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Investigation of miRNA Makeup in Iraqi Patients with Chronic Lymphocytic Leukemia.

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ABSTRACT

B-cell chronic lymphocytic leukemia (CLL) is the most common human leukemia in adults MicroRNAs is considered as new tool in cancer detection, diagnosis and treatment. MicroRNA expression can be used to predict patients fate. this study aimed to evaluate miR15a, miR16-1, miR155, miR,34a, b, and c expression to predict its role as a biomarker in Chronis Lymphocytic Leukemia patients before and after treatment with Fludarabine. Total RNA was extracted by trizol from the blood plasma of patients comparing to healthy group individuals. Complementary DNA was used to measure the expression of those miRs by RT-qPCR. The results were indicated: it was the expression level of miR-15a\16-1 was slightly low in the CLL patients. MiRA34b/miR34c and miR-34a which are tumor suppressor genes, was slightly low in the patients CLL. miR-155 levels were significantly high in patients before treatment and it may use as a prognostic bio marker for the worse fate to the patients with chronic lymphocytic leukemia

Keywords: MicroRNA(miR), Chronic Lymphocytic Leukemia.

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INTRODUCTION

Chronic lymphocytic leukemia is the most common malignant leukemia in adults, it occurs in the western world more than the Asian world (1,2,3) representing (25%-30%) of all leukemia types(4,5). Chronic lymphocytic leukemia classified according to its risk stratifications into indolent and aggressive depending on molecular and cytogenetic features associated with each form (1). Both of these forms are characterized by general symptoms: fever, fatigue, night sweats and loss Weight as well as accumulation of immune incompetent CD 5+, CD 19+ and CD 23+, B lymphocytes in the bone marrow, secondary lymph organs and peripheral blood due to failed in apoptosis process (6,7) Patients survival in CLL still around 66% of all patients overall the word during 5 years of diagnosis. This type of leukemia affects elderly people, with highest incidence among ages (60-80) years, and rarely affects people younger than 50 years of age (8). and its prevalence in men more than (2:1.5%) of women (9). Two major systems were used for risk assessment and disease staging, those are Ria that distributes patients into five stages(0, I,II,III and IV) and Binet which devided into three stages (A -low risk, B-moderate and C- high) systems those were suggested in 1975 and 1977 respectively. According to Ria and Binet, Patients classified as low and moderate risk (score I and II) will develop lymphocytosis (more than 5000 monoclonal lymphocytes/ml), with or without lymphadenopathy and hepatosplenomegaly, patients' lives could be extended for more than 10 years after diagnosis with minimal treatment. This indolent pattern associated with frequent chromosomal 13 deletions (Del 13q14) which occurs in more than 50% of CLL patients and deletion in long arm of chromosome 11 (Del 11q) in (15-20%) of CLL patients (10). In other side high risk patients show the aggressive form of the disease (stage III/IV) when 3-5 areas developed lymphadenopathy, anemia or thrombocytopenia or both, with overall survival rate between 2-4 years (1). The aggressive form of chronic lymphocytic leukemia is correlated with the deletion of the short arm of chromosome 17 (Del 17p) which occurs in (5-10%) of CLL patients. This numerical chromosomal aberration associated with final bad fate due to the fact that deleted region contains the tumor suppressor gene (P53) that plays a vital role in direct the incompetent lymphocytes into apoptosis (11).

MicroRNA has gained great interest since its discovery in 1993 by Lee and her colloquies, for its role in regulating cell fate, life and functions (12), by interpretation in many cellular processes such as development, differentiation, survival, reproduction, and cell death, and it gained a special place in the medical field as a tool for diagnosis and classification of tumors. There are evidences refer to the role of microRNA in cancer initiation and progression through enhance cell proliferation and reduce cell programmed death (12,13). These tiny molecules can enhance or inhibit the development of malignancy resembling way to oncogenes and tumor suppressor gene products actions. This indicates that microRNA can be the exact RNA (OncomiR) or tumor suppression (14).

MicroRNA is a short piece of non-coding RNA molecules composed of 19 to 22 nucleotides in length (15,16,17,18), containing a seed area at the 5`, that composes of 1-7 or 2-8 bases that strongly required for the precise selection of target mRNA (19). These tiny molecules which encoded by single or cluster of genes, regulate the gene expression by inhibiting translation, deadenylation and degradation to reduces the stability of the targeted mRNA (19,20,21). the complete integration of microRNA with the 3`UTR of the target mRNA leads to the destruction of this mRNA, while the semi-complete integration inhibits the translation of the target mRNA (22). The first link between pathogenicity of CLL and microRNAs was demonstrated by Calin and his group in 2002. They found that: the expression of miR-15a/16-1 is low in patients who suffer from chronic lymphocytic leukemia. Then it was discovered that: miR-15a / 16-1 cluster gene located at the deleted region at chromosome 13q14. Extensive studies elucidated that: miR-34b / miR-34c lies in the long arm of the 11 (Del 11q) chromosome which is closer to the deleted region in patients with chronic lymphocytic leukemia. Also; miR-34b / miR-34c expression is controlled directly by P53. So, deletion of TP53 on the short arm of the chr.17 (Del 17p) was found associated with high levels of miR-34b / miR-34c in CLL patients. Now there a complicated network composed of at least 18 different microRNAs associated with CLL pathogenicity (23). This study aimed to evaluate several microRNA as a biomarker for CLL prognosis.

MATERIAL AND METHODS

This study included (81) samples collected from two groups: patients group composed of (51) of patients with chronic lymphocytic leukemia (CLL), (21) of them were newly diagnostic and without treatment yet with Fludarabin and (30) patients after treatment, the diagnosis of patients were confirmed by hematologists in the educational Baghdad Hospital /medical city and The National Center of Hematology /Al

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Mustansria University according to detection of B-cell cluster of differentiation by Flow cytometry to identify lymphocyte CD markers (CD 5,19,20,22 and 23), complete blood picture and bone marrow biopsies. Patients age ranged between (35-75) years. The second group was a control group, consisted of (30) apparently healthy individuals. The members of this group were similar to those of age and sex. Total RNA was extracted from the blood plasma of patients and healthy individuals by trizol according to the instructions of the Kit. Then extracted RNA were converted to cDNA by specific primers for miR15a/16-1 and miR155, miR 34a/34b and miR 34c. table (1) shows primers sequences. RT-PCR technique was used to measure changes in gene expression, RNU expression used as a housekeeping gene to normalize the expression of miRs. Fisher exact test were done as statistical analysis to the miR15a/16-1 and miR155, miR 34a/34b and miR 34c, In patients before and after treatment comparing to the control group.

Table 1: MiRs primers used in RT-qPCR

Primers	Sequences
miR-15a	F: GGG TAG CAG CAC ATA ATAT R: GTT GGC TCT GGT GCA GGG TCC GAG GTATCC GCA CCA GAG CCA ACC ACA AA
miR-16-1	F:GTT TGG TAG CAG CAC GTA AAT A R:GTT GGC TCT GGT GCA GGG TCC GAG GTA TTC GCA CCA GAG CCA ACC GCC AA
miR-34a	F:GTG TGG CAG TGT CTT AGC " R:GTT GGC TCT GGT GCA GGG TCC GAG GTA TTC GCA CCA GAG CCA ACA ACT CA
miR-34b	F:GGT AGG CAG TGT CAT TAG R:GTT GGC TCT GGT GCA GGG TCC GAG GTA TTC GCA CCA GAG CCA ACC AAT CA
miR-34c	F:GGA GGC AGT GAT GTT AGC " R:GTT GGC TCT GGT GCA GGG TCC GAG GTA TTC GCA CCA GAG CCA CAA TC
miR-155	F: GTG GGT TAA TGC TAA TCG TGA " R:GTT GGC TCT GGT GCA GGG TCC GAG GTA TTC GCA CCA GAG CCA CCC CT
RNU-1	F: GTGAACTTATTGACGGGCG R: GTT GGC TCT GGT GCA GGG TCC GAG GTA TTC GCA CCA GAG CCA ACA ATC AG

RESULTS

In this study, patients with CLL who diagnosed and treated in Baghdad Hospital /Medical City/ Ministry of Health and the National Hematology Center /Al Mustanserhia University, were classified according to Ria and Binet systems depending on the clinical features of the patients, peripheral lymphocytes CD markers and complete blood investigation. As shown in figure (1) lymphocyte CD markers in newly diagnostic CLL patients were distributed as CD5 about 80%, CD19 about 84%, CD20 about 75%, CD22 about 61% and CD23 was 56%. Patients age were (35-75 years) with median 55 years.

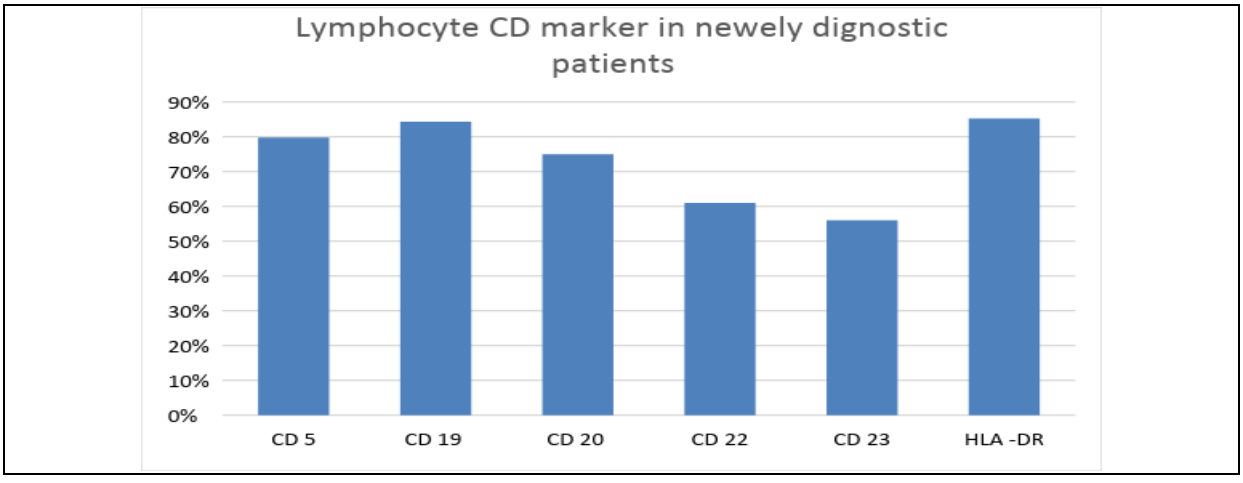

Figure 1: Distribution of CD markers on the surface of lymphocytes in newly diagnostic CLL patients.

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It's found also that (19 patients whom composed 90.5%) of newly diagnostic patients had splenomegaly, 7(33.3%) of them had hepato-spleno megaly and 15 (71.4%) had lymphadenopathy as shown in table (2), the percentages of CLL patients in each stage of CLL. It's found that one third of patients in at diagnosis time were relatively in low and moderate risk stages (one patient at Binet A/ Ria 0, I and six patients at Binet B/ RIA II) and the other two third were at relatively high risk (4 at Binet B/Ria III, 4 at Binet C/Ria III and Binet C/Ria IV). Also one third of patients who received Fludarabin as a treatment (3-10 doses) were considered at low risk of CLL (two at BinetA/ Ria 0, I and seven at Binet B/ RIA II). Patients with high risk under treatment with Fludarabin were (9 at Binet B/Ria III, 9 at Binet C/Ria III and 4 at Binet C/Ria IV).

Table 2: Chronic Lymphocytic Leukemia patients staging according to Ria and Binet.

Relative risk	Stage	% of Newly diagnosed patients	% of patients After treatment
Low risk	Binet A/ RIA 0,I	1 (4.7%)	2 (6.6%)
Moderate risk	Binet B/ RIA II	6 (28.5%)	7 (23.3%)
High risk	Binet B/ RIA III	4 (19%)	9 (30%)
	Binet C/ RIA III	4 (19%)	9 (30%)
	Binet C/ RIA IV	6 (28.5%)	3 (10%)
	Total	21	30

MicroRNA expression

The expression of the level of microRNAs (miR-15a, miR16-1, miR155, miR34a, miR34b and miR34c) were measured in the healthy groups and CLL patients. A slightly lower level of miR-16-1 in the patients before treatment (0.064 ± 0.004), than the healthy group (0.0772 ± 0.022) with no significant differences in the expression, but patients after treatment show elevated level of miR16-1(0.104 ±0.013) with significant differences than the control group as shown in figure (2).

The level of miR-15a was (0.04± 0.017) for the patients before treatment while it was (0.03± 0.001) for the healthy controls with no significant differences. While patients after treatments showed slightly higher expression level (0.04± 0.002) with no significant differences than the expression of the patients before treatment, as shown in figure (2).

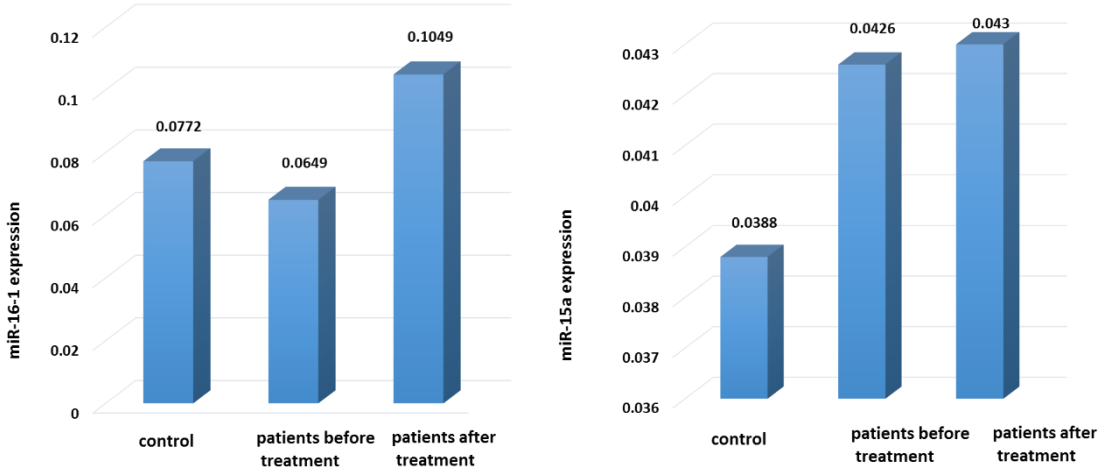

Figure 2: Expression of miR16-1 and miR15-a in patients before and after treatment comparing with the control group.

The results of miR-155 showed that there was significant difference in the expression between patients before treatment (0.03± 0.004) and the healthy group (0.0166± 0.002) and it was significantly higher

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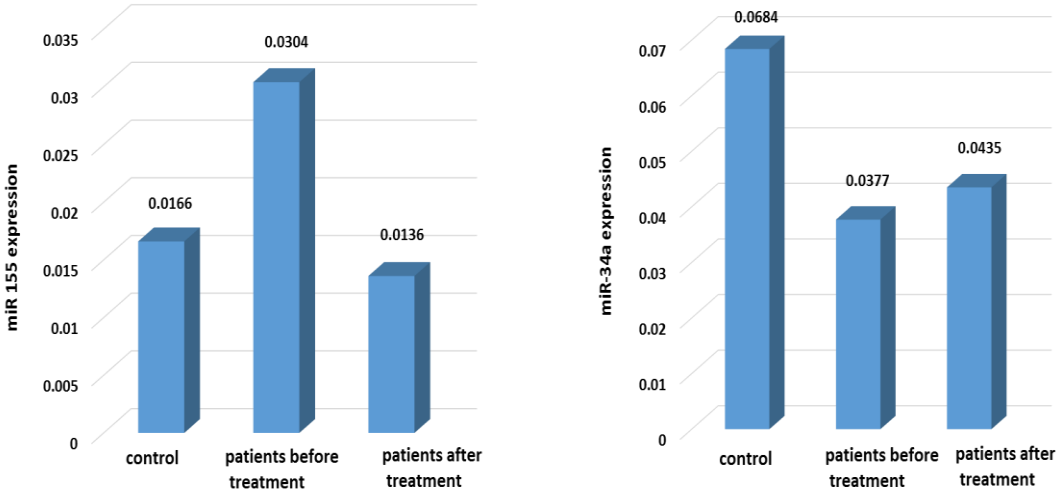
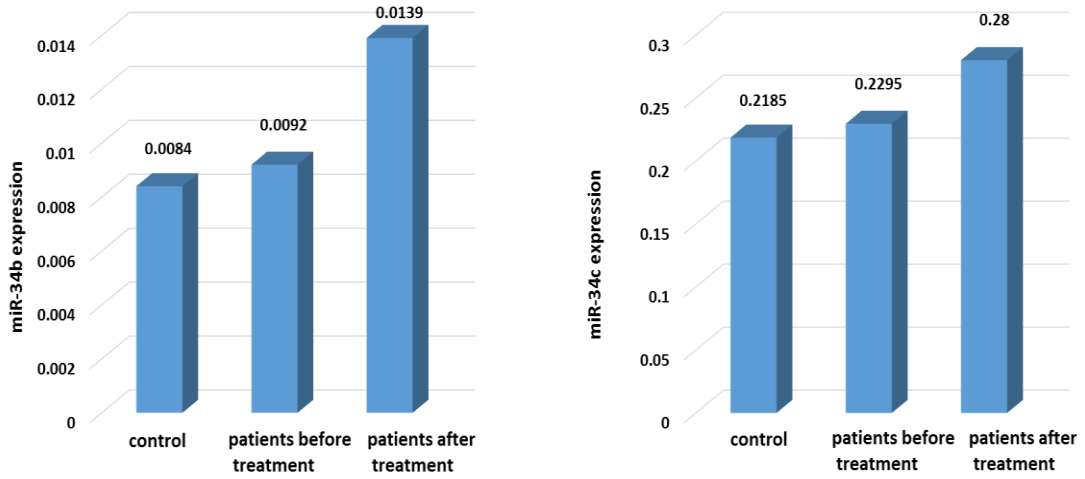
than the expression after treatment (0.0136± 0.002). The results of the miR-34a expression showed a significant increase in the level of miR-34a expression for the healthy group (0.0684 ± 0.003) than the patients before treatment (0.037 ± 0.002) and patients after treatment (0.435 ±0.003) while there are no significant differences between patients before and after treatment as shown in figure (3).



Figur e 3: Expression of miR-155 and miR-34a in patients before and after treatment comparing with the control group.

While the miR-34b levels explained in figure (4) showed no significant differences between the healthy groups (0.0084 ± 0.001) and patients before treatment 0.009± 0.003 and patients after treatment (0.01± 0.003) as well as there is no significant differences between them. the expression levels of miR 34c were shown in figure(3).



Figur e 4: Expression of miR-34band miR-34c in patients before and after treatment comparing with the control group.

DISCUSSION

Chronic lymphocytic leukemia is one of most common blood malicious disease in adults. The newly diagnostic patients in this study showed typical symptoms of the disease that characterized with high percentage of B-lymphocytes with CD 5, CD19,CD20,CD22 and CD23 which were 80%, 84%, 75%, 61% and 56% respectively, and 90.5 % of them had splenomegaly and 71.4% lymphadenopathy with (3-5) of enlarged

lymph nodes, and only 7(33.3%) had hepatomegaly. These findings and the findings of Ria and Binet systems, reflects the stage and the progression of the disease. Its characterized by accumulation of malignant B-cells in peripheral blood, bone marrow, secondary lymph nodes and spleen. Patient treated with purines' analog drug (Fludarabine) to enhance patient's response against malignant B-cell proliferation (24)

In current study the expression of miR-16-1, miR15a, miR155, mir34a, miR43b and mir34c expression were measured by real time PCR technique, in patient with CLL to evaluate miRs ability to reflect CLL progression and deducing studied microRNAs as a prognostic biomarker for CLL. Micro RNAs are short sequence of RNA that controls and regulate enormous cell functions by reduce mRNA stability via complete or partial complementary binding with mRNA leading to destruction of mRNA and break protein synthesis series. It was found that miRs react in similar way to products of tumor suppressor genes or oncogene in controlling cell cycle and programmed cell death as well as proliferation of cells. Clian and his colleagues in 2002, were pointed for the first time, to the pathogenic role of non-coding microRNA in chronic lymphocytic leukemia during looking for the hypothesized tumor suppressor genes at chromosome 13q14 in CLL patients. Instead they found that miR15a/miR 16-1 gene clusters were located at that region of chromosome 13q14, the most often deleted region in CLL patients (25). They found also that miR15a/miR 16-1 expression is down regulated and in revers proportion with BCL2 (the anti-apoptotic protein) in CLL patients. In current study expression of miR16-1/miR15a in patients before treatment was slightly lower than the expression of the control group without significant differences , that may be related to deletion of chromosome 13q14 region in some patients but the expression in the patients after treatment returns to its expected levels significantly at($p \leq 0.05$) after treatments due to the role of Fludarabine drug in killing the malignant B-lymphocytes that carry this chromosomal aberration, and allowing the normal karyotype B-cell to take their protective roles. As the expression of miR16-1/miR15a is not significantly lower than the control group we think that's related to two suspected reasons, the first one is that only some of the patients from patients before treatments had deletion in the chromosome 13q14 region so not all of them had reduced miR16-1 and miR15a levels (26,27,28,29,30). The second causes may be due to mutation in p53 gene rather that p53 deletion which included in chromosome 17p13 region which is observed in 50% of patients with chronic lymphocytic leukemia, the RNA miR-15a / 16 -1 (31,32). the mutation in p53 lead to reduce its function but not losing all its controlling role to miR16-1/miR15a Those two reasons may lead to slightly lower the expression of miR16-1/miR15a.

The results of miR-155 showed that there was significant difference in the expression between patients before treatment (0.03± 0.004) and the healthy group (0.0166± 0.002) and it was significantly higher than the expression after treatment (0.0136± 0.002). The role of microRNA (miR-155) acts as an oncogene that stimulates B lymphocytes division and migration and inhibit cell cycle arrest (33). It was also observed that the high level of RNA expression (miR-155) In patients with chronic lymphocytic leukemia is associated with worse fate to the patients more than those with a lower level of expression (34,35). A decrease in the levels of miR-34a gene expression in patients with chronic lymphocytic leukemia due to a mutation in the TP53 gene leads to a decrease in programmed death (36,37), and may indicate that patients they may carry different chromosomal patterns, and some patients may carry the GG-type inhibitor inhibit splitting (38) Observed in the cancer of the colon and rectum (39) and stomach cancer (40). The microRNA (miR-34b\34c) is a tumor suppressor gene that controls the cell cycle and programmed death in the B-cell. The high level of expression is associated with chronic lymphocytic leukemia and is the deletion of chromosome 11 (Del 11q23) is one of the main reasons for the low microRNA expression (miR-34b \ 34c) in patients with chronic lymphocytic leukemia (41). This type of microRNA is also found under the healthy TP53 gene. On the short arm in chromosome 17 (17P) or its mutation, which was observed in patients with chronic lymphocytic leukemia (42), this (MiR-34b \ 34c) inhibits gene expression of genes BCL-2, Notch1, cyclin E2, cyclin-dependent kinases 4 and 6 (CDK4 and CDK6), through its association with the microRNA of these genes, and in the case of the deletion or mutation of the TP53 gene that controls the miR-34 family, this causes the loss of control of the cellular cycle and the programmed death control (43).

CONCLUSION

Mir 15al, miR16-1,mir 34a,b and c in patients before treatment had not have significantly differences than control groups so they did not give an valuable results as a bio markers, while miR-155 had the most prognostic value and it could be used as a biomarker for progression chronic lymphocytic leukemia and may give an insight to patients fate.

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