	0.623	0.77	0.40	0.947
	Weibull	0.81	0.40	0.677	1.12	1.99	0.947	--	--	--
	Log-normalτ	1.19	0.44	0.639	0.96	0.31	0.925	--	--	--
	Log-logistic$\pounds$	1.26	1.47	0.551	0.86	1.44	0.689	0.89	1.41	0.747
CC	Exponential	0.20	0.15	0.035*	0.19	0.15	0.042*	0.19	0.15	0.041*
	Weibull	0.17	0.17	0.022*	0.01	0.001	0.257	--	--	--
	Log-normalτ	4.75	2.54	0.004*	5.30	2.05	<0.001*	--	--	--
	Log-logistic$\pounds$	4.53	1.71	0.006*	4.66	1.43	<0.001*	4.88	1.43	<0.001*
AA				Reference category						
				BAX G-248A (rs4645878)						
AG	Exponential	1.60	0.85	0.374	1.65	0.94	0.373	1.64	0.92	0.374
	Weibull	1.60	0.85	0.374	1.49	2.33	0.799	--	--	--
	Log-normalτ	0.69	0.32	0.431	0.90	0.37	0.816	0.90	0.30	0.772
	Log-logistic$\pounds$	0.64	1.59	0.357	0.95	1.52	0.917	0.99	1.46	0.987
GG	Exponential	1.07	0.71	0.918	1.09	0.77	0.899	1.08	0.76	0.903
	Weibull	1.04	0.70	0.947	3.06	5.50	0.534	--	--	--
	Log-normalτ	0.88	0.50	0.831	0.74	0.36	0.553	0.90	0.39	0.826
	Log-logistic$\pounds$	0.92	1.82	0.895	0.66	1.66	0.420	0.70	1.59	0.471
AA				Reference category						

¥ Hazard ratio
† Standard error
*Significant at 0.05 level
$\pounds$ Results of these models are proportional odds with their standard errors.
τ Results of these models are time ratio with their standard errors.

AG allele in *BAX* polymorphism was a significant factor in survival rate (2.05(1.26); *P*=0.003)). However, the effect of the GG allele in *BAX* polymorphism was not statistically significant in survival rate. Also, the survival of patients with CC allele was 6.0 times more than AA allele in *BCL-2* polymorphism (6.0 (1.47); *P*<0.001)).

Table 2: Results of multivariate survival model with and without frailty

Variable	*Model*	*Without frailty*			*Gamma frailty*			*Inv-Gussian frailty*		
		HR¥	SE†	P	HR¥	SE†	P	HR¥	SE†	P
WBC (abnormal)	Exponential	3.56	2.05	0.028*	3.56	2.05	0.028*	3.56	2.05	0.028*
	Weibull	6.31	4.31	0.007*	--	--	--	--	--	--
	Log-normalτ	0.39	0.14	0.013*	--	--	--	--	--	--
	Log-logistic£	0.36	1.49	0.013*	0.52	1.20	<0.001*	--	--	--
CD3 (ng/mL)	Exponential	0.13	0.77	0.730	0.13	0.77	0.730	0.13	0.77	0.730
	Weibull	0.18	1.21	0.798	--	--	--	--	--	--
	Log-normalτ	1.11	4.23	0.977	--	--	--	--	--	--
	Log-logistic£	1.39	3.88	0.932	<0.001	2.47	0.005*	--	--	--
CD7 (ng/mL)	Exponential	1.03	0.08	0.639	1.03	0.08	0.639	1.03	0.08	0.639
	Weibull	1.03	0.093	0.677	--	--	--	--	--	--
	Log-normalτ	0.99	0.05	0.920	--	--	--	--	--	--
	Log-logistic£	0.98	1.05	0.846	1.10	1.03	0.003*	--	--	--
Age (years)	Exponential	1.13	0.08	0.078	1.13	0.08	0.078	1.13	0.08	0.078
	Weibull	1.18	0.09	0.034*	--	--	--	--	--	--
	Log-normalτ	0.91	0.04	0.069	--	--	--	--	--	--
	Log-logistic£	0.90	1.04	0.045*	0.99	1.02	0.681	--	--	--
Gender (female)	Exponential	1.04	0.56	0.934	1.04	0.56	0.934	1.04	0.56	0.934
	Weibull	1.08	0.59	0.880	--	--	--	--	--	--
	Log-normalτ	0.89	0.30	0.739	--	--	--	--	--	--
	Log-logistic£	0.88	1.43	0.734	0.71	1.19	0.061	--	--	--
				BCL2 C-938A (rs2279115)						
AC	Exponential	0.84	0.43	0.739	0.84	0.43	0.739	0.84	0.43	0.739
	Weibull	0.90	0.47	0.857	--	--	--	--	--	--
	Log-normalτ	1.12	0.38	0.736	--	--	--	--	--	--
	Log-logistic£	1.09	1.41	0.796	0.57	1.23	0.009*	--	--	--
CC	Exponential	0.16	0.13	0.032*	0.16	0.13	0.032*	0.16	0.13	0.032*
	Weibull	0.08	0.08	0.008*	--	--	--	--	--	--
	Log-normalτ	5.24	2.91	0.003*	--	--	--	--	--	--
	Log-logistic£	4.94	1.71	0.003*	6.00	1.47	<0.001*	--	--	--
AA				Reference category						
				BAX G-248A (rs4645878)						
AG	Exponential	1.69	1.00	0.378	1.69	1.00	0.378	1.69	1.00	0.378
	Weibull	1.88	1.47	0.305	--	--	--	--	--	--
	Log-normalτ	0.77	0.29	0.508	--	--	--	--	--	--
	Log-logistic£	0.69	1.50	0.376	2.05	1.26	0.003*	--	--	--
GG	Exponential	1.50	1.11	0.583	1.50	1.11	0.583	1.50	1.11	0.583
	Weibull	1.86	1.43	0.415	--	--	--	--	--	--
	Log-normalτ	0.89	0.43	0.816	--	--	--	--	--	--
	Log-logistic£	0.750	1.64	0.562	1.90	1.45	0.083	--	--	--
AA				Reference category						

¥ Hazard ratio
† Standard error
*Significant at 0.05 level
τ Results of these models are time ratio with their standard errors.
£ Results of these models are proportional odds with their standard errors.

AIC and BIC criterions applied for the multivariate survival models in Table 3. The conclusion of these criterions showed that the Log-logistic with frailty gamma has the best fit among other models. The frailty term in this model had a high level of significance among the other models (P=0.009).

Table 3: AIC and BIC criterion of the different models of acute lymphoblastic leukemia Patients

Model	*BIC*	*AIC*	*RANK*
Without Heterogeneity			
Exponential	135.542	114.271	5
Weibull	134.096	110.698	4
Log-normal	132.337	108.939	2
Log-logistic	133.267	109.868	3
Gamma Heterogeneity			
Exponential	139.670	116.271	6
Log-logistic*	131.719	106.193	1
Inverse Gaussian Heterogeneity			
Exponential	139.670	116.5795	6

* Best model with high level of significant in frailty term.

Discussion

Diagnosis of key proteins in the apoptotic process can be effective in controlling cancer progression. Finding how to affect these proteins in the apoptotic pathways may lead to the best treatments. *BAX* and *BCL2* polymorphism in controlling apoptosis are important factors.

In this paper, we studied the effective association between the survival of acute lymphoblastic leukemia patients and several most common prognosis factors such as alleles of *BCL2* C-938A (rs2279115) SNP polymorphism, *BAX* G-248A (rs4645878) *SNP* polymorphism, age at diagnosis, WBC and gender. We used frailty models to study heterogeneity among individuals.

Frailty models account for the presence of a latent multiplicative effect on the hazard function. This effect is not directly estimated from the data. When the standard models cannot account for all the variability in the failure times, frailty models can be used instead of standard models. Concept of frailty was discussed in many studies (22-24).

In the study, frailty term was significant. Parametric survival models had good fitting rather than semi-parametric models (25). AIC and BIC criteria indicated that the Log-logistic with frailty gamma model are the best models in multivariate analysis.

We found that age and WBC were effective factors under the most of models. Gender was not a significant factor in the survival rate. Allele CC of the *BCL2* polymorphism appears a significant factor in all fitted models, this implies that patients with the CC allele had higher survival time than other patients. The effect of AC allele in *BCL-2* polymorphism is a significant factor in the survival rate. However, only the AG allele in *BAX* polymorphism was an effective factor in survival rate. This showed that *BCL-2* polymorphism is an important factor in survival than *BAX* polymorphism.

Several studies have shown a correlation between high *BCL-2* expression and poor response to therapy in specific tumors; against many studies that have shown low *BCL-2* expression is related to poor response and shortened survival in lung cancer and childhood acute lymphoblastic leukemia (12, 15, 26-28). A study on acute myeloid leukemia (AML) showed that expression of *BAX* and *BCL-2* does not differ significantly among AML patients in terms of remission, relapse and overall survival (29). *BCL-2* effects in remission rates in B-cell chronic lymphocytic leukemia (B-CLL) (30). Other studies confirm the important role of the *BCL-2* protein in B-CLL (31, 32). The expression

pattern of *BAX*, *BCL-2*, and their ratio differs between various cancers and within the same cancer. Some items such as type of cancer, the source, the sample size, the data variance, the treatment modalities, and the techniques used are effective in the results.

Limitation

Survival analysis was performed for one year after. Long-term follow-up with a larger sample size is required for results that are more accurate. We focused only on *BAX* and *BCL-2* polymorphism. However, different proteins are involved in the apoptosis process. Given the conflicting results, further studies are needed.

Conclusion

With controlling heterogeneity between individuals, the effect of CC allele in *BCL-2* polymorphism is more than other alleles. AG allele of *BAX* polymorphism is a single effective allele at this polymorphism in survival rate. Generally, both of these genes are significant in the survival of patients. WBC and age in prognostic are effective factors. Patients with normal WBC counts and young patients showed better survival.

Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of interest

No potential conflict of interest was reported by the authors.

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