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MicroRNA 21 Expression Levels in HIV Negative and HIV Positive Diffuse Large B Cell Lymphoma

Phillips P[1], **Mcgrath E**[1], **Sekar D**[2], **Sookhayi R**[1], **Govender D**[1], **Mohamed Z**[3], **Zerbini LF**[2] **and Naidoo R**[1]

[1]*Division of Anatomical Pathology, Department of Clinical Laboratory Sciences, Faculty of Health Sciences, University of Cape Town/National Health Laboratory Service, Groote Schuur Hospital, South Africa*

[2]*Cancer Genomics Group, International Centre for Genetic Engineering and Biotechnology (ICGEB), South Africa*

[3]*Department of Radiation Oncology, University of Cape Town, Groote Schuur Hospital, South Africa*

[*]**Corresponding author:** Richard Naidoo, Division of Anatomical Pathology, Department of Clinical Laboratory Sciences Faculty of Health Sciences University of Cape Town Anzio Road, Observatory7925, Cape Town, Western Cape, South Africa, Tel: 27214047615, Fax: 27214047611, E-mail: Richard.Naidoo@uct.ac.za

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Abstract

Diffuse large B cell lymphoma (DLBCL) is a heterogeneous disease with various morphological and molecular subtypes. It commonly occurs in the elderly as well as in people infected with HIV. According to the Hans' algorithm DLBCL can be classified prognostically into the favourable germinal centre (GC) subtype and the unfavourable non-GC subtype. The disease tends to be more aggressive in HIV infected individuals. Research on DLBCL has been largely conducted on HIV negative samples and it is still unclear how HIV affects the molecular mechanisms of the disease. We conducted a study to compare the expression levels of miR-21 in HIV negative and HIV positive DLBCL patients. Our results suggest that miR-21 expression levels are higher in HIV positive cases of DLBCL. We did not observe any statistically significant difference in miR-21 levels between DLBCL subtypes in both HIV negative and positive cases. Favourable prognosis in HIV negative patients was associated with high miR-21 expression and the reverse was true in HIV positive patients. In conclusion, our results indicate that HIV infection may affect the expression of miR-21 in DLBCL. This highlights the need for further studies on HIV positive DLBCL as the prognostic significance of miR-21 expression level may differ depending on the HIV status.

Keywords: B-cell lymphoma; Micro RNA; Prognosis; HIV; miR-21; DLBCL

Introduction

Diffuse large B cell lymphoma (DLBCL) is the most common subtype of non-Hodgkin lymphoma (NHL) comprising of 30% of all NHL [1]. The disease shows marked heterogeneity, with morphological and molecular variants [2,3], making it difficult to treat. DLBCL is divided into two histological subtypes with prognostic significance according to the Hans' algorithm. The non-germinal centre (non-GC) subtype has a worse prognosis than the germinal centre B cell like (GC) subtype [4].

Recent data suggests that HIV gp120 induces B cells expressing mannose C type lectin receptors (MCLRs) to undergo immunoglobulin class switching by increasing the expression of activation-induced cytidine deaminase [5]. Chronic B-cell activation in HIV infection is driven by the production of B-cell stimulating cytokines which are secreted by gp120 bound monocytes [6]. These cytokines act by upregulating MCLRs on the surface of B cells, thus creating chronic B cell activation [5]. Therefore, people infected with HIV are at an increased risk of developing B cell NHL [7]. DLBCL is the most common NHL diagnosed in HIV infected individuals [8,9].

MicroRNAs are non-coding RNA molecules that silence the expression of target genes [10]. Gene silencing is achieved by imperfect or perfect pairing of microRNA with3'UTR of target mRNA thereby resulting in inhibition of translation or degradation, respectively [11,12]. They are transcribed as long transcripts and processed by the enzymes Drosha and Dicer into microRNA duplexes. The functional strand of the duplex is loaded into the RNA- induced silencing complex enzyme (RISC) for gene silencing [10]. MicroRNAs play a role in diverse biological processes including development, cell growth and apoptosis [11,13,14]. Their expression levels are usually disrupted during malignancy [12], making them crucial regulators of the cell cycle and potential biomarkers for cancer [15].

MicroRNA 21 (miR-21) is overexpressed in most solid malignant neoplasms such as carcinomas of the breast, pancreas, lung, stomach and prostate, and glioblastomas [11,16-18]. miR-21 is involved in tumourigenesis [17], tumour progression [19], and metastasis [20]. Data generated by Thapa and colleagues reveal that the levels of miR-21 are elevated in circulating B cells of HIV positive individuals who eventually develop NHL in less than three years [15]. An above normal increase in the expression of miR-21 has been observed in cell lines, tissue [21] and serum [22,23] of DLBCL patients. Given the high rate of HIV infection in Southern Africa and the increased risk of acquiring DLBCL in HIV positive individuals; there is little research on miR-21 expression in DLBCL of HIV positive individuals. Therefore, we conducted a study to compare the expression levels of miR-21 in DLBCL in HIV positive and HIV negative individuals, and also to determine its prognostic potential in HIV positive DLBCL since no data is currently available in this particular cohort.

Materials and Methods

Ethics approval

The project was approved by the University of Cape Town, Faculty of Health Sciences Human Research Ethics committee, reference number: 261/2010.

Sample selection

DLBCL cases from confirmed HIV negative (n=29) and HIV positive (n=23) individuals were retrieved using the laboratory information system. The pathological diagnoses originally established were subsequently reviewed according to the morphological and immunohistochemical criteria of the WHO classification. The corresponding tissue blocks were retrieved from the archives of the Division of Anatomical Pathology, National Health Laboratory Service (NHLS), Groote Schuur hospital. Reactive lymph nodes from HIV negative (n=6) and HIV positive (n=5) patients were used as controls.

miR-21 quantification

The quantification of miR-21 was achieved by utilising a single reverse transcription reaction for extracted microRNAs, combined with real time PCR using two miR-21-specific DNA primers. Primer annealing temperatures were optimized by adding a DNA tail to the primers [24].

Total RNA was extracted from FFPE tissues using the Roche High pure FFPET RNA isolation kit as per manufacturer's instructions. A poly (A) tail was added to the isolated RNA prior to cDNA synthesis. The reaction mix (10 µl) consisting of 100 ng total RNA, 1x poly(A) polymerase buffer, 0.1 mM ATP, and 1 unit of poly(A) polymerase (New England Biolabs) was incubated at 37°C for 75 minutes. This was followed by poly (A) polymerase inactivation at 65°C for 25 minutes. cDNA was synthesised using the Roche transcriptor first strand cDNA synthesis kit with buffer, 1 µM poly (T) primer: 5-CAGGTCCAG(T)15ACA-3 (adaptors), 1 mM dNTP mix, 25 units reverse transcriptase, and20 units RNAse inhibitors. The reaction was incubated at 42°C for 135 minutes followed by 85°C for 10 minutes. Quantification of miR-21 was performed using the following primers:

Forward Primer 5-AACACCAGTCGATGGGCT-3

Reverse Primer 5-GGTCCAGTTTTTTTTTTTTTTTTACA-3

MiR-21 levels were normalized to 18sRNA levels using the 2ΔΔCt model. Briefly, for the equation 2-ΔΔCt, ΔΔCt (miR-21-18s)=(Cpsample-CpcontrolmiR-21)-(Cpsample-Cpcontrol18s)

Statistical analysis

Differences in miR-21 expression levels between controls and DLBCL were detected using the Mann–Whitney non-parametric t-test in GraphPad Prism 6 software. The differences in miR-21 levels between non-GC and GC subtypes were detected using one way ANOVA with a Dunn's multiple comparisons test. Kaplan–Meier survival analysis was carried out on overall survival (OS) times of DLBCL cases as a function of microRNA expression, using the median value as cut-off. OS was calculated from the time of diagnosis until the date of clinical relapse, death or last contact. Patients who were disease-free at time of last contact were censored for analysis. Curves were compared by logrank (Mantel-Cox) analysis using GraphPad Prism 6. An association between mir-21 expression levels and clinicopathological characteristics was determined using the Chi-square test in Stata 12.

Results

The effect of HIV on miR-21 levels in DLBCL

MiR-21 levels were significantly higher in DLBCL tissue from HIV positive cases (2.22 ± 4.6878 vs. 19.88 ± 30.93) (Figure 1). We did not observe any statistically significant difference in miR-21 expression levels between the subtypes of DLBCL in both cohorts (Figure 2). In HIV negative DLBCL cohort, the non-GC subtype seemed to have higher expression levels of miR-21 (0.59 vs. 1.24 units). In comparison, the GC subtype seemed to express higher expression levels of miR-21(27.08 vs. 8.66 units) in the HIV positive DLBCL cohort.

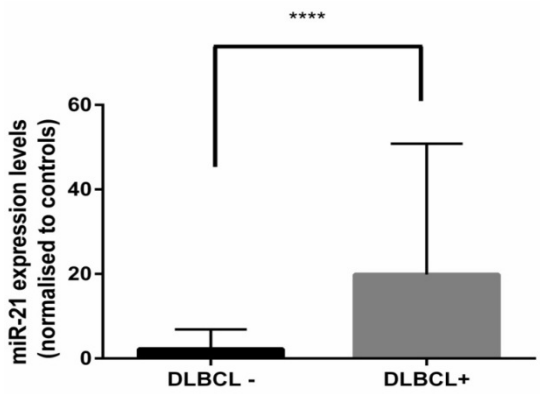

Figure 1: Comparison of miR- 21 levels in DLBCL from HIV negative and HIV positive patients. miR-21 expression levels from HIV positive patients were higher than those from HIV negative patients (Mann- Whitney test, *P* value<0.001). Graph depicts mean with SD.

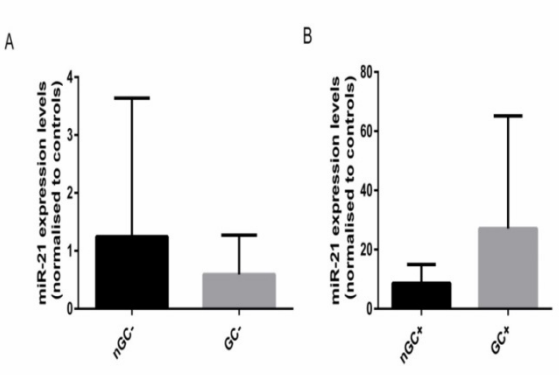

Figure 2: miR- 21 expression levels in the subtypes of DLBCL in HIV negative (A) and HIV positive (B) cases. The expression levels of miR-21 in GC vs. nGC subtypes were not statistically significant in both the HIV negative and positive cohorts (Mann Whitney t test, $p>0.05$).

The prognostic significance of miR-21 levelsThe patients were divided according to miR-21 levels, relative to the median value (0.3240 and 6.64 for HIV negative and HIV positive, respectively) into 'low miR-21' and 'high miR-21' expressing groups. 'Low miR-21' was values below or equal to the median miR-21 expression level and 'high miR-21' were values above the median expression value. We did not find any significant differences in overall survival between low miR-21 and high miR-21 expression in both HIV negative and positive DLBCL (Figure 3). However, the data suggest that high miR-21 expression levels in HIV negative DLBCL patients are associated with favourable prognosis (median survival of 66.8 vs. 31.2 months) (Figure 3A). In comparison, high miR-21 expression levels are associated with a poor prognosis in HIV positive patients (median survival of 7 vs. 60.83 months) (Figure 4B). In addition, high miR-21 expression levels were associated with high LDH levels (p=0.006) (Table 1) in HIV positive patients. When comparing survival curves based on the DLBCL subtypes, the GC subtype had favourable prognosis in HIV negative patients (median survival of 31 vs. undefined months, p=0.02), while this subtype exhibited poorer prognosis in HIV positive patients (median survival of 8.5 vs. 34 months, p=0.97) (Figure 4).

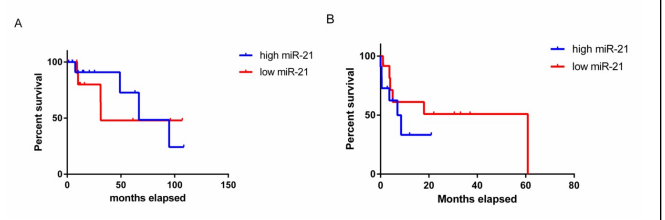

Figure 3: Kaplan-Meier curve for overall survival in DLBCL cases treated with CHOP. A. Analysis of the prognostic significance of miR-21 levels in HIV negative DLBCL patients. B. The prognostic significance of miR-21 levels in HIV positive DLBCL patients. There was no statistically significance found (Mantel-Cox test, p>0.05).

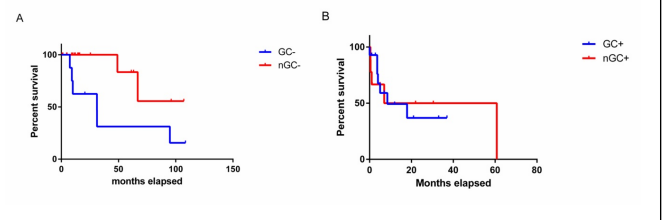

Figure 4: Kaplan-Meier curve for overall survival in DLBCL cases treated with CHOP. A. Analysis of survival in the subtypes of DLBCL in HIV negative patients. The GC subtype has a favourable prognosis (p<0.05). B. Analysis of survival in the subtypes of DLBCL in HIV positive patients. There was no statistically significance found (Mantel-Cox test, p>0.05).

Discussion

This study found that miR-21 expression levels in HIV positive DLBCL tissues were significantly higher than those expressed in HIV negative DLBCL. Our results support the data of Thapa et al., who observed an increase in miR-21 levels in HIV positive individuals who developed lymphoma within 3 years compared to the circulating B cells of HIV negative controls (15). We did not observe any statistically significant differences in miR-21 levels between non-GC and GC subtypes in both cohorts. The non-GC subtype has previously been shown to expresses higher miR-21 levels than the GC subtypes in HIV negative DLBCL [21,22]. The lack of statistical significance in our study may be due to the small sample size. The GC subtype exhibited a favourable prognosis in HIV negative DLBCL (p<0.05). These results support previously published data which suggest that the GC subtype has a better prognosis in HIV negative DLBCL [25].

	HIV negative	Correlation with miR-21 expression level (p value)	HIV positive	Correlation with miR-21 expression level (p value)
N	29		23	
Age (mean)	57.3		39	
Gender (male:female)	16:12		10:13	
PSa 0	2		0	
1	9		7	
2	2	0.139	4	0.199
3	4		2	
4	3		0	
Stage I/II	11	0.66	9	0.2
III/IV	12		10	
LDH low	10	0.141	6	0.006
LDH high	15		15	
GC/NON-GC	Aug-20	1	14-Sep	0.795
CD4 count<200	NDb		13	0.104
≥ 200	NDb		8	

Table 1: The clinicopathological and demographical characteristics of the study population. An association of clinicopathological characteristics with miR-21 expression levels was determined using Chi square test.

The prognostic significance of miR-21 expression levels was determined by looking at the overall survival of the patients. We did not observe any significant differences in overall survival. However, we used the data to postulate trends in overall survival. The large differences in median survival times support a possible prognostic role for miR-21 expression levels. Patients with high miR-21 expression levels had higher median survival time in the HIV negative cohort. Our results are similar to Lawrie et al., and Chen et al., who showed that high miR-21 levels have favourable prognosis in HIV negative DLBCL [21,22]. Patients with high miR-21 expression levels in the HIV positive cohort had a lower median survival time. This result is further supported by the association of high miR-21 levels with high LDH levels in the HIV positive cohort. High LDH levels are associated

with an unfavourable prognosis [26]. The prognostic significance of miR-21 expression levels seems to change depending on the HIV status of the patient. This may suggest that the levels of miR-21 may signify different prognosis depending on the context. This notion is supported by a meta analysis review that revealed high levels of miR-21 correlated with poor survival in squamous cell carcinomas[27] whereas the opposite is true for DLBCL [21,22].

In conclusion, this is the first report comparing the miR-21 expression levels in HIV positive and HIV negative DLBCL tissues. Our results demonstrate that miR-21 expression levels are significantly higher in HIV associated DLBCL. This suggests that HIV infection may play a role in the regulation of miR-21 in DLBCL. There was no statistical difference in miR-21 expression levels between non-GC and GC subtypes of DLBCL in our study. We also did not observe statistically significant difference in overall survival in 'low' and 'high' miR-21 expressing groups. This study highlights the need for further studies on HIV associated DLBCL as the prognostic significance of miR-21 expression levels may differ depending on HIV status.

Acknowledgements

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