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Research Paper

Evidence and Nature of a Novel miRNA Encoded by HIV-1

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Evidence is provided for the existence of a novel viral miRNA precursor that is exclusively and conspicuously present in HIV-1 genome. This miRNA precursor was found to exploit cellular machinery to generate matured miRNA that interferes with apoptosis antagonizing transcription factor (AATF) mRNA-stability leading to its defective expression which in turn facilitates cell death as well as down-regulation of Dicer gene that is known to play crucial role in providing cellular immunity against any viral invasion. Expression of this HIV-1 specific matured miRNA, observed in the blood mononuclear cells derived from untreated full blown AIDS patients, was accompanied by similar defect in AATF mRNA stability as well as Dicer gene expression within these cells. Based upon these results we propose that HIV-1 encoded miRNA, reported here, may be responsible for the depletion of $CD4^+$ T cells through its inherent capacity to downregulate AATF gene expression within these cells.

Key Words: HIV-1 miRNA, AATF, Dicer, AIDS, $CD4^+$ T-cell depletion, Apoptosis

Introduction

Since the beginning of AIDS pandemic, there exists incontrovertible evidence to support the view that $CD4^+$ T cell depletion contributes to the development of AIDS [1]. Although a direct association between HIV burden and $CD4^+$ T cell loss has been established [1,2], the precise mechanism by which HIV causes a decrease in $CD4^+$ T cells is far from clear [3]. The non-coding RNAs (e.g. siRNA and miRNA) have been recognized to bind complimentary sites of the target genes leading to regulation of their protein expression through unknown mechanism [4-6]. Keeping in view the fact that these small non-coding RNAs provide natural host defence against invasive sequences of viral origin [7,8], our study was addressed to understand three basic questions. First, as there are reports of miRNA expression by HIV-1 genome [9,10] does HIV 3' UTR encodes RNA sequences capable of generating miRNAs within human cells? Second, if such a miRNA exists in blood cells derived from AIDS patients, does it regulate any gene(s) involved in cellular apoptosis as well as Dicer gene regulation that are known to be of crucial importance in imparting cellular immunity against any viral invasion? Third, if yes, does this HIV-1 specific miRNA pathway get reflected in blood mononuclear cells derived from full-blown AIDS patients?

Material and Methods

Cellular-model employed: Mononuclear cells were obtained from normal healthy volunteers, who were fasting for 12 hours and abstained from any medication for two weeks before blood donation, using Ficoll-Hypaque gradient centrifugation [11] and subsequently maintained in RPMI-1640 medium supplemented with 10% FCS in humidified 5% CO_2 atmosphere at 37^0C. Blood mononuclear cells were also obtained from untreated full blown AIDS patients and corresponding controls (from our institute's AIDS clinic with informed consent), subsequently subjected to FACS analysis and RNA extraction procedure.

Computer Analysis of HIV Genome: A generalization based on previously known miRNA in retroviral genomes was derived (9,10). The miRNA sequences of the viral genome for generalization were mined from miRNA registry hosted by Sangar institute (http://microrna.sanger.ac.uk/sequences/index.shtml). The generalization was derived keeping in mind; a) the stem length and loop size of the precursor miRNAs sequences; b) the thermodynamics signatures of precursor miRNAs present in retroviral genome [12]. The genomic sequences corresponding to HIV-1 and HIV-2, obtained from (http://www.ncbi.nlm.nih.gov), were screened for novel miRNA precursors using a computational programme written in PERL, developed by us, based on consensus precursor miRNA structure (based on generalization) with provisions: a) G-T pairing was allowed in DNA sequences to foresee G-U pairing in the corresponding RNA structure; b) variable values for stem-length and loop-length were committed; c) More than 75 percent complementary was a prerequisite in the stem-region. The sequences that were repeatedly coming as output even with variable loop-length were short-listed

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and subsequently subjected to RNA structure prediction as well as thermodynamic analysis using Vienna RNA package (http://www.tbi.univic.ac.at/~ivo/RNA/) [13]. The short-listed viral sequence that exhibited miRNA precursor-like feature [12] was subjected to BLAST analysis (http://www.ensembl.org/multi/blastview) against human genome; other viruses as well as reported miRNA precursor-sequences in order to find out homology if any so as to find out putative targets on human genome and to figure out any similarity with already reported miRNAs. In silico approach was adopted to perform analysis of the viral genome and consequently the target gene identification in human genome.[14]

Transfection Experiments: Transfection-ready expression cassette, consisting of miRNA precursor HIV-1 sequence coupled to RNA pol III promoter sequence, was designed and prepared using PCR technology. This expression cassette (EC) was purified by QIAquick PCR purification kit (QIAGEN). Mononuclear cells (maintained in vitro culture) were transfected with this "EC" using siPORTTM reagent (Ambion) and subsequently the cells were maintained at 37^0C in 5% CO2-atmosphere for 5 days. Control cells were transfected with "EC" consisting of scrambled sequence coupled with RNA pol III promoter sequence and subjected to incubation conditions as in the case of test cells. At the end of 5th day of incubation, the cells from each well were processed for RNA isolation and FACS analysis using propedium iodide (PI) as tracking dye foe exploring the extent of cell death.

AATF & Dicer mRNA Expression: Total RNA was extracted, from cultured cells, Jurkat cell lines as well as mononuclear cells obtained from untreated full blown AIDS patients (n=10), by acid guanidinium-phenol-chloroform method [15]. RNA yield and purity was determined spectrophotometricaly and integrity of RNA verified by electrophoresis through agarose gels stained with ethidium bromide. First strand cDNA was synthesized from extracted RNA samples using the Revert AidTM cDNA synthesis kit (Fermentas). The cDNA was then amplified using primers specific for **Exon-1:** Forward 5'-GACACGGACAAAAGGTATTGG-3'; Reverse 5'- AGACCCAGTCCCTCTGAAATCT-3' and **Exon-12:** Forward 5-GCTCTCTTTTTGGCCAGCTC-3'; Reverse 5'-TTGCGTGTATTAGGCACAGG-3' of AATF gene. Dicer gene was amplified using specific primers: Forward 5'-AAGCATGCCATCACCACATA- 3'; Reverse: 5-CACATCAGGCTCTCCTCCTC-3'. GAPDH gene was also amplified using its specific primers and this gene was used as invariant control. PCR products were resolved on 2.5% agarose gels stained with ethidium bromide. The gels were visualized by Gel-Doc system and band intensities were evaluated using SCION IMAGE analysis software. The relative level of AATF mRNA expression was quantified by ratio of AATF band intensity to GAPDH band intensity.

miRNA Expression in Normal and HIV-1 Infected Cells

Non-coding short RNAs were isolated from normal and HIV-1 infected cells by using mirVanaTM miRNA isolation kit (Ambion). A 5'phosphorylated 3'adaptor oligonucleotide (5-Pho-AAGGAGTGAAA-ddc-3') was ligated to these non-coding short RNAs using T4 RNA ligase. cDNA synthesis was done using the primer having sequence as 5'-TAATACGACTCACTATAGGTTTCACTCCTT-3'. The PCR reaction was carried using one common primer (5'-TAATACGACTCACTA-3') and HIV miRNA specific primer (5'-CCAGGGAGGCGTGCCTGGGC-3'). The PCR products were resolved on 15% polyacrylamide gels and subsequently stained with ethidium bromide. The above mentioned PCR based identification of miRNAs developed by us in our laboratory gave reproducible results with other known cell specific miRNAs that were tested by us.

Results

Computer-directed systematic screening of short sequences (80-90 bp long), present in HIV-1 & 2 genomes, revealed the existence of a novel sequence that was exclusively and conspicuously present in HIV-1 genome and had the inherent capacity to encode a hairpin-loop structure with thermodynamic signatures resembling that of miRNAs (Fig. 1). BLAST analysis of this sequence (found in HIV-1 genome) revealed specific pairing affinity for intronic region between 11^{th} and 12^{th} Exons of gene coding for apoptosis antagonizing transcription factor (AATF). This 20bp sequence, that showed highly specific pairing with AATF hnRNA, was designated by us as matured miRNA (Fig. 1). In order to explore the relationship between this HIV-1 encoded

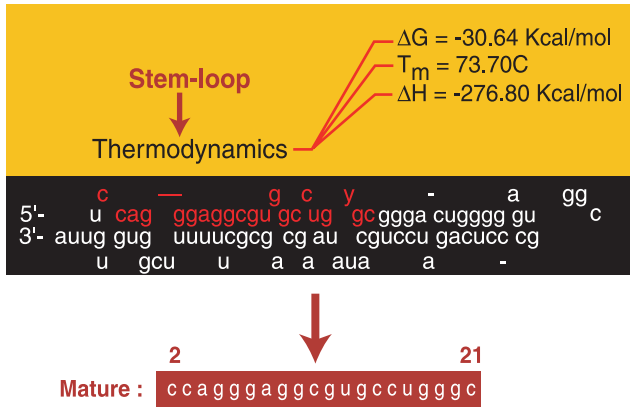

Fig. 1: miRNA precursor-sequence, present conspicuously in 3' LTR region of HIV-1 genome and its thermodynamic characteristics. Predicted matured miRNA sequence is also depicted

miRNA and AATF RNA processing, this HIV-1-derived sequence (having ability to encode for miRNA) was transfected in human blood mononuclear cells maintained in vitro culture. Such a study (Fig. 2) revealed: a) In transfected cells, the disappearance of 12th exon from AATF mRNA was paralleled by its reduced expression (Fig.2d) indicating thereby that transfected HIV-1 derived sequence is able to exploit cellular machinery for the generation of matured miRNA which in turn helps in the deletion of 12th exon from AATF hnRNA; b) Down-regulation of AATF mRNA expression in transfected cells was accompanied by significantly lower viability of these cells as compared to untransfected cells (Fig. 2c). Experiments directed to explore whether or not blood mononuclear cells, derived from full blown AIDS patients, also exhibited significant AATF mRNA down regulation as a result of its 12th exon deletion, confirmed this possibility in cells from AIDS patients as compared to corresponding control cells (Fig. 3). To ascertain whether or not HIV-1 infected cells (from AIDS patients) have the ability to express conspicuously and selectively the matured miRNA (reported here), the expression of this miRNA was studied in uninfected normal blood mononuclear cells as well as that in the HIV-1 infected cells. The results of this study revealed that HIV infected cells indeed selectively express this miRNA with respect to the control cells (Fig. 4). In order to explore if there existed any interrelationship between genes coding for AATF and Dicer, we examined the expression of Dicer gene in cells (that were transfected with HIV-1 encoded miRNA as well as that were derived from untreated HIV-1 infected patients with AIDS) and Jurkat cells (that were found by us to over-express AATF gene). The results of such a study revealed AATF gene over-expression was always accompanied by overexpression of dicer gene and vice versa (Fig. 5).

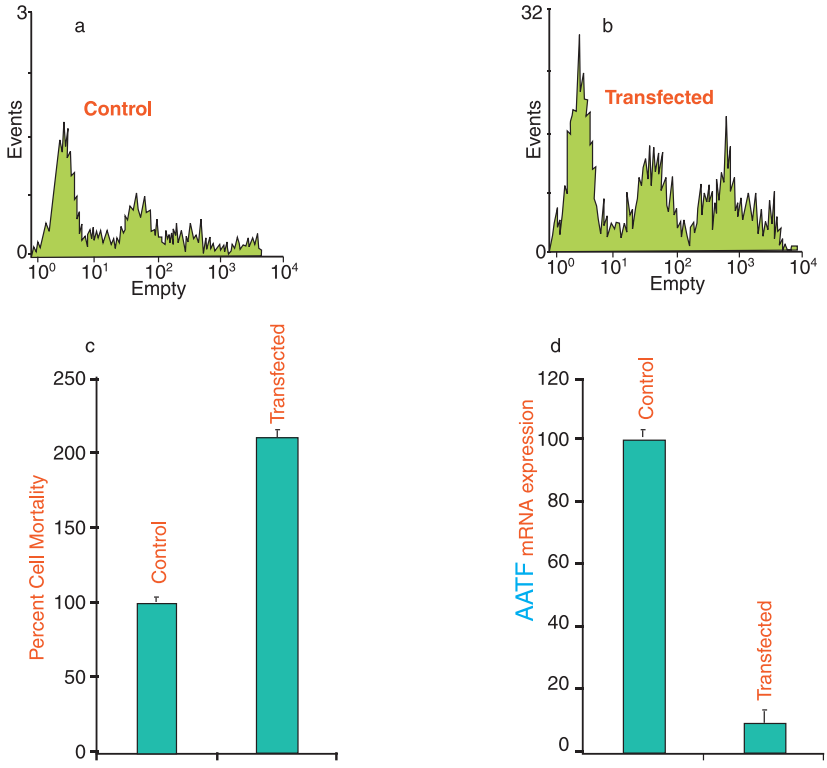

Fig. 2: *FACS analysis of mononuclear cells: Control cells transfected with scrambled sequence (a) cells transfected with miRNA precursor-sequence (b) and percent cell mortality (c) of miRNA precursor-sequence transfected cells with respect to corresponding control cells. Expession of AATF mRNA (d) in miRNA precursor transfected cells with respect to corresponding control cells. Each experiment was repeated five times to ensure reproducibility*

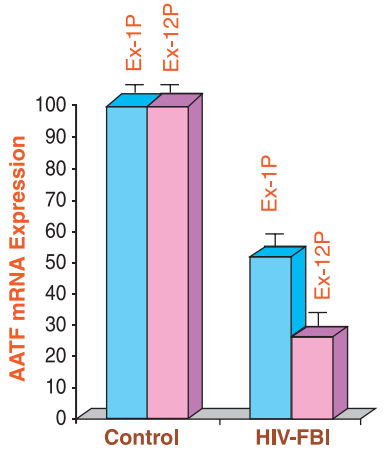

Fig. 3: *Representative graph showing AATF mRNA expression in mononuclear cells derived from normal as well as untreated full blown AIDS patients, using specific primers against 1st and 12th exons of this gene*

Discussion

Keeping in view the findings that HIV-1 encoded miRNA is not only expressed in blood cells derived from AIDS patients (Fig. 4) but also interferes with AATF mRNA processing (Fig. 6) leading to down regulation of this gene; it becomes pertinent to understand as what role AATF plays in HIV-1 dependent pathway. AATF (also termed che-1) protein has a modular structure containing acidic region characteristic of several transcriptional factors as well as leucine zippers and three LxxLL motifs having ability to act as co-activators of nuclear hormone receptors [18]. AATF is not only shown to inhibit cellular apoptosis but also binds RNA polymerase II and inhibits the Rb growth suppressing function by interfering with Rb-mediated histone deacetylases (HDAC1) recruitment on E2F gene promoters [19]. Consequently, it is in this context the results reported here assume importance because of instability of AATF mRNA induced by HIV-1 encoded miRNA can lead to depletion of CD4$^+$ T cells (Fig. 5). Further it is interesting to note that RNA-induced

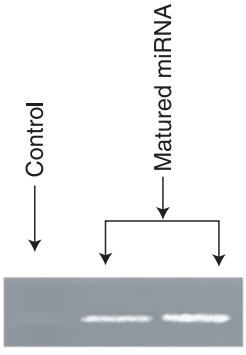

Fig. 4: *Representative photograph showing the expression of HIV-1 specific matured miRNA in normal human blood mononuclear cells (Control) and blood mononuclear cells derived from AIDS patients. The results were verified in triplicates*

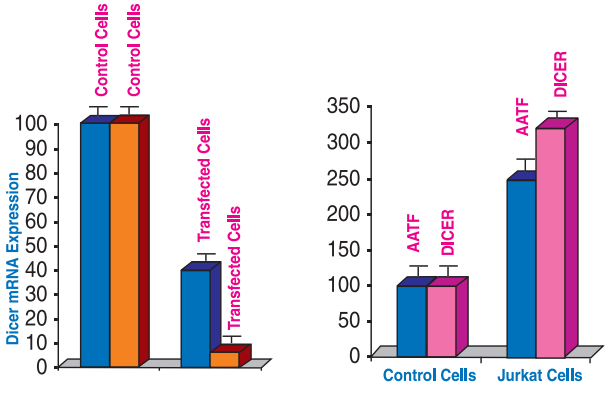

Fig. 5: *Representative graph showing Dicer mRNA expression in: a) human mononuclear cells transfected with the novel miRNA coded by HIV-1 infected AIDS patients; b) Jurkat cell line (found by us to over express AATF gene) and corresponding normal human mononuclear cells revealing that over expression of AATF gene is accompanied by over expression of dicer within Jurkat cells as compared to corresponding controls*

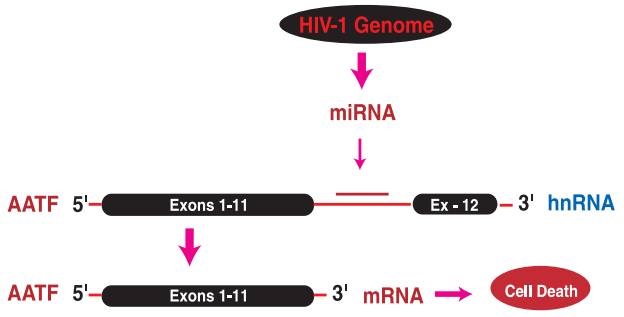

Fig.6: *Proposed mechanism through which matured miRNA, generated from miRNA precursor-sequence within the cell, can induce cellular apoptosis through its ability to interfere with AATF mRNA processing*

transcriptional silencing (RITs) has been recognized to interact with the transcript not only through base-pairing but also by recognizing an RNA polymerase II specific modification of mRNA such as cap structure or poly(A) tail [19,20] as well as our finding that E2F transcription site is present in the promoter of Dicer gene (Fig. 7). Thus it is likely that HIV-1 encoded miRNA-dependent down regulation of AATF gene may serve two purposes. First it can impair siRNA machinery within CD4$^+$ T cells because AATF is known to compete with retinoblastoma gene product (Rb) for its E2F binding site leading to the release of E2F transcription factor which in turn has the ability to upregulate Dicer gene expression at the transcriptional level [18] (Fig. 7). Secondly, AATF is known to interact with tumor susceptibility gene product TSG 101 which has been recognized to play crucial in HIV budding and release phenomenon [16]. Consequently, down regulation of AATF gene expression can facilitate CD4 $^+$ T cell depletion, by promoting, HIV replication and budding leading to excessive viral load within these cells [6, 16].

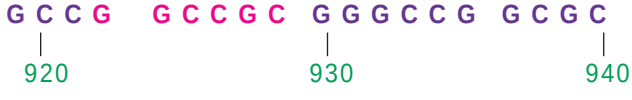

Fig. 7: *Location of E2F binding site in the promoter sequence of Dicer gene*

Conclusion

In conclusion, the novel miRNA, encoded by HIV-1, may be responsible for CD4 $^+$ T cells deletion through its ability to down regulate AATF gene expression.

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