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## ORIGINAL ARTICLE

# Prognostic Values of MicroRNA-21 and Ki-67 in Diffuse Large B-Cell Lymphoma Patients: Egyptian Experience

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### SUMMARY

***Background:*** **MicroRNA-21 (miR-21) is a small non-coding RNA which influences tumorigenesis by inhibiting the expression of target genes. Ki-67 is a nucleolar antigen highly correlated with the rate of proliferating cells. In this study, we aimed to evaluate the prognostic impact of miR-21 and Ki-67 in DLBCL disease in a cohort of Egyptian patients.**
***Methods:*** **We prospectively enrolled 53 newly diagnosed DLBCL patients. RT-PCR was used to evaluate the plasma expression levels of miR-21. Tissue Ki-67 was assessed using immunohistochemistry (IHC) of lymph node biopsy sections. Overall survival (OS) and progression free survival (PFS) were the primary outcomes.**
***Results:*** **miR-21 expression was significantly higher in patients with DLBCL in comparison to controls ($p < 0.001$). The median Ki-67 expression was 70% and positivity ranged from 25% to 100%. Response to treatment was achieved in 23 patients (43.4%). Higher miR-21 was associated with poor response to treatment ($p = 0.03$). Although patients' age was a significant predictor of OS in univariate analysis, none of the studied factors could predict OS in multivariate analysis. However, we found that Ki-67 expression was a significant predictor of PFS in both univariate and multivariate analyses.**
***Conclusions:*** **The study suggested that plasma miR-21 might be a valuable non-invasive prognostic marker of response to treatment in DLBCL patients. Moreover, Ki-67 is a potential significant predictor of both OS and PFS in those patients.**
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### KEY WORDS

diffuse large B cell lymphoma, miR-21, Ki-67

### INTRODUCTION

Non-Hodgkin lymphoma is a lympho-proliferative neoplasm with diffuse large B-cell lymphoma (DLBCL) accounting for about 40% of all cases. In spite of the recent advances in diagnostic and therapeutic strategies, large number of cases still have poor prognosis. Thus, introduction of novel biomarkers may improve diagnostic and therapeutic potentials [1,2].
MicroRNAs (miRs) are non-coding RNAs comprising with approximately 22 nucleotides. Their main function

is post transcriptional modification of gene expression to inhibit polypeptide translation. Altered expression of miRs has been linked with different malignancies with some acting as oncogenes and others as tumor suppressors [3,4]. Accumulating evidence showed their significance in both B-cell differentiation and lymphomagenesis [5,6]. Several studies reported miR-21 over-expression in many cancers including solid tumors (lung, stomach, prostate) as well as chronic lymphocytic leukemia [5]. In addition, its association with the pathogenesis of DLBCL was recently studied [7].

Ki-67 is a nuclear antigen expressed in all active stages of the cell cycle [8]. Some studies confirmed the correlation between the proliferation index of Ki-67 and different grades and clinical behavior of tumors. Evaluation of its expression has become a routine part of cancer workup especially breast cancer and lymphoid neoplasms [9-11] and its tissue levels were correlated with miR-21 expression [12]. In the present study, we aimed to examine the plasma miR-21 expression level and Ki-67 expression in lymph node sections of DLBCL patients and correlate them with clinical and pathological features in a cohort of Egyptian patients. In addition, we assessed if miR-21 expression is related to Ki-67 expression.

## MATERIALS AND METHODS

The present study was conducted in the period from January 2017 through December 2018. The study protocol was approved by the local ethical committee and informed consent was obtained from all participants.

The study included 53 newly diagnosed DLBCL patients. Patients in all stages of the disease were included. Fifty age and gender matched healthy blood donners were enrolled as controls. A medical history and clinical and laboratory data were collected from all patients, especially age, gender, performance status, presence of B symptoms, presence of extra nodal sites, Ann Arbor clinical stages, serum lactate dehydrogenase (LDH) level, and the International Prognostic Index (IPI) score.

Plasma samples were collected from both patients and healthy controls at the time of diagnosis. Molecular study to assess expression of plasma miR-21 was performed using quantitative real-time polymerase chain reaction (RT-PCR). In addition, lymph node biopsy or lymphoid tissue samples were obtained for detection of Ki-67 expression using immunohistochemistry.

### miR-21 expression analysis

Before receiving chemotherapy, 3 - 5 mL of venous blood was collected under complete sterile conditions. Plasma samples were isolated after centrifugation and preserved at -80°C until analysis.

Total RNA, including miR, was extracted following steps of the manufacturer's instructions using miRNeasy Serum/Plasma Kit (cat. no. 4427975) supplied by QIAGEN.

Five µL of extracted total RNA was used as a template for synthesis of cDNA performed using TaqMan MicroRNA reverse Transcription Kit (cat. no. 4366596) (Applied Bio-system). As recommended in the assay instructions provided, 15 µL of reverse transcription reaction components was used as a total volume.

This was followed by quantitative real-time PCR for detection of miR-21 expression using TaqMan® Small RNA Assay kit (cin-217004) and TaqMan® universal Master Mix II kit (cat. no. 4440043) (Applied Bio-system). The total reaction volume was 20 µL, and subsequent reaction conditions were as follows: 95°C for 10 minutes then 45 cycles at 95°C for 15 seconds followed by 60°C for 1 minute.

PCR primers were supplied by Applied Bio-systems; miR-21 (hsa-miR-21; ID 000397) (target gene) and U6 (U6 snRNA; ID 001973) (reference control gene).

The expression of the miR-21 gene was analyzed using the relative quantification method and the results were expressed by $2^{-\Delta\Delta CT}$ where CT is cycle threshold and $\Delta\Delta CT$ = (CT miR-21 - CT U6) patient sample - (CT miR-21 - CT U6) control sample [13].

### Immunohistochemistry detection of Ki-67

Paraffin sections obtained from lymph node/lymphoid tissue blocks were made at 4 mm thickness and mounted on positive charged glass slides followed by incubation for an hour at 37°C and another at 65°C for accurate adhesion. Immunostaining was done for all sections using Automated Bench-Mark ULTRA IHC/ISH system using the following steps:

- De-paraffinization followed by cell conditioning for 80 minutes and a reaction buffer added (PH 7.4 - 7.8) for antigen retrieval. The slides were then incubated with 100 µL of the diluted antibody (1:100 dilution) of concentrated mouse monoclonal anti Ki-67 (Clone GM-010 by Genemed, Isotype IgG2b) at 42°C for 32 minutes.
- Di-amino-benzidine (DAB) was applied as a chromogen (NexES Ultra View DAB Detection Kit) and counterstained with hematoxylin II for 8 minutes. Finally, post counter staining with blueing reagent for 4 minutes. Then slides were extracted, washed for 5 minutes, dehydrated in alcohol for 5 minutes and cleared in Xylene.
- Phosphate-Buffered Saline (PBS) was incubated with the tissue sections instead of primary antibody and used as negative controls where other sections known to be immune positive were used as positive controls.
- For each slide, results were scored semi-quantitatively. Stained nuclei were considered positive regardless of its intensity. Hot spots of neoplastic cells were determined and the proportion of Ki-67 positive cells out of 1,000 cells were calculated as a percentage (Figures 1, 2).

Other assessed parameters included performance status, Ann Arbor staging, and international prognostic index (IPI). Patients were treated using the standard CHOP regimen with a median observation time of 10.42 months. The primary study outcomes included treat-

**Table 1. Relationship between treatment response and clinical and laboratory data.**

| | All patients n = 53 | CR n = 23 | No CR n = 30 | p-value |
|---|---|---|---|---|
| Age (years) median (IQR) | 58.0 (47.0 - 67.0) | 59.0 (43.0 - 68.0) | 57.5 (48.0 - 65.8) | 0.91 |
| Females n (%) | 28 (52.8) | 12 (52.2) | 16 (53.3) | 1.0 |
| Performance status n (%) | | | | |
| I | 36 (67.9) | 16 (69.6) | 20 (66.7) | 0.2 |
| II | 12 (22.6) | 6 (26.1) | 6 (20.0) | |
| III | 4 (7.5) | - | 4 (13.3) | |
| IV | 1 (1.9) | 1 (4.3) | - | |
| Extranodal infiltration n (%) | 23 (43.4) | 8 (34.8) | 15 (50.0) | 0.41 |
| Tumor stage n (%) | | | | |
| I - II | 16 (30.2) | 9 (39.1) | 7 (23.3) | 0.35 |
| III - IV | 37 (69.8) | 14 (60.9) | 23 (76.7) | |
| LDH (U/L) median (IQR) | 381.0 (249.0 - 552.5) | 290.0 (223.0 - 395.0) | 453.0 (320.0 - 690.0) | 0.004 |
| International prognostic index n (%) | | | | |
| Low risk | 14 (26.4) | 9 (39.1) | 5 (16.7) | 0.16 |
| Intermediate risk | 26 ( 49.1) | 11 (47.8) | 15 (50.0) | |
| High risk | 13 ( 24.5) | 3 (13.1) | 10 (33.3) | |
| miR-21 median (range) | 2.96 (0.1 - 34.5) | 2.4 (0.1 - 10.0) | 3.5 (0.2 - 34.7) | 0.03 |
| Ki-67 median (range) | 70.0 (25.0 - 100.0) | 60.0 (25.0 - 98.0) | 70.0 (25.0 - 100.0) | 0.15 |

**Table 2. Cox regression analysis for predictors of overall survival.**

| | Univariate analysis | | Multivariate analysis | |
|---|---|---|---|---|
| | HR (95% CI) | p-value | HR (95% CI) | p-value |
| Age | 1.05 (1.4 - 1.1) | 0.035 | 1.05 (0.99 - 1.1) | 0.096 |
| Gender | | | | |
| Male | Ref. | - | - | - |
| Female | 0.88 (0.28 - 2.7) | 0.83 | - | - |
| Extranodal infiltration | 0.4 (0.11 - 1.5) | 0.17 | - | - |
| LDH | 0.99 (0.99 - 1.1) | 0.26 | - | - |
| Ki-67 | 1.02 (0.99 - 1.1) | 0.13 | 1.02 (0.99 - 1.05) | 0.25 |
| miR-21 | 0.97 (0.88 - 1.08) | 0.62 | - | - |

**Table 3. Cox regression analysis for predictors of progression free survival.**

| | Univariate analysis | | Multivariate analysis | |
|---|---|---|---|---|
| | HR (95% CI) | p-value | HR (95% CI) | p-value |
| Age | 1.03 (0.99 - 1.07) | 0.16 | 1.02 (0.97 - 1.07) | 0.45 |
| Gender | | | | |
| Male | Ref. | - | - | - |
| Female | 1.02 (0.43 - 2.44) | 0.96 | - | - |
| Extranodal infiltration | 0.95 (0.39 - 2.39) | 0.9 | - | - |
| LDH | 1.0 (0.99 - 1.1) | 0.65 | - | - |
| Ki-67 | 1.03 (1.4 - 1.06) | 0.024 | 1.03 (1.01 - 1.06) | 0.03 |
| miR-21 | 1.8 (0.94 - 1.08) | 0.81 | - | - |

ment response and patients' survival at the end of follow-up.

The collected data were analyzed using SPSS 25 (IBM, USA). Numerical data were presented as mean and standard deviation or median and range while categorical data were expressed as number and percent. Statistical comparisons were achieved using *t*-test, Mann-Whitney U test or chi-squared test. Kaplan-Meier test was used for survival analysis. Cox regression analysis of factors potentially related to survival was performed to identify the independent factors of survival. All reported p-values were two-tailed and $p < 0.05$ was significant.

## RESULTS

The present study was conducted on 53 newly diagnosed DLBCL patients. The control group comprised 50 healthy adults. miR-21 expression was significantly higher in patients in comparison to controls ($p < 0.001$). Regarding Ki-67 expression, we found that the median Ki-67 expression was 70% and positivity ranged from 25% to 100%. We defined the patient's subgroups as high expressors and low expressors for both miR-21 and Ki-67 using the median where values higher than the median were considered high expressors and those lower than it were low expressors.

All patients received the standard CHOP treatment protocol and patients were evaluated according to the response criteria of lymphoma. The response to treatment was achieved in 23 patients (43.4%) and only four patients relapsed. Comparison between the responders and non-responders revealed significant higher miR-21 expression among the non-responders (median (IQR): 3.5 (0.2 - 34.7) versus 2.4 (0.1 - 10.0), $p = 0.03$). Moreover, it was shown that the non-responder group had significantly higher LDH levels (median (IQR): 453.0 (320.0 - 690.0) versus 290.0 (223.0 - 395.0) U/L, $p = 0.004$) (Table 1). No significant correlation was found between miR-21 expression and Ki-67 expression ($p = 0.81$).

Logistic regression analysis for prediction of non-response to chemotherapy within the studied DLBCL cohort revealed that higher miR-21 expression was considered as an independent prognostic factor for prediction of poor response to treatment ($p = 0.042$).

At the end of the study, 90.6% of patients were still alive (48/53 patients). The whole cohort had a mean overall survival (OS) time of 16.54 months (95% CI: 14.86 - 18.23) and a mean progression free survival (PFS) time of 11.41 months (95% CI: 9.6 - 13.2).

Comparison between low and high miR-21 expressors revealed no significant differences between both groups regarding OS and PFS times (Figures 3, 4). However, low Ki-67 expressors had significantly longer OS and PFS versus high Ki-67 expressors [mean (95% CI): 18.9 (17.8 - 19.9) versus 13.25 (10.4 - 16.11) months, $p = 0.006$ for OS] [mean (95% CI): 14.08 (12.23 - 15.93) versus 7.32 (5.858 - 8.79) months, $p = 0.001$ for PFS] (Figures 5, 6).

In the Cox regression analysis, although patients' age was a significant predictor of overall survival in univariate analysis (HR: 1.054, 95% CI (1.4 - 1.11) $p = 0.035$), none of the studied factors could predict overall survival in multivariate analysis (Table 2).

However, we found that Ki-67 expression was a significant predictor of PFS in both univariate [HR (95% CI): 1.030 (1.4 - 1.057), $p = 0.024$] and multivariate [HR (95% CI): 1.03 (1.01 - 1.06), $p = 0.03$] analyses (Table 3).

## DISCUSSION

The prognostic value of miR-21 and Ki-67 in DLBCL was evaluated in the current study. To the best of our knowledge, this is the first study that tried to assess the relationship between miR-21 expression and Ki-67 expression in DLBCL patients. However, the present study found no significant association between both markers. Conflicting results were reported in other cancers. In invasive ductal carcinoma of the breast, high miR-21 expression was associated with increased Ki-67 expression [14,15]. In other tumors including colorectal cancer [16] and neuroendocrine tumors [17], no relationship was found.

We found that miR-21 is significantly upregulated in the DLBCL group compared to controls ($p < 0.001$), in concordance with Sun and Luan [18], El-Halawani, et al. [19], and Glickman, et al. [20].

Also, we found that higher miR-21 expression was associated with poor response to treatment. El-Halawani, et al. [19] in his study, agreed with us that higher miR-21 expression levels were associated with poor response to chemotherapy [20]. Also, the study of Sun and Luan [18] which detected the expression of miR-21 in B-NHL patients revealed significant association between lower miR-21 levels and achieving CR [18]. In fact, miR-21 proved to contribute to decreased apoptosis and increased viability of DLBCL cells by targeting the tumor suppressive molecule B cell lymphoma-2 (Bcl-2) [21]. Also, it was found that miR-21 can modulate the PI3K/AKT/mTOR/FOXO1 pathway in DLBCL patients, thus promoting survival of tumor cells. [22] On the other hand, we did not find a significant relationship between having CR and Ki-67 expression; this was in line with the findings of Zhou, et al. [23].

Regarding OS and PFS, our analysis revealed that high Ki-67 was significantly associated with adverse OS and PFS. Univariate and multivariat cox regression analyses revealed that Ki-67 is considered an independent prognostic factor for prediction of PFS. One meta-analysis involving 27 studies (3,902 patients) done by He, et al. [24] found that high Ki-67 index is negatively correlated with OS and DFS in harmony with our findings. Also, Li, et al. [25] demonstrated a significant correlation between high Ki-67 expression and a poor survival outcome in B-NHL patients [25].

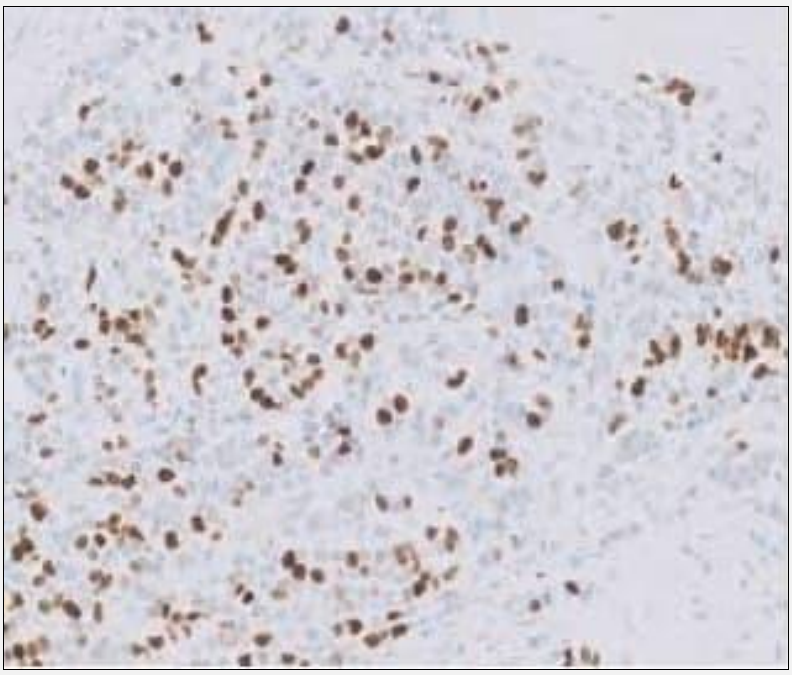

**Figure 1. Low Ki-67 expression in LNB (Ki-67 PI: 30%).**

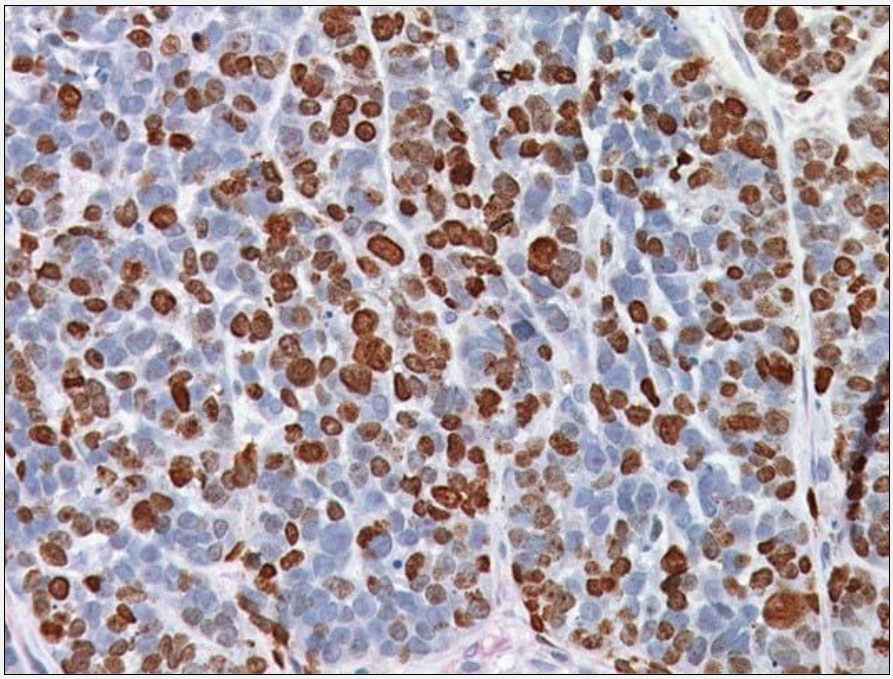

**Figure 2. High Ki-67 expression in LNB (Ki-67 PI: 80%).**

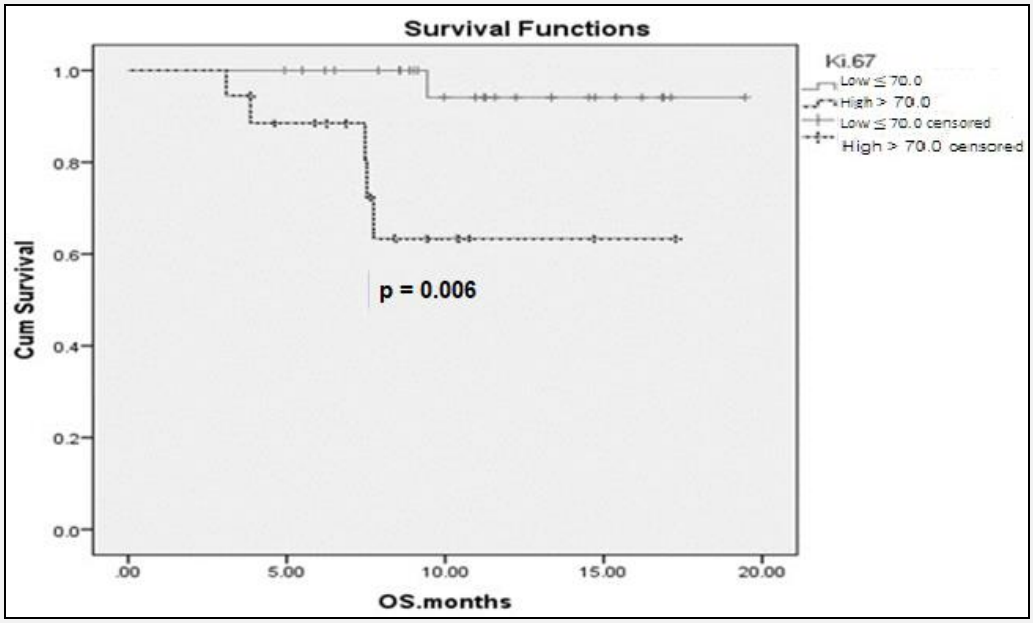


**Figure 3. Kaplan Meier OS of high Ki-67 expression vs. low Ki-67 using median 70% as cutoff.**

**The analysis revealed that patients with high Ki-67 expression had worse OS than those with low Ki-67 expression (p = 0.006).**

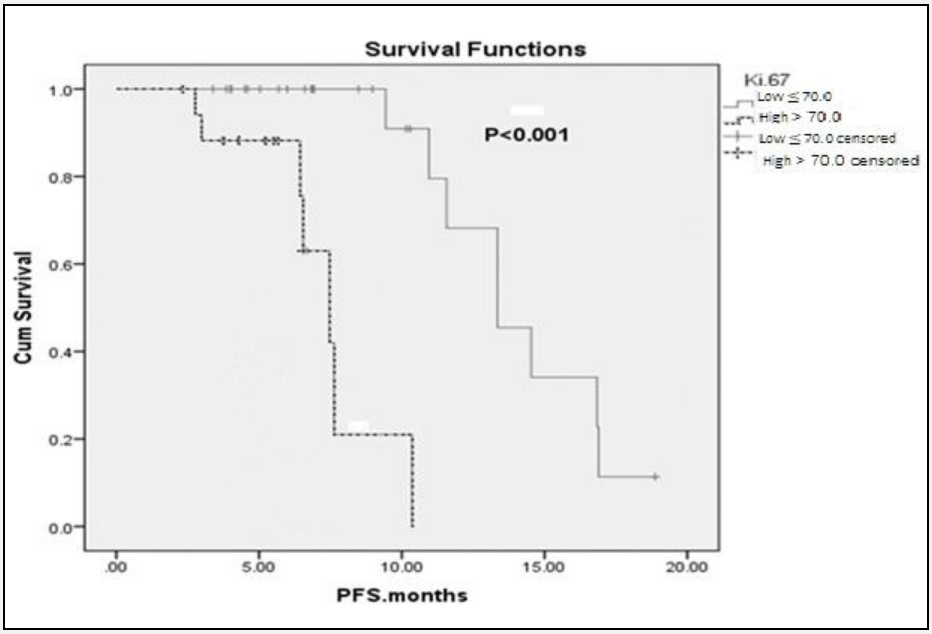


**Figure 4. Kaplan Meier PFS of high Ki-67 expression vs. low Ki-67 using median 70% as cutoff.**

**The analysis revealed that patients with high Ki-67 expression had worse PFS than those with low Ki-67 expression (p < 0.001).**

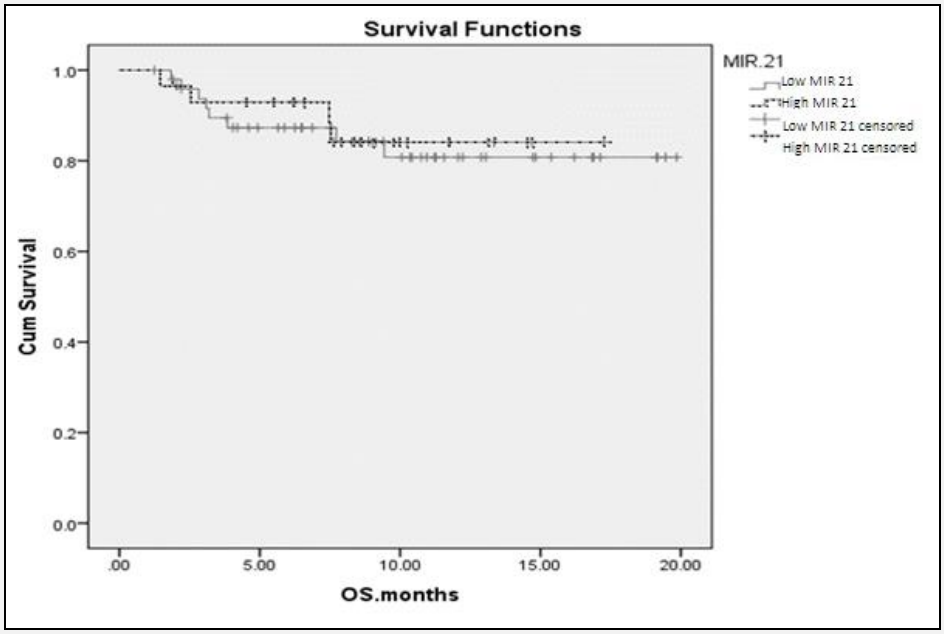


**Figure 5. Kaplan Meier OS of high MIR-21 expression vs. low MIR-21 using mean 5 as cutoff.**

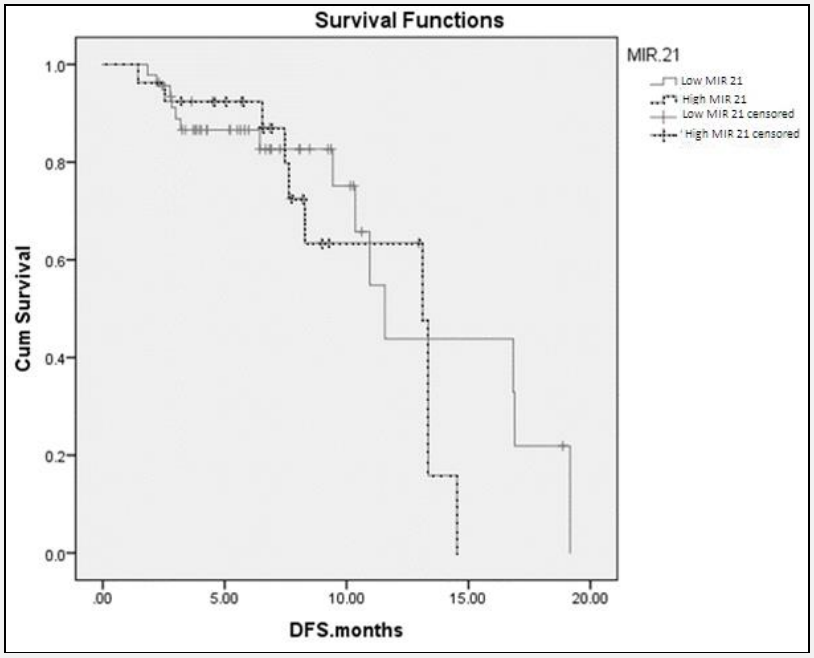


**Figure 6. Kaplan Meier DFS of high MIR-21 expression vs. low MIR-21 using mean 5 as cutoff.**

In the present study, comparison between patients with low and high miR-21 expressions revealed no significant differences regarding OS and PFS times. In contrast, Gohar, et al. [26] revealed that OS was significantly worse in DLBCL patients with high miR-21 expression compared to those with low expression levels [26]. Similarly, Glickman, et al. [20] found that the survival rates of the 112 DLBCL patients with high miR-21 levels were significantly lower than those with low levels ($p = 0.000$) [20]. The possible explanations of this discrepancy could be small sample size and the short term follow-up.

Our preliminary findings, denote that the consideration of both markers in DLBCL patients requires further long term follow-up studies with larger sample size, multi-centric, and international contribution to clarify their role as important prognostic markers.

## CONCLUSION

miR-21 is significantly upregulated in Egyptian DLBCL patients and high miR-21 expression can be considered a predictor of poor response to treatment. In addition, high Ki-67 is significantly associated with adverse overall survival and progression free survival in DLBCL.

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None.

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None.

**Declaration of Interest:**
None.

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