**Assessment of miR-150-5 p as diagnostic and prognostic markers of B- chronic Lymphocytic Leukemia in chronic hepatitis C**

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**Abstract:**

**Aims:** the current study aimed to investigate the role of miR-150-5 p expression as a diagnostic and prognostic marker of B- CLL among Egyptian patients with chronic hepatitis C and its impact on risk stratification and response to therapy.

**Patients & Methods**: This study was conducted on 25 patients with chronic hepatitis C recently diagnosed as CLL and 25 healthy control All studied patients were subjected to entire history, taking the complete clinical examination, and laboratory investigations including serum LDH, serum β2 microglobulin (β2M), CD38 and ZAP70 expression by flow cytometry. We measured miR-150-5 p expression by qRT-PCR.

**Results:** The results of this analysis indicated that CLL patients had a significantly lower level of miR-150-5 p expression (0.464±0.16) compared to the control group (0.8±0.76), P ˂0.001\* Additionally, the expression levels of miR-150-5 p were significantly inversely correlated with laboratory and clinicopathological features of CLL as well as the severity of CLL according to Modified Rai staging system and Binet staging system. Interestingly, concerning the treatment of CLL, there were significantly lower levels of miR-150-5 p in no treatment subgroup (n=18, 0.49±0.13) compared to successfully treated subgroups (n=7, 0.77±0.07), P ˂0.001\*

**Conclusions**: miR-150-5 p expression level was downregulated in CLL patients, particularly patients with HCV infection, and significantly inversely correlated with the severity of CLL. Thus, it could be helpful for diagnostic and prognostic purposes in CLL.

***Keywords:*** HCV; CLL; MicroRNAs; MiR-150-5 p, Expression; CD38; ZAP70; qRT-PCR; β2 microglobulin; Flow cytometry.

**Introduction**

Hepatitis C virus (HCV) is a hepatotropic and lymphotropic virus, and its infection affects the B lymphocyte compartments. HCV has been implicated in the pathogenesis of chronic lymphocytic leukemia (CLL) [1]. A vast and growing literature firmly supports the hypothesis that CLL is the most frequent hematological cancer noted in the aging group of patients [2].

There is gathering proof that CLL is deemed as the dysregulated proliferation of lymphocytes in the blood and other organs [3,4]. But interestingly, It is becoming more widely accepted that there is a wide range of clinical and pathological features of CLL due to its complexity [5,6 ].

MicroRNAs (miRNAs) are a class of endogenous non-coding RNA molecules that regulate vital biological processes, including cell development and malignant transformation. For example, in cancer, miRNAs can act as oncogenes or tumor suppressor genes by regulating the expression of genes that control cell proliferation, differentiation, apoptosis, invasion, and metastasis [7].

Accumulated experimental and clinical evidence indicates that miR-150 is an immuno-miR regulating immune functions, such as proliferation, apoptosis, and differentiation of NK, T, and B cells [8] and lymphocyte activation [9].

It is well established that miRNAs could be used not only as diagnostic and prognostic biomarkers but also as predictive biomarkers. Elegant studies have evaluated the role of early cancer diagnosis in successful and effective treatment. However, inopportunely, many cancer patients are diagnosed late due to insufficient early screening [10]. Thus, this study aimed to gain further insights into the role of miR-150-5 p expression as a diagnostic and prognostic marker of B- CLL among Egyptian patients with chronic hepatitis C and its impact on risk stratification and response to therapy.

**Subjects**

This case-control study was conducted on 25 adult patients of both sexes with CLL, and in addition to the 25 healthy control individuals,' the age and sex matched the cases. The diagnosis of CLL was based on current WHO guidelines [11]. Among CLL, we selected 18 Egyptian patients (72%) who were either newly diagnosed with CLL or in the “watch and wait” phase. Those patients were assigned to wait and see strategy and were monitored periodically every three months for disease progression. Interestingly, seven Egyptian patients (28%) were indicated for therapy. The treatment modalities included FCR. (Fludarabine, cyclophosphamide, and rituximab) And ibrutinib and the response to treatment were assessed according to IWCLL [11]. The risk stratification of the patients was done according to modified Rai and Binet staging systems. The Ethics Committee of the Faculty of Medicine, Zagazig University, approved the study protocol with (IRB no. 10213). All participants were assigned informed agreement before their inclusion. The flow chart of the study is demonstrated in figure 1.



Fig . **Flowchart of the study.**

**Laboratory tests**

Blood samples were taken from all registered participants from the Departments of Internal medicine, Clinical Oncology, Nuclear Medicine, and Tropical Medicine. Testing was done according to operating techniques in Zagazig university hospital and Medical Biochemistry laboratories, as shown in figure1.

**miR-150 expression levels by real-time PCR**

According to the company's instructions, the RNA was extracted from EDTA peripheral blood samples. The mRNA expression of the sequences of primers: miR-150 forward: 5′-GCTCTCCCAACCCTTGTACC -3′, reverse: 5′-GTGCAGGGTCCGAGGT-3 and U6 was used as a housekeeping gene; the relative expression level was determined using the 2-ΔΔCT method.

**Statistical analysis**

Statistical analyses were performed by SPSS-26 (SPSS Inc, United States). Mean ± SD, or median, was used to express all the data in this study. A chi-square test was used for categorical variables to compare different groups. Student’s t-test was used for quantitative variables to compare between two studied groups. Analysis for receiver operating characteristic (ROC) with the area under the curve (AUC) was used to evaluate the diagnostic value of miR-150-5 p. We considered P significant at <0.05 with a 95% confidence interval (CI).

**Results**

As expected, there were significant differences between the studied groups regarding CLL patients' clinical characteristics, as demonstrated in Table 1.

Table . clinical characteristics of studied patients.

|  |  |  |  |
| --- | --- | --- | --- |
| **Parameters**  | **Control group, (n=25)** | **CLL group, (n=25)** | **P value**  |
| **Age (years)** | 66.86±5.93 | 63.57±6.32 | 0.065 |
| **Sex [n (%)]:**MaleFemale | 13(52%)12(48%) | 11(44%)14(56.7%) | 0.389 |
| **B symptoms**NoYes | 25 (100%)0 (0%) | 10(40%)15(60%) | ˂0.001\* |
| **Fever:**NoYes | 25 (100%)0 (0%) | 10(40%)15(60%) | ˂0.001\* |
| **Weight loss:**NoYes | 25 (100%)0 (0%) | 14 (56.7%)11(44%) | ˂0.001\* |
| **Bleeding:**NoYes | 25 (100%)0 (0%) | 19 (76%)6 (24%) | ˂0.001\* |
| **Spleen:**NoYes | 25 (100%)0 (0%) | 13(52%)12(48%) | ˂0.001\* |
| **Lymphadenopathy:**NoYes | 25 (100%)0 (0%) | 19(76%)6(24%) | ˂0.001\* |
| **Anemia:**NoYes | 25 (100%)0 (0%) | 7 (28%)18 (72%) | ˂0.001\* |
| **Thrombocytopenia:**NoYes | 25 (100%)0 (0%) | 16 (64%)9 (36%) | ˂0.001\* |
| **AIHA**NoYes | 25 (100%)0 (0%) | 23 (92%)2 (8%) | ˂0.001\* |
| **ITP**NoYes | 25 (100%)0 (0%) | 23 (92%)2 (8%) | ˂0.001\* |

CLL; chronic Lymphocytic Leukemia, AIHA; autoimmune hemolytic anemia, ITP; immune thrombocytopenia, p-value significant when p ≤ 0.05.

The current research concerning laboratory tests showed significant differences between the studied group's regards. WBC, ALC, hemoglobin, platelets. As expected, there were significantly higher values of WBC and ALC in the CLL group compared to controls. On the other hand, there were significantly lower values of hemoglobin and platelets in the CLL group compared to the control group. Concerning severity and diagnostic markers, LDH, serum β2M, ZAP-70% 17p del/ p53 mutation, and CD38% were significantly higher in the CLL group than in controls. Regards risk stratification, according to the Rai staging system, 10(40%) patients had low risk, 9(36%) patients had intermediate risk, and 6(24%) patients were high-risk. (Table 2, P˂0.01\*). We also used the Binet staging system to evaluate CLL’s severity further. According to Binet staging system, 11(44%) patients in stage A, 8(32%) patients in stage B and 6(24%) patients in stage C. (Table 2, P˂0.01\*).

Table . laboratory characteristics of the studied patient.

|  |  |  |  |
| --- | --- | --- | --- |
| **Parameters**  | **Control group, (n=25)** | **CLL****group, (n=25)** | **P value**  |
| WBC (× 109/L) | 6.13±1.93 | 78.70±55.11 | ˂0.001\* |
| ALC (× 109/L) | 2.73±0.93 | 58.484±46.32 | ˂0.001\* |
| Hemoglobin (g/dl) | 13.5±0.61 | 10.3±1.96 | ˂0.001\* |
| Platelets (× 109/L) | 237.2± 35.3 | 192.4±96.5 | ˂0.001\* |
| AST (IU/L) | 18.59±3.9 | 19.4±3.2 | 0.719 |
| ALT(IU/L) | 21.93±3.5 | 22.9±5.3 | 0.412 |
| Total bilirubin (mg/dl) | 0.988±0.133 | 1.001±0.272 | 0.7943 |
| Albumin (g/dl) | 4.14±0.16 | 4.10±0.22 | 0.465 |
| S. creatinine (mg/dl) | 0.72±0.22 | 0.74±0.25 | 0.576 |
| Alpha-fetoprotein(ng/ml) | 8.89±2.9  | 8.9 ±5.2 | 0.592 |
| LDH | 200.2± 41.3 | 422.4±137.5 | ˂0.001\* |
| Serum β2M (ng/dl) | 2.52 ± 0.77 | 5.80 ± 1.26 | ˂0.001\* |
| ZAP-70%ZAP-70- (<20%)ZAP-70+(≥ 20%) | 25(100%)0(0%) | 17(68%)8(32%) | ˂0.001\* |
| CD38%CD38- (<20%)CD38+ (≥20%) | 25(100%)0(0%) | 16 (64%)9 (36%) | ˂0.001\* |
| 17p del/ p53 mutationPositiveNegative | 0(0%)25(100%) | 3(12%)22(88%) | ˂0.001\* |
| Modified Rai staging system:Low riskIntermediate riskHigh-risk | 0(0%) | 10(40%)9(36%)6(24%) | ˂0.001\* |
| Binet staging system.Stage AStage BStage C | 0(0%) | 11(44%)8(32%)6(24%) | ˂0.001\* |

Hb; hemoglobin, WBC; white blood cells, ALC; absolute lymphocyte count, AST; aspartate aminotransferase, ALT; alanine transaminase, LDH; lactate Dehydrogenase ZAP-70%; Zeta-chain-associated protein kinase 70. A chi-square test was used for categorical variables to compare different groups. Student’s t-test was used for quantitative variables. \*P < 0.05 when compared with the control group.

**The Circulatory miR-150-5 p expression levels in studied groups.**

CLL patients (0.464±0.16) had a significantly lower level of miR-150-5 p expression than the control group (0.88±0.12), figure 2, p ˂0.001\*.



Figure . **Comparison between studied groups regards relative expression level of miR-150-5 p.**

**The relationship between the relative expression level of miR-150-5 p and clinicopathological characteristics in CLL patients.**

According to the current study, there were statistically significant differences regards HCV infection percentage among CLL patients, with about 19 (76%) of CLL patients not infected with HCV and 6 (24%) of CLL patients infected with HCV P ˂0.001\*. In addition, 11(55%) of patients had B symptoms, 6(24%) of patients had Lymphadenopathy and 12(48%) of patients had HSM. (Table 3).

Table . The relationship between the relative expression level of miR-150-5 pand clinicopathological characteristics in CLL patients.

|  |  |  |
| --- | --- | --- |
| **Parameters**  | **CLL patients (n=25)** | **miR-150-5 p** |
| Non-HCV HCV P value | 19 (76%)6 (24%)˂0.001\* | 0.466±0.2060.61±0.160.051 |
| B symptoms [n (%)]PresentAbsentP value | 10(40%)15(60%) | 0.57±0.150.49±0.070.289 |
| Site of involvement [N (%)]LymphadenopathyHSM P value | 6 (24%)12(48%) | 0.47±0.800.51±0.010.604 |
| ZAP-70%ZAP-70- (<20%)ZAP-70+(≥ 20%)P value | 17(68%)8(32%) | 0.77±0.150.52±0.190.003 |
| CD38%CD38- (<20%)CD38+ (≥20%)P value | 16 (64%)9 (36%) | 0.75±0.140.53±0.190.006 |
| 17p del/ p53 mutationPositiveNegative | 3(12%)22(88%) | 0.533±0.050.71±0.200.138 |
| Modified Rai staging system:Low riskIntermediate riskHigh-riskP value | 10(40%)9(36%)6(24%) | 0/75±0. 20.59±0.010.46±0.20.008 |
| **Binet staging system.**Stage AStage BStage CP value | 11(44%)8(32%)6(24%) | 0.76±0.30.57±0.20.46±0.10.004 |
| **Treatment of CLL**No treatment Chemotherapy P value | 18(72%)7(28%)  | 0.49±0.130.77±0.07˂0.001\* |

**The relationship between the relative expression level of miR-150-5 p and treatment in CLL patients.**

In the current study, 18(72%) patients were newly diagnosed with CLL or in the “watch and wait” phase. Those patients were assigned to wait and see strategy and were monitored periodically every three months for disease progression. As a result, seven Egyptian patients (28%) were indicated for therapy and successfully treated with a good response.

The treatment modalities included FCR. (Fludarabine, cyclophosphamide, and rituximab) and ibrutinib. Interestingly, there were significantly lower miR-150-5 p in the no-treatment subgroup (n=18, 0.49±0.13) compared to successfully treated subgroups with good response (n=7, 0.77±0.07), table 3.

**Pearson correlations between** **the relative expression level of miR-150-5 p with clinical and** **laboratory parameters among the CLL group**

The relative expression level of miR-150-5 p was negatively correlated with WBC ALC, serum β2M, ZAP-70%, CD38%, and LDH. In contrast, miR-150-5 expression level was positively associated with hemoglobin, and platelet, P ˂0.001\* (Table 4).

Table . **Correlation of serum levels of miR-150-5 p with clinical and laboratory characteristics in CLL patients.**

|  |  |
| --- | --- |
| **parameters** | **miR-150-5 p** |
| ***r*** | **p** |
| WBC | -0.842 | ˂0.001\* |
| ALC  | -0.820 | ˂0.001\* |
| Hemoglobin  | 0.723 | ˂0.001\* |
| Platelets  | 0.446 | ˂0.001\* |
| AST  | -0.138 | 0.341 |
| ALT | -0.162 | 0.260 |
| Total bilirubin  | -0.179 | 0.213 |
| Albumin  | 0.158 | 0.274 |
| S. creatinine  | 0.076 | 0.599 |
| Alpha-fetoprotein | -0.098 | 0.374 |
| LDH | -0.528 | ˂0.001\* |
| Serum β2M | -0.478 | ˂0.001\* |
| ZAP-70% | -0.384 | ˂0.001\* |
| CD38% | -0.503 | ˂0.001\* |
| 17p del/ p53 mutation | -0.276 | 0.182 |

**linear regression analysis in the CLL group**

According to the current findings, the miR-150-5 p levels had significant correlations with different laboratory parameters. To address this point, we applied linear regression analysis. As a result, we revealed that ALC and platelets were the main predictors of the relative expression levels of miR-150-5 p among other laboratory tests, P ˂0.001\* (Table 5).

Table . linear regression analyses to examine the main independent variables against the relative expression level of miR-150-5 pwithin CLL patients.

| Model | Unstandardized Coefficients | Standardized Coefficients | t | P value  | 95% C.I. |
| --- | --- | --- | --- | --- | --- |
| B | S.E. | Beta | Lower Bound | Upper Bound |
| 1 | Constant | -0.704 | 0.187 |  | -3.764 | 0.000 | -1.080 | -0.328 |
| WBC | 0.001 | 0.001 | 0.109 | 0.952 | 0.346 | -0.001 | .002 |
| ALC | 0.102 | 0.018 | 0.670 | 5.841 | ˂0.001\* | 0.067 | 00.138 |
| Hemoglobin | 0.001 | 0.001 | 0.147 | 1.222 | 0.228 | 0.000 | 0.002 |
| Platelets | 0.077 | 0.030 | 0.505 | 2.574 | ˂0.001\* | 0.017 | 0.137 |
| LDH | 0.000 | 0.000 | -0.181 | -1.040 | 0.303 | -0.001 | 0.000 |

**The accuracy of** **miR-150-5 p expression levels for discriminating CLL from control by ROC analysis**

The exciting finding of the current research is that the power of miR-150-5 p expression levels to differentiate CLL from control was evaluated using ROC analysis. The AUC was 0.965 (95% CI = 0.901-1.000) with sensitivity = 96.2%, specificity = 98 %, and the cutoff values (0.553), figure 3, P-value ˂0.001\*.



Fig . **Receiver operating characteristic curve of the relative expression levels of miR-150-5 p for diagnosis CLL.**

**Discussion**

Several studies reported that HCV infects not only hepatocytes but also extrahepatic cells, for example, B lymphocytes [12]. The oligoclonal proliferation of the infected B-cells and the presence of HCV in lymphocytes could initiate growth dysregulation and predispose the lymphocyte to develop molecular changes and malignant B-cell lymphoproliferative disease, for example, CLL [13]. Indeed, the prevalence of monoclonal B-cell lymphocytosis (MBL) in HCV-infected patients was higher than in the general population [14].

CLL is more prevalent in the elderly, and to overcome any drawbacks in the results, we selected a control group matched to the CLL group regarding age and sex. Therefore, the present study aimed to explore the prevalence of HCV infection among studied CLL patients, and we detected that there were statistically significant differences regards HCV infection percentage among CLL patients, with about 19 (76%) of CLL patients not infected with HCV and 6 (24%) of CLL patients were infected with HCV.

Similarly, Vladareanu et al. observed a higher frequency of chronic lymphoproliferative disorders related to HCV infections in particular older women [15]. They contributed their findings to persistent activation by HCV leading to B–clone proliferation in the bone marrow and liver and fatal alteration. HCV antigens such as E2 protein have the binding site CD 81, representing the specific hepatocyte and B–lymphocyte receptor for HCV[16].

On the contrary, other studies detected non-statistically significant differences regards the prevalence of HCV infection among CLL [patients [17,18]. This difference could be due to the small sample size and the low prevalence of HCV in the countries where these studies were conducted [19].

This is consistent with a previous finding that miRNAs abundantly expressed in CLL may regulate the expression of genes encoding proteins participating in the critical molecular pathways, such as those involved in BCR signaling [20]. Interestingly, the most important results of this analysis indicated that CLL patients had a significantly lower level of miR-150-5 p expression than other studied groups. Additionally, the expression values of miR-150-5 p were significantly inversely correlated with laboratory and clinicopathological features of CLL as well as the severity of CLL according to the Modified Rai staging system and Binet staging system.

In line with this, Ling et al. study found miR-150 as a tumor suppressor in osteosarcoma. Concerning the influence of treatment with doxorubicin-induced apoptosis by targeting RUNX2, they revealed that its level improved after treatment. Remarkably, these findings propose that miR-150 could be a potential therapy in future osteosarcoma treatment [21]. Similar results detected by Mraz et al. postulated that low-level expression of miR-150 was associated with unfavorable clinical and prognostic markers of CLL [22].

Similar effects were observed in Szurián et al. research, which investigated miR-150 in progenitor B- and T-cells and observed under-expression in them. Nevertheless, miR-150 overexpressed in mature cells. Intriguingly they detected that the epigenetic biomarker was correlated with poor prognosis in CLL [23].

However, compared to our data, Chiaretti et al. observed a significant upregulation of mir-150 in CLL patients compared to the healthy group and recommended that mir-150 could initiate or progress proliferation in CLL. However, they recommended further investigations to elucidate these findings [23]

Interestingly, concerning the treatment of CLL, In the current study, 18(72%) patients did not receive treatment as we mentioned before, and seven Egyptian patients (28%) received treatment modalities, including FCR. (Fludarabine, cyclophosphamide, and rituximab) and ibrutinib. Interestingly, there were significantly lower miR-150-5 p in the non-treatment subgroup than in successfully treated subgroups. But on the other hand, treating patients with CLL increases miR-150-5 p levels. So, we could use this epigenetic marker as a diagnostic and prognostic marker.

Despite these pieces of evidence, there is a substantial gap in our knowledge about the diagnostic and prognostic role of miR-150-5 p in hematological cancers. To address these issues, we further evaluated our findings with Pearson correlations. We detected that the relative expression level of miR-150-5 p was negatively correlated with WBC ALC, serum β2M, and ZAP-70%, respectively CD38%, and LDH. In contrast, miR-150-5 expression level was positively correlated with hemoglobin and platelet. In addition, linear regression analysis revealed that ALC and platelets were the main predictors of the relative expression levels of miR-150-5 p among other laboratory tests.

To further understand the observations, we performed a ROC analysis. The power of miR-150-5 p expression levels to differentiate CLL from control had sensitivity (96.2%) and specificity ( 98 % ).

**Conclusions**: The current research findings revealed that miR-150-5p values were under-expressed in CLL patients, particularly patients with HCV infection, and significantly inversely correlated with clinical and laboratory characteristics as well as the severity of CLL. Thus, it could be helpful for diagnostic and prognostic purposes in CLL.

**The limitation and recommendations of the study**

The fundamental limitations of the current study were the small sample size of the analysis and the case-control study design, as we needed to assess the prevalence of HCV in CLL. Therefore, we recommend further extensive cross-sectional studies and multicenter to confirm the study results so we can use mir-150-5p diagnostic and prognostic epigenetic markers in CLL.

**List of abbreviations**

CLL: Chronic lymphocytic leukemia.

HCV: hepatitis C virus.

MiRNAs: MicroRNAs.

β2M: β2 microglobulin .

CD38: Cluster of differentiation 38

ZAP70: Zeta-chain-associated protein kinase 70

RT-PCR: A real-time polymerase chain reaction

IWCLL: International Workshop on Chronic Lymphocytic Leukemia

ALC: absolute lymphocyte count

**Footnotes.**

**Peer-Reviewers:** Amr Hanafy (Professor of internal medicine), Samia Hussien (Assistant Professor of biochemistry), Marwa Shabana (Assistant Professor of clinical pathology), Ola Elfarargey (Professor of medical oncology).

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**Data availability**

All the data obtained and analyzed are included in this manuscript.

**Conflicts of interest**

The authors declare that they have no competing interests.

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**Authors, contributions:** all authors participated in collecting data, writing the manuscript, and revising it.

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