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Investigation of Programmed death-1 Expression Levels in Newly Diagnosed HIV (+) Individuals

Yeni Tanı Almış HIV (+) Bireylerde Programmed death-1 Ekspresyon Düzeylerinin İrdelenmesi

Hande TOPTAN[1], Atılay AKDOĞAN[2], Gülay TRAK[1], Mehmet KÖROĞLU[3], Oğuz KARABAY[4], Mustafa ALTINDİŞ[3]

[1]Sakarya Training and Research Hospital, Clinic of Medical Microbiology, Sakarya, Turkey
[2]Sakarya University Institute of Health Sciences, Department of Medical Microbiology, Sakarya, Turkey
[3]Sakarya University Faculty of Medicine, Department of Medical Microbiology, Sakarya, Turkey
[4]Sakarya University Faculty of Medicine, Department of Infectious Diseases and Clinical Microbiology, Sakarya, Turkey

Abstract

Introduction: Antiretroviral therapy (ART) increases life expectancy by positively affecting viral load and immune system cells in individuals infected with human immunodeficiency virus (HIV). In this study, it was aimed to examine the correlation between CD4 T cell count and viral load changes and Programmed death-1 (PD-1) level before and after ART in newly diagnosed HIV (+) individuals.
Materials and Methods: Thirty HIV-infected individuals who were admitted to Sakarya University Training and Research Hospital Infectious Diseases Outpatient Clinic, had no other chronic disease, were newly diagnosed, and were to be treated for the first time with this study were included. In the follow-up of all patients, before the treatment and in the later stages of the treatment (in the 1st and 3rd months); quantitative HIV RNA levels and CD4 T and CD8 T cells counts were determined. In our study, PD-1 levels expressed from CD4 and CD8 T cells were determined by flow cytometry method.
Results: A positive, moderate correlation and a statistically significant correlation (n=80, rs=0.49614, p<0.001) was found between the rate of PD-1 expressed in CD4 T cells and viral loads. A positive, moderate correlation and a statistically significant correlation (n=80, rs=0.50954, p<0.001) was found between the rate of PD-1 expressed in CD8 T lymphocytes and viral loads.
Conclusion: As a result, it was concluded that PD-1 might be a marker of viral load and might be useful for monitoring viremia and changes in treatment response.
Keywords: HIV, antiretroviral therapy, programmed death-1

Öz

Giriş: Antiretroviral tedavi (ART), insan immün yetmezlik virüsü (HIV) ile enfekte bireylerde viral yük ve immün sistem hücrelerini olumlu yönde etkileyerek yaşam beklentisini arttırmaktadır. Bu çalışmada yeni tanı almış HIV (+) bireylerde ART öncesi ve sonrası CD4 T hücre sayısı ve viral yük değişimleri ile Programmed death-1 (PD-1) düzeyi korelasyonunun irdelenmesi amaçlanmıştır.
Gereç ve Yöntem: Çalışmamıza Sakarya Üniversitesi Eğitim ve Araştırma Hastanesi Enfeksiyon Hastalıkları Polikliniği'ne başvuran, başka bir kronik hastalığı olmayan, yeni tanı almış ve ilk kez bu çalışma ile tedavisine başlanacak olan 30 HIV ile enfekte birey dahil edilmiştir. Tüm hastaların izlem ve tedavi takibinde, tedavi öncesi ve tedavinin ilerleyen dönemlerinde (1. ve 3. aylarda); kantitatif HIV RNA düzeyleri ve CD4 T ve CD8 T hücre seviyeleri belirlenmiştir. Çalışmamızda CD4 ve CD8 T hücrelerinden eksprese edilen PD-1 düzeyleri akım sitometrisi yöntemi ile belirlenmiştir.
Bulgular: CD4 T hücrelerinden eksprese edilen PD-1 oranı ile viral yük değerleri arasında pozitif yönlü, orta düzeyde korelasyon ve istatistiksel olarak anlamlı bir ilişki (n=80, rs=0,49614, p<0,001) bulunmuştur. CD8 T lenfositlerden eksprese edilen PD-1 oranı ile viral yük değerleri arasında pozitif yönlü, orta düzeyde korelasyon ve istatistiksel olarak anlamlı bir ilişki (n=80, rs=0,50954, p<0,001) bulunmuştur.
Sonuç: Sonuç olarak PD-1'in viral yükün bir belirteci olabileceği, viremi ve tedavi yanıtındaki değişiklikleri izlemek için yararlı olabileceği sonucuna varılmıştır.
Anahtar Kelimeler: HIV, antiretroviral tedavi, programmed death-1

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Address for Correspondence/Yazışma Adresi: Hande TOPTAN MD, Sakarya Training and Research Hospital, Clinic of Medical Microbiology, Sakarya, Turkey
Phone: +90 264 800 44 44 **E-mail:** hande_cakar@hotmail.com **ORCID ID:** orcid.org/0000-0001-6893-8490
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Introduction

Human immunodeficiency virus (HIV) infection, which is caused by HIV and progresses to acquired immunodeficiency syndrome (AIDS) if not treated, is an important public health problem. Infections caused by HIV have a wide clinical presentation ranging from asymptomatic carriers to life-threatening opportunistic infections and the development of malignancy[1]. An estimated 37.7 million people worldwide are living with HIV, and with the advent of combined antiretroviral therapy (ART), the average life expectancy of people living with HIV has increased significantly[2].

Human immunodeficiency virus is a globular virus that binds with its surface glycoproteins to its primary target, CD4 T cells. The virus then integrates its chromosomal material into the host cell and uses the host cell machinery for replication and protein synthesis. Eventually the host cell dies and more and more CD4 T cells become infected. Neutralizing antibodies do not eliminate the virus as it has a high frequency of somatic mutations, but the viral loads decrease and the blood CD4 T cell count partially recovers. This asymptomatic latent phase reflects the progressive destruction of CD4 T cells in the untreated person and the subsequent elevation of HIV viral load in the blood. The number of CD4 cells in the affected individual will decrease by approximately 50-80 cells/µl per year without initiation of ART, and the decrease may be even faster when the number falls below 200 cells/µl[3,4]. After exposure to the virus, cellular immunity, especially CD4 T lymphocytes, is progressively suppressed[5].

Diagnosis of infection, timely initiation of ART to the infected person, and prevention of transmission to other individuals are important. In the laboratory diagnosis of HIV infection, reactive results with high-sensitivity screening tests should be confirmed with an alternative specific test[6]. The level of HIV-1 RNA in the plasma is related to the level of the disease, and a higher viral load is observed in symptomatic patients or patients in AIDS stage compared to asymptomatic patients. Viral load measurement is now routinely used for initiating and evaluating the effectiveness of ART[7]. In addition, CD4 T cell count is required as a complementary parameter to viral load monitoring in determining the stage of the disease, initiating ART and opportunistic infection prophylaxis practices, and evaluating the efficacy of treatment[8,9].

In chronic viral infections such as HIV infection that cannot be controlled by the immune system, continuous viral antigen stimulation causes progressive loss of T cell functions such as T cell proliferation and cytokine secretion, which is called "T cell depletion"[10,11]. This leads to ineffective immune responses and defects in viral clearance. Studies in animal models and humans have shown that immune functions are suppressed in cases of chronic antigen exposure through upregulation of inhibitory pathways that cause active and potentially reversible T cell disruptions[12,13]. Since the first reports highlighting the role of Programmed death-1 (PD-1), one of these immunoregulatory molecules, as a major factor in T-cell depletion, there have been significant advances in understanding the complex web of molecular events that shape T-cell dysfunction in HIV infection[14]. In the acute phase of HIV infection, an increase in HIV viral load and an increase in PD-1 receptor levels in HIV-specific T cells have been found[13]. In the chronic phase of HIV infection, high PD-1 expression levels and T cell depletion are observed due to HIV antigens that are constantly changing with the mutations that occur. With treatment, PD-1 expression in HIV-specific T cells decreases as the viral antigen levels decrease along with the decrease of plasma viral load to levels below the detection limit[15].

Although information about the basic immunology of the PD-1 pathway and its role in HIV pathogenesis continues to increase, there are still many unknowns. In combination with ART, new and effective therapeutic strategies can be developed to block the PD-1 pathway or other regulatory molecules. The aims of this study were to determine the correlations of PD-1 marker with other parameters such as CD4 T cell count and viral load before treatment and in the later stages of treatment (0, 1 and 3 months), the changes over time, and the importance of them on the clinical course and prognosis in newly diagnosed naive HIV patients.

Materials and Methods

Study Group, Ethics Committee Approval and Collection of Samples

The study included 30 patients who were admitted to Sakarya University Sakarya Training and Research Hospital Infectious Diseases Outpatient Clinic, were diagnosed as having HIV infection for the first time, who were in compliance with the study criteria, whose treatment and follow-up would be newly started, and who read and signed the Informed Voluntary Consent Form.

This study was approved by the Sakarya University Faculty of Medicine Dean's Office, Ethics Committee Presidency with the decision number 16214662/050.01.04/26. The study was carried out in Sakarya University Training and Research Hospital Medical Microbiology Laboratory.

Samples of patients with clinical suspicion and recurrent 4th generation ELISA test positivity were sent to the National Reference Microbiology Laboratory and confirmed with HIV ½ antibody discrimination rapid test, and the diagnosis of HIV infection was confirmed. Patients with a negative confirmatory

test result or whose diagnosis could not be confirmed were excluded from the study. In the first control (0 month) of the patients whose diagnosis was confirmed, blood samples taken into tubes containing 2 pieces of EDTA (ethylenediamine tetraacetic acid) for flow cytometry studies and HIV-RNA measure were sent to the medical microbiology laboratory of our hospital. While flow cytometry studies were performed without waiting in whole blood from the samples accepted to the laboratory, for NAT, the plasma part of the blood sample which was centrifuged at 4000 rpm for 10 minutes was separated and the tests were studied in the plasma. In cases where it was not possible to immediately study NAT from plasma samples, samples were stored at -80˚C until studied.

Determination of HIV RNA Level

One ml of the samples were centrifuged and the plasma was separated and studied in the GeneXpert® System (Cepheid, California, USA) device with the Xpert HIV-1 viral load (Cepheid, California, USA) kit. In this automated system, RNA extraction, reverse transcription, real-time detection of the 5'-LTR region of HIV-1 and quantitative detection by PCR were performed in a completely closed cartridge system. Approximately 90 minutes after the plasma sample was loaded into the cartridge, the result was automatically analyzed and reflected on the computer screen. The kit's lower limit of detection was specified by the manufacturer as <40 copies/ml in the kit insert.

Determination of PD-1 Expression Levels in CD4 T and CD8 T Cells

The PD-1 level was analyzed together with the parameters of CD4 T and CD8 T cells ratio and HIV RNA level before treatment and at one and three months after treatment initiation in naive patients. Blood samples were first incubated at room temperature with monoclonal antibodies against CD3, CD4, CD8 and PD-1, according to the manufacturer's instructions. After the washing and fixation processes, the analysis phase was started by flow cytometry.

Flow Cytometry Analysis

In order to detect CD3, CD4, CD8 and PD-1 levels in the flow cytometry method, antibodies (Beckman Coulter, USA) were first pipetted into tubes as 10 µl. 100 µl of whole blood sample was pipetted on them and vortexed. After 15 minutes of incubation at room temperature in the dark, 500 µl of Optolyse C was added to the tubes. After 10 minutes of incubation at room temperature in the dark, 500 µl of PBS was added to the tubes. It was incubated for 10 minutes at room temperature in the dark and centrifuged for 5 minutes at 300 x rcf. The tubes were poured slowly and filtered, the mouth part was dried. 2 ml of PBS were added and vortexed. It was centrifuged for 5 minutes at 300 x rcf. The supernatant was discarded. 500 µl of PBS/Formaldehyde was added to the tubes and stored at +4 °C until flow cytometry analysis. Samples were run on the Navios Ex (Beckman Coulter, USA) flow cytometry instrument.

Statistical Analysis

Obtained data were analyzed using the Statistical Package for the Social Sciences version 18.0 (IBM, Chicago, IL, USA). The correlation coefficient of PD-1 marker and other parameters used in follow-up [CD4 (+) T, CD8 (+) T cell ratio, HIV RNA level] was determined by Spearman's correlation test. In addition, Wilcoxon signed-rank test was applied for the analysis of non-parametric dependent variables.

Results

The patients included in the study were aged 20-70 years with a mean age of 35.9±4.8 (±13.44%) and 7 (23.3%) of the patients were female and 23 (76.7%) were male.

Increase in CD4 T ratio ($p=0.0009$), decrease in CD8 T ratio ($p=0.003$), decrease in viral load ($p<0.00001$) between the pre- and post-treatment periods were found to be statistically significant.

As a result of the correlation analysis, a positive, moderate correlation and a statistically significant correlation ($n=80$, $rs=0.49614$, $p<0.001$) was found between the rate of PD-1 expressed in CD4 T cells and viral loads. A positive, moderate correlation and a statistically significant correlation ($n=80$, $rs=0.50954$, $p<0.001$) was found between the rate of PD-1 expressed in CD8 T cells and viral loads. The ratio of PD-1 molecule expressed from CD4 T and CD8 T cells and the variation of viral load at 0, 1 and 3 months of treatment are presented in Table 1.

Discussion

Despite the developments in antiretroviral treatment protocols in recent years, the treatment applied cannot fully restore the immune system to its previous state, as a result, complications related to inflammation such as cardiovascular disease and cancer or complications of immune deficiency continue to be important[16]. During the acute phase of HIV infection, the majority of individuals experience a dramatic increase in HIV viral load and increased levels of PD-1 receptors on HIV-specific T cells. Untreated patients with high viral load progress to the chronic phase of HIV infection. Exposure to constantly changing HIV antigens at this stage results in high PD-1 expression levels and T cell depletion. ART significantly inhibits viral replication in most patients, resulting in a decrease in plasma viral load to levels below the detection limit by standard testing. When low levels of viral antigen are present, PD-1 expression drops to lower levels in HIV-specific T cells[15]. In our study, we examined

Table 1. Change of PD-1 level and viral loads with treatment

Patient No	Pre-treatment			Post-treatment					
				1st month			3rd month		
	PD1/CD4* (%)	PD1/CD8** (%)	Viral load (copy/ml)	PD1/CD4 (%)	PD1/CD8 (%)	Viral load (copy/ml)	PD1/CD4 (%)	PD1/CD8 (%)	Viral load (copy/ml)
1	98.59	98.97	3140000	99.30	88.87	8370	99.79	95.04	169
2	94.81	98.10	10000000	88.44	78.49	240	70.94	64.21	0
3	94.48	97.41	33600	40.46	58.90	139	28.36	41.52	0
4	61.29	57.63	17500	22.57	41.43	60	25.11	16.15	0
5	52.68	39.28	194000	45.62	37.97	476	34.02	19.12	0
6	60.89	99.23	10000000	56.55	98.70	226	37.84	40.83	0
7	99.52	79.22	295000	39.00	69.84	595	17.53	21.28	0
8	47.74	56.72	81900	34.19	35.09	275	23.78	28.35	0
9	89.65	99.12	79300	49.21	97.53	891	30.46	88.11	340
10	96.81	95.35	431000	45.58	91.53	40	33.88	67.64	0
11	50.99	46.32	119000	60.27	51.16	64	54.02	52.57	65
12	64.64	91.20	32100	29.27	75.95	60	24.81	37.34	0
13	94.29	99.05	163000	95.24	94.32	164	63.21	57.23	0
14	97.17	99.60	74900	48.54	93.79	66	44.27	64.05	0
15	94.81	97.78	14700	30.82	36.13	50	22.60	26.82	0
16	39.39	93.80	442000	67.36	98.75	5580	58.45	75.55	209
17	57.93	97.46	115000	46.04	87.98	569	37.92	72.78	0
18	99.80	95.58	715000	83.83	96.00	5580	34.65	35.84	0
19	95.94	98.36	27500	50.01	77.82	40	38.67	54.47	0
20	92.38	46.04	157000	71.26	52.11	4690	61.77	52.22	110
21	90.77	98.64	37400	40.30	47.15	72	35.60	40.54	0
22	80.66	98.26	57500	72.82	94.23	151	44.52	80.12	0
23	33.87	77.84	376000	19.35	98.16	506	20.50	96.88	98
24	31.70	62.41	14000	35.65	61.34	418	15.16	32.98	0
25	51.12	62.82	1420000	26.27	35.41	1350	20.48	24.56	0
26	49.50	71.60	684000	33.05	55.94	4640	24.18	36.44	0
27	30.29	62.45	140000	27.65	49.62	40	22.24	36.55	0
28	90.69	89.19	357000	77.90	67.21	148	55.18	40.44	0
29	33.30	58.00	380000	17.96	28.49	410	13.44	20.56	0
30	80.06	91.22	710000	61.53	74.80	610	44.34	60.12	0

*Percentage of CD4 T cells expressing PD-1, **Percentage of CD8 T cells expressing PD-1.

PD-1: Programmed death-1

the changes in PD-1 levels before starting the treatment and during the follow-up period of newly diagnosed naive HIV patients and investigated its correlation with other parameters.

Virus-specific CD8 T cells play an important role in controlling viral infections such as HIV infection. However, during chronic HIV infection, virus-specific CD8 T cells are functionally depleted, lose their effector functions, and become inadequate in controlling viral infection[17]. PD-1 expression in HIV-specific CD8 T cells correlates with HIV viral load. PD-1 has been shown to attenuate cytotoxic T lymphocyte function by contributing to the reduced immune response of HIV infection. However, controlling viral load through conventional therapy results in a decrease in PD-1 expression[14]. It has been suggested that high level of PD-1 expression in CD4 and CD8 T cells is also associated with the inability to reconstruct immunity after successful viral control with ART[17-19]. In this study, an increase in the number of CD8 T cells and a decrease in the rate of PD-1 expressed on CD8 T were observed at the end of the 3rd month in naïve patients followed for three months ($p<0.05$).

The CD4 T cell count and viral load are very important markers in all international guidelines in the follow-up of patients infected with HIV. In addition to these tests, some biomarkers such as PD-1, which have been shown to have roles in HIV immunopathogenesis, will help the development of alternatives to high-cost molecular methods in the future by conducting studies that show their changes with treatment. Although there

are conflicting results in the first studies on the expression of PD-1 in CD4 T cells under treatment in the literature, recent studies clearly show that PD-1 molecule expression decreases with ART and that antibodies that block the PD-1 molecule can be used as therapeutic agents in the treatment of patients infected with HIV[20-23]. In our study, an increase in the number of CD4 T cells and a decrease in the rate of PD-1 expressed in CD4 T cells were observed at the end of the 3rd month ($p<0.05$).

Macatangay et al.[24] reported that pre-ART PD-1 (+) CD4 T cell count was positively correlated with viral load and negatively correlated with total CD4 T cell count, and the proportion of PD-1 (+) CD4 T cells decreased at the end of the 1st year under ART, but remained stable after four years in 99 patients with newly diagnosed HIV. In our study, it was determined that PD-1 expression level decreased significantly with ART and showed a moderate correlation with viral load. Since HIV-specific CD4 T cells could not be examined in our study, PD-1 expressed on total CD4 T cells was examined, and similarly, a decrease in expression levels was observed.

Two recent studies have shown that high levels of PD-1 expression in total CD4 and CD8 T-cell subsets are associated with failure of immune reconstitution after successful viral control with ART[25,26]. PD-1 (+) T cells remain in the blood for a long time despite ART suppression and are associated with HIV RNA levels. It remains to be determined whether PD-1 is the cause or consequence of the observed low CD4 T-cell counts. If PD-1 upregulation is a contributing factor to permanent immune damage, manipulation of the PD-1 pathway may have potential as adjuvant therapy to promote immune restoration in these patients. In our study, although an increase in CD4 T and CD8 T cell numbers and a decrease in viral load and PD-1 expression level were found to be significant after three months of treatment, in some patients (for example, patient number: 1) the viral load decreased to very low levels but did not reset and it was found that the levels of PD-1 expressed on CD8 T cells did not decrease as much as other patients. Due to the small sample size, it would be appropriate to conduct an analysis on a larger cohort, including treatment regimens and drug resistance outcomes, before making an overall conclusion for these patients.

The failure of current ARTs to completely clear HIV is mainly attributed to the persistence of HIV DNA integrated into the host cell DNA. The main obstacle to the eradication of HIV is the latently infected memory CD4 T cell population that harbors the virus and persists despite long-term and effective viral suppression by ART[27]. Therefore, in order to design new therapeutic approaches that could eradicate HIV or allow ART to be discontinued without repeating viral replication, it is imperative to identify the key mechanisms that cause the generation and persistence of latently infected cells.

The PD-1 has recently emerged as a potential target on the path to HIV eradication. Among memory CD4 T-cells, cells expressing high levels of PD-1 contain more proviral DNA than cells with low PD-1[24]. Additional studies suggest that blocking PD-1 triggers HIV replication and can therefore reactivate the latent virus, giving the body an opportunity to eliminate productively infected cells that occur via cell death and/or HIV-specific immune responses[28]. In light of these results, further research will be needed to understand which genes modulated by the PD-1 pathway regulate HIV replication.

Although knowledge about the basic immunology of the PD-1 pathway and its role in HIV pathogenesis continues to increase, there is still a significant amount of unknowns. In combination with ART, new and effective therapeutic strategies can be developed to block the PD-1 pathway or other regulatory molecules. In recent years, publications on the detection and killing of HIV-infected cells and cancer cells by PD-1 blockade by monoclonal antibodies have gained momentum. It is thought that long-term follow-up of PD-1 expression levels and clarification of this pathway may be an important step on the path to achieve sterilizing cure of HIV[29].

Study Limitations

There are some limitations of our study. PD-1s expressed on total CD4 and CD8T cells, but not PD-1s expressed on HIV-specific CD4 and CD8T cells, were evaluated by flow cytometry. In addition, the fact that the patients could not be followed longer than the 3rd month after the treatment was another limitation of the study.

Conclusion

In conclusion, in our study, a significant increase in the number of CD4 T and CD8 T cells and a significant decrease in the levels of PD-1 expressed from these cells were found at the end of the 3rd month in patients who were started on ART after the first diagnosis. It was concluded that PD-1 might be a marker of viral load and might be useful for monitoring viremia and changes in treatment response.

Ethics

Ethics Committee Approval: The study was approved by the Sakarya University Faculty of Medicine Dean's Office, Ethics Committee Presidency (protocol no: 16214662/050.01.04/26, date: 08.02.2019).

Informed Consent: Consent form was filled out by all participants.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: H.T., G.T., O.K., Concept: H.T., M.K., M.A., Design: H.T., M.K., M.A., Data Collection or Processing: H.T.,

A.A., G.T., O.K., Analysis or Interpretation: A.A., M.K., Literature Search: H.T., M.K., Writing: H.T., M.K.

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