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Modulatory Effect of Human Immunodeficiency Virus on Circulating p53, miR-21, and miR-125b: Any Diagnostic Implication?

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Abstract: Identifying immunocompromised women who are at risk of developing cervical cancer remains a challenge for clinicians. In an effort to identify the role of HIV in cervical carcinogenesis, this study evaluated the levels of normally downregulated oncomirs (miR-21, miR-146a, miR-155, miR-182, and miR-200c) and normally upregulated tumor suppressors (miR-let-7b, miR-125b, miR-143, miR-145, and p53 expression) associated with cervical cancer in the serum of women living with HIV (HIV+) and without HIV (HIV. Method: This case-control study included 173 women; confirmed HIV+ (n = 103) and HIV− (n = 70). Serum levels of miRNAs and p53 were determined using reverse transcriptase PCR. *t*-test and Pearson's correlation analyses were carried out on the generated data. Result: A higher level of miR-21 was observed among HIV+ women compared with their HIV− counterpart ($p = 0.028$), whereas lower levels of miR-125, and p53 gene were observed among HIV+ women compared with HIV− women at $p = 0.050$ and 0.049, respectively. Significant direct relationships were observed between miR-21 and other oncomirs ($p < 0.05$) among HIV+ women. Conclusion: This study revealed that HIV contributes to cervical carcinogenesis by modulating circulating levels of miR-21, p53, and miR-125b. It suggests that these biomarkers could be used to identify at high risk for developing cervical cancer.

Keywords: human immunodeficiency virus; cervical cancer; biomarkers; microRNA; monitoring; testing

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1. Introduction

Globally, cervical cancer is the fourth most common cancer among women [1]. A recent WHO report shows that the risk ratio of developing cervical cancer among women living with and without Human immunodeficiency virus (HIV) is 6:1 [1]. Cervical cancer is a significant global health concern, particularly among immunocompromised women, such as those with HIV. In Nigeria, even though the prevalence of HIV declined from 5.0% to 3.2% from 2003 to 2014, the negative impact of HIV on the trend of cervical cancer is still being felt [2]. From 2005 to 2014, the incidence of the disease in Nigeria increased from 25 per to 28.3 per 10,000 [3,4]. As of 2018, reports show that the prevalence of cervical cancer attributable to HIV was higher in Africa than in other continents [2]. Evidence shows that 25% of women living with HIV develop premalignant lesions and 12–30% of such women develop invasive cervical cancer despite receiving antiviral therapy [5]. Through its viral protein R (vpR), HIV alters the function of the p53 gene and telomerase which eventually results in the immortalization of cells with damaged DNA [6,7]. Identifying

reliable biomarkers is vital for the effective monitoring of high-risk groups, especially in low-resource settings. This would ultimately reduce late-stage presentation and cervical cancer-related death. In this study, we evaluated the levels of circulating miR-21, miR-146a, miR-155, miR-182, miR-200c, miR-let-7b, miR-125b, miR-143, miR-145, and p53 among HIV+ and HIV− women to identify early biomarkers of genetic instability [8,9]. For the first time, we demonstrated the singular modulatory effect of HIV on oncogenes and tumour suppressors among Human papillomavirus (HPV) and Epstein-Barr virus (EBV) seronegative women.

2. Materials and Methods

This case-control study was carried out between May (2017) and April (2019). It included HIV uninfected women (HIV−; n = 70) and women living with HIV (HIV+; n = 103) living in Abeokuta, Nigeria.

2.1. Sample Collection, Handling, and Assays

Participants were screened for cervical cancer using a conventional Pap smear. Blood samples were collected at the Family Planning and HIV clinics at State Hospital Ijaiye, Ogun State. The blood samples were centrifuged, and the sera were separated. The sera obtained from the blood samples were separated and transferred to plain tubes. These tubes were then stored at −20 °C in the Department of Physiology, Babcock University, until further analysis. Five (5) mL of peripheral blood was also collected into an ethylenediaminetetraacetic acid vacutainer tube for CD4+ T-cell counts and analyzed within 2 h of collection using the CD4 easy count kit and Cyflow Counter in the Department of Chemical Pathology, Babcock University Teaching Hospital, Ogun State. To test for antibodies against HIV1/2, HPV, and EBV ELISA kits (from Qingdao Hightop Biotech Co., Ltd., Qingdao, China, and Calbiotech Inc., El Cajon, CA, USA) were used according to the manufacturer's instructions. ositive and negative cut-off values for HIV-1/2 antibodies were 1.077 and 1.076, respectively. Individuals with any history of Human papillomavirus and Epstein-Barr virus infection, cancer, especially cervical, breast, and oral cancers were excluded [9,10].

2.1.1. RNA Isolation from Serum

The optimized phenol-chloroform method was used for RNA extraction in the Centre for Biocomputing and Drug Development, Adekunle Ajasin University [10]. Fifty (50) µL of serum was added to Eppendorf tubes containing 50 µL Trizol reagent and vortexed at 2500 rpm for 15 mins using a vortex mixer. The supernatants containing the RNA were aspirated into newly labeled tubes. Iso-amyl alcohol (100 µL) was added to the supernatant with subsequent centrifugation for 30 min at 1500 revolutions per minute. For the cervical cells, 50 µL of Liquid-based Prep infranatant from each woman was homogenized in a different Eppendorf tube containing 50 µL Trizol reagent by pipetting up and down and later vortexed as well. Chloroform (100 µL) was added to the mixture which was subsequently vortexed and centrifuged for 30 min at 1500 revolutions per minute. The RNA was recovered in pellet form following decantation of the supernatant containing initially containing serum and cervical cells. The RNA pellet was washed thrice with 70% ethanol. Fifty µL of 70% ethanol was added to the tube and the tube was centrifuged for 5 min at 1500 revolutions per minute. Next, the supernatant was decanted. After washing, all tubes were allowed to air dry. Fifty microlitres (µL) of nuclease-free water was added to the total RNA to form the RNA solution. The total RNA concentration (48 µL of deionized distilled water + 2 µL of RNA solution) was quantified using a spectrophotometer at 260 nanometres. The RNA limit of importance was set at 0.05–1.00. Acceptable RNA quality was set at 1.8–2.2 (based on OD260/OD280 calculation) [9,10].

2.1.2. Complementary DNA Synthesis

The cDNA synthesis was carried out by adding a miRNA-universal stem-loop primer cocktail (containing nuclease-free water, the reverse transcriptase buffer, the reverse tran-

scriptase, miRNA-universal stem-loop primer specific oligos, and oligo deoxyribonucleotide triphosphate) to 20 µL of total RNA of each sample. The samples were then incubated at room temperature overnight. The concentration of complementary DNA was determined spectrophotometrically at 260 nanometres and homogeneity was carried out on every sample. To establish homogeneity of cDNA (HcDNA) concentration across all samples, the formula HcDNA = VB − VA was applied; where VB (dilution volume) = CA × VA/CB; CA = absorbance reading of RNA × 40 × 25, VA = needed volume of cDNA (25) and CB = least absorbance reading. All samples were diluted to the same concentration [9,10].

2.1.3. Reverse Transcriptase Polymerase Chain Reaction

The primers for miRNA quantification included: miR-Let-7b forward (5′-GTTTC GGGGTGAGGTAGTA-3′), miR-16 forward (5′-GTTGTCAGCAGTGCCTTAG-3′), miR-21 forward (5′-GGTGTCGGGTAGCTTATCA-3′), miR-125b forward (5′-GTTTTGCGCTCCTCT CAGT-3′), miR-143 forward (5′-TTTTTGCGCAGCGCCCTG-3′), miR-145 forward (5′-GTTTCACCTTGTCCTCACG-3′), miR-155 forward (5′-GTTTCTGTTAATG CTAATCGT GATA-3′), miR-182 forward (5′-GTTTTAGAACTCACACGTGTGA-3′), miR-200c forward (5′-GTTTCCCTCGTCTTACCCA-3′), universal reverse primer (5′-GTGCAGGGTCCGAGG T-3′), wild type p53 forward (5′-GCTCAAGACTGGCGCTAAAA-3′), wild type p53 reverse (5′-GTGACTCAGAGAGGACTCAT-3′); β-actin forward (5′-ACACTTTCTACAATGAGCTG CG-3′) β-actin reverse (5′-ACCAGAGGC ATACAGGACAAC-3′) and miRNA-USLP (Universal stem-loop primer; 5′-AGTGCAGGGTCCGAG GTATTCGCACCAGAGCCAACATGT CACG-3′). The amplification was performed using optimization. Template (cDNA) 2 µL, nuclease-free water 3 µL, forward primers 0.5 µL and reverse primers 0.5 µL (Inqaba Biotechnical industries Ltd., Pretoria, South Africa) and master mix 4 µL (Biolabs, Midrand, South Africa). All reagents were added to each sample for a complete enzymatic reaction, and PCR was carried out. Amplification conditions were: 94 °C pre-denaturation for 5 min, 94 °C for 30 sec, annealing 55 °C for 30 s, and Extension 72 °C for 30 s and then 5 min at 72 °C by 45 cycles) [9,10].

2.1.4. Gel Electrophoresis

Products from PCR were electrophoresed in 0.5% of agarose gel using 0.5× TBE buffer of pH 8.3 (2.6 g of Tris base, 5.0 g of Tris boric acid, and 2 mL of 0.5 M EDTA) with 0.2 µL ethidium as a fluorescent tag. Snapshots of amplicons (Figure 1) were densitometrically analyzed using ImageJ software (1.49 V). Figure 1 shows the fluorescent bands of the circulating genes from the HIV+ women (A) and HIV− women (B) in gel (g-) following PCR. The relative expression of miRNAs and p53 gene were calculated following endogenous normalization using miR-16 for miRNAs and β-actin for the p53 gene) [9,10].

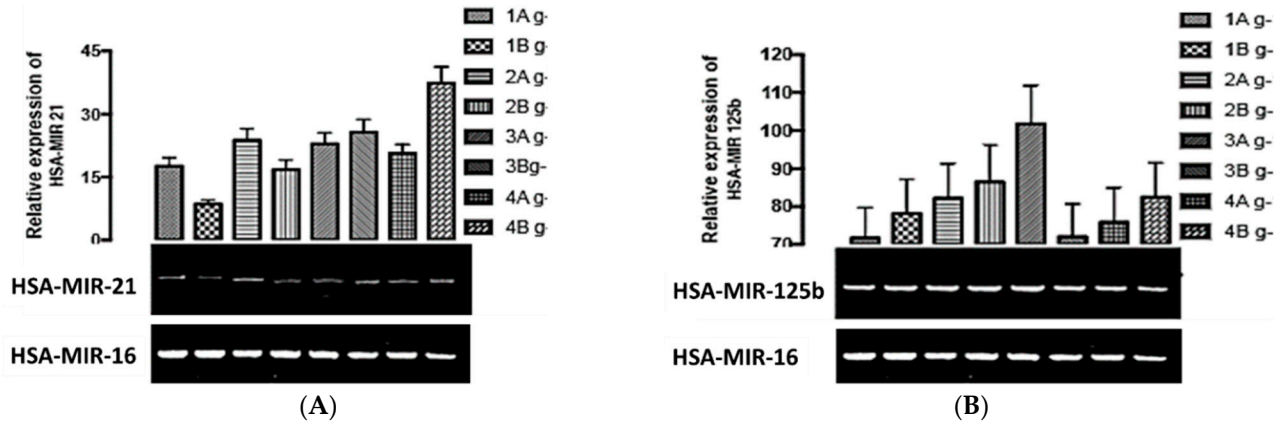

Figure 1. Gel electrophoretic image and expression of miR-21 (**A**) and miR-125b (**B**).

2.2. Statistical Analyses

The relative expression of miRNAs and p53 from two groups—Group 1 (HIV uninfected women) and Group 2 (Women living with HIV)—were compared using the *t*-test and Pearson's (bivariate) correlation using SPSS (version 21). Results were presented as mean ± standard error of the mean (SEM). Statistical significance was set at $p \leq 0.05$.

3. Results

The mean age of HIV− women (38.33 ± 10.04 years) was insignificantly lower compared with HIV+ women (41.36 ± 10.23 years) at $p = 0.290$. The CD4+ T-cell counts were insignificantly higher among HIV− women (1690 cells/μL) compared with HIV+ women (763.1 cells/μL) at $p = 0.055$.

In Figure 1, the expression products are visualized as bands (amplicons) using UV-transilluminator. The representative figure shows the variation in miR-21 and miR-125b levels in the serum of HIV+ women (1A g- to 4A g-) and HIV− women (1B g- to 4B g-) in the first four samples.

In Figure 2, a higher level of miR-21 can be observed among HIV+ women compared with HIV− women ($p < 0.05$) while lower levels of miR-146a, miR-155, miR-182, and miR-200c are observed among HIV+ women compared with HIV− women at $p < 0.05$, $p > 0.05$, $p > 0.05$, and $p > 0.05$, respectively. Also, a higher level of miR-145 is observed among HIV+ women compared with HIV− women ($p > 0.05$).

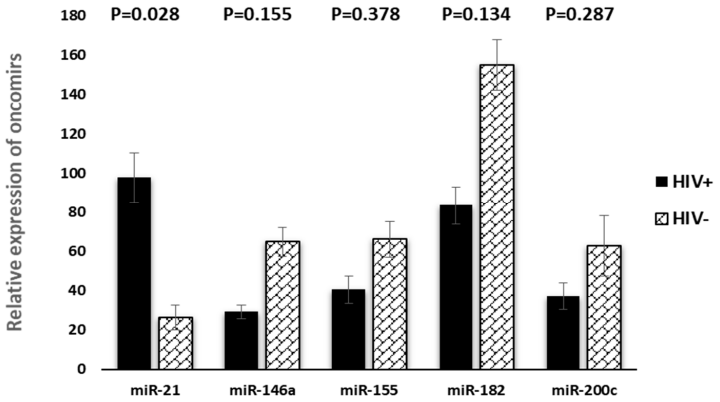

Figure 2. Comparative analysis of serum level of oncogenes between HIV+ and HIV− women.

In Figure 3, lower levels of miR-let-7b, miR-125b, miR-143, and p53 gene are observed among HIV+ women compared with HIV− women at $p > 0.05$, $p = 0.05$, $p > 0.05$, and $p < 0.05$, respectively. The low levels of miR-let-7b, miR-125b, miR-143, and p53 among HIV+ women could be due to immune exhaustion.

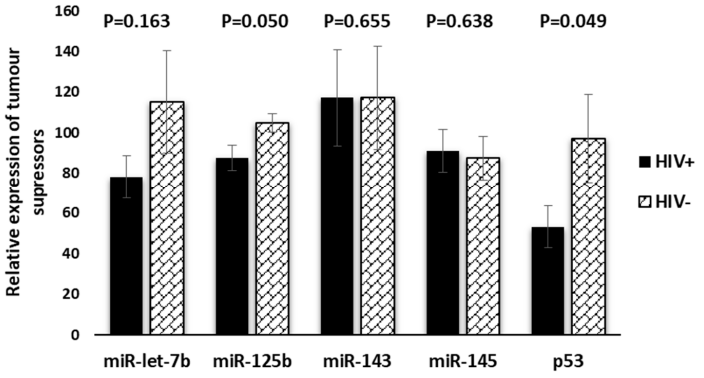

Figure 3. Comparative analysis of tumour suppressor levels in serum of HIV+ and HIV− women.

Relationships between Tumour Suppressors and Oncomirs

In the serum of both HIV+ and HIV− women, significant direct relationships were between miR-21 and miR-155 ($p = 0.002$ and $p = 0.014$), miR-21 and 182 ($p = 0.041$ and $p = 0.045$, respectively) while a significant inverse relationship was observed between miR-21 and miR-let-7b ($p = 0.013$ and $p = 0.049$, respectively). Among HIV+ women, significant inverse relationships were observed between miR-200c and miR-let-7b ($p = 0.039$), and miR-let-7b and miR-145 ($p = 0.006$), while a direct relationship was observed between miR-155 and miR-145 ($p = 0.049$), miR-155 and miR-182 ($p = 0.001$), and miR-21 and miR-200c ($p = 0.048$). Among HIV− women, significant direct relationships were observed between miR-146a and miR-145 ($p = 0.033$), miR-146a and p53 ($p = 0.030$), miR-155 and miR-200c ($p = 0.006$), miR-155 and p53 ($p = 0.012$), and miR-200c and p53 gene ($p = 0.003$).

4. Discussion

Several studies have have demonstrated the significant contribution of HPV and EBV to cervical carcinogenesis, either independently or synergistically [1–4,11]. However, unlike other studies, this study excluded women with detectable levels of HPV and EBV antigen to evaluate the singular modulatory effect of HIV on some selected oncogenes and tumour suppressors among HIV+ and HIV− women. An earlier study revealed a higher peripheral blood level of miR-21 among HIV+ individuals compared with their HIV− counterparts [12]. This is consistent with the findings of this study and could be due to sustained stimulation, dysfunction, and apoptosis of CD4+ T cells among HIV+ women [13]. It could also be attributable to a low level of circulating miR-let-7b since there was an inverse relationship between miR-21 and miR-let-7b [8]. Thus, miR-21 could be used as an early indicator of oncoviral activity and epithelial transformation among HIV+ women. Likewise, in this study, a significantly lower level of miR-125b was observed in the serum of HIV+ women compared with their HIV− counterparts. This could be due to immunosuppression following enhanced HIV replication [14], CD4+ T cell activation [15], or the overwhelming effect of viral co-infections such as Human papillomavirus (HPV) and Epstein-Barr virus (EBV) [11]. This suggests that the level of miR-125b could also be used to monitor immunosuppression-dependent epithelial transformation associated with HIV infection, irrespective of CD4+ T-cell counts and the nature of the sample.

Jin et al. reported a significantly higher plasma level of miR-155 among HIV1+ participants who were highly active anti-retroviral therapy (HAART) naïve, and HAART non-responders compared with HAART responders and HIV− participants [14]. In this study, there was an insignificant lower serum level of miR-155 among HIV+ women compared with HIV− women. The variation between the findings of Jin et al. and the findings of this study could be related to the nature of the sample assayed; plasma versus serum. MiR-155 is produced not only by mononuclear cells but also by CD4+ and CD8+ T lymphocytes found in plasma [16]. The cells are abundant in the microenvironment of the cervical epithelium. Researchers have shown that infection with Epstein-Barr virus (EBV) infection results in cellular expression of miR-155 which in turn immortalizes B-cells [17]. Thus, in the event of EBV infection, the already existing high level of cellular miR-155 in HIV+ women creates a fertile ground for the development or progression of cervical cancer and a common AIDS-associated malignancy known as diffuse large B-cell lymphomas. Similarly, in this study, the level of p53 was lower in the serum of HIV+ women compared with their HIV− counterparts. The low serum level of p53 in this study is supported by the findings of Akwiwu et al. who reported a lower level of serum p53 protein and CD4+ T cell counts among HIV+ HAART naïve women followed by HIV+ HAART-experienced women compared with HIV− women [18]. The low circulating level of the p53 gene may forestall p21, DRAM1, and Bax-linked apoptosis, autophagy, and senescence in HIV+ women in the event of epithelial transformation and ensures cancer-cell survival and invasion [19].

Parikh et al. reported a higher level of miR-145 in HIV+ individuals compared with HIV− counterparts. Even though most of the participants in their study were on ART, the mean CD4+ T-cell counts of their participants were 484 cells/μL [12]. In this study, although

miR-145 was slightly lower in the serum of HIV+ women compared with HIV− women, no significant difference was observed between the samples from the two groups. Taken together, the insignificant differences in serum level of tumor suppressors between HIV+ and HIV− women in this study, especially miR-145, could be due to the responsiveness of the HIV+ women to HAART. This is underscored by the fact that the CD4+ T-cell counts among the HIV+ women in this study are higher compared with the CD4+ T-cell count among the HIV+ participants in the study carried out by Parikh et al. [12].

In humans, miR-143 exerts anti-inflammatory effects and its low serum level is predictive of poor prognosis among critically ill patients [20,21]. Since inflammation precedes and co-exists with cancer, the low serum level of miR-143 among HIV+ women in this study suggests that they are at risk of developing cervical cancer. Egana-Gorrono et al. and Nahand et al. reported higher levels of miR146a and miR-200c in mononuclear cells of viraemic HIV+ individuals, especially treatment naïve individuals, compared with uninfected individuals and elite controllers (ART naïve individuals with <50 HIV−1 RNA copies/mL and CD4 T cell count of 200 to 1000 cells/lL) [22,23]. Thus, the low levels of miR-146a, miR-182, and miR-200c in the serum of HIV+ women could be attributed to viral latency, reduced viral load, or low rate of T cell reactivation. Interestingly, viral latency forestalls HIV elimination in reservoir cells among HAART-experienced persons [7,24].

The viral load in blood was not determined. This is a limitation in this study as the viral load may have some impact on the level of the oncogenes and tumour suppressors in serum. Our screening for HPV and EBV was also based on the ELISA method, which could be another limitation. It could also be argued that a positive viral ELISA test and negative viral DNA test are associated with an imminent higher risk of epithelial transformation compared with a positive DNA test and negative ELISA test [25]. A positive ELISA test is suggestive of a viral-host interaction that could alter mRNA-miRNA networks attributable to genetic instability. We did not differentiate between individuals with HIV1 and HIV2. Each virus may have different pathways for inducing genomic instability. The insignificant differences in the levels of miR-let-7b, miR-143, miR-145, miR-146a, miR-155, miR-182, and miR-200c observed between HIV+ and HIV− women could be due to the small sample size. Finally, this study is limited by the fact that it was carried out only in South-Western Nigeria. A multiregional study may provide robust data for conclusive generalization. Despite these limitations, the data from this study could be used as a baseline for further studies.

5. Conclusions

This study unveils compelling findings regarding the circulating levels of miR-21 and miR-125b among HIV-positive women in Southern Nigeria. This study revealed a high circulating level of miR-21 and a low circulating level of miR-125b among HIV+ women in Southern Nigeria. These biomarkers hold the potential for identifying individuals at risk of developing cervical cancer, particularly among immunocompromised patients. Importantly, this research also It also highlights the importance of exploring novel therapeutic approaches aimed at modulating these microRNAs to prevent or delay the progression of cervical cancer in this vulnerable population.

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Institutional Review Board Statement: Ethical Consideration and Informed Consent. For this study: ethical approvals were obtained from the State Hospital Abeokuta Ethics Committee (SHA/RES/VOL.2/177) and Babcock University Health Research Committee (BUHREC549/18). The study was conducted in accordance with the Declaration of Helsinki.

Informed Consent Statement: As recommended by the ethics committee, Informed consent was obtained from all subjects involved in the study. Written informed consent has been obtained from the patients to publish this paper.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to ongoing research.

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Conflicts of Interest: The authors declare no conflict of interest.

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