Background and Objective: Various cellular factors affect the process of HIV activity. One of these cellular factors are structures known as microRN that are expected to be involved in controlling HIV replication and infectivity. The expression of one or a set of them may represent the patient's clinical conditions. In this study, the expression of miR-29a and miR-29b involved in regulating viral genes' expression was evaluated in three HIV-positive groups and a healthy control group. Later, the expression level of these microRNAs was compared between the cases and controls.

Methods: Total RNA extraction was performed on the collected samples using RNA-plus kit and then the microRNA expression levels were evaluated using Relative Real-time PCR. The obtained data was entered into SPSS 22 and Graphpad softwares and analyzed using Kruskal-Wallis and Mann-Whitney tests. P-value of less than 0.05 was considered as statistical significance level.

Results: The expression level of miR-29a was reduced in patients under treatment and drug-resistant patients (P ≤ 0.05). All three HIV-positive groups including people without drug treatment, patients under treatment and drug-resistant patients showed reduced miR-29b expression level compared to control group (P ≤ 0.05).

Conclusion: The decreased expression of miR-29a and miR-29b in patients under treatment and drug-resistant patients indicates an increased viral replication and reduced CD4 cell count. It may be possible to predict the progression of the disease by miRNA measurement or control viral replication using these mir-RNAs that requires further studies.

Keywords: HIV, Expression, Mir-29a, Mir-29b.
INTRODUCTION

Human immunodeficiency virus (HIV) is a lentivirus from the retrovirus family causing acquired immune deficiency syndrome (AIDS) in humans. HIV infects CD4+ T-cells and its infection is divided into two phases including an initial phase that lasts about 1-2 months and a chronic phase that may take 10-20 years before the onset of AIDS. HIV is an RNA virus with 9.7 kb long genome containing several secondary structures (LTR, TAR, RRE, PE, SLIP, CRS, INS) and 9 genes (gag, env, tat, rev, nef, vif, vpr, vpu) that produce 19 proteins (1). Nef is a 27-25 kDa myristoylated protein that plays an essential role in HIV replication and pathogenesis. This is a pathogenic protein known as the virulence factor that alters intracellular mechanisms and provides the necessary conditions for replication and survival of HIV. Nef expression in infected cells induces several changes including control of protein expression in the cell membrane and alteration of the cytoskeleton and signaling system. Nef reduces the stimulation threshold of CD4+ T-cells and activates them that facilitates the replication of HIV. Nef protein decreases the expression of CD4, LCK and CTLA4 on the surface of CD4+ T-cells and thus controls the cell signal transduction pathways (2,3). MiRNA, expressed in most eukaryotes, is a small RNA with a length of about 22 nucleotides and acts as the key regulator of gene expression through binding to target mRNA. It is estimated that almost 30-90% of protein-encoding genes are targeted by mammalian miRNAs that have been identified until now. A mature 22 nucleotide-long mRNA includes a guide strand and a passenger strand. The key area of guide strand, a 6-8 nucleotides region, is situated at the 5′-end of the guide strand. Binding of this area to complementary sequence in the 3′UTR of mRNA activates the RISC complex and depletes or inhibits the translation of mRNA (1, 4-7). MiRNA expression changes in viral infections that indicates their important role in the interaction between virus and host (8). In addition to the protein-RNA and RNA-RNA interactions, recent studies have revealed a remarkable correlation between the viral proteins involved in the pathogenesis of HIV-1 and RNA silencing, similar to the tat and TRBP relation (5). It has been demonstrated that during viral infection, some proteins with suppressor of RNA silencing (SRS) feature are produced that act against RNAi pathway in host (9, 10). A study has shown that a group of miRNAs are involved in controlling the infectivity of HIV in infected people and the reduced-expression of these miRNAs results in an increased viral replication. It was suggested that these anti-HIV miRNAs can have an important role in the control of HIV latency mode (11).

MiR-29 is one of the miRNAs that directly binds to the 3′UTR of viral mRNA. This binding to HIV miRNAs directs MiR-29 toward P-bodies (Processing body) and thus prevents the translation of viral proteins and inhibits the replication (12). It also has been shown that miR-29a inhibition significantly increases HIV infection that indicates the reduction of Nef viral protein expression by miR-29a (13).

Some clinical studies also suggest that miRNA expression profiles in elite suppressor and healthy individuals compared to patients with viremia is significant and plays an important role in immunity against the virus. miRNAs’ expression is mostly correlated with the reduced CD4+ T-cell count during HIV infection and can reflect the progression of HIV infection. In this study, several miRNAs including miR-125b, miR-150 and miR-29 family were shown to have a regulatory role in HIV replication. The results of this study suggest that the role of miRNA in the creation of latent mode of HIV infection and infection control should be widely investigated. The results of this study also showed the decreased expression of miR-29a, miR-29b and miR29c in viremic individuals (14).

Therefore, this study aimed to evaluate the expression of miR-29a and miR-29b in three HIV-positive groups of patients and a control group.

MATERIAL AND METHODS

The samples used in this study consisted of 70 whole blood samples from three groups of patients and a control group. The first three groups had 20 people each, and the fourth group had 10 people. Group 1: Control (healthy individuals)

Group 2: HIV-positives without taking medication. Group 3: HIV-positives treated with highly active antiretroviral therapy (HAART) drugs

Group 4: First line drug-resistant HIV-positives treated with HAART dr
The RNA extraction was performed from 2 ml of whole blood using RNx-PLUS kit (Sinagene Company) according to the manufacturer’s protocol. In order to assess the purity and quality of the extracted RNA, the absorbance (Optical density) ratio of RNA samples was measured at 260 nm to 280 nm and then the samples with absorbance of 1.8-2 at 260/280 nm were used. Also, the concentration of the extracted RNAs was assessed using a bio-photometer (Eppendorf, Germany). DNA was removed by DNase enzyme (Fermentase, Germany) and in the same stage, integration of primary extraction also took place. MiRNAs’ amplification was done by Relative Real time-PCR method and Parsgenome MiR-Amp kit, according to the manufacturer’s protocols that are explained briefly. The poly A tail was added to miRs and then the first cDNA strand was synthetized using a primer with a poly T section in the 3’ end and a general sequence at the 5’ end. Finally, real time PCR was performed using specific primers for the investigated miRs. Internal control (5s rRNA) was used due to the relativity of the method. To perform the real time PCR, 10 µl SYBER Green master mix, 0.5 µl ROX dye, 5 µl of the synthetized cDNA and 4 µl miR specific primer were mixed and then reached the volume of 20 µl. Real time PCR was carried out by the ABI 7300 model (Applied Biosystems) using an optimal temperature protocol. At this stage, two miRNAs and an Internal control (5s rRNA) were amplified in duplicate and then the obtained data was recorded.

First, Gene expression level was calculated using the ΔΔCT method according to the following formulae:

$$\Delta \Delta CT = \left[(C_{T\text{gene of interest}}} - C_{T\text{internal control}})\right]_{\text{patients}} - \left[(C_{T\text{gene of interest}}} - C_{T\text{internal control}})\right]_{\text{controls}}$$

Then, SPSS statistical software (version 22) and Graph Pad were used for data analysis. Kruskal-Wallis was used to compare miRNA expression levels between the test groups.

Figure 1- Comparison of relative expression of miR-29a in the study groups

Figure 2- Comparison of relative expression of miR-29a in the study groups.
RESULTS
Among the participants of the control group, 20% were female and 80% were male with an average age of 28.7 years. The patient groups included 50 patients, 36 of whom were male and 14 female with an average age of 38.7 years. In this study, the expression of miR-29a and miR-29b was evaluated in a control group and three HIV-positive groups (HIV-positives without taking medication, HIV-positive people treated with HAART and those resistant to HAART).

DISCUSSION
In this study, the expression level of miR-29a and miR-29b was evaluated in three HIV-positive groups of patients and a control group (miR-29a and miR-29b which target the Nef region of the HIV genome). The results showed reduced expression of miR-29a to 18.5-fold in patients treated with HAART and 31.8-fold in the drug resistant group when compared with the control group. The results demonstrated decreased expression of miR-29b to 12.8-fold in HIV-positive individuals without medication, 2.1-fold in treated group with HAART and 225-fold in the HAART resistant group. Studies have shown reduced HIV-infectivity and replication in the presence of miR-29a. Also, viral strains with mutations in the miR-29a target region increase the production of viral particles and thus infectivity of the produced virus particles will increase compared to the wild types. MiR-29a can target HIV genes and affect the replication and infectivity. It can also interact directly with viral transcripts and directs them toward p-body which will limit the virus (12). The negative effect of miR-29a in HIV replication in a study by Ahluwalia et al. was examined and it was found that the over-expression of miR-29a result in declined production and HIV-infectivity. Their results also showed the inhibition of viral genes expression by miRNA and limitation of HIV replication. Nevertheless, the same study suggested that HIV is expected to develop defense mechanisms against this intracellular process (13). In a study on clinical samples by Witwer et al., it was demonstrated that miRNA expression profiles are significantly different in elite suppressors and healthy individuals compared to viremic patients. It was also shown that miRNA expression is associated with the reduction of CD4+ T-cells’ count during HIV infection which can reflect the progression of HIV infection. In the mentioned study, several miRNAs, including miR-125b, miR-150 and miR-29 family have been shown to have a regulatory role in the HIV replication. The results of the aforementioned study showed decreased expression level of miR-29a and miR-29b in patients with viremia which is in agreement with our study findings (14). In a study by Houzet et al., 36 patients were categorized into 4 groups according to their CD4+ T-cell count and viral load and then later compared with a control group. miRNAs with 2-fold increase or decrease in comparison to the control group in at least 50% of patients, were assessed. Their results showed reduced miR-29a expression in the three groups of patients as well as in the HIV-infected PBMCs. This indicates that there is a believable model based on patients’ conditions for miRNAs expression (15). Their results are in agreement with our study findings. In a study by Sun et al., based on data from the PITA software, 256 miRNAs with sequences complementary to the Nef-3’-LTR were detected. Based on the miRNAs expression in CD4+ T-cells, miRNA binding energy and target sequence, a list of miRNAs of the host with the ability to target viral sequences in the 3’LTR, including miR-29a were prepared. Decreased expression of miR-29a was reported in the mentioned study. The relative expression level of miR-29b in HIV-infected PBMCs was investigated using microarray and RT-PCR methods and the results showed declined expression of miR-29b in the infected PBMCs (16) which further confirms our results. In Reynoso et al. study, miRNA expression profiles in plasma of HIV-positive patients, healthy controls and elite controller individuals were compared and their results showed that the expression level of miR-29b in patients with low CD4 count have been reduced when compared with the control group and the elite controllers. In a study by Dey et al. in India, the expression levels of miR-29a in 75 HIV-positive patients who were classified into three groups were studied. Groups included those with long-term illnesses but with less progression, patients with normal progression and individuals with rapid disease progression who were compared with a healthy control group. Their results showed that the expression of miR-29a in these three groups was higher than the control group (17) which is not consistent with our results.
This may be due to proper management and control of the disease during the study and monitored drug administration by assessing drug resistance in the studied patients. As it was demonstrated in our previous studies, drugs are administered and prescribed without studying and determination of drug resistance in patients. Such cases may explain this discrepancy. The Nef protein plays an important role in the replication and pathogenesis of HIV which can deplete CD4 and MHC in T-cells and also miR-29a and miR-29b, target the Nef gene and control its expression level in HIV. Moreover, tat protein of HIV due to increased expression of NFxB through interaction with SIRT-1 and SIRT-1 blocking ability for deacetylation of lysine 310 in the P65 subunit, can inhibit miR-29a. Thus the decreased expression of miR-29a may indicate an increase in the activity of the HIV. Infectivity level and viral replication can be affected by the regulatory activities of miR-29a and miR-29b. This suggests that where viral replication and infectivity of the virus are increased, the expression of miR-29a and miR-29b are reduced. While drug-resistant and patients under treatment have increased viral activity and the virus dominance on the immune system is accompanied by reduction of D4+ cells, reduced expression of miR-29a and miR-29b is expected. In this study, it was also demonstrated that miR-29a and miR-29b reduction is in the direction of disease progression and drug-resistant patients and patients under treatment with low CD4 count, showed reduced miR-29a and miR-29b expression. Based on the results of this study and previous studies, it can be concluded that miR-29a and miR-29b may be suitable biomarkers for assessing the disease progression in patients.

CONCLUSION

According to the results of this study and its comparison with previous studies, it seem that there are significant changes in miRNA expression in HIV-positive individuals which is also significantly associated with patients’ clinical conditions. Moreover, some miRNAs such as miR-29a and miR-29b which are altered in the direction of disease progression, can be used to assess patients’ conditions and lead the treatment process. However, it is noteworthy that the miRNA expression level alone cannot reflect and justify the patients’ conditions and it is required to be used along with other factors, affecting the patient’s clinical conditions. Also, due to the influence of several factors on miRNA expression, determination of their association with clinical conditions and the degree of miRNA’s impact on patients’ conditions and viral activity require further studies.

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CONFLICT OF INTEREST

There are no conflicts of interest.