



# Upregulation of Apoptosis Pathway Genes in Peripheral Blood Mononuclear Cells of HIV-Infected Individuals with Antiretroviral Therapy-Associated Mitochondrial Toxicity

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**ABSTRACT** A case-control study of the effect of antiretroviral therapy (ART) on apoptosis pathway genes comprising 16 cases (HIV infected with mitochondrial toxicity) and 16 controls (HIV uninfected) was conducted. A total of 26 of 84 genes of the apoptosis pathway were differentially expressed. Two of the upregulated genes, DFFA and TNFRSF1A, classified 75% of study participants correctly as either a case or control. Thus, apoptosis may be in the causal pathway of ART-associated mitochondrial toxicity. These two genes could be markers for detecting and monitoring ART-induced mitochondrial toxicity.

**KEYWORDS** antiretroviral therapy, mitochondrial toxicity, apoptosis, biomarker

Antiretroviral therapy (ART) has markedly improved outcomes of HIV infection (1–4). However, the therapeutic benefit of ART is sometimes limited by long-term toxicities (5, 6). Although newer and next-generation ART may have less toxicity, ART-induced toxicity remains a substantial burden globally (7, 8). There are reports that contemporary ART, such as some integrase-based regimens, may be associated with neuropsychiatric toxicity (9, 10). The underlying mechanisms of ART-induced toxicity are still debatable. Nucleoside reverse transcriptase inhibitors (NRTIs) have been associated with toxicities such as skeletal muscle myopathy, lactic acidosis, lipodystrophy, peripheral neuropathies, cardiomyopathies, and pancytopenia (11–14). These toxicities have been implicated in the inhibition of mitochondrial DNA (mtDNA) polymerase gamma (Pol- $\gamma$ ) by NRTIs (15). A recent review of emerging studies concluded that mitochondrial dysfunction cannot be explained solely by Pol- $\gamma$  inhibition (16). Furthermore, protease inhibitors (PIs) and nonnucleoside reverse transcriptase inhibitors (NNRTIs) do not inhibit Pol- $\gamma$ , and yet they also cause toxicities similar to those associated with mitochondrial dysfunction. Moreover, HIV-infected patients are at increased risk for chronic conditions, such as cardiovascular disease, renal disease, bone disease, and diabetes mellitus, compared to non-HIV patients (17). These toxicities have been associated with ART (18–20).

We recently found elevated plasma concentrations of cytochrome c (CYCS) protein in HIV treatment-experienced patients with toxicity compared with HIV treatment-experienced patients without toxicity (21). CYCS is a mitochondrial protein and proapoptotic signal that, when released from the mitochondria, activates effector caspases 3 and 7, resulting in apoptosis (programmed cell death) (22). Other investigators have found that several components of ART can cause apoptosis, which is a function of the mitochondrion (23, 24). Thus, biomarkers of apoptosis could be used to detect and

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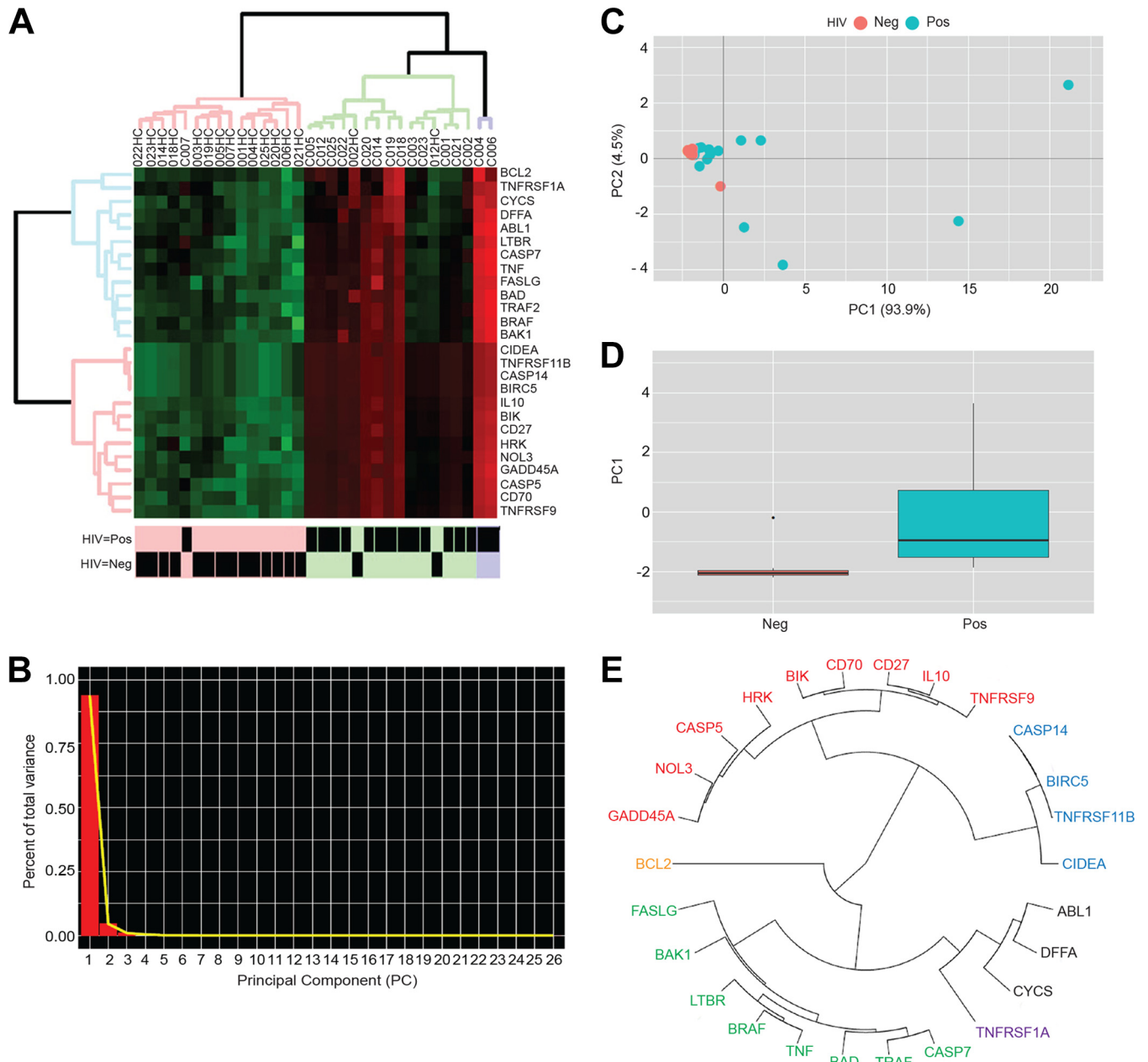
**TABLE 1** Demographic and clinical characteristics of study participants

Variable	HIV-uninfected participants <sup>a</sup>	HIV-infected participants with toxicity <sup>a</sup>
Gender ( <i>n</i> )		
Female	5	5
Male	11	11
Age (range) (yrs)	33–66	33–66
Race (%)		
White non-Hispanic	25	25
White Hispanic	6	6
African American	69	69
Mean CD4 count (range) (count/ $\mu$ l)	NA <sup>b</sup>	523 (86–1336)
Mean viral load (range) (copies/ml)	NA	2,087 (20–29297)
Mean duration of exposure to nucleoside analogs (range) (yrs)	NA	8.18 (1–17)
Toxicities (%)		
Single	NA	63
Multiple	NA	38
Basis of treatment regimen (%)		
NNRTI	NA	25
PI	NA	44
Integrase	NA	6
PI/NNRTI	NA	13
PI/integrase	NA	6
Integrase/NNRTI	NA	6

<sup>a</sup>Sample size (*n*) = 16.<sup>b</sup>NA, not applicable.

monitor ART-induced toxicity (25, 26). In the current study, we sought to investigate the differential expression profile of genes of the apoptosis pathway in HIV-infected patients with ART-associated mitochondrial toxicity (cases) versus HIV-uninfected individuals (controls).

We included 32 participants in this case-control study of the effect of ART on apoptosis pathway genes. A case comprised an HIV-infected individual (*n* = 16) diagnosed with ART-associated mitochondrial toxicity by his or her provider based on clinical and/or laboratory evidence of one or more of the following ART toxicities: hyperlipidemia, anemia, elevated liver function tests, thrombocytopenia, lactic acidosis, elevated blood urea nitrogen or creatinine, peripheral neuropathy, lipodystrophy, and/or pancytopenia (27). Cases were matched to HIV-uninfected controls (*n* = 16) by age, sex, and race/ethnicity. The study protocol was approved by the institutional review board of the Yale School of Medicine. All participants gave their written informed consent before participation in the study. The demographic and clinical characteristics of cases are listed in Table 1. Out of the 16 cases, only 3 had detectable viremia: C02 (29,297 copies/ml), C019 (1,508 copies/ml), and C023 (215 copies/ml). To investigate the impact of ART on apoptosis pathway-specific genes, quantitative PCRs were done using the Human Apoptosis RT<sup>2</sup> Profiler PCR Array kit (SuperArray Biosciences) as previously published (28). We identified differentially expressed genes on the basis of false discovery rate (FDR) adjusted *P* value using empirical Bayes moderated tests. The FDR was controlled using Benjamini and Hochberg algorithm. We identified 26 out of the 84 genes that were differentially expressed between the cases and controls (Fig. 1A). Of note, the gene profiles of one case (C007) and 2 controls (002HC and 012HC) segregated with controls and cases, respectively. There was no significant association between gene expression and disease characteristics (e.g., viral and CD4<sup>+</sup> T-cell count) among cases. Of the 26 genes, 18 were proapoptotic (TNFRSF1A, CYCS, DFFA, ABL1, LTBR, CASP7, FASLG, BAD, TRAF2, BAK1, CIDEA, TNFRSF11B, CASP14, BIK,



**FIG 1** Differentially expressed apoptosis pathway genes. (A) Heat map of significantly differentially expressed genes between HIV-infected patients with mitochondrial toxicity (HIV = Pos) and HIV-uninfected patients (HIV = Neg). Columns represent the subjects (identification numbers with “HC” at the end represent HIV-uninfected patients, and identification numbers with “C” in front represent HIV-infected patients), and rows represent genes. Gene expression levels are color-coded; red, black, and green represent high, medium, and low expression levels, respectively. (B) Screen plot depicting the proportion of the total variance accounted for by each of the principal components. (C) Score plot of the first principle component (PC1) versus the second principle component (PC2). (D) Box plots comparing scores of the first principle component (PC1) between the two groups. (E) Clusters formed by performing unsupervised analyses on the transcriptional profile data of the 26 genes using hierarchical clustering analysis.

GADD45A, CASP5, CD70, and TNFRSF9), 5 were antiapoptotic (BCL2, BRAF, BIRC5, IL-10, and NOL3), and 3 had overlapping functions (CD27, HRK, and TNF).

Principal-component analysis (PCA) was used to group the 26 differentially expressed correlated genes to uncorrelated summary variables called principal components (PC) (Fig. 1B). The PCA revealed that cases and controls formed two distinct groups, which were primarily separated on the first principal component (PC1) (Fig. 1C). PC1 accounted for 94% of the total variance of the 26 genes, with high positive correlation with all 26 genes (Fig. 1C). As illustrated in Fig. 1D, the overall gene expression score (PC1) was significantly higher in cases than in controls ( $P < 0.05$ ).

Furthermore, unsupervised hierarchical clustering analysis divided the 26 differentially expressed genes into four major groups (Fig. 1E). To further identify the key genes which contributed to the differences in profile, penalized regression was used to select the best subset of genes from the 26 genes which were differentially expressed between the two groups. This analysis selected two genes: DFFA and TNFRSF1A. DFFA is a proapoptotic gene in the executioner pathway, and TNFRSF1A is a proapoptotic gene in the extrinsic pathway. To assess the discriminatory power of DFFA and TNFRSF1A, we then developed a classifier model to classify study participants into groups based on these two selected genes. The classifier model correctly classified 75% of the participants into their respective groups.

In this exploratory study, we found 26 out of the 84 genes that were differentially expressed between the two groups. To the best of our knowledge, this is the first report of the association of these 26 genes with ART-associated mitochondrial toxicity. Although these genes belong to the three main apoptosis pathways (i.e., intrinsic, extrinsic and executioner), we observed a preponderance of proapoptotic genes ( $n = 18$ ) to antiapoptotic genes ( $n = 5$ ). The relative ratio of expression of proapoptotic to antiapoptotic genes determines the fate of a cell (29). Our findings suggest that apoptosis may be part of the causal pathway of ART-associated mitochondrial toxicity.

In many cell types, the extrinsic and intrinsic pathways converge to induce apoptosis, which requires the involvement of the mitochondria (30). The extrinsic signaling pathway of apoptosis is initiated by transmembrane death receptors, which are members of the tumor necrosis factor (TNF) receptor gene superfamily (TNFRSF) (31). Members of the TNF receptor family bind to extrinsic ligands, leading to the activation of the initiator caspase-8, which eventually results in the death of the cell (31). In our study, "case patients" had upregulation of TNFRSF9, LTBR, TNF, TNFRSF11B, and TNFRSF1A; proteins encoded by these genes are members of the TNF receptor superfamily. We also observed an upregulation of TNF receptor-associated factor (TRAF) gene. The intrinsic apoptotic pathway is initiated by a diverse array of non-receptor-mediated stimuli such as DNA damage, growth factor withdrawal, radiation, toxins, hypoxia, hyperthermia, viral infections, and free radicals (32). This pathway is closely regulated by the BCL-2 family, a group of related proteins which regulate the integrity of the mitochondrial membrane (32). Damaged mitochondrial outer membranes cannot produce energy by oxidative phosphorylation and leak apoptogenic factors, such as CYCS and Smac/DIABLO into the cytosol (29). CYCS, among other factors, is thought to induce caspase activation, which leads to apoptosis (33). We observed upregulation of proapoptotic genes, such as BAD, BAK1, and BIK. We also observed an upregulation of the CYCS gene.

Studies of apoptotic gene expression in HIV infection using both *in vitro* and *in vivo* models in the absence of treatment have demonstrated increased expression of proapoptotic genes of intrinsic, extrinsic, and executioner pathways (34, 35). However, on initiation of ART, studies have reported downregulation of proapoptotic genes (36) and upregulation of antiapoptotic genes (37). The aforementioned studies of HIV treatment-experienced patients focused on either a single pathway of apoptosis or a restricted number of apoptosis-related genes. Balestrieri et al. studied the expression profile of 19 apoptosis-related genes in 12 treatment-naïve patients initiating ART. They found that after 12 months of ART, the expressions of proapoptotic genes (FAS, FAS-L, FAF-1, FADD, CASPASE-8, DR3, TRAIL, TRADD, and BAX) were significantly downregulated compared with those at time zero, while the expressions of antiapoptotic genes (BCL-2, BCL-XL, and MCL-1) were significantly upregulated (38). Furthermore, Pitrak et al. recently observed increased activation of caspase 8 (extrinsic pathway), caspase 9 (intrinsic pathway), and caspases 3 and 7 (executioner pathway) at baseline compared to 6 months after initiating ART in 10 HIV treatment-naïve patients (39). Altogether, considering these data, we surmise that HIV infection increases apoptosis, while ART reduces apoptosis. The effect of ART on apoptosis in these studies is contrary to our finding of increased expression of proapoptotic genes. This may be explained by the difference in the study populations. Our study compared HIV treatment-experienced

patients who were diagnosed with ART-associated mitochondrial toxicity with HIV-uninfected patients. However, our finding is consistent with findings from other studies, both *in vitro* and *in vivo*, that reported increased apoptosis in association with ART-induced clinical toxicities (40–42). The findings of these studies suggest that markers of apoptosis could be more sensitive and specific biomarkers of ART toxicity (25). In our exploratory study, upregulation of 2 (DFFA and TNFRSF1A) out of the 84 genes successfully classified 75% of study participants correctly as either a case or control. Thus, if these genes are validated in further studies, quantitative PCR assays of these genes could serve as biomarkers for ART-induced toxicity.

Our study has several limitations. First, like all cross-sectional studies, we have not provided incontrovertible proof that the upregulated genes were caused by ART-induced toxicity in the HIV-infected individuals, as we did not have their gene profile prior to starting ART. Second, the diagnosis of mitochondrial toxicity was not confirmed with tissue biopsy. Third, the small sample size limits the generalization of our findings. Fourth, we did not study HIV-infected patients on ART without toxicity as controls. However, our findings are consistent with those of other investigators, suggesting that apoptosis may be part of the causal pathway of ART-associated mitochondrial toxicity. Therefore, further well-designed prospective studies are needed.

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