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RESEARCH ARTICLE

Evaluation of the Expression Pattern of 4 microRNAs and their Correlation with Cellular/viral Factors in PBMCs of Long Term Non-progressors and HIV Infected Naïve Individuals

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Abstract: ***Background*:** Long-term non-progressors (LTNPs) are small subsets of HIV-infected subjects that can control HIV-1 replication for several years without receiving ART. The exact mechanism of HIV-1 suppression has not yet been completely elucidated. Although the modulatory role of microRNAs (miRNAs) in HIV-1 replication has been reported, their importance in LTNPs is unclear.

***Objective*:** The aim of this cross-sectional study was to assess the expression pattern of miR-27b, -29, -150, and -221, as well as their relationship with CD4+ T-cell count, HIV-1 viral load, and nef gene expression in peripheral blood mononuclear cells (PBMCs) of untreated viremic patients and in LTNPs.

***Methods*:** MiRNAs expression levels were evaluated with real-time PCR assay using RNA isolated from PBMCs of LTNPs, HIV-1 infected naive patients, and healthy people. Moreover, CD4 T-cell count, HIV viral load, and nef gene expression were assessed.

***Results*:** The expression level of all miRNAs significantly decreased in the HIV-1 patient group compared to the control group, while the expression pattern of miRNAs in the LNTPs group was similar to that in the healthy subject group. In addition, there were significant correlations between some miRNA expression with viral load, CD4+ T-cell count, and nef gene expression.

***Conclusion*:** The significant similarity and difference of the miRNA expression pattern between LNTPs and healthy individuals as well as between elite controllers and HIV-infected patients, respectively, showed that these miRNAs could be used as diagnostic biomarkers. Further, positive and negative correlations between miRNAs expression and viral/cellular factors could justify the role of these miRNAs in HIV-1 disease monitoring.

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1. INTRODUCTION

Human immunodeficiency virus-1 (HIV-1) belongs to the genus *Lentivirus* and the Retroviruses family (*Retroviridae*) and leads to lifelong diseases in humans. HIV-1 can infect human CD4+ T lymphocytes and if patients are not treated with antiretroviral therapy (ART), they will more likely progress to acquired immune deficiency syndrome (AIDS) [1-3]. Over the past two decades, various definitions have been proposed to categorize HIV-infected subjects, including viremic progressors (VPs), viremic controllers (VCs), and elite controllers (ECs) [4, 5].

Viremic progressors are HIV-positive individuals who have a high level of HIV viral load and progress to AIDS if not treated with ART [6, 7]. Viremic controllers and ECs are small subsets of HIV-infected subjects that can control HIV-1 replication for several years without receiving ART and are known as 'long-term non-progressors' (LTNPs) [4, 6]. They make up about 5% to 15% of subjects infected with

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HIV-1 [8-12]. Without therapy, viral load and CD4-cell count in the EC group can reach an undetectable level (less than 50 copies/mL) and normal level (200 -1000/μL), respectively [13-17]. However, in the VC group, the viral load level is between 200 and 2000 copies/mL (detectable level) [4, 14]. Some factors, *i.e.*, host immune system, some genetic factors, and mutation in some viral genes may be involved in this phenomenon. The exact mechanism of HIV-1 suppression has not yet been completely elucidated [18-20]. In some studies, the prevalence of ECs has been evaluated among the HIV positive population [4, 21-23] The documented evidence suggests that long-term control of HIV viremia usually occurs in <1% of patients [17, 24]. However, the prevalence of ECs has not been so far assessed in the Iranian population.

MicroRNAs (miRNAs) are a class of small non-coding RNAs with a length of about 18-24 nucleotides that have a critical role in the causation and development of different diseases through regulating a wide range of physiological and developmental processes [25-27]. It has been shown that the expression pattern of a specific miRNA can change in various physiological cell conditions, different tissue types and various diseases, such as viral infection, autoimmune disease and cancer [28-32]. Thus, miRNAs have been recently been considered as novel potential therapeutic and diagnostic biomarkers [33, 34]. Many studies have reported that miRNAs are involved in the progress of HIV-1 infections through modulating viral proteins and/or affecting host parameters associated with viral replication [35]. In addition, some miRNAs can inhibit the HIV-1 replication [36]. Anti-HIV-1 miRNAs restrict HIV activity by several mechanisms, such as targeting the HIV-1 receptor and directly targeting env, pol, gag, vif, tat, and nef genes (viral genes) [37]. Furthermore, some researches have shown that miR-221 and miR-222 block the CD4 receptor and suppress the HIV-1 entry into the cell [38]. It has also been revealed that miR-29a [39, 40], miR-28, miR-150, miR-223 and miR-382 [34, 41], and miR-1236 [42] can inhibit HIV replication and that miR-27 regulates HIV replication in resting CD4+T lymphocytes [43-45]. Moreover, the family of miR-223 [46] and miR29a [47] is upregulated in peripheral blood mononuclear cells (PBMCs) and by targeting Nef-3′UTR that leads to a decrease in the Nef protein expression and interferes with HIV replication [47]. The accessory nef gene is unique to HIV-1 and encodes a 27–32 kDa protein. Moreover, the nef gene is an essential pathogenic factor for virus replication and pathogenesis of HIV/AIDS disease [48]. Nevertheless, impairment has been reported in the enhancement of HIV-1 replication and infectivity mediated by Nef in EC subjects [49, 50]. Although some studies have reported that mutations in the nef gene have been observed in EC individuals, it has not been proven in all ECs [19, 51] and the cause is not yet well understood. Thus, understanding the expression profile and role of miRNAs in the restriction of HIV replication in EC populations can be helpful in controlling them, identifying different stages of HIV/AIDS disease and distinguishing ECs from other subjects infected with HIV-1. The aim of this study was to evaluate the expression pattern of miR-27b, -29, -150, and -221 with regard to cellular/viral factors in the PBMC of Iranian LNTPs and HIV-1 infected naive people.

2. MATERIALS AND METHODS

2.1. Study Population

From March 2014 to November 2019, 157 consecutive treatment-naïve HIV-1-infected patients admitted in hospitals and clinics in Tehran, Iran, were recruited in the present cross-sectional survey. Without any treatment, the viral load and CD4-cell count of 12 HIV-infected patients were less than 2000 copies/mL and normal level (200 -1000/μL), respectively. The statuses of the subjects were monitored in terms of viral load and CD4-cell count for some time, and it was found that they were LTNPs. Forty-two subjects were divided into three groups, including 12 HIV-1 LTNPs (with the HIV-1 viral load of less than 2000 copies/mL) and HIV-1 ECs (with the HIV-1 viral load of less than 50 copies/mL), 15 HIV-1 infected naive individuals and 15 healthy people.

2.2. Collection of Samples and Determination of HIV-1 viral Load

Five mL of peripheral blood was collected from each subject into an EDTA-containing vacutainer tube. After centrifugation of the blood samples, plasma was separated and frozen at –80°C until RNA isolation. The HIV-1 viral load was determined, as previously described in detail [52]. Blood samples from five HIV-1 infected people and five healthy individuals were used as positive and negative controls, respectively.

2.3. Isolation and Processing of Peripheral Blood Mononuclear Cells (PBMCs)

Peripheral blood mononuclear cells were separated using the Ficoll Hypaque (Lympholyte-H, Cedarlane, Hornby, Canada) density gradient centrifugation method washed more than twice with phosphate-buffered saline (PBS, pH=7.3 ±0.1). Afterward, the isolated PBMCs were counted and resuspended in 300μL of RNALater solution (Ambion, Inc., Austin, TX) and then were kept at –20°C for total RNA extraction.

2.4. Total RNA Extraction and Analysis of miRNA Expression

Total RNA was isolated from 1-3×10^6 PBMC samples with the use of the miRNeasy Mini Kit (reference 217004, Qiagen, CA), following the manufacturer's protocols, and stored at -20 °C until testing. The cDNA synthesis was performed on 5μg of total RNA using the miScript® II RT Kit (Qiagen, Germany). It should be noted that according to the previous studies, we selected the miRNAs with antiviral properties against HIV-1 for this study: miR-221 block the CD4 receptor and suppresses the entry of HIV-1 into the cell [53]. miR-29 family by targeting of HIV Nef- 3′UTR leads to decrease the expression of Nef protein and interferes with

HIV replication [47]. Also, miR-27 can decrease the replication of HIV strain NL4.3 [43]. In addition to, miR-150 is an essential factor involved in T-cell activation and may serve as a biomarker for HIV disease progression [54, 55].

The real time polymerase chain reaction (PCR) method was carried out using the miScript SYBR Green PCR Kit (Qiagen, Valencia, CA; #218073), according to the manufacturer's instructions. This assay was performed at 95°C for 2 min, followed by 40 cycles of 94°C for 15 sec, 60°C for 20 sec, and 70°C for 25 sec, using the Rotor-Gene® Q (Qiagen, Hilden, Germany) real time PCR instrument. All the reactions were conducted in triplicate, and SNORD47 was selected as miRNA endogenous controls for relative quantification.

Moreover, the relative gene expression was calculated with the $2^{-\Delta\Delta CT}$ method using the QIAGEN's online data analysis tool, Gene Globe (http://www.qiagen.com/us/shop/ genes-and-pathways/data-analysis-center-overview-page/). Intergroup comparisons were carried out, and top differentially expressed miRNAs were recognized using miScript miRNA array data analysis (Qiagen, Valencia, CA), according to the following criteria: 1) a *p*-value of ≤0.05 for significance and 2) being greater than four-fold differences.

2.5. Expression Level of HIV-1 Nef Gene

For quantifying nef gene levels in two HIV infected groups in the current survey, the real-time PCR assay was optimized and carried out with the Rotor-Gene® Q (Qiagen, Hilden, Germany) system using the SYBR® Premix Ex Taq (Tli Plus) Master Mix (TaKaRa Bio Inc. Shiga, Japan). For determination of HIV-1 nef gene expression level, synthesis of cDNA was performed, as previously described in detail [56].

The real time PCR for the determination of nef gene expression was performed in 25 µL reaction mixture including 12.5 µL 2X SYBR® Premix Ex Taq (Tli Plus) Master Mix (TaKaRa Bio Inc. Shiga, Japan), 10 pmol of each primer for nef region of HIV-1 (forward primer; Nef-F 5′- TGA GAC GAG CTG AGC CAG CAG-3′ and reverse primer; Nef-R 5′- CTG AGG TGT GAC TGG AAA ACC CA-3′ for HIV-1 subtype B [57] (Table **1**) and forward primer; Nef-F 5′-CAG TCA GGC CAC AAG TAC CA -3′ and reverse primer; and Nef-R 5′-AAT CAG GGA ARW AGC CCT GT-3′ for HIV-1 CRF35_AD and CRF35_AE) (Table **1**), 5 µL of cDNA as template, and nuclease-free distilled water up to 25 µL. Amplification steps were performed as follows: initial denaturing for 4 min at 94°C, followed by 40 cycles of denaturation for10 sec at 94°C, annealing for 15 sec at 58°C, and extension for 20 sec at 72°C. Then, the melting curves determination were carried out at temperatures ranging from 55 to 99°C to ensure particular amplification of the target sequence. Moreover, the copy number of the nef gene was estimated based on Kiddle *et al.*'s method [58]. Briefly, this method was calculated based on the fact that the molecular weight of a base pair (bp) is equal to 650 Daltons, or in other words, one mole of a bp is weighed 650 g. The molecular weight of double-strand cDNA (× 2) was estimated by taking the product of its length (in bp) and 650. the inverse of the calculated molecular weight is equivalent to the number of moles per gram and that using Avogadro's constant (6.022×10^{23} molecules/mole) gives the copies of template per gram sample. It is necessary to mention, the molecular weight of cDNA synthesized was measured by Nanodrop (ng/µL). Finally, the number of copies in the sample can be estimated by multiplying by 10^9 to convert to ng. http://www.uri.edu/research/gsc/resources/cndna.html.

Copies of target per genome= (ng double strand DNA) × (6.022×10^{23}) / (length in bp × 10^9 × 650) × 2

2.6. Statistical Analysis

The clinical characteristics of the subjects for continuous variables were described as median or mean ± standard deviation. The sample t-test or the Mann-Whitney U-test was used for among-group comparison. The categorical variables were displayed as N (number) and percentage (%) and were compared using the Fisher's exact test or chi-square analysis. The analysis of miRNAs expression was performed using Kruskal-Wallis and one-way ANOVA tests. However, correlation analysis was carried out using Spearman's correlation coefficient to examine the association between miRNA expression, nef, viral load, CD4 and age. In addition, the results were corrected according to the Benjamini and Hochberg false discovery rate. All data were analyzed with a statistical software package using SPSS version 16.0 (SPSS Inc., Chicago, IL) and Graph Pad Prism version 6 (CA, USA). Receiver-operator characteristic (ROC) curve analysis was performed for each miRNA to identify the sensitivity and specificity of miRNAs as biomarkers of HIV-infected groups. Statistical significance was considered as *p*-values ≤0.05.

3. RESULTS

3.1. Characteristics of Participants

One hundred and fifty-seven consecutive treatment naïve HIV-1-infected patients were enrolled in this cross-sectional study. The mean age of the participants was 34.9 ± 11.2 (ranging between 1-70 years). Of the 157 participants, 104 (66.2%) were male. The demographic, laboratory, and epidemiological characteristics of the studied individuals are summarized in Table **2**.

The viral load and CD4-cell count of 12 treatment naïve HIV-1-infected subjects were less than 2000 copies/mL and normal level (200-1000/µL), respectively. The statuses of the subjects were monitored in terms of viral load and CD4-cell count for some time, and it was found that they were LTNPs.

Forty-two subjects were divided into three groups, including 12 HIV-1 VC (with the HIV-1 viral load of less than 2000 copies/mL) and HIV-1 ECs (with the HIV-1 viral load of less than 50 copies/mL), 15 HIV-1 infected naive individuals and 15 healthy people. The demographic, laboratory, and epidemiological properties of the studied individuals are shown in Table **2**.

Table 1. The thermal cycling conditions of qPCR and primers used in this research for nef gene of HIV-1.

Cycle	Time	Temperature (˚C)	Stages	Size/bp	Sequences	Name
1	4 min	94	Initial Denaturation	161/bp	5′- TGA GAC GAG CTG AGC CAG CAG-3′	Nef-F (Subtype B)
40	10 s	94	Denaturation		5′- CTG AGG TGT GAC TGG AAA ACC CA-3′	Nef-R (Subtype B)
	15 s	58	Annealing	165/bp	5′-CAG TCA GGC CAC AAG TAC CA -3′	Nef-F (CRF35_AD, AE)
	20 s	72	Extension			
0	5s Intervals	50 - 99	Melt		5′-AAT CAG GGA ARW AGC CCT GT-3′	Nef-R (CRF35_AD, AE)

Table 2. Demographic, Laboratory, and Epidemiological Characteristics of Iranian HIV-1 Infected Individuals and healthy people.

Parameters		All HIV-1 Infected Individuals *n*=157	HIV-1 VPs[1] *n*=15	HIV-1 ECs[2] *n*=4	HIV-1 VCs[2] *n*=8	Healthy People *n*=15
Age /Year ± SD		**34.9 ± 11.2 (1-70)**	**30.7 ± 6.3 (20-41)**	**38.0 ± 10.5 (29-52)**	**30.6 ± 12.6 (16-59)**	**35.8 ± 12.1 (20-65)**
No. of patients	Male	104 (66.2%)	9 (60.0%)	4 (100.7%)	3 (37.5%)	8 (53.3%)
	Female	51 (32.5%)	6 (40.0%)	0 (0.0%)	5 (62.5%)	7 (46.7%)
	Transgender	2 (1.3%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Laboratory parameters						
Viral Load IU/mL Median		206082 (0-19575684)	262716 (6012-2109348)	48 (0-50)	978 (120-1692)	-
CD4 count		485 ± 307 (16-2489)	536 ± 253 (233-1077)	780 ± 179 (574-978)	844 ± 357 (388-1434)	874 ± 178 (633-1322)
Epidemiological Characteristics						
Intravenous drug user		52 (33.1%)	0 (0.0%)	3 (75.0%)	5 (62.5%)	0 (0.0%)
injection drug user sexual partner		24 (15.3%)	6 (40.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
History of imprisonment		43 (27.4%)	0 (0.0%)	3 (75.0%)	3 (37.5%)	0 (0.0%)
History of partner imprisonment		15 (9.6%)	3 (20.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
History of unprotected sex		107 (68.2%)	13 (86.7%)	2 (50.0%)	7 (87.50%)	0 (0.0%)
History of blood transfusion		0 (0.0%)	1 (6.7%)	0 (0.0%)	0 (0.0%)	1 (6.7%)
History of surgery		45 (28.7%)	4 (26.7%)	0 (0.0%)	2 (25.0%)	7 (46.7%)
History of tattooing		32 (20.4%)	1 (6.7%)	2 (50.0%)	1 (12.5%)	0 (0.0%)
History of needle stick		19 (12.1%)	0 (0.0%)	3 (75.0%)	1 (12.5%)	1 (6.7%)
History of transplantation		11 (7.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Education	Under diploma	81 (51.6%)	2 (13.3%)	2 (50.0%)	5 (62.5%)	1 (6.7%)
	Diploma	53 (33.8%)	9 (60.0%)	2 (50.0%)	1 (12.5%)	4 (26.7%)
	Upper diploma	23 (14.6%)	4 (26.7%)	0 (0.0%)	2 (25.0%)	10 (66.7%)
Marital Status	Single	71 (45.2%)	7 (46.7%)	1 (25.0%)	7 (87.5%)	5 (33.3%)
	Married	66 (42.0%)	7 (46.7%)	2 (50.0%)	1 (12.5%)	10 (66.7%)
	Divorced	12 (7.6%)	1 (6.7%)	1 (25.0%)	0 (0.0%)	0 (0.0%)
	Widow	8 (5.1%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)

1. Viremic progressors (VPs).
2. Elite controllers (ECs).
3. Viremic controllers (VCs).

It is noteworthy that none of the studied HIV-1-infected participants was infected with hepatitis B (HBV-DNA and HBsAg negative) and hepatitis C (HCV-RNA and anti HCV Abs negative) viruses.

3.2. Determination of microRNA Expression

In the present study, the expression patterns of four miRNAs including miR-27b, -29, -150, and -221 were quantified in the PBMC samples of two groups of HIV-1 infected subjects (HIV-1 LTNPs and HIV-1 infected viremic progressor naive individuals) and compared with those of the healthy cases.

SNORD47 was used as reference genes to normalize the miR-27b, -29, -150 and -221 data, and adequate normalization strategies for statistical analysis were applied for the determination of the mentioned expression patterns of the miRNAs in LNTP (EC and VC) groups compared to those of the healthy group. The expression level of all the miRNAs was

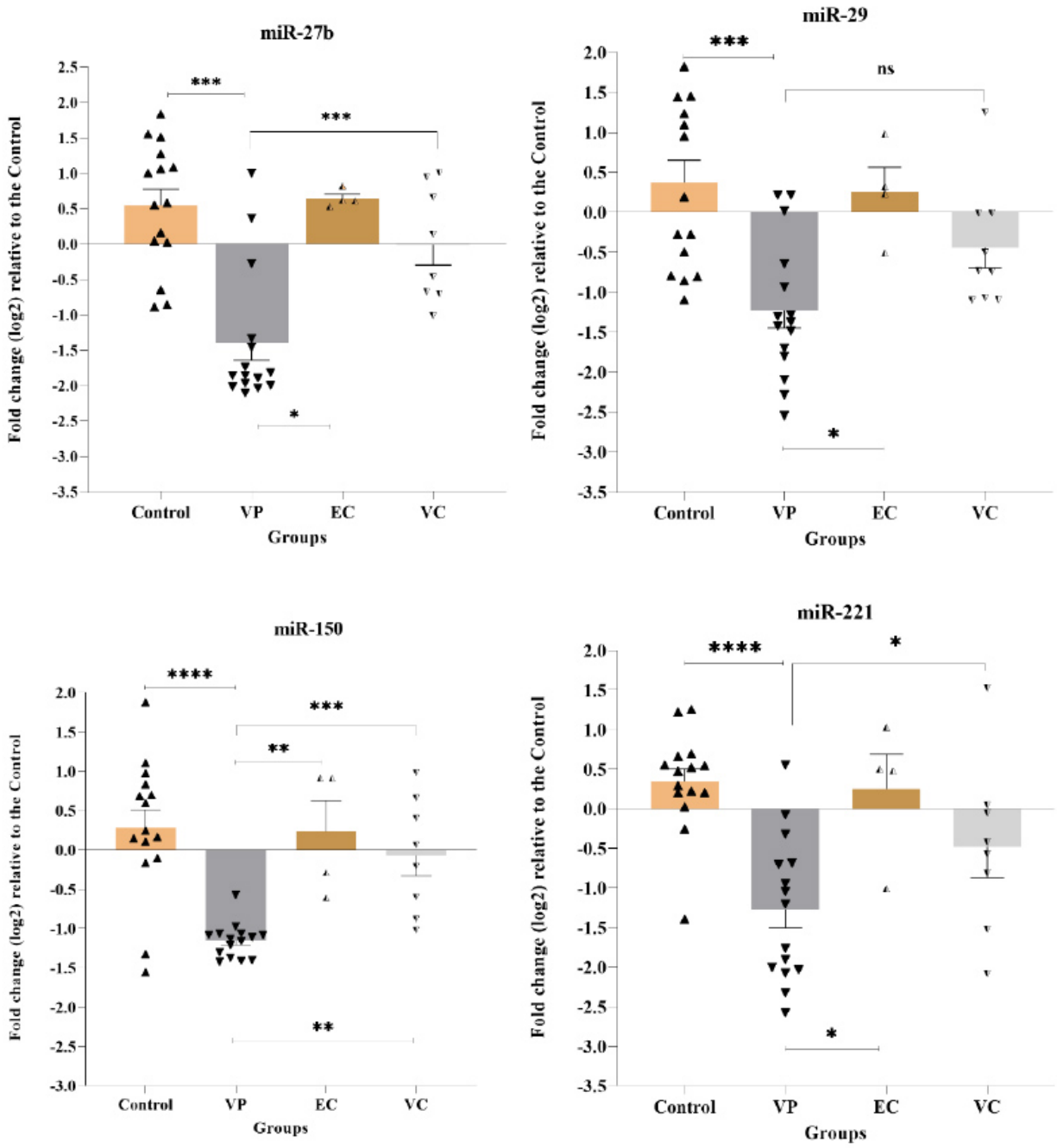

Fig. (1). Differential expression of the indicated miR-27b, 29, 150 and -221 in the PBMCs of healthy controls, VP, EC, and VC. The values in the bar graphs are given as mean ± SEM, and asterisks denote statistically significant differences ($P > 0.05$. *. $P \leq 0.05$. **. $P \leq 0.01$. ***. $P \leq 0.001$. ****. $P \leq 0.0001$) for the indicated groups (ANOVA tests) (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

significantly downregulated in the VP group. Moreover, the expression pattern of all miRNAs was statistically similar in both the EC control groups and their expression level was significantly higher than the VP group. The highest expression level was related to miR-27b and miR-221 in ECs (Fig. **1**). As can be observed in Table **3** and Fig. (**1**), overall, there were no significant differences among the EC, VC and control groups in terms of the expression pattern of the miRNAs. The expression level of miR-150 increased in VC compared to VP, while no significant differences were found in the expression pattern of miR-27b, -29 and -221 between VC and VP. Furthermore, in the VP group compared to the

Table 3. Comparison of miRNAs expression level between VP, EC, and VC patients with healthy controls (control group as a reference group).

microRNA	Healthy Controls (*n*=15)		VP (*n*=15)		EC (*n*=4)		VC (*n*=8)	
-	Difference (Mean ± SEM)	*p*-value	Difference (Mean ± SEM)	*p*-value	Difference (Mean ± SEM)	*p*-value	Difference (Mean ± SEM)	*p*-value
miR-27b	1 (0.55 ± 0.22)	NA	-3.52 (-1.3 ± 0.24)	0.0002	+0.16 (0.64 ± 0.06)	ns	-1.01 (-0.01 ± 0.2)	ns
miR-29	1 (0.37 ± 0.28)	NA	-4.29 (-1.2 ± 0.22)	0.0002	-0.32 (0.25 ± 0.3)	ns	-2.18 (-0.4 ± 0.25)	ns
miR-150	1 (0.28 ± 0.22)	NA	-5.1 (-1.15 ± 0.05)	<0.0001	-0.17 (0.23 ± 0.3)	ns	-1.25 (-0.07 ± 0.25)	ns
miR-221	1 (0.34 ± 0.16)	NA	-4.7 (-1.2 ± 0.23)	<0.0001	-0.26 (0.25 ± 0.4)	ns	-2.4 (-0.48 ± 0.3)	ns

Each cell indicated as difference, adjusted *P*-value; *P*-value adjusted by Benjamini-Hochberg method for multiple comparisons; NA: not applicable, ns: not significant.

VC group, the expression levels of miR-27b (*p*-value: 0.0008), -150 (*p*-value: 0.0007), and -221 (*p*-value: 0.047) significantly were downregulated. More information is presented in Table **3** and Fig. (**1**).

The correlation between the expression and cellular/viral factors of the miRNAs in EC group is shown in Fig. (**2**). The results of the analysis showed that in the VP group, significant negative correlations were found between the expression level of miR-29 and the nef gene expression level (r=-0.87, $p<0.0001$). Further, a statistically significant inverse correlation was observed between miR-221 and HIV viral load in this group (r= -0.67, $p=0.007$). However, no significant relationship was observed between the expression levels of the miRNAs and the mentioned factors in the EC group (Fig. **2**). Furthermore, in the VC group, the positive correlation between the expression levels of miR-150 and T-cell CD4 count was statistically significant (r=0.83, $p=0.01$).

3.3. ROC Curve Analysis

ROC curves can be used to find the circulating cellular miRNAs as biomarkers to discriminate different subsets of the HIV-infected subjects from healthy individuals. AUC was done, miRNAs with AUC value >0.8 [59] were considered as good potential biomarkers to discriminate among the mentioned groups. According to the ROC results, miR-27b (AUC: 0.92; 95% CI: 0.81 to 1.0; $P<0.0001$), miR-29 (AUC: 0.87; 95% CI:0.75 to 1.0; $P=0.0005$), miR-150 (AUC: 0.89; 95% CI:0.74 to 1.0; $P=0.0003$) and miR-221 (AUC: 0.91; 95% CI: 0.8 to 1.0; $P=0.0001$) could be serve as useful markers for discriminating between the healthy control group and VP group. Furthermore, miR-221 (AUC: 0.808; 95% CI: 0.57 to 1.0; $p= 0.016$) with optimal sensitivity and specificity were potentially useful biomarkers to differentiate the healthy control group from the VC group.

Furthermore, miR-29 (AUC: 0.8; 95% CI: 0.55 to 1.0; p-value: 0.04) as well as miR-27b (AUC: 0.93; 95% CI: 0.8 to 1.0; *p*-value: 0.009), miR-29 (AUC: 0.95; 95% CI: 0.84 to 1.0; *p*-value: 0.006), miR-150 (AUC: 0.98; 95% CI: 0.93 to 1.0; *p*-value: 0.003) and miR-221 (AUC: 0.86; 95% CI: 0.67 to 1.0; *p*-value: 0.02) with optimal sensitivity and specificity were potentially useful biomarkers to differentiate EC group from VC groups and VP groups, respectively.

Also, to assess the diagnostic value of the studied miRNAs in PBMC to discriminate VP from VC groups, ROC curve analysis showed that miR-27 (AUC: 0.93; 95% CI: 0.8–1.0, $p=0.009$) and miR-150 (AUC: 0.96; 95% CI: 0.9–1.0, $p=0.003$) were a useful marker for discriminating VP group from the VC group.

4. DISCUSSION

We evaluated the expression pattern of four miRNAs, including miR-27b, -29, -150 and -221 in the PBMCs of LTNPs controlling viremia without ART therapy compared to untreated HIV-1 infected individuals. In addition, the correlation was assessed among HIV-1 viral load, the expression of the HIV nef gene, CD4+ T-cell counts and the expression level of the mentioned miRNAs. According to the results, the difference in miR-27b, -29, -150 and -221 expression profiles was statistically significant among individuals classified into four groups (P-value < 0.01). The expression levels of all the miRNAs were higher in EC and VC than in VP, while they were similar to those in the healthy group.

The interaction between viruses and cellular miRNAs has recently received attention, and it has been observed that viruses can use cellular conditions to their benefit by deregulation of host miRNAs [27, 53, 60-64]. Many studies have reported that miRNAs are involved in the progression of HIV-1 infections through modulating viral proteins and/or affecting host parameters associated with viral replication [35, 36, 38-40]. Moreover, it has been shown that the cellular miR-221 and -222 are upregulated in HIV□infected bystander macrophages, and through negative regulation of CD4 expression, cause to restrict the entry of HIV-1 [53, 65], in return, the HIV-Tat protein can inhibit these miRNAs [38]. In this study, we observed that miR-221 was significantly downregulated in VPs compared with the LTNP (*i.e.*, EC and VC) and healthy groups (P-value <0.01). The reason is that some of the HIV-1 genetic mutations (*e.g.*, mutation in the encoding region of Vif, Nef, Tat, Vpr, *etc.*) have been reported to be probably associated with non-progression to AIDS [66]. Thus, the defect of these genes in LNTPs may impair their inhibitory effect on the expression of cellular miRNAs and increase the miRNA expression levels. However, no studies have been conducted in this regard and further research is required.

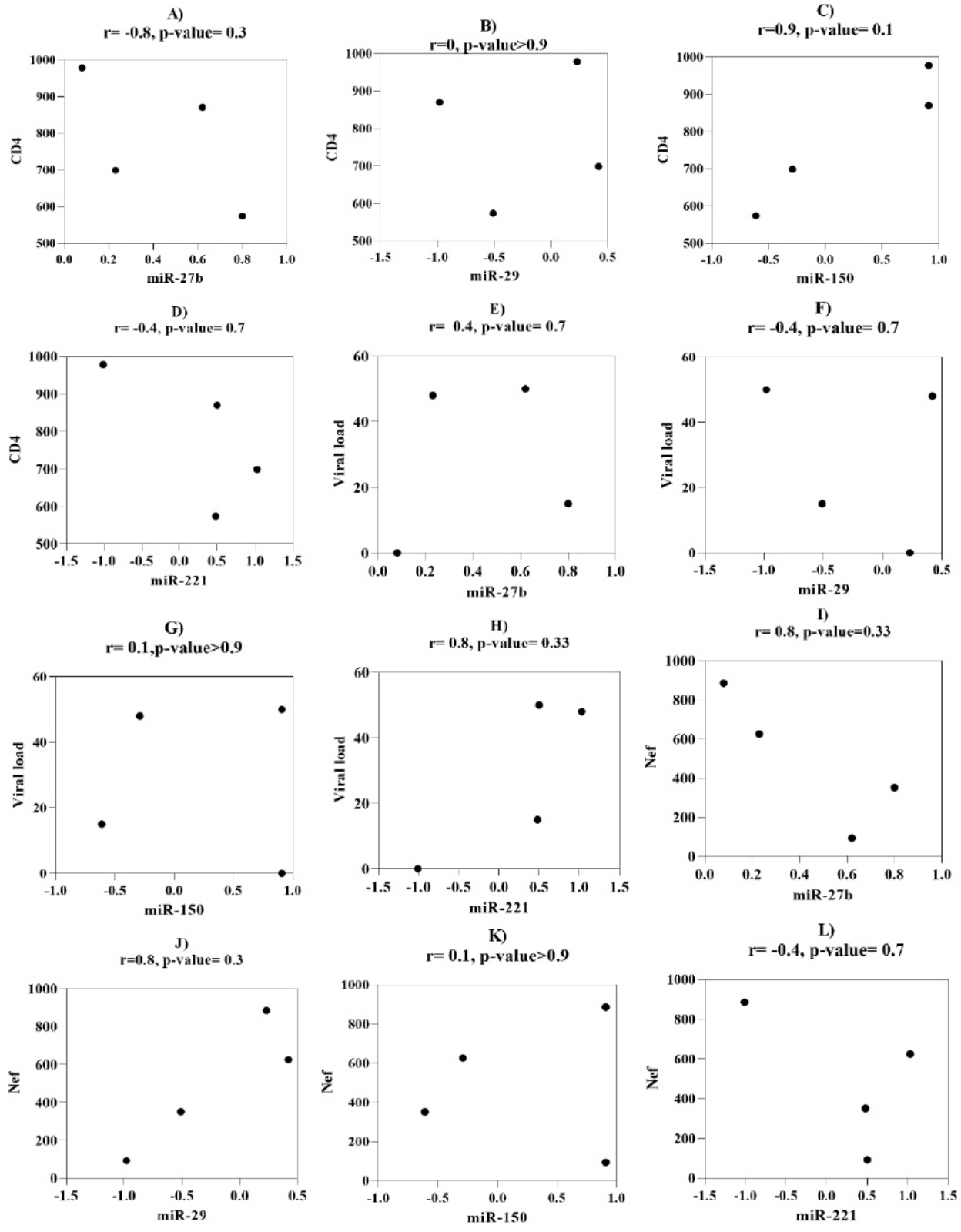

Fig. (2). Spearman's correlation coefficient between the miRNA's expression level with CD4 counts, the expression level of nef gene and viral load in EC group. The x-axis shows the Log2 of miRNA expression and the y-axis shows the CD4 count (**A**-**D**), viral load (**E**-**H**) and Nef expression level (**I**-**L**).

HIV-1 negative factor (Nef protein) is one of the HIV accessory proteins and is a critical factor for highs viral loads, induction of host cell destruction, and for timely progression to acquired immunodeficiency syndrome (AIDS). Besides, Nef has multiple functions, such as it a) inhibits the degradation of viral virions in the acidic environment of the endosome, b) can stimulate apoptosis of host cell by interfering with cell death pathways as well as c) can enhance viral DNA synthesis by regulating different cellular pathways [67-69]. In addition, some studies reported that mutations in the nef gene had been observed in ECs individuals, which may lead to decreases in HIV replication, but this event has not been proven in all ECs [19, 51] and the cause is not yet well understood. Furthermore, experimental studies have shown that among HIV-1 genes, the nef gene is more targeted than other HIV-1 genes by cellular miRNAs [35, 70]. For this reason, the expression level of the nef gene and the correlation between this gene with selected miRNAs were evaluated in the current study.

Huang *et al.* [71] showed that the clusters of miRNAs, including miR-150, -382, -223, 125b and -28 could bind to 3' UTR of HIV mRNA and inhibit its translation [71]. In another study, Huang *et al.* [41] examined the expression level of miR-125b-5p, -28-5p, -150- 5p, -223-3p and -382-5p in monocytes cells. They reported that these miRNAs were overexpressed in monocyte-derived macrophages and that knockdowns of these miRNAs facilitated infectivity of HIV-1 in monocytes. They also found that these miRNAs had the ability to suppress the replication of HIV-1 in resting CD4 T cells [41]. Consequently, it is possible that these miRNAs potentially result in disrupting effects in HIV replication and also deregulation of these miRNAs by HIV is expected. One crucial step for the HIV replication cycle is integration in the host genome, allowing the persistent expression of viral genes [72, 73]. Transcription of the integrated HIV-1 genome, known as provirus, is dependent on the host-cell positive transcription elongation factor b (P-TEFb). P-TEFb is a critical factor in regulating the elongation of cellular RNA polymerase II transcripts [74]. The P-TEFb heterodimer is composed of a kinase, (CDK9), and one of the three cyclin subunits (*i.e.*, cyclin T1, cyclin T2A, and cyclin T2B) [75-77]. The Cdk9/cyclin T1 P-TEFb complex activates transcription of the provirus and subsequently, the cyclin T1 subunit binds to HIV-transactivator protein Tat and supports transcription of the provirus by cellular RNA polymerase II [78]. Chiang *et al.* [43] investigated the effect of miR-27b, -29b, -150, and -223 on the translation of cyclin T1 in resting CD4+ T cells. They observed that miR-27b, -29b, -150, and -223 could decrease expression levels of cyclin T1 protein. Furthermore, they observed that miR-27b was directly bound to 3′-UTR of cyclin T1 mRNA and enforced expression of miR-27b, which was associated with the decrease in the HIV-1 reporter virus expression level. They reported, however, that miR-29b, -150 and -223 had an indirect effect on the expression levels of cyclin T1 [43]. Our results demonstrated that the expression of miR-27b, -29, and -150 was significantly downregulated in PBMC samples of VPs, and that the expression pattern of these miRNAs in LNTP is similar to uninfected people. Thus, the results make the role of these miRNAs more prominent in controlling HIV progression.

Some factors, including mutation in viral genes, host immune system, and host genetic factors, may justify control of HIV replication in LNTPs. However, since these characteristics are not the same in all LNTPs, it is expected that other mechanisms are involved in the control of HIV disease [18-20]. On the other hand, the role of miRNAs in controlling virus replication in LNTPs has not yet been investigated and limited studies have reported the expression pattern of miRNAs in these subjects. Recently, Witwer *et al.* [79] examined the expression profiling of miRNAs in PBMC samples of elite suppression (ES or elite control) and viremic patients using the TaqMan low-density array (TLDA), NanoString platform and stem-loop quantitative PCR. According to the NanoString result, an expression level of mir-150 and -29 families (*i.e.*, miR-29a, b, and c) decreased in viremic patients in comparison with the control group. Moreover, according to all the three profiling methods, the expression level of miR-150, -125b, and -31 were statistically significantly lower in both the ES and viremic patients than in the healthy group. In this study, it is suggested that ongoing effects of HIV infection, despite viral inhibition by EC or the durability of host miRNA responses, can explain the similarity of expression patterns of miRNAs between EC and viremic patients [79]. Contrary to the results of this study, our results showed that the expression level of miR-150 and -29 was higher in EC and VC than in VP, and no statistically significant differences were observed in the expression level of these miRNAs in comparison with control groups. Different genotype distribution of HIV-1, sample size, sampling, and methodological differences may be the cause of differences in the results of the studies.

Similar to our results, the expression pattern of miR-27, -29, and -221 has been reported in a study by Egana-Gorrono *et al.* [80]. They screened 286 miRNAs in the stimulated PBMC from LNTPs, VPs, patients receiving ART therapy and healthy groups using the TLDA assay. The result of their study showed that the level of miR-27a, -27b, -29b, and -221 was overexpressed in ECs and uninfected subjects and that these miRNAs were downregulated in VPs and patients under ART treatment. These results suggest the similarity of miRNA expression patterns between controllers and uninfected individuals [80]. It should be noted, although some studies have shown that the expression pattern of some cellular miRNAs in LNTPs individuals is similar to that in the healthy groups [70], expression patterns of cellular miRNAs can be different between LNTPs and healthy subjects [70, 81]. Moreover, besides the viral infections, other factors such as stress, antiviral drugs, co-infections with other pathogens [82-85] and *etc.*, can alter the expression pattern of cellular miRNAs. For example, Murray *et al.* [86] reported that expression levels of miR-122 and -200a were significantly upregulated in HIV/HCV co-infected group compared to the HIV-1 mono-infected subjects [86]. As a result, the expression pattern of cellular miRNAs between the mentioned groups can be similar or different and so further experimental studies are needed to understand this topic.

Hulka *et al.* defined biomarkers as "cellular, biochemical or molecular alterations, that could be measured in biological media, such as human cells, tissues or fluids" [87]. MiRNAs have useful characteristics such as high stability and are measurable in the body fluids (urine, serum, plasma, saliva, and PBMCs) and tissues [88]. For this reason, many researchers have mentioned that miRNAs can potentially act as useful diagnostic and prognostic biomarkers with high sensitivity and specificity [89, 90]. Munshi *et al.* evaluated the expression pattern of miR-150, -16, -146b-5p, -191 and -223 in order to recognize novel biomarkers in PBMCs and plasma of different stages of HIV/AIDS infection [54]. They observed that the miR-150 expression significantly decreased in ART-naive AIDS patients compared to the control group. Moreover, after 6 months of therapy with ART in these patients, miR-150 expression levels obtained levels similar to healthy subjects. This study demonstrated that the expression pattern of miR-150 in plasma and PBMCs could be a good diagnostic biomarker of the status of HIV disease [54]. The results of our study showed that there was a significant difference in the expression level of miR-150 between VPs, healthy individuals and LTNPs (ECs and VCs). It was also revealed that among miRNAs examined in the study, a significant difference was only observed in the expression level of miR-150 between VPs and VCs. Consequently, this miRNA can serve as a biomarker in distinguishing VPs from LNTPs. Of course, more studies are required to prove this claim. Therefore, cellular miRNAs can consider as viable biomarkers to detect subsets of the HIV-infected subjects. For this purpose, we used ROC curve analysis. According to the ROC results, miR-27b, -29, -150, and -221 with optimal sensitivity and specificity were potentially useful biomarkers to differentiate the healthy control group and EC from the VP group. According to the results, miR-27b, and -miR-150 can be used as tools to discriminate between VP and VC groups.

In some studies, the correlation was analyzed between cellular miRNAs and cellular/viral factors in virus-infected patients. For example, for miR-125b and miR-146b-5p levels, a positive and a negative correlation was observed with HIV-viral load, respectively [54, 79]. Witwer *et al.* analyzed the correlation between miRNAs and HIV viral load in HIV-1-infected elite suppressors and viremic patients. They reported that there was a positive correlation between miR-125b and -150 with viral load [79]. In contrast to this study, no significant relationship was observed between miR-150 and viral load in our study. However, a negative correlation was observed in our study between expression fold differences of miR-221 and HIV-viral load in the VP group. Our results are also supported by a previous study [34], where miR-150 expression level was positively correlated with CD4 count and a significant positive correlation was observed between the expression of miR-150 and the CD4 count in the VP group. As mentioned above, cellular miRNAs can have effects on HIV replication by either targeting RNA or encoding a viral protein that have necessary products for viral replication [46]. It has been demonstrated that miR-29 potentially targets 3'-UTR of nef and decreases the Nef protein expression [47]. In addition, in some studies, positive and negative correlations were reported between miR-29 expression with CD4 count and the nef gene expression level [34, 91]. Based on our data an inverse correlation between the miR-29 expression level and the nef expression level in the VC group. This probably highlights the role of this miRNA in the regulation of nef transcription. However, no significant relationship was found between miR-29 expression and CD4 count. The difference in the expression pattern of cellular miRNAs can be useful to distinguish non-infected people or LTNPs from HIV infected patients and also to distinguish different stages of HIV/AIDS disease. In conclusion, the present research was the first report about the comparison of expression patterns of four miRNAs, namely miR-27b, -29, -150, and -221, between HIV-1 infected naive patients and LNTPs with healthy individuals in Iran.

CONCLUSION

This study reported an expression profile of cellular miRNAs in HIV naïve patients, ECs, and VCs, as compared to non-infected individuals. Distinguishing HIV-infected people from LTNPs and healthy people requires the discovery of biomarkers that specifically categorize these subjects. Determination of the role and expression pattern of miRNAs in these groups can open new gates to control HIV disease or differentiate HIV-infected subgroups.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This cross-sectional study was established to be in agreement with the ethical principles as well as the standards and national norms for conducting medical studies in Iran. Research ethical approval was received from the ethics committee of the Iran University of Medical Sciences (IUMS), Tehran, Iran (ethical code: IR. IUMS. FMD. REC 1398. 051). The research was performed in accordance with the Second Declaration of Helsinki, and all the subjects provided informed consent (both written and verbal) to participate in the research.

HUMAN AND ANIMAL RIGHTS

No animals were used in this survey. All humans procedures were followed in accordance with the standards set forth in the Declaration of Helsinki program of 1975, as revised in 2013 (http://ethics.iit.edu/ecodes/node/3931).

CONSENT FOR PUBLICATION

The studied subjects were informed about the current survey, and a written consent form was obtained from all volunteers before their enrollment.

STANDARD OF REPORTING

The survey conforms to the STROBE guidelines.

AVAILABILITY OF DATA AND MATERIALS

The authors of this study confirm that the data supporting the results and findings of this survey are available within the article.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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