Original Research Article

Assessing the frequency of t (12; 21) (p13; q22) in patients with acute lymphoblastic leukemia in northeastern of Iran

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DOI: 10.22034/HBB.2018.26

Received: September 15, 2018; **Accepted:** December 15, 2018

ABSTRACT

Studying the results of the prevalence of a particular genetic translocation of acute lymphoblastic leukemia (ALL) could predict the incidence rate of overall survival (OS) and event-free survival (EFS). 283 patients from northeastern of Iran with new diagnosis of ALL by bone marrow aspiration or peripheral blood sample were enrolled in the study. The expression of TEL-AML1 fusion were studied by TaqMan probe qualitative real time PCR compared with ABL reference gene. Then, the five-year survival rates were surveyed in the region of Khorasan Razavi and were analyzed by kaplan-meier test. Prevalence of t (12, 21) in patients with ALL was 14.4 %, survival rate was 75 %, and the average five-year survival rate for patients was 4.77 ± 39.76 months by the log-rank test. Prevalence of t (12, 21) in patients with ALL in the north east of Iran was lower than the world average.

***Keywords*:** Acute lymphoblastic leukemia lymphoma, TEL-AML1, t (12, 21)

INTRODUCTION

Over-expression of proto-oncogenes, changes in tumor suppressors genes and genes regulating apoptosis and those which contribute to DNA repair or the occurrence of trans-locations, are all among the causes that can lead to a malignant process [4,5]. Acute lymphoblastic leukemia (ALL) causes 80 % of children leukemia and is one of the most common malignancies in people under the age of 15 years old and they contain 30 % of all cancers in this age. The incidence of ALL in children aged 1 to 14 in the United States is 41 cases per million [6]. In ALL, the number of leukemic blast cells increases and the accumulation of these cells in the bone marrow impair the development and normal functioning of the blood cells, which disrupts the defense against infection. PBC-ALL (Precursor B-cell ALL) is known as the most common form of ALL especially in children aged 1 to 15 years and the cause of 30% of this disease is hyper diploid cell clones with t (12;21) and t (1;19) translocation. Pro-leukemic cells in this disease require environmental stimuli to become completely cancerous and although these environmental stimuli and the exact cause of the ALL are not known, malignancy is likely to occur due to the accumulation of genetic mutations in the genome[7]. In t (12;21) p13; q2 translocation, the AML gene on chromosome 21 fuses to TEL gene on chromosome 12. The AML gene encodes the RUNT region which makes the C terminal of a transcription activator domain called CBR protein. The complex is actually the result of the transcription of the AML-1/CBFB gene which results in a transcriptional activator that plays a role in a large number of hematopoietic cells. The TEL gene encodes a HLH domain associated with one of the transcription factors called ETS-DNA binding. The protein resulting from the fusion of these two genes leads to incomplete maturity of the B lymphocytes. However, there are other causes of ineffective maturation of lymphocyte B like the deposition of the proto-oncogene C-MYC near the immunoglobulin kappa chain coding gene, which is called t (2; 8) (p12; q24) [8].

The aim of this study was the better categorization of different subgroups of acute lymphoblastic leukemia, diagnosis of the overall prognosis of the disease and its 5 year survival.

MATERIALS AND METHODS

This study was conducted during the years from 2010 to 2016 in the molecular cytogenetic-pathology department of Ghaem Hospital in Mashhad, sponsored by the Pathological Molecular Research Center of this hospital. The subjects included all

patients with ALL in the North East of Iran which were assessed in terms of five year survival and the presence or absence of t (12,21) (as the most common molecular abnormality in children).

Results were registered in SPSS version 18 and Stata version 11.2 software and were analyzed by log-rank test and Kaplan-meier survival curves. Also, ALL patients with t (12.21) positive or t (12.21).

Sample size

For logical estimation of the sample size, we used previous studies in the field of ALL and translocation of t (12;21) (p13; q22) [6,9,10]. The sample size (283 samples) was obtained based on the test for estimating a proportion in society, according to previous studies with a confidence level of 95 % and d = 1/5p. After the primary diagnosis of the disease by CBC, PBS and BMA, in order to investigate the presence of TEL-AML1 fusion in ALL, mono nuclear cells were isolated from bone marrow aspiration by Ficoll gradient centrifugation method.

Isolation of mono nuclear cells (BMNC)

In this method, bone marrow aspiration was added to the ficoll solution in the test tube and centrifuged for 20 minutes with 3000 rpm. After centrifugation, plasma, mono nuclear cells and ficoll were layered from top to bottom, respectively. Then the mono nuclear layer was transferred to another sterilized tube. The isolated BMNC was washed with 3 cc phosphate saline buffer (PBS) in a centrifuge with 3000 rpm for 10 minutes. Supernatant was extracted and 200 λ of PBS was added to the sediment.

RNA extraction

To RNA extraction, we used Tri-pure solution (Roche Company) and then added chloroform to Tri-pure solution, the final solution was layered into three phases: RNA, DNA, protein and organic compounds.

RNA purification

In order to the RNA purification, the higher phase was added to another micro tube and 0.5 ml of isopropanol was added to that. Then we upped and downed them for several times and placed in a refrigerator temperature for 10 minutes, until a RNA sediment be obtained. After incubation, the micro tubes were centrifuged at 12000 to 15000 (14,000 rpm) for 15 min at a temperature of 2 to 8 °C. To wash RNA, 1 ml of 75 % ethanol (made with water and free of RNase) was added to the micro tube and centrifuged for 5 min at 7500 g (9000 rpm) and 2 to 8 °C. After centrifugation, the superficial ethanol was dispersed and the tubes were placed in open area for 5 to 10 min in a vacuum of 56 °C to get dried. Extracted RNA was dissolved in 40 µl of RNase free water adjacent to DEPC water (Diethyl Pyrocarbonate) and was

placed in the oven with 55 °C which could be used immediately or be stored in -70 °C.

RNA quality and quantity

2 µl of extracted RNA was loaded on agarose 2 % and electrophoresed for 15 minutes. Because the 28 srRNA and 18 srRNA bands were quite strong and the intensity of the 28srRNA band was twice that of 18srRNA, the RNA quality was confirmed.

DNA removal

In order to prevent unwanted DNA replication in the real time PCR phase, before the synthesis of CDNA, DNA was removed. Deoxyribonuclease (DNasei) was used in order to remove the DNA.

cDNA synthesis

In this study, RevertAid ™ H minus First Strand cDNA Synthesis kit (Fermentes Company) was used to synthesize cDNA.

cDNA quality

In order to assess the quality of the cDNA, the light absorbance by cDNA was measured at 260 nm using the product of Reverse Transcriptase and Nano drop instrument. The concentration of cDNA was about 50-50 ng/µl.

Real time PCR

Primer and probe: The sequence of probes and primers for the cDNA derived from TEL-AML1 fusion mRNA was as follows (table1). This sequence was selected according to the reference number [11] and its specificity was checked in NCBI [12].

ABL gene was used as a reference gene and its primers and probes are mentioned below (table2).The real time PCR reaction was carried by ABI Thermal cycler ABI (USA). After obtaining the results, a positive and negative control samples were tested to check the presence or absence of the TEL-AML1 fusion. In this study, Taq Man probes were used to check for the presence or absence of a TEL-AML1 fusion and the ABL control gene was used to ensure the quality of the extracted RNA.

Data analysis

Data analysis was performed by SPSS version 18 and Stata version 11.2. Thus, to describe the characteristics of the sample, descriptive statistics such as frequency tables, mean and standard deviation were used. Since a part of this study has been carried out retrospectively, survival analysis was used to measure the significant difference between the survival times of the studied groups. The significance level of these tests was considered to be P value <0.05. Data analysis was performed based on the Kaplan-Meier survival curve, ranked test, independent t-test and Chi-square table.

Table1. Primer sequence of forward TEL-AML1, reverse TEL-AML1 and TEL-AML1 probe

TEL-AML1Forward	*5′-CTCTGTCTCCCCGCCTGAA-3′*
Reverse *TEL-AML1*	5′-CGGCTCGTGCTGGCAT-3′
***TEL-AML1* Probe**	TCCCAATGGGCATGGCGTGC

Table 2. Primer sequences of forward ABL, reverse ABL and ABL probe

Forward ABL	*5′TGGAGATAACACTCTAAGCATAACTAAAGGT3′*
Revers ABL	5′GATGTAGTTGCTTGGGACCCA3′
ABL probe	F-CCATTTTTGGTTTGGGCTTCACACCATT-T

Table 3. Annual incidence of ALL in the KhorasanRazavi area in children aged 2-10 years

Annual incidence of ALL	**The new ALL outbreak number**	**The total number of children**	**Year**
28/73 cases per million people	28	974,461	2010
35/73 cases per million people	34	951,531	2011
47/72 cases per million people	47	984,751	2012
26/27 cases per million people	26	989,767	2013

Table 4. Death event incidence in t (12.21) positive ALL patients in northeast Iran by considering the age group

Death event	Age group (in terms of years)	P-value
8	2-10	0.99
2	>10	

RESULTS

In data analysis using descriptive tests, the average of annual incidence of ALL in Khorasan Razavi province was 34.61 people per million according to Khorasan Razavi from 2010 to 2013 (table 3). Among 283 ALL patients in northeastern parts of Iran, 41 patients (14.4 %) (figure 1) were (12.21) positive. In terms of the age of ALL patients with t (12,21) positive , 25 % of all patients (first quartile) were under 3 years old, 50% of the total population (second quartile) were under 5 years old and 75 % of patients were under 7 years old. It should be noted that, most ALL with t (12, 21) were 3 years old. The mean age of ALL patients t (12,21) positive, regardless of age limit was 5.83 ± 4.43 years and the average age of ALL patients with an age range of 1-11 years was 4.89 ± 2.61 years. The mean age of ALL patients with t (12.21) negative was 13.07 ± 14.84 years. Independent t-test with PV = 0.003 showed a significant difference between the two groups of patients with ALL with t (12.21) positive and ALL patients with t (12.21) negative in terms of age distribution. The minimum age of patients with ALL with t (12.21) positive was 0.75 years and the maximum age was 23 years.

To facilitate the data analysis, the age of patients was divided into two groups. 78.9 % of ALL patients with t (12.21) positive, 10.5 years old and 21.1 % of them were smaller than 10 years old. 55 % of ALL patients with negative t (12.21) were 10.5 years old and 45 % of them were smaller than 10 years old.

In terms of gender, in the group of ALL patients with t (12.21) positive, 46.7 % of patients were female and 53.3 % of them were male. In patients with ALL with t (12.21) negative, 33.3 % of patients were females and 66.7 % of them were male. Chi square test did not show a significant difference between the two groups in terms of sex distribution.

In terms of clinical symptoms, 22.58 % of the patients with t (12.21) positive showed splenomegaly, 24.19 % showed hepatomegaly and 77.4 % of the population showed other clinical symptoms such as fever, fatigue, anemia, bruising, CNS involvement, Testicular involvement etc. Regarding laboratory tests, the findings were as follows:

The average rate of five-year survival in ALL patients with t (12.21) positive in north east of Iran, was 39.77 ± 4.77 months based on the log test and the 95 % confidence interval for the average survival of patients was calculated (49.11 and 30.41) and the Kaplan-Meier survival curve showed a five-year survival rate for patients with t (12.21) positive about 75 %. In order to facilitate the investigation

of survival, 40 ALL patients with t (12.21) positive were classified for age, sex, white blood cell count (WBC count) and platelet count (figures 2,3,4).

After classification of ALL patients with t (12.21) positive in two age groups of 2-10 years and more than 10 years, to determine the survival rate, 80 % of patients were in the age group of 2-10 years (group 1) and 20% of them were in the age group of more than 10 years (group 2). The average survival rate in the first group was 39.9 ± 5.27 months and in the second group was 9.95 ± 35.5 months. The 95 % confidence interval for the average survival of patients was obtained in the first group (50.22 and 29.55) months and in the second group (55.00 and 15.96) months. 10 deaths were observed in 40 ALL patients with t (12.21) positive. 8 deaths occurred in the first group and 2 deaths in the second group. In assessing the survival rate of t (12.21) positive ALL patients, 8 deaths were observed in female group and 2 deaths were observed in males group. The average survival rate for 18 female ALL patients with (12, 21) positive were 4.81 ± 41.87 months and for 22 males were 7.18 ± 33.83 months, respectively. The confidence interval for the female group was (51.31 and 32.44) and for the male group was (47.91 and 19.76). In examining the survival rates in four groups of white blood cell count, 6 deaths in group 4 with cell count, 2 deaths in group 3 with cell counting (50,000-100,000 µl/ L) and two death events were observed in group 2 with cell count (10,000-50,000 µl/ L) and no death was occurred in group one with cell count (10000 µl/ L).

The average survival rate in the first group of white blood cells was obtained 47 months with confidence intervals (47 and 46.98), In the second group, 45.6 ± 6 months with confidence intervals (34, (55) and 32), In the third group, 12.2 ± 31 months with confidence interval (7-55) and in the fourth group 18 ± 9 months with confidence intervals (35.5 and 0.65). In survival studies in three groups of platelet count, 8 death were observed in the first group with a count of less than 50,000 µl/ L platelets, Two death events were observed in the second group with a count of 150,000 to 50,000 µl/ L platelets and no death was occurred in the third group with the counting of more than 150,000 µl/ L.

The survival average in the first group of platelet counts was 33.8 ± 7.1 months with a confidence interval of 47.9 and 19.7. In the second group was 6.3 ± 36 months with a confidence interval of 48.5 and 23.5, and in the third group was 46.9 months with a

confidence interval of 46.9 and 46.9 months were obtained, respectively.

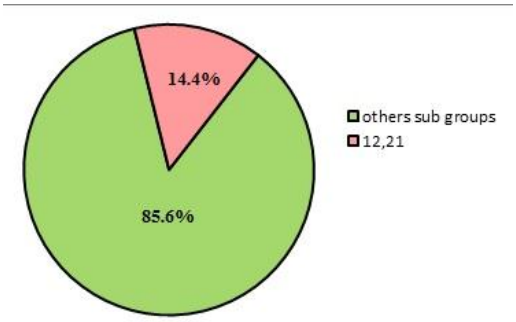

Figure 1. The incidence of t (12, 21) in patients with ALL in the North East of Iran.

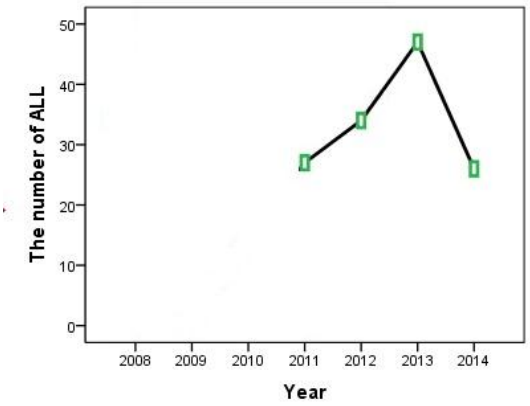

Figure 2. Frequency of annual ALL numbers during the years 2011-2014 in children aged 2-10 years in northeastern Iran

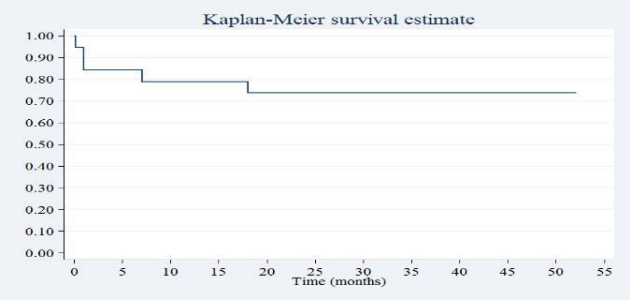

Figure 3. Survival rate in t (12.21) positive ALL patients in northeast Iran.

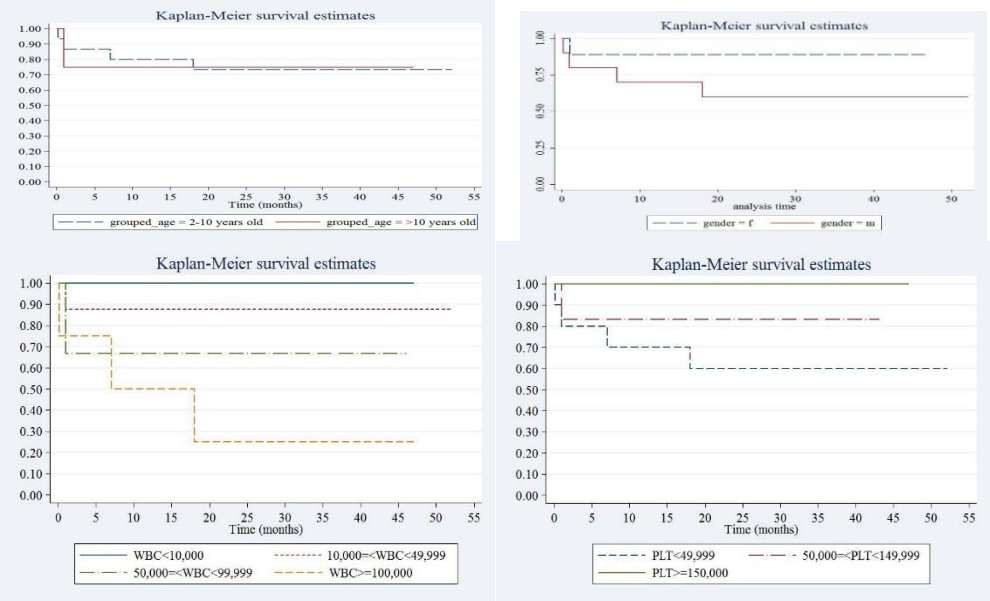

Figure 4. Survival rates in t (12.21) positive ALL patients in northeast Iran by age group; survival rate in terms of sex in t (12.21) positive ALL patients in northeast Iran; relationship between survival rate in t (12.21) positive ALL patients in northeast Iran with white blood cell counts; survival rate in T (12.21) Positive ALL Patients in North East of Iran.

DISCUSSION

ALL causes 80 % of children leukemia and is the most common malignancy in people under the age of 15. It continues 30 % of all cancers in this age group [13].

Although the incidence of t (12,21) in the censuses of the World Health Organization was about 25 % [14] and Ching-Hon in 2004 reported the prevalence of this recurrent genetic abnormality in the New England Journal of Medicine about 22 % [15], but the prevalence was 14.4 % in this study which was lower than the global average. Since the presence of fusion mRNA is associated with prognosis and appropriate response to treatment [15-17] and leads the application of the Standard Risk chemotherapy protocol with a lower probability of a patient's disease and reduces the need for bone marrow transplantation (BMT) [15].

Therefore, the lower prevalence of this translocation than the global mean, suggests the possibility of an increase in the prevalence of acute lymphoblastic leukemia with no specific genetic abnormalities (NOS

subgroup) [18] or other recurrent genetic abnormalities in ALL which some of them like t (9.22) or the involvement of chromosome 11 are accompanied with poor prognosis and requiring severe treatment protocols like bone marrow transplantation.

Then we divided the patients into two male and female groups to compare the frequency of this genetic abnormality and its effect on the survival rate of patients.

While in this study the prevalence of t (12.21) in ALL male patients was 53.3 % and in female patients was 46.7 % and the survival rate was lower in men than in women, we concluded that our male patients were more likely to be more frequent with lower prognosis and survival rates and that is because the average survival time for female ALL patients with t (12.21) positive was 41.87 ± 4.81 months and for male patients was 33.83 ± 7.18 months.

It is worth noting that other studies have not suggested gender as an effective factor in the prognosis of patients and only Mr. Jorge E Cortes and his colleagues in the review article stated that the incidence of ALL in all age groups in males is higher than that of females [16]. Age in people with ALL with t (12.21) is considered as a prognostic factor, and people between one and ten years have a better prognosis than those who are outside this age range [15,17,19].

Ching-Hon and his colleagues in 2004, in the New England Journal of Medicine, suggested age as an important factor in the prognosis of ALL patients [15].

In the study of the St Jude Children's Research Hospital, 847 ALL children with four therapeutic protocols were enrolled from 1991 to 2006, and the results of the study showed that children aged 1 to 9 years were more successful than infants and adults. The five-year EFS reported the survival rate for patients aged 1 to 9 years 88 %, for patients aged from10 to 15 years 73 %, for patients over 15 years of age 69 %, and for children less than 12 months, 44 %. Infants less than 6 months of age had significantly poor prognosis [17].

Jorge E. Cortes and his colleagues reported age distribution in ALL patients as a bimodal pattern, with an initial peak of 5-3 years of age and a secondary peak of over 50 years of age [16].

As the average age of ALL patients with t (12.21) positive was 2.61 ± 4.89 years, our findings confirmed that the incidence of t (12.21) in children is higher than that of adults, as 50 % Of the total population we studied (second quarter) were under 5 years old and 75 % of the total affected population (third quarter) were under the age of 7 years. As indicated, age is a prognostic factor in people with ALL with t (12.21) and people

between one and ten years old have better prognosis and therapeutic treatment than those who are outside of this age range, which of course it was not confirmed in our study due to the low sample size, and Kaplan-Meier tests showed that there was no significant difference between the survival rate and the age group of our subjects.

According to the WHO definition, when the Hb concentration, hematocrit (PCV), or red blood cell count reaches below the reference (according to age, sex, and geographical location), the person has anemia [14]. According to this, when the hemoglobin level is less than 13.5 g/dl in males and less than 12 g/dl in females, when the hematocrit level is less than 39 % in males and less than 36 % in females and when the red blood cell count is less than 5.5 million in males and less than 4 million in female, disease is considered as an anemia [14].

From other influential factors in making appropriate treatment protocol, the patient white blood cell count can be mentioned, So as the patient white blood cell count is closer to the normal range, the disease's prognosis will be better, the survival rate will be higher and with fewer deaths, and the standard treatment protocol can be adopted for him [15,17,20]. Hoelzer described WBC30≥$\times 10^9$/ L, being over 35 years of old and CR for more than 4 weeks as unfavorable criteria of ALL patients [6]. Katarjian and his colleagues presented the disadvantaged criteria with the WBC≥ 50×10^9/ L in addition to positive Philadelphia chromosomes. Gaynor et al. introduced WBC≥ 20×10^9 /L, being over 60 years of age and CR over five weeks with positive Philadelphia chromosome as factors affecting poor prognosis [16].

In this study, the average white blood cell count in t (12.21) positive ALL patients in northeastern Iran was 39.77 ± 62.36 ($10^9 \times 10^9$).

Also, based on the white blood cell count, patients were classified to less than 10 WBC (10^9/L) in group 1, 10-49 WBC (10^9/L) in group 2, 50-99 WBC (10^9/L) in group 3 and more than 100 WBC (10^9/L) in group 4. Most of the patients (53.33 %) were in the second group and 21.67 % of the patients were in second group.

We then examined the white blood cell count and the survival rate in the patients. As a next step, we used the Kaplan-Meier analysis and the standard log test. The highest death event (60 %) was observed in the fourth group and no death was observed in the first group. In the Kaplan Meier chart, four groups of white blood cell count were different in terms of survival rate, so that the highest survival rate was observed in the first group (47 months) and the lowest survival rate was in the fourth

group (18 ± 9 months). So, as expected, with the increase in the number of white blood cells relative to the reference level, the survival rate was reduced in patients.

The result of the log rank test with $P_V = 0.06$ shows that if the study limits are eliminated, there is probably a significant difference between the survival rate and the WBC count.

When the relationship between the death event and platelet count was assessed by ranking leg test in t (12.21) positive patients in northeastern parts of Iran, it was found that the highest death rate was occurred in the first group with a platelet count of less than 50,000 (µl) (80 %) and an average survival rate was 33.8 ± 7.1 months and in the third group, which had more than 150,000 (/µl) of platelets, there was no death and the survival rate was 46.9 months to the endpoint of our study.

Therefore, as the platelet count is closer to the normal range, the survival rate will be higher in patients which is important in the adoption of appropriate supportive therapies, such as platelet injections. Although the Kaplan-Meier survival curve confirmed this issue in our study, no significant difference was seen by the log rank test with PV=0.43.

Unlike chronic leukemia is often accompanied with organomegaly, the incidence of organomegaly is lower in acute leukemia [14].

This was confirmed in our study, so that 22.58 % of t (12.21) positive patients were involved with splenomegaly and 24.19 % of patients had hepatomegaly, while Jorge E. Cortes et al. reported an incidence of lymphadenopathy and splenomegaly 80 % and 75% respectively which was completely contradictory with our findings [16].

Following the repeated emphasis on a good prognosis of t (12.21) in ALL patients [15-21], we examined the average survival time between 1392 and 1392 in mentioned patients. During a telephone conversation with t (12.21) positive ALL patients, we collected the information such as the date of diagnosis, the date of death or the last follow-up, the applied treatment protocol and the recovery process.

Following the analysis and interpretation of the results, the log rank test showed that the average survival time during the period of experiment was 39.76 ± 4.77 months and the 95 % confidence interval for the survival of patients was equal to 49.11 and 30.41. Based on the Kaplan-Meier test, the survival rate of patients was estimated at 75% which was consistent with other studies and results in this regard (figure 3). Ching-Hon Pui and colleagues reported a survival rate of 80 %

and Jorge E. Cortes 90 % for 4 years survival rate [15,16].

CONCLUSION

In this study, the prevalence of t (12.21) in ALL patients in northeast Iran was 14.4 %, which was lower than the global average. As the presence of fusion mRNA Is accompanied with a proper response and prognosis (51-53) and cause the using of Standars Risk Chemotherapy therapeutic protocol with the lower probability of developing a disease for the patient and reduces the need for bone marrow transplantation, Therefore, the lower prevalence of this translocation than the global average brings up the probability of an increase in the prevalence of acute lymphoblastic leukemia-lymphoma without any specific genetic abnormalities (NOS subgroup) or other recurrent genetic abnormalities in ALL including t (9.22) or the involvement of chromosome 11 with poor prognosis and requires severe treatment protocols and even bone marrow transplantation. On the other hand, the survival rate of 75 % and the average five-year survival rate in these patients was 39.77 ± 4.77 months based on log rank test which indicates a good therapeutic potential and prognosis of this disease.

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