miR-28-3p Is a Cellular Restriction Factor That Inhibits Human T Cell Leukemia Virus, Type 1 (HTLV-1) Replication and Virus Infection*

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Background: HTLV-1 has not been reported to be regulated by microRNA. 
Results: We discovered that cellular miR-28-3p reduces virus replication and infection.  
Conclusion: miR-28-3p may play an important role in limiting virus spreading.  
Significance: miR-28-3p may represent a therapeutic target for HTLV-1-infected patients.

Human T cell leukemia virus, type 1 (HTLV-1) replication and spread are controlled by different viral and cellular factors. Although several anti-HIV cellular microRNAs have been described, such a regulation for HTLV-1 has not been reported. In this study, we found that miR-28-3p inhibits HTLV-1 virus expression and its replication by targeting a specific site within the genomic gag/pol viral mRNA. Because miR-28-3p is highly expressed in resting T cells, which are resistant to HTLV-1 infection, we investigated a potential protective role of miR-28-3p against de novo HTLV-1 infection. To this end, we developed a new sensitive and quantitative assay on the basis of the detection of products of reverse transcription. We demonstrate that miR-28-3p does not prevent virus receptor interaction or virus entry but, instead, induces a post-entry block at the reverse transcription level. In addition, we found that HTLV-1, subtype 1A isolates corresponding to the Japanese strain ATK-1 present a natural, single-nucleotide polymorphism within the miR-28-3p target site. As a result of this polymorphism, the ATK-1 virus sequence was not inhibited by miR-28. Interestingly, genetic studies on the transmission of the virus has shown that the ATK-1 strain, which carries a Thr-to-Cys transition mutation, is transmitted efficiently between spouses, suggesting that miR-28 may play an important role in HTLV-1 transmission.

Human T-cell Leukemia virus, type 1 (HTLV-1)2 infection is associated with a disease with poor prognosis known as adult T cell leukemia/lymphoma or HTLV-1-associated myelopathy (HAM/TSP) (1–5). Because the HTLV-1 virus is poorly infectious and has a very low antigenic variability, reducing the expression of viral antigens is critical in virus maintenance in vivo. Several studies suggest a tight dynamic between virus expression and immune control of proviral loads in HTLV-1-infected patients (6, 7). The discovery of p30-mediated repression of HTLV-1 replication and its role in virus silencing suggests the existence of viral factors to help the virus persist in the host (8, 9). Other HTLV-1 proteins (Tax, basic leucine zipper (HBZ), p13, and p12) have also been reported to control virus expression (10–13).

In addition to viral factors, it is well established that the cellular environment has a profound impact on virus infection and replication. Many cellular genes can act as innate immunity factors to prevent replication and virus dissemination. In mammalian cells, viral infection is a potent trigger of the IFN response and activation of an antiviral state (14). Viruses have evolved multiple strategies to escape IFN (15). Recently, much attention has been focused on the role of non-coding RNA in virus pathogenesis. Virus-derived microRNAs can favor viral gene expression, virus replication, and virus infectivity (16) or even antagonize the IFN response (17). This mechanism has been described extensively for herpesviruses such as HSV1, Kaposi sarcoma-associated herpesvirus (KSHV), human cytomegalovirus (hCMV), and EBV (18–20) but appears to be absent in the HTLV-1 genome. In addition, some cellular microRNA may promote virus replication, as seen in the case of miR-122 and the hepatitis C virus (HCV) (21, 22), or a protective role, as reported for primate foamy virus, type 1 replication, which is inhibited by miR-32 (23). However, microRNA can also negatively regulate virus expression and infectivity. In fact, several anti-HIV cellular microRNAs (miR-28-5p, miR-150, miR-223, and miR-382) that target the HIV-1 genome have been reported to act in this way (24). These microRNAs are believed to participate in HIV latency because their level of expression correlates inversely with that of HIV-1 (25, 26).

To date, HTLV-1 has not been shown to encode or be directly targeted by microRNA (27). HTLV-1 genome analyses revealed no conserved site for anti-HIV microRNAs miR-28-5p, miR-150, miR-223, and miR-382. However, we identified an octamer target site for miR-28-3p. The same miR-28 hairpin structure can be processed into mature products derived from each strand, termed miR-28-5p and miR-28-3p, which can then target different microRNAs. This study is the first to report the existence and mechanism used by a cellular
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miRNA to control HTLV-1 virus expression and to prevent virus transmission.

EXPERIMENTAL PROCEDURES

Cell Culture, Transfections, and Luciferase Assays—293T (ATCC) and BHK1E6 (28) cells were grown in DMEM with 10% FBS. Jurkat cells (ATCC) and the HTLV-1-transformed cell line MT-2 (16) were cultured in RPMI 1640 medium with 10% FBS. 293T, COS7 and BHK1E6 cells were transfected with Polyfect (Qiagen). Luciferase activities were assayed 48 h after transfection using the Dual-Luciferase reporter assay system (Promega). Peripheral blood mononuclear cells from healthy donors and HTLV-1-infected acute adult T-cell leukemia blood samples (29) were obtained after written informed consent and approved by consent in a study approved by the Institutional Review Board of the National Cancer Institute/National Institutes of Health. Plasmids—Pri-miR-28 was amplified from healthy donor peripheral blood mononuclear cell DNA with the CTGGATC-CTGAAGGCAGGCCCTCAGAAG (forward) and AAGATTC-TCCCATGTACCAGGCTCACGAGA (reverse) primers and cloned into pCDNA3.1, pSIH-H1-copGFP, and pSIH-H1-puro vectors. The gag fragment from nucleotide 4836–5442 in HTLV-1 genomic RNA was amplified from the pBST primers CTCTAGAGTTTCTGGAATTTTATTC (forward) and AGAATTCTGGTATAGAGGGCTCGAGGA-CTCTAGAGTTTCTGGAATTTTATTC (reverse) and cloned into the pGL3-promoter vector. All mutants were made using the QuikChange® site-directed mutagenesis kit (Stratagene). To generate the miR-28-3p mutant, only the miR-28-3p sequence was mutated, and miR-28-5p remained wild-type. All constructs were verified by sequencing. The HTLV-1 envelope-expressing vector, CMV-Env-LTR, HTLV-LTR-luc, p-BST, and pc-Tax have been described previously (30–32).

Western Blot Analyses—293T cells were lysed in radioimmunoprecipitation assay buffer (50 mm Tris-Cl (pH 7.5), 150 mm NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, and 0.1% SDS) containing a complete protease inhibitor mixture (Roche Diagnostics). Cell lysate concentrations were determined by a modified Bradford assay (Bio-Rad). Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The membranes were probed with anti-HTLV-1 p24 (catalog no. TP-7, ZeptoMetrix), anti-HTLV-1 p24, and anti-β-actin (catalog no. C-11, Santa Cruz Biotechnology). All secondary antibodies were from Santa Cruz Biotechnology.

Lentiviral Packaging and Infections—293T cells were transfected with 20 H1-puro-miR28 viruses were generated as described previously as those used for real-time PCR. Prior to coculture with HTLV-1-producing cells, BHK1E6 and Jurkat cells were pretreated with AZT for 3 or 15 h, respectively. AZT was maintained in the culture medium during the coculture experiment.

RNA Extraction, RT-PCR, and Real-time PCR—Total RNA was extracted from cells using TRIzol (Invitrogen) and treated with DNase I to remove the DNA contamination. The reverse transcription was performed using high-capacity cDNA reverse transcription kits (Applied Biosystems) according to the instructions of the manufacturer. Real-time PCR was carried out using TaqMan primers (Applied Biosystems) and TaqMan probes (Fam, 6-FAM, or VIC) for HTLV-1 gag and other HTLV-1 genes.
out using iTaq™ SYBR® Green Supermix (Bio-Rad) with the following sets of primers: GAPDH, GAAGGTGAAGGTCGGAGTC (forward) and GAAGATGGTGATGGGATTTC (reverse); LIM domain containing preferred translocation partner in lipoma (LPP), GTGCAATGTGTGTTCCAAGC (forward) and TGGCATAATAGGCTCCTTGC (reverse). Real-time PCR for mature miR-28-5p and miR-28-3p was performed with the miScript PCR system according to the instructions of the manufacturer. The miScript HiSpec buffer was used to prepare cDNA. The forward primers used for miR28-5p and miR-28-3p were AAGGAGCTCACAGTCTATTGAG and CACTAGATGGTGACTCTTGGA, respectively. The expression of mature miR-28-5p and miR-28-3p was normalized to human RNU6B, which was provided in the kit. The expression of miR-28 in the stable cell lines was performed with pSIH-F and pri-miR-28-R primers.

RESULTS

Analyses revealed a conserved target site for miR-28-3p but not miR-28-5p in the proviral genome of HTLV-1 (Fig. 1A). This binding site is located between nucleotides 5031 and 5038 of the HTLV-1 genomic mRNA, 196 bp after the stop codon of the polymerase gene. There is no conserved site for miR-28-3p in the genomes of HTLV-2 or HTLV-3. To confirm the functionality of the miR-28-3p site, we transiently transfected the HTLV-1 molecular clone pBST (35) in the absence or presence of a miR-28 expression plasmid. The results from these experiments demonstrated a dose-dependent decrease in HTLV-1 gag p19 and p24 products expressed from the full-length HTLV-1 genomic RNA (Fig. 1B). Because Tax controls the expression of viral genes by transactivating the viral LTR, we next wanted to demonstrate that a decrease in gag expression was not directly related to a miR-28-induced change in Tax expression. The results from these experiments demonstrated a dose-dependent decrease in HTLV-1 gag p19 and p24 products expressed from the full-length HTLV-1 genomic RNA (Fig. 1B).
Tax cDNA sequence. In agreement with these data, Tax expression detected by Western blot analysis was not affected by the presence or absence of miR-28-3p (Fig. 1D).

To further confirm the specificity of miR-28-3p and its direct effect on the target sequence identified in the HTLV-1 provirus, we cloned a fragment of the HTLV-1 genome encompassing the miR-28-3p site. We cloned miR-28 and also created a mutated control sequence of miR-28-3p (Fig. 1E), and verified that this mutated miR-28-3p vector (miR-28-3 pm) does not have a target site in the HTLV-1 provirus. As expected, the luciferase reporter vector containing the miR-28-3p target fragment was affected in a dose-dependent manner by coexpression of miR-28-3p, whereas the miR-28 mutant (miR-28-3 pm) was not (Fig. 1F). The increase observed in the presence of a mutated miR-28-3p may be related to interference with endogenous wild-type miRNA28. Consistent with these data, the transiently expressed HTLV-1 molecular clone was affected by miR-28-3p but not the miR-28-3p mutant, as shown by the reduction in both p19 and p24 gag only in the presence of wild-type miR-28-3p (Fig. 1G).

Real-time RT-PCR analyses of the LIM domain containing preferred translocation partner in lipoma (LPP), the locus encompassing the miR-28-3p and miR-28-5p sequences (36), revealed differential expression between activated and resting peripheral blood mononuclear cells (24). To further confirm these results, we directly measured the mature miR-28-5p and miR-28-3p in both resting and activated cells. Our results confirmed a 5- to 10-fold reduction of miR-28-3p expression in activated cells (Fig. 2A). It is well established that HTLV-1 virus particles can infect activated T cells but cannot infect resting T cells, raising the possibility that miR-28-3p acts as a restriction factor for HTLV-1 infection. We next investigated the natural
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divergence of the miR-28-3p site in various HTLV-1 strains. The miR-28-3p target site was highly conserved in HTLV-1 subtypes B and C. However, the Japanese ATK-1 strain, subtype 1A, presented a natural polymorphism and Thr-to-Cys mutation within the miR-28-3p target site (Fig. 2B). The mutation is silent (AAT to AAC) and does not change the amino acid sequence in that region. Genetic studies of the transmission of the virus between spouses have shown that a virus carrying a Cys mutation is transmitted efficiently (37). We decided to test whether the Thr-to-Cys mutation affected the ability of miR-28-3p to suppress HTLV-1 virus replication. To this end, we introduced the Thr-to-Cys mutation in our gag-UTR reporter vector and transfected either the wild-type or mutated sequence in the absence or presence of miR-28. Importantly, the natural polymorphism occurring in the Japanese subtype 1A was more resistant to miR-28-3p inhibition (Fig. 2C). The transcriptional activity of the LPP/miR-28 promoter is induced by constitutive activation of STAT5, which recruits transcriptionally active p53 to the LPP/miR-28 promoter. Both active STAT5 and p53 are required for activation of the LPP/miR-28 promoter (38). Although STAT5 is constitutively active in HTLV-1-transformed cells in vitro and ATL cells in vivo (39–41), studies have shown that p53 is generally inactive in HTLV-1-transformed cells in vitro (42). However, p53 function is reduced but functional in ATL cells in vivo (43). Consistent with these observations, we found that LPP (a surrogate marker of miR-28 expression) (44) was generally expressed at least 10-fold higher in vivo ATL samples compared with HTLV-1-transformed cells in vitro (Fig. 2D). Because the LPP gene is the host gene of miR-28-3p and their expressions are related (44), high LPP gene expression means high miR-28-3p expression. Interestingly, these data parallel virus expression, which is usually undetectable from ATL cells in vitro but abundant in transformed cells in vitro. These observations raise the possibility that miR-28-3p participates in silencing the virus in vivo to facilitate immune escape and virus persistence, and this warrants additional studies.

Because miR28-3p targets the genomic viral RNA, we next hypothesized that miR-28-3p may restrict de novo infection by HTLV-1 virus particles. To test this hypothesis, we used a previously characterized reporter cell line stably transfected with an HTLV-1-LTR-Lac Z vector (28). Because the full-length HTLV-1 LTR is integrated in these cells, basal activity is extremely low, and only infected cells are revealed by a blue color after X-gal staining. We used this cell line to stably express pSI-H1-GFP or pSI-H1miR-28-3p (Fig. 3, A and B). These cell lines, BHK1E6, BHK-GFP, and BHK-miR-28, were cocultured in the presence of an HTLV-1 virus-producing cell line, MT-2, for 1 day. After staining, the number of blue cells was quantified visually. Results from several independent experiments suggest that miR-28-3p-expressing cells were resistant to infection, as shown by a significant decrease in the number of blue cells in BHK-miR-28-3p cocultured with MT-2 (Fig. 3, C and D). We next demonstrated that the reduced Lac Z expression was not the result of a decreased ability of Tax to transactivate the integrated HTLV-1 LTR. Because we have shown previously that miR-28-3p is unable to target the Tax cDNA sequence (Fig. 1, C and D), we transfected the Tax expression vector. Results from independent experiments indicated a similar number of blue cells in BHK, BHK-GFP, and BHK-miR-28-3p upon transfection of Tax (Fig. 3, E and F), suggesting that stable expression of miR-28-3p does not affect Tax expression or Tax ability to transactivate the HTLV-1 LTR or expression of Lac Z.

We next wanted to identify the infection step that is inhibited by miR-28-3p. In the absence of a cell-free infection system for HTLV-1, the effect of miR-28-3p on viral entry was tested using a lentiviral vector (pSI-H1-GFP) pseudotyped with either the HTLV-1 envelope (Env1) or the vesicular stomatitis virus envelope (VSV-G) as a control. The fact that miR-28-3p had no effect on VSV-G pseudotype particles (Fig. 4, C and D) suggests that miR-28-3p does not have a target site in the pSI-H1 vector sequence. Therefore, if miR-28-3p can affect HTLV-1 receptor expression or HTLV-1 viral entry, then we should see a difference in the number of infected cells. In fact, there was no significant difference in the efficiency of infection between control and miR-28-3p-expressing cells (Fig. 4, A and B). The VSV-G pseudotype was used as a control to confirm the specificity of our neutralization assays using HTLV-1 infected TSP/HAM patient serum to block infection by the Env-1 virus. As shown in Fig. 4, E and F, the HTLV-1 TSP/HAM serum effectively inhibited Env-1 pseudotype virus particle infection. As expected, the serum had no significant effect on particles pseudotyped with the VSV-G envelope (Fig. 4, G and H). Together, our results suggest that miR-28-3p does not affect viral entry but targets a post-entry step.

A major limitation in the HTLV-1 field is the absence of a reliable system to measure de novo infection. Despite a report of cell-free virion infection in dendritic cells (45), this system is difficult and relatively inefficient. HTLV-1 cell-free virus preparations are largely not infectious. HTLV-1 is mainly transmitted upon cell-cell contact (46, 47), and, as a result, it is difficult to discriminate between producing cells and newly infected cells. We developed a new sensitive assay for the detection and quantification of newly infected cells by HTLV-1. Specific primers were designed in the pX and gag regions so that only products of reverse transcription in newly infected cells could be amplified (Fig. 5A). Both single LTR circles and two-LTR circles are present at very low levels in MT-2 cells (Fig. 5A). Although two-LTR circles were also detected in infected cells, the frequency of these products was much lower than the single LTR circles, and, unlike single LTR circles, the relative amounts of two-LTR circles did not increase in infected cells (Fig. 5B). Therefore, the increase in single LTR circles (products of reverse transcription) can be used as a readout of newly infected cells. To demonstrate the specificity of our assay, we cocultured HTLV-1 virus-producing MT-2 cells with either BHK or Jurkat cells. Single-LTR circle DNA was not detected in any of these cell lines alone under the conditions described under “Experimental Procedures.” Products of reverse transcription were easily detected following coculture with BHK or Jurkat cells (Fig. 5C). This was specific for newly infected cells because as treatment with the reverse transcriptase inhibitor AZT inhibited infection and the detection of single LTR products in cocultures (Fig. 5C). Importantly, our results demonstrate that the single-LTR circle detection method described here allows very sensitive and semiquantitative measurement of infection
in coculture systems. We next wanted to confirm the effect of miR-28-3p in both non-T cells and T cells. To this end, we generated a Jurkat cell line stably transfected with miR-28-3p (Fig. 5D). The presence of single LTR circles was monitored 0, 3, 6, and 9 h after initiation of coculture with HTLV-1-producing MT-2 cells. Infection of Jurkat cells, identified by reverse transcriptase product single-LTR circles, was detected as early as 3 h after contact with HTLV-1 through coculture with MT-2 cells for 24 h. 24 h after infection, cells were stained with X-gal to measure the infection. The images were taken at ×100 magnification in a bright field. Representative images of the X-gal staining are shown. AZT (10 μM) was used as a control to prevent HTLV-1 infection. D, the average number of infected cells (blue cells) for BHK1E6, BHK1-GFP, and BHK1-miR-28 per 6-well plate. Mean ± S.D. was calculated from two independent counts. The data are representative of three independent experiments. Results are statistically significant (Student’s t test, ***, p = 0.015). E, BHK1E6, BHK1-GFP, and BHK1-miR-28 cells were transfected with 0.1 μg of pc-Tax. The cells were fixed 2 days later and stained with X-gal. The images were taken at ×100 magnification in a bright field. Representative images of the X-gal staining are shown. F, the number of blue cells for BHK1E6, BHK1-GFP, and BHK1-miR-28 that were transfected with pc-Tax. Mean ± S.D. was calculated from two independent counts. The data are representative of two independent experiments.

DISCUSSION

In this study, we identified a novel mechanism for the control of HTLV-1 expression and the infection of target cells. The cellular microRNA miR-28-3p was found to target a sequence localized within the viral gag/pol genomic viral mRNA and reduce viral replication and gene expression in transiently transfected cells with an HTLV-1 molecular clone. All viral
mRNAs that are derived from the genomic gag/pol RNA miR-28-3p can potentially reduce expression of all viral proteins. To demonstrate that reduced levels of p19 and p24 were not the result of a direct effect of miR-28-3p on Tax activation of the viral LTR, we performed luciferase assays and Western blot analyses using a Tax cDNA expression vector. The results from these experiments showed no effect of miR-28-3p on Tax-mediated transactivation or Tax expression, demonstrating that inhibition of HTLV-1 replication by miR-28-3p was independent of Tax.

HTLV-1 has a large tropism in vitro and in vivo. We next demonstrated that cells expressing miR-28-3p are refractory to HTLV-1 infection in cocultivation assays using both T cell and non-T cell target cells. Inhibition was not linked to receptor interaction and entry, as demonstrated by pseudotyping of the HTLV-1 provirus with a VSV envelope. In the absence of an efficient cell-free infection system, we developed a new technique for the detection of newly infected cells in a coculture system. This assay is on the basis of the detection of reverse transcription intermediate single-LTR circles. Consistent with a previous report (48), our data show no increase in the presence of double-LTR circles following infection but, rather, a specific and significant increase in the presence of single-LTR circles as early as 2 h after mixing target and virus-producing cells (data not shown). The fact that miR-28-3p restricts HTLV-1 expression and infection is consistent with the high levels of miR-28-3p found in resting T cells and the inability of these cells to be infected by HTLV-1 without prior activation.

Interestingly, a natural feedback loop exists to control miR-28-3p expression in response to virus infection. Although de novo infection of target T cells activates the IFN antiviral response, miR-28-3p expression is increased significantly upon stimulation with IFN-α or IFN-γ (49). It is tempting to hypothesize that stimulation of miR-28-3p expression may, in turn, contribute to restrict virus expansion to neighboring cells by reducing virus expression. This may play a role in reducing local
inflammation and, possibly, the initial establishment of a latent reservoir. Expression of antagomiRs directed against anti-HIV micro-RNAs (miR-28-5p, miR-125b, miR-150, miR-223, and miR-382) reactivated virus from latently infected T cells isolated from patients on suppressive, highly active antiretroviral therapy (50). Studies show that the miR-28-5p seed-matching sequence is the best conserved of all anti-HIV microRNAs, with 95% conserved of more than 5500 isolates (51). It is remarkable that miR-28 encodes two distinct miRNAs, miR-28-3p and miR-28-5p, targeting HTLV-1 and HIV-I, respectively. Other cellular microRNA regulating HIV-I (miR-125b, miR-150, miR-223, and miR-382) had no conserved site within the HTLV-1 genome.

We found a natural polymorphism Thr-to-Cys mutation within the miR-28-3p target site in the Japanese ATK-1 viral genome strain, subtype 1A. Our studies demonstrate that the ATK-1 strain is relatively resistant to miR-28 expression, raising the possibility that this strain might be transmitted to resting T cells and dendritic cells more efficiently, and this warrants further studies. Importantly, the miR-28-3p target site is very well conserved in the HTLV-1 genome (90%). Target sequences identified by a Blast search, using as queries the miRNA-pairing sequences, revealed that, among 603 worldwide distributed sequences, 66 were identical to ATK1 in the miR-28-3p site. In Brazil, a new study showed that 95.5% are cosmopolitan transcontinental sub-subtypes and 4.5% are the ATK1 type (52).

Dynamic modulation of miR-28 expression is an attractive concept for HTLV-1 virus spreading because virus particles are able to transiently activate resting T cells, thereby reducing miR-28 expression and favoring infection. However, because IFN response is a strong inducer of miR-28 expression, the initial antiviral response may backfire, helping to conceal virus expression and to protect newly infected cells from being eliminated. Additional studies aimed at blocking miR-28-3p expres-
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