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Expression of MicroRNA-21 in Chronic Lymphoproliferative Disorders: A Cross-Sectional Study from India

Running Title: *Expression of MicroRNA 21 in CLPD*

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Abstract: ***This cross-sectional study evaluated the expression of microRNA-21 (miRNA-21) in patients newly diagnosed with chronic lymphoproliferative disorders (CLPD) at a tertiary hospital in Delhi, India, between April 2023 and April 2024. A total of 26 patients were included and miRNA-21 levels were measured using quantitative PCR. Although overall miRNA-21 expression was elevated in CLPD compared to standard values, statistical significance was observed only in patients with Hodgkin and Non-Hodgkin lymphomas. No significant correlation was found between miRNA-21 expression and clinical staging systems, including Rai, Ann Arbor, and ISS. These findings suggest a potential role for miRNA-21 in lymphoproliferative diagnostics, warranting further studies with larger sample sizes for validation.***

Keywords: miRNA-21, chronic lymphoproliferative disorders, lymphoma, cancer biomarker

1. Introduction

The term lymphoproliferative diseases refer to a group of illnesses characterised by the uncontrolled growth of lymphocytes, which can cause bone marrow infiltration, lymphadenopathy and monoclonal lymphocytosis. These disorders commonly affect immunocompromised individuals. They arise from the unregulated growth of T, B or NK lymphocytes, resulting in immunoproliferative diseases associated with immune system dysfunction and lymphocyte dysregulation [1].

Naturally occurring, non-coding and single-stranded RNAs known as microRNAs are essential for controlling cell development, proliferation, apoptosis and cancer development [2]. At the post-transcriptional level, miRNAs control gene expression. Aberrant miRNA expression has been linked to many different types of cancer. OncomiRNA21, known for its strong carcinogenic properties, has been found to be upregulated in multiple cancers, with its role first identified in gliomas. In addition to suppressing tumor suppressor genes, miRNA21 is associated with resistance to various cancer treatments. Recent research suggests that miRNA21 can be used as a treatment target for malignancy as well as a biomarker for early detection of malignancy.

This study aims to evaluate the expression of miRNA-21 in chronic lymphoproliferative disorders and assess its association with clinical staging in different subtypes.

Lacunae in existing knowledge

There is a paucity of published studies pertaining to expression of miRNA-21 in chronic lymphoproliferative disorders from India.

Rationale of the study

Compared to standard invasive biopsy used for lymphoma diagnosis, which is often uncomfortable, painful and risky for patients, circulating miRNA are non-invasive, less painful and less risky. Repeated blood sampling can be done in a patient undergoing cancer treatment for screening /monitoring of cancer. Identifying non-invasive biomarkers like miRNA-21 may improve early detection and disease monitoring in hematological malignancies, offering an alternative to invasive diagnostic procedures.

2. Material and Methods

This cross-sectional observational study was conducted among newly diagnosed CLPD presented to tertiary care hospital, Delhi from April 2023 to April 2024. The study included 26 patients adhering to the following inclusion and exclusion criteria:

Inclusion criteria

1) Newly diagnosed CLPD patients aged 12 years or older.
2) Patients who provided informed consent.

Exclusion criteria

1) Patients with acute leukemia.
2) Patients with chronic myeloproliferative neoplasms.

Sample Size

The study enrolled 26 patients who met the inclusion and exclusion criteria during the study period.

Data Collection and Investigations

Patient demographic and clinical characteristics were recorded using a predesigned proforma. A thorough clinical evaluation was done, followed by blood investigations, radiological imaging, bone marrow or lymph node biopsies were conducted as needed. Clinical staging was done for each patient. CLL staging was done using Rai staging, Lymphoma staging was done using Ann Arbor staging and Multiple Myeloma staging was done using ISS staging.

For miRNA extraction and expression analysis, 3 mL of venous blood was collected in EDTA vials. miRNA-21 was extracted using the miRNeasy Micro Kit and its expression was measured via quantitative real-time PCR, with miR-103 as the endogenous control. To standardize the assay, blood samples from 10 healthy volunteers were analyzed, yielding a median ΔCt value of 0.29.

Expression Analysis

The following formulas were applied:

- $\Delta Ct = Ct\ (miR\text{-}21) - Ct\ (miR\text{-}103)$.
- Fold change = $2^{-\Delta\Delta\ ct}$,
  where $\Delta\ \Delta\ ct = \Delta$ ct of sample (test or control)- average Δ ct of control.
- miR-21 relative expression was calculated as the average fold change within each group.

Statistical Analysis

Data were analyzed using SPSS version 25. The Mann-Whitney U test assessed differences in miR-21 expression, while Spearman's correlation was used to determined relationships between miR-21 levels and clinical staging of CLL and lymphoma. A p-value < 0.05 was considered statistically significant.

Ethical Considerations

The study was approved by the Institutional Ethics Committee of Maulana Azad Medical College (F.1/IEC/MAMC/MD/MS 96/02/2023/No. 163).

3. Results

Patients Demographics and characteristics

All the subjects included in the study were above 12 years of age. The mean age of the study population was 47.4 years (range was 15 – 75 years). Patients were Indian and predominantly from northern eastern states. Out of the 26 patients recruited in the study, 16 patients (61.5 %) were male and 10 patients (38.4%) were female. The Male: Female ratio was 1.6 :1 in the study population.

Comorbidities

Most common comorbidity was hypertension (4 patients, 15.3%). 2 patients had history of coronary artery disease (7.6%), 1 patient was hypothyroid (3.8%), 1 patient was diabetic (3.8%), 1 patient was prediabetic (3.8%) and 1 patient was HBsAg positive (3.8%).

Clinical features at presentation

Of the 26 patients enrolled in the study, 5 (19.2%) presented with ascites, 4 (15.3%) with hepatosplenomegaly, 9 (34.6%) with splenomegaly, 7 (26.9%) with hepatomegaly, 15 (57.6%) with lymphadenopathy, 11 (42.3%) with pallor and 4 (15.3%) with B symptoms.

Diagnosis

Figure 1 depicts diagnosis of study population.

Staging

Among the 7 CLL patients, 1 was in Rai stage 0, 1 in Rai stage I, 4 were in Rai stage II and 1 was in Rai stage III. None of the patients were classified as Rai stage IV. Among the 15 lymphoma patients, 1 was in Ann Arbor stage I, 2 were in stage II, 3 were in stage III and 9 were in stage IV. Among the 4 Hodgkin lymphoma patients, none were in Ann Arbor stage I or III, 2 were in stage II and 2 were in stage IV. Among the 11 Non-Hodgkin lymphoma patients, 1 was in Ann Arbor stage I, none were in stage II, 3 were in stage III and 7 were in stage IV. One patient was diagnosed as Hairy cell leukemia. No formal staging available for Hairy cell leukemia .Among the 3 Multiple Myeloma patients, none were in ISS stage I, 2 were in stage II and 1 was in stage III.

Treatment Outcome

Out of 26 patients, 6 patients of CLL (Rai stage 0,1 and 2) had no indications for chemotherapy and were advised to undergo follow-up, while rest of the 20 patients were advised for treatment. Among 20 patients advised for treatment, 19 patients opted for treatment while 1 patient of B-cell NHL declined cancer treatment. Among those who opted for treatment response to treatment was noted. 11 patients showed a complete response, 2 had a partial response, 5 had stable disease, 0 experienced disease progression and there were 7 cases of mortality.

Expression of miRNA-21 in CLPD

Table 1 summarises expression of miRNA -21 in CLPD. Figure 2 depicts Box plot graph for miRNA-21 relative expression in CLPD.Table 2 summarises Mann Whitney U test among various CLPD subgroups and standard value.

There was statistically significant increased expression of miRNA-21 in HL and NHL as compared to standard value. Figure 3 depicts box plot graph for miRNA-21 relative expression in HL and NHL.

Correlation of miRNA-21 expression with clinical staging of CLPD

CLL

Spearman correlation test was done: correlation between Rai stage and miR-21fold change is not statistically significant (p value=0.96) due to small sample size, highlighting the need for a larger sample in future studies. Figure 4 depicts Spearman correlation between Rai staging of CLL and miR-21 fold change.

Lymphoma

Spearman correlation test was done: Spearman Correlation Coefficient= 0.318 and P-Value= 0.248. The correlation coefficient (0.318) indicates a weak positive correlation between Ann Arbor stages and miR-21 fold change values.However, due to small sample size this correlation is not statistically significant (p-value =0.248), highlighting the need for a larger sample in future studies. Figure 5 depicts Spearman correlation between Ann Arbor staging of Lymphoma and miR-21 fold change

Multiple Myeloma

Mann Whitney U test was done which revealed that there is no statistically significant difference between the fold change of miRNA-21 for ISS Stage 2 and Stage 3 in this dataset (p value = 0.667) due to small sample size, highlighting the need for a larger sample in future studies.

Table 1: Expression of miRNA -21 in CLPD

	miR-21 relative expression	Standard deviation
Standard value for miRNA kit used	1.545	1.632
CLPD	5.963	11.009
MM	0.539	0.668
CLL	0.650	0.323
HL	11.039	8.290
NHL	12.407	16.316
Lymphoma	12.065	14.469

Table 2: Mann Whitney U test among fold change various CLPD subgroups and standard value

Mann Whitney U test between fold change of standard value and fold change of following subtype of CLPD	p value
CLPD	0.297
CLL	0.364
Multiple Myeloma	0.161
Hodgkin Lymphoma	0.008
Non Hodgkin Lymphoma	0.044
Lymphoma	0.008

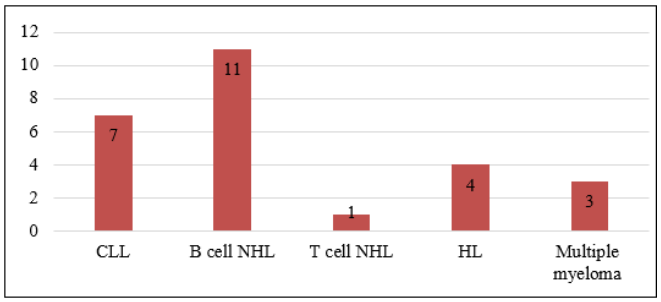

Figure 1: Bar diagram - Diagnosis of study population

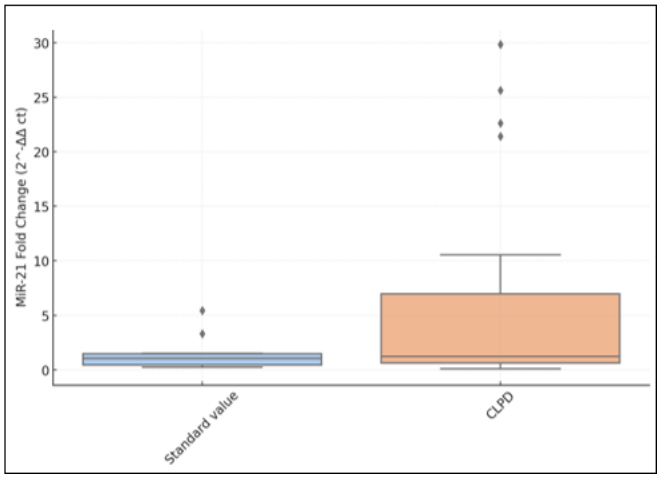

Figure 2: Box plot graph- miRNA-21 relative expression in chronic lymphoproliferative disorders

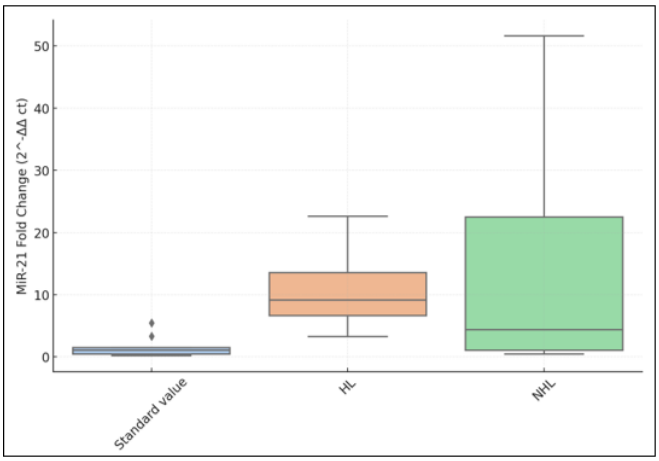

Figure 3: Box plot graph- miRNA-21 relative expression in HL and NHL

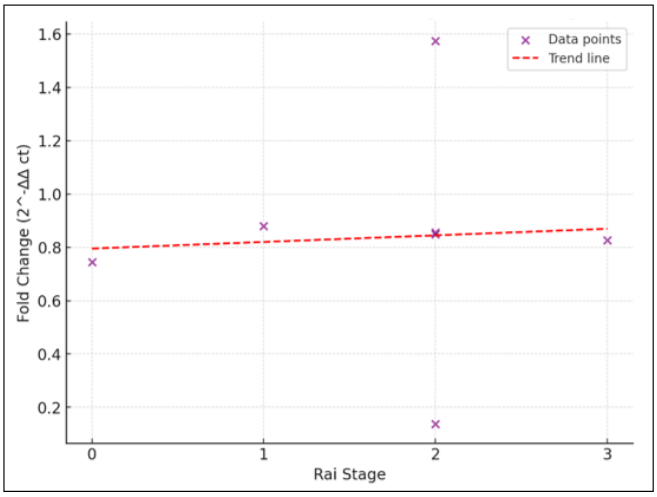

Figure 4: Spearman correlation between Rai staging of CLL and miR-21 fold change

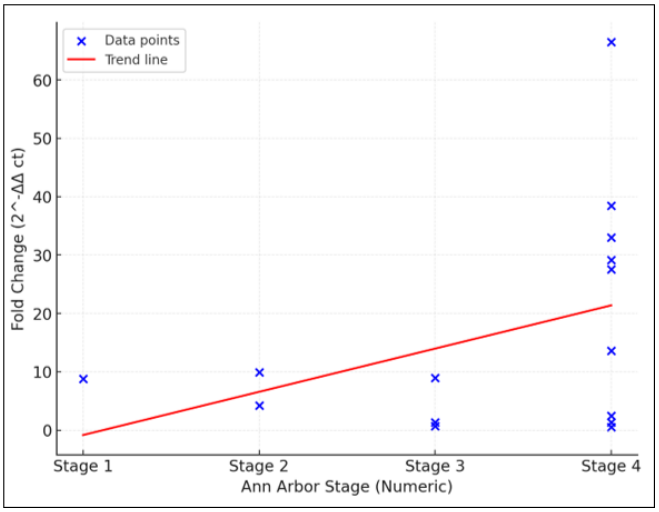

Figure 5: Spearman correlation between Ann Arbor staging of Lymphoma and miR-21 fold change

4. Discussion

MiRNA -21 expression in chronic lymphoproliferative disorders

In our study, miRNA-21 relative expression in CLPD was higher than standard value but due to small sample size it was not statistically significant. MiRNA-21 relative expression in HL and NHL was statistically higher than the standard value and miRNA-21 relative expression in CLL and MM was lower than the standard value, though this difference was not statistically significant due to small sample size.

Several studies have explored the expression of miRNA-21 in various lymphoproliferative disorders. A.L. Gkioka et al. [3] in Greece, in a study involving 48 multiple myeloma patients, reported that plasma miRNA-21 expression at diagnosis and before treatment initiation was significantly lower than in healthy subjects. W. Chen et al. [4] in China found significantly increased serum miRNA-21 expression in 62 DLBCL patients. L. Gu et al. [5], also in China, demonstrated that miRNA-21 was significantly upregulated in activated B-cell-like DLBCL compared to germinal center-like DLBCL cells and inhibition of miRNA-21 led to suppressed proliferation and invasion, alongside increased apoptosis in DLBCL. H. Go et al. [6] in South Korea, in a study of 200 DLBCL patients, found significantly upregulated miRNA-21 expression in DLBCL tissue compared to control tonsils. J. Li et al. [7] in China, in a study involving 112 DLBCL patients, showed markedly higher serum miRNA-21 expression in patients compared to healthy controls. Similarly, Q. Ji et al. [8] in China reported significantly elevated serum miRNA-21 expression in 156 patients with primary gastrointestinal DLBCL compared to healthy controls. K. Jones et al. [9] in Australia found higher circulating cell-free miRNA-21 levels in 42 patients with classical Hodgkin lymphoma. In CLL, N. R.-Lafuente et al. [10] in Spain observed miRNA-21 expression levels similar to normal B cells in a study of 16 CLL patients. However, V. Fulci et al. [11] in Italy reported a 3.4- to 4-fold overexpression of miRNA-21 in 56 CLL patients.

C. H. Lawrie et al. [12][13] in the United Kingdom conducted two studies on DLBCL. The first, involving 60 patients, found significantly higher serum miRNA-21 levels in DLBCL patients compared to controls ($p = 0.04$). The second study, which included 98 patients with DLBCL and FL, showed a 2.11-fold overexpression of miRNA-21 compared to normal lymphocyte populations. Francisco J. et al. [14] in Spain, in a study of 111 HIV-1-infected patients, including 37 with classical HL, found plasma miRNA-21 levels to be overexpressed in classical HL patients at diagnosis.

These studies highlight the significance of miRNA-21 as a biomarker. Larger studies are needed to further explore the clinical significance of miRNA-21 expression in CLPD and related disorders.

5. Limitations of this Study

The sample size of our study was small and hence, the study was not powered enough to achieve statistically significant correlations. Further studies are needed to investigate the correlation between miRNA-21 expression and individual subgroups of CLPD, using larger sample sizes, to enable comparison of miRNA-21 with invasive diagnostic tests. Further studies are needed to investigate the correlation between miRNA-21 expression and treatment outcomes of CLPD, using larger sample sizes.

6. Conclusion

This study highlights the increased expression of miRNA-21 in patients with chronic lymphoproliferative disorders, particularly in Hodgkin and Non-Hodgkin lymphomas. While no significant correlations were observed with clinical staging systems, the findings reinforce the potential of miRNA-21 as a non-invasive biomarker. Future research involving larger patient cohorts is essential to validate these initial observations and to explore its diagnostic and prognostic utility more comprehensively.

Ethical Consideration

Each subject was given a consent form to fill. Subjects were explained the purpose of study and he/she had the right to quit at any time without providing any reasons. Patient's information was dealt with confidentiality. Subjects between 12-17 years of age were given an assent form to their parent/guardian for consent. The study was approved by the Institutional Ethics committee (IEC) of Maulana Azad Medical College and associated hospitals vide F.1/IEC/MAMC/MD/MS 96/02/2023/No.163.

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Abbreviations

1) CLL – Chronic Lymphocytic Leukemia
2) CLPD – Chronic Lymphoproliferative Disorder
3) HL – Hodgkin Lymphoma
4) ISS – International Staging System
5) MM – Multiple Myeloma
6) NK cell – Natural Killer Cell
7) NHL – Non-Hodgkin Lymphoma
8) SPSS – Statistical Package for the Social Sciences

Conflict of interest: None

Financial disclosure: None

Running Head: Expression of miRNA 21 in CLPD

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