CELLULAR PATHWAY DEREGULATION AND POTENTIAL TARGETED THERAPY FOR ADULT T-CELL LEUKEMIA

By Ramona Moles
© 2017
M.Sc., University of Bologna, 2011
B.Sc., University of Bologna, 2008

Submitted to the graduate degree program in Pathology and Laboratory Medicine and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

______________________________
Chairperson: Dr. Christophe Nicot

______________________________
Dr. Timothy Fields

______________________________
Dr. M.A. Karim Rumi

______________________________
Dr. Joan Conaway

______________________________
Dr. Kenneth R. Peterson

Date Defended: 31 October 2017
The dissertation committee for Ramona Moles certifies that this is the approved version of the following dissertation:

Cellular pathway deregulation and potential targeted therapy for Adult T-cell Leukemia

__________________________________________

Chairperson: Dr. Christophe Nicot

Date Approved: 07 November 2017
Abstract

Human T-cell leukemia virus type 1 (HTLV-1) is the etiologic agent of Adult T-cell Leukemia (ATL), a lymphoproliferative disorder with a very poor prognosis. While approximatively 5% of individuals infected with HTLV-1 develop ATL within twenty years of first infection, the molecular mechanism that the virus uses to induce ATL is not entirely understood. microRNAs are posttranscriptional regulators involved in a wide range of biological processes. Based on biological functions, alteration of their expression can potentially contribute to tumor initiation and progression. The purpose of this thesis is to study changes in miRNA expression and their role in the deregulation of cellular pathways essential in HTLV-1-transformed and ATL cells.

Moreover, the absence of an effective treatment for patients led to investigation of potential therapeutic strategies for ATL. Since previous findings show that DNA repair is impaired in HTLV-1-transformed cells, this thesis is focused on targeting DNA repair as a new therapeutic option in ATL. More specifically, the aim is the study of antiproliferative effects and cytotoxicity of PARP and helicase inhibitors in HTLV-1-transformed and ATL cells.
# Table of Contents

Acceptance page ............................................................................................................................. ii

Abstract .......................................................................................................................................... iii

List of Figures and Tables............................................................................................................. xii

Chapter I: General introduction .......................................................................................................1
  Retroviridae................................................................................................................................. 2
  Retrovirus structure .................................................................................................................. 2
  Retrovirus replication ............................................................................................................... 4
  The HTLV family ...................................................................................................................... 7
  HTLV structure and gene products .......................................................................................... 8
  Gag ........................................................................................................................................... 10
  Pol ........................................................................................................................................... 10
  Env ......................................................................................................................................... 11
  Tax .......................................................................................................................................... 11
  Rex .......................................................................................................................................... 12
  HBZ .......................................................................................................................................... 14
  pX region ORFs I and II .......................................................................................................... 15
  LTR ......................................................................................................................................... 16
  Genetic Variation in the HTLV family ...................................................................................... 18
  HTLV-1 infectivity ..................................................................................................................... 19
  HTLV-1 receptor ...................................................................................................................... 21
  Role of Tax in T-cell immortalization .................................................................................... 21
Chapter III: Clinical significance of microRNAs in chronic and acute human leukemia ........115

Abstract ......................................................................................................................116

Chronic lymphocytic leukemia (CLL) .......................................................................116
miRNA signature in CLL ...........................................................................................117
miRNA expression and drug response in CLL patients .............................................119

Chronic myeloid leukemia (CML) .............................................................................120
miRNA signature in CML .........................................................................................120
miRNA expression and drug response in CML patients ...........................................122

Acute lymphoblastic leukemia (ALL) .......................................................................124
miRNA signature in ALL .........................................................................................124
miRNA expression and drug response in ALL patients ............................................125

Acute myeloid leukemia ............................................................................................127
miRNA signature in AML .........................................................................................127

MicroRNAs in the diagnosis of AML ........................................................................129
MicroRNA expression associated with favorable prognosis in AML .......................130
miRNA expression associated with unfavorable prognosis in AML .................131
miRNA expression and drug response in AML patients ...........................................133

Adult T-cell Leukemia (ATL): signature and prognosis ...........................................135

Role of circulating RNA ..................................................................................................137

Clinical significance of other non-coding RNAs: IncRNA and snoRNAs ...............139

References ..................................................................................................................141

Chapter IV: STAT1: A Novel Target of miR-150 and miR-223 Is Involved in the Proliferation of HTLV-I-Transformed and ATL Cells ..........................................................165

Abstract ......................................................................................................................168

Introduction ................................................................................................................169

Experimental Procedure .............................................................................................172

  miRNA Target Predictions ......................................................................................172
  Cell Culture and Patient Samples ...........................................................................172
  Plasmids ..................................................................................................................173
  3′UTR and Luciferase Reporter Assay .....................................................................173
  Transfection, Viruses, and Generation of Stable miR-150 and miR-223
  Inducible Cell Lines .................................................................................................174
  RNA Extraction and Real-Time Quantitative PCR .................................................175
  Western Blot Analysis ..............................................................................................175

Results .........................................................................................................................177

  miRNAs miR-150 and miR-223 Target the STAT1 3′UTR ..................................177
  miR-150 and miR-223 Decrease STAT1 Protein Expression and STAT1 Signaling .................................................................................................................................182
miR-150 and miR-223 Expression Is Inversely Correlated With STAT1 Expression in HTLV-I–Transformed and ATL Cell Lines

Enforced Expression of miR-150 and miR-223 in Human T Cells Decreases STAT1 Expression and STAT1-Dependent Transcription

STAT1 Is Required for the Proliferation of HTLV-I–Transformed and ATL Cells

Increased STAT1 and MHC-I Expression in ATL Cells

Discussion

References

Chapter V: Small PARP inhibitor PJ-34 induces cell cycle arrest and apoptosis of adult T-cell leukemia cells

Abstract

Introduction

Materials and methods

Cell lines and reagents

Cell cycle and cell proliferation

Cell tracking assay

Apoptosis assays and mitochondrial membrane potential

Western blotting

Real-time RT-PCR

Results

PJ-34 induces cell cycle arrest in HTLV-I-transformed and ATLL-derived cell lines
PJ-34 treatment leads to reactivation of p53 functions in HTLV-I-transformed cells ......................................................... 232

Resistance of HTLV-I-transformed cells to PJ-34-induced apoptosis is associated with defective caspase-3 activation .................... 234

Resistance to PJ-34 in HTLV-I-transformed MT-2 cells coincides with increased expression of RelA/p65 .............................................. 239

Discussion ............................................................................................................................................................................. 241

Footnotes ............................................................................................................................................................................... 243

References .......................................................................................................................................................................... 244

Chapter VI: WRN-targeted therapy using inhibitors NSC 19630 and NSC 617145 induce apoptosis in HTLV-1-transformed adult T-cell leukemia cell ............................................................... 251

Abstract ............................................................................................................................................................................. 252

Background ......................................................................................................................................................................... 253

Methods ............................................................................................................................................................................. 255

Cell lines and reagents ........................................................................................................................................................... 255

Cell cycle and cell proliferation ................................................................................................................................................ 255

Apoptosis assay and mitochondrial membrane potential ....................................................................................................... 256

Western blotting .................................................................................................................................................................. 256

Immunofluorescence ............................................................................................................................................................ 257

Statistical analysis ................................................................................................................................................................ 257

Results .................................................................................................................................................................................. 258

NSC 19630 inhibitor induces S-phase cell cycle arrest ..................................................................................................... 258
NSC 19630 inhibits cellular proliferation and induces apoptosis in HTLV-1-transformed and patient-derived cells...................................................263
NSC 19630-resistant ATL-55T and LMY1 cell lines are sensitive to NSC 617145......................................................................................................270
Discussion..................................................................................................................276
Conclusions................................................................................................................277
References..................................................................................................................278
Chapter VII: Mutation of epigenetic regulators TET2 and MLL3 in patients with HTLV-I-induced acute adult T-cell leukemia ............................................................286
Abstract ......................................................................................................................287
Background................................................................................................................288
Methods......................................................................................................................290
ATL patient samples ...............................................................................................290
Next generation sequencing (NGS) .........................................................................290
Direct PCR-sequencing and TA cloning for analyses ..............................................293
Results.......................................................................................................................295
Discussion................................................................................................................299
Conclusions..............................................................................................................301
306
Chapter VIII: miRNA-based therapy: future directions ..................................................312
References..............................................................................................................315
Chapter IX: DNA Repair inhibitions: future directions.................................................317
References..............................................................................................................220
List of Tables and Figures

Chapter I: General introduction

Figure 1. Schematic representation of structure and composition of a retrovirus. ....4
Figure 2. Representation of retrovirus life cycle ........................................................... 6
Figure 3. Schematic representation of the HTLV genome ........................................... 9
Figure 4. HTLV-1 transmission requires the formation of a virological synapse. .....20
Figure 5. The natural history of HTLV-1 inducing ATL ............................................ 24
Figure 6. Adult T-cell leukemia .................................................................................. 28
Figure 7. HTLV-1-associated myelopathy/tropical spastic paraparesis .................. 30

Chapter II: The Emerging Role of miRNAs in HTLV-1 Infection and ATLL Pathogenesis

Figure 1. Human T-cell leukemia virus HTLV-1 interferes with cellular miRNA machinery ......................................................................................................................... 68
Figure 2. MiR-28-3p targets the HTLV-1 genome .................................................... 71
Figure 3. MiRNAs promote cell proliferation ............................................................. 73
Figure 4. MiRNAs induce resistance to apoptosis ..................................................... 76
Figure 5. MiR-149 and miR-873 promote chromatin remodeling ............................... 82
Figure 6. MiRNAs induce genetic instability ............................................................. 87

Chapter III: Clinical significance of microRNAs in chronic and acute human leukemia

Table 1. microRNAs deregulated and associated with clinical outcome in human Leukemia ................................................................................................................................. 123
Table 2. microRNAs deregulated in human leukemia and their predicted target genes ................................................................................................................................. 136
Chapter IV: STAT1: A Novel Target of miR-150 and miR-223 Is Involved in the Proliferation of HTLV-I-Transformed and ATL Cells

Table 1. Predicted miRNA Target Sites of Human STAT1. ........................................176
Figure 1. STAT1 is a direct target of miRNA miR-150.............................................179
Figure 2. STAT1 is a direct target of miRNA miR-223. ...........................................181
Figure 3. miR-150 and miR-223 hamper the IFN-mediated response by inhibition of STAT1 activity.............................................................................................................184
Figure 4. miR-150 and miR-223 expression correlates with STAT1 expression in HTLV-I and ATL cell lines. .................................................................187
Figure 5. miR-150 and miR-223 inhibit STAT1 in an HTLV-I line. .........................191
Figure 6. miR-150 and miR-223 inhibit STAT1 in the non–HTLV-I cell line, Jurkat..........................................................193
Figure 7. Exogenous expression of miR-150 and miR-223 inhibits proliferation of HTLV-I cells.................................................................196
Figure 8. Inhibition of STAT1 expression in the HTLV-I cell, MT-2, results in cell cycle disruption.................................................................198
Figure 9. Inhibition of STAT1 expression results in a decrease in cellular proliferation in HTLV-I cells.....................................................................................200
Figure 10. STAT1 expression correlates with increased MHC-I expression in ATL patient samples..........................................................202

Chapter V: Small PARP inhibitor PJ-34 induces cell cycle arrest and apoptosis of adult T-cell

Figure 1. PJ-34 induces S/G2M cell cycle arrest.......................................................228
Figure 2. Antiproliferative effect of PJ-34.................................................................229
Figure 3. PJ-34 induces cell apoptosis in most HTLV-I-transformed and ATL cells except for C91PL and MT-2 cells.................................................................231

Figure 4. PJ-34 induces cell apoptosis in most of HTLV-1-transformed and ATL cells except for C91PL and MT-2 cells.................................................................233

Figure 5. PARPi olaparib (AZD2281)........................................................................236

Figure 6. PJ-34 causes DNA damage and p53 pathway activation even in MT-2 and C91PL cells.............................................................................................................237

Figure 7. The defect of caspase-3 activation in MT-2 and C91PL contributes to the resistance of PJ-34 in MT-2 and C91PL cells. .........................................................238

Figure 8. The expressions of known resistant markers in MT-2 and sensitive cell lines.....................................................................................................................240

Chapter VI: WRN-targeted therapy using inhibitors NSC 19630 and NSC 617145 induce apoptosis in HTLV-1-transformed adult T-cell leukemia cell

Figure 1. NSC 19630 inhibitor induces S-phase cell cycle arrest............................259

Figure 2. NSC 19630 inhibits cellular proliferation in patient-derived cells..........261

Figure 3. NSC 19630 induces apoptosis in HTLV-1-transformed and patient-derived cells..................................................................................................................268

Figure 4. ATL-55T and LMY1 cell lines are sensitive to NSC 617145...............271

Table 1. Estimated IC50 of NSC 19630 in HTLV-1-transformed and patient-derived cells ..........................................................................................................................274

Table 2. Estimated IC50 of NSC 617145 in HTLV-1-transformed and patient-derived cells..........................................................................................................................275
Chapter VII: Mutation of epigenetic regulators TET2 and MLL3 in patients with HTLV-I-induced acute adult T-cell leukemia

Figure 1. HTLV-1 proviral load in cell lines and patients..........................................................289

Figure 2. Schematic representation of MLL3 protein and distribution of mutations found in ATL patients................................................................................................................292

Figure 3. Schematic representation of TET2 protein and distribution of mutations found in ATL patients................................................................................................................294

Figure 4. TET2 mutations were found in 6/18 ATL patients by TA-cloning and direct PCR sequencing. ...........................................................................................................................299

Table 1. Novel mutations identified in epigenetic regulators EZH1, EZH2, EED, SUZ12, DNMT1, DNMT3A, DNMT3B, TET1, TET2, TET3, IDH1/2, MLL, MLL2, MLL3, MLL4 and ASXL1 in ATL patients..........................................................302

Table 2. Sequence alignment of MLL3 gene and genome regions of very high homology found in chromosome 1, 2, 3, 13 and 21. ...............................................................304
Chapter I: General Introduction
Retroviridae

In recent years, retroviruses have received increased attention, not only for their importance as human pathogens but also for their exceptional value as experimental objects. Their unique replication leads to distinctive biological characteristics.

Retroviruses display a large variety of interaction with hosts, ranging from benign infections to fatal pathogenesis, however, among this family, major pathogens of all vertebrates are included. Human Immunodeficiency Virus type I, HIV, belongs to this family and is responsible for Acquired Immunodeficiency Syndrome (AIDS), which has caused more deaths than all pathogens in contemporary history [1-3]. Retroviruses have a genome encoded for Reverse Transcriptase and Integrase, which quickly become useful tools for nucleic acid manipulation in vitro and in vivo, and they can also be used to insert therapeutic genes into target cells [4, 5]. Currently, retroviruses serve modern medicine as a valuable tool for gene therapies. Moreover, retrovirus sequences can be utilized as a marker of evolutionary history. The insertion of the provirus in the germ line can be used as a Mendelian tag to study speciation, migration, and divergence of species [6].

Retrovirus structure

The retrovirus family includes a large number of viruses, primarily of vertebrates, which are responsible for different diseases, including human malignancies, neurological disorders and immunodeficiencies. Retrovirus structure is similar to general virions, enveloped in a 100 nm diameter. The surface presents a single protein structure, generally assembled in a trimer of two protein subunits, encoded by the Env gene. The nucleocapsid, also named core, can be spherical.
or conical made by three to four protein products encoded by the Gag genes. Additionally, in the nucleocapsid are located catalytic proteins involved in the replication of the virus, including protease and the products of the pol gene – reverse transcriptase and integrase. The reverse transcriptase converts the viral genetic information from single strand RNA to double strand DNA (provirus); on the other hand, the integrase is required to join the provirus to the host cellular DNA. The retrovirus genome consists of two copies, generally identical, of a single strand RNA ranging from 7-10 kb, reminiscent to cellular RNA, including 3′ polyadenylation and 5′ capping. The genome encoded for Gag, Pol, and Env [7, 8].

The cleavage of Gag, Pol, and Env gene products leads to the formation of the mature virions. These proteins are called by two letters as follows: CA - capsid, NC - nucleocapsid, PR - proteinase, DU - dUTPase, RT - reverse transcriptase, IN - integrase, SU - surface protein, and TM - transmembrane protein. A schematic representation of a retrovirus is elucidated in Figure 1.
Figure 1. Schematic representation of structure and composition of a retrovirus

The core contains the viral genome and the RT - reverse transcriptase, IN - integrase, and the PR - protease. The core is enclosed by the envelope, which presents transmembrane - TM and receptor binding - SU.

Retrovirus replication

In the replication of retroviruses two steps can be distinguished. The first phase includes the entry of the nucleocapsid into the cytoplasm, synthesis of double strand DNA using viral single strand RNA as a template, transfer of the provirus into the nucleus, and its integration into the host genome (Figure 2). This process is mediated by different viral enzymes, such as reverse
transcriptase and integrase. The second phase is characterized by synthesis and processing of the viral genome (by cellular RNA polymerases and translation machinery), and virus assembly by encapsulation of the genome by gag and gag/pol fusion proteins with the cell membrane, which lead to the release of the new viruses by budding [9, 10] (Figure 2).

The retrovirus life cycle requires the following events:

- receptor binding and membrane fusion
- internalization and uncoating
- reverse transcription of the RNA viral genome into double strand DNA (provirus)
- integration of the provirus in the host genome
- transcription of the provirus
- splicing and nuclear export of the RNAs
- translation of the RNAs to viral proteins
- assembly of the virion and packaging of the genome
- budding and release of the viral progeny
Figure 2. Representation of retrovirus life cycle

Top Left: In the early phase of the life cycle, the virus replicates its genome in the cytoplasm of the host cells. Subsequently, the provirus undergoes integration into the host genome. In the late stage of the life cycle, viral progeny is produced and released.
The HTLV family

Human T-cell Leukemia Virus type -1 and -2 have been studied in the past decades based on their association with specific pathological conditions, such as hematopoietic malignancies and neuropathy [11-15].

Adult T-cell leukemia, also named ATL, was described for the first time in 1977 in Japan. After that, HTLV-1 was reported in other geographic areas. Seroepidemiological studies allow for identification of the etiological role of HTLV-1 in ATL. Interestingly, an association between the virus and the hematopoietic malignancy was described in patients affected by ATL [16-22].

HTLV-1 was first identified in T-lymphoblastic cell lines, HUT 102, which was established from a patient affected by cutaneous T-cell lymphoma. In an independent study from 1981 HTLV-1 virus was isolated from MT-1 cell lines established from an ATL patient. The two viruses isolated from these cell lines were later identified as the same virus, HTLV-1, and associated with ATL and other diseases. It is now well-established that HTLV-1 is the etiological agent of ATL and neuropathies, including tropical spastic paraparesis (TSP) and HTLV-1-associated myelopathy (HAM) [23, 24].

In 1978, HTLV-2 was identified for the first time in a T-cell line, Mo-T, established from the splenic tissue of a patient affected by hairy-cell leukemia. Based on serological cross-reactivity, the virus isolated was found to be similar to HTLV-1, and was then called HTLV-2. HTLV viruses both infected and transformed T cells in vitro. Only a few patients infected by HTLV-2 have been studied and characterized and limited pieces of evidence are provided from these studies investigating the association between HTLV-2 and hairy-cell leukemia. Moreover, few clinical cases of neurological diseases have been described in HTLV-2-infected individuals. The
reduced number of subjects infected with HTLV-2 affected by specific diseases preclude
demonstrating the etiological role of HTLV-2 in human pathogenesis [25-28].

**HTLV structure and gene products**

As a retrovirus, HTLV encodes for proteins typical of every retrovirus, which includes structural
and enzymatic proteins: Gag, Pol, and Env. In addition to that, a unique region was described at
the 3' end of the viral genome; this portion was named X-region because its role was unknown.
The X-region presents four open reading frames (ORFs), two of which are now well
characterized, and they encode for the viral regulatory proteins Tax, Rex and HBZ. Tax is
essential for the cellular transformation and for this reason is reported as a viral oncoprotein [29-
33]. The Tax gene encodes for a protein responsible for the transcriptional activation of LTR,
long terminal repeat. On the other hand, Rex is involved in the expression of the viral proteins
and partially overlaps the oncoprotein Tax in the alternative reading frame. The full-length
messenger RNA, mRNA, synthesizes for gag and pol gene products and is packed into the
virions. The reverse transcriptase uses the cellular tRNA-Pro as a primer for the viral genomic
RNA and produces a single spliced subgenomic mRNA that encodes for the Env gene, a doubly
or complementary spliced subgenomic mRNA that has two introns removed and encodes for Rex
and Tax genes. The Env initiation codon is the same as Tax and is located in the second exon of
Tax/Rex mRNA. In contrast, the Rex start codon is located in the same exon, more precisely 50
nucleotides upstream [34-38]. Many minor alternative spliced mRNA species have been reported
by using RNase protection assay or RT-PCR in cells isolated from ATL or HAM/TSP patients.
Figure 3 shows the arrangement of the ORFs, gene products and HTLV-1 genome.

**Figure 3. Schematic representation of the HTLV genome**

The top figure illustrates the structure and organization of the HTLV genome and the gene products. In the HTLV genome, Gag, Pol, and Env genes are flanked by 5' and 3' long terminal repeats (LTRs). Interestingly, the 5' LTR is reported to be deleted or methylated in some ATL patients; however, the 3' LTR invariably remains intact in all cases. The figure illustrates that the pX region, located at the 3'side of the genome, encodes for several proteins - Tax, Rex, p21, p12, p13, p30, and the HTLV-1 basic leucine zipper factor, termed HBZ - by using various reading frames.
Gag

Generally, in retroviruses the gag protein is translated as a polyprotein precursor; HTLV-1 is not an exception. Gag polyprotein p55 undergoes a proteolytic cleavage, which leads to the release of 19 kD matrix - MA, 24 kD capsid - CA, and 15 kD nucleocapsid - NC. Consistent with other retroviruses, p19 undergoes transcriptional modification, which results in the addition of myristic acid to the N-terminus of the protein. Myristoylation targets the precursor polyprotein Gag to the inner layer of the cellular membrane. This molecular event is particularly important in the assembly and budding of virus from the infected cells [37, 39, 40].

Moreover, additional viral Gag-related proteins have been described in an HTLV-1 context, which includes species with 28, 29, 37, 55 and 70 kD. Further analysis has shown that these species are immature precursors of three different Gag proteins. For instance, p29 and p55 have determinants related to the mature p19 and p24. However, pulse-case experiments clearly show that p55 is the precursor molecule. The 3’ end of Gag open reading frame overlaps with the 5’ part of Pol, which encodes for the protease. Gag polyprotein precursor carries out the synthesis of the protease by ribosomal frameshift. The role of the protease in an HTLV-1 context has been well documented; its function is to process the Gag products. Moreover, by catalyzing its self-cleavage, the protease induces the activation of its mature form [41-44].

Pol

The polymerase region can potentially encode for 982 and 896 amino acid products in HTLV-1 and HTLV-2, respectively, although a frameshift event is required to express the Pol gene, leading to an 863 amino acid open reading frame in HTLV-1 and 864 in HTLV-2. Moreover, the
pol gene encodes for reverse transcriptase and integrase genes, which are located in the 5’ part of the Pol gene [45].

**Env**

The Env gene encodes for a glycoprotein of 61-69 kD; the size varies in the different cell lines. The precursor of the Env genes is reported to be detectable on the cellular surface of HTLV-1-infected cells. The precursor envelope protein undergoes proteolytic cleavage, which leads to reliance on the mature forms: SU (gp46), the 46 kD surface glycoprotein, and TM (p21), the 21 kD transmembrane protein. Interestingly, the culture media of HTLV-1-infected cells presents a significant level of free gp46, which are released from the cellular surface [46].

**Tax**

The regulatory protein Tax and Rex are unique to the HTLV family and are involved in virus replication and cellular transformation. The HTLV-1 Tax gene encodes for a 40 kD phosphoprotein (Tax-1); on the other hand, in an HTLV-2 context the Tax gene encodes for a 37 kD phosphoprotein (Tax-2). The homology between these two proteins is very high, around 77-85%, and both localize in the nucleus of infected cells, with RNA polymerase II, slicing complex and specific transcription factors. Structural and functional analyses have shown that the Tax protein presents at least four domains. At the amino-terminal of the Tax protein from the residue 2 to 50 is localized an activation domain, which performs its function mostly in the nucleus [30-33, 47, 48].
Moreover, amino-terminal presents another domain, a zinc binding domain, also named zinc finger, from the residues 23 to 49, which is believed to be involved in protein-protein interaction. From the residues 161 to 211, the Tax protein contains a structural domain, and from the residues 289 to 322 an activation domain. At the Serines residue 300 and 301 of the carboxyl terminus of Tax-1 are localized the major phosphorylation sites. The phosphorylation of one or both Serines results in the localization of Tax in the nucleus and mediated Tax-transcriptional activation. Tax activates the transcription and it promotes its initiation from the promoter in the 5’ of LTR of the provirus genome [47, 48]. The oncprotein Tax has an essential role in HTLV replication and T-cell immortalization and its function is discussed in more detail in section 6.

**Rex**

In the HTLV-1 genome, the Rex gene encodes for 21 or 27 kD protein, whereas in HTLV-2 it encodes for a 26 or 27 kD product. The larger p24 and p26 are phosphorylated, which is considered essential for their function. The larger Rex products have been reported to be involved in the control of HTLV gene expression, however, the role of smaller Rex proteins, such as p21, p20/p22, are not entirely understood. Rex is necessary for virus replication and transcriptionally regulates viral gene expression. Moreover, the Rex protein increases the ratio of incomplete or complete splicing of mRNA in the cytoplasm of HTLV-infected cells [49, 50].

Rex activity is mediated by specific cis-acting sequences in the LTR promoter. Rex requires two different cis-acting sequences, one named Rex-responsive element, RxRE, which is located at the 3’ LTR in HTLV-1, and the other one called cis-acting repressing sequence, CRS, localized in the U5 region of LTR. RxRE allows overcoming the repression induced by CRS. The
phosphorylated Rex proteins are located in the nucleus, more precisely in the nucleoli of HTLV-1/HTLV-2 infected cells. Functional studies of Rex have shown that it presents three different regions. The first domain contains an arginine-rich region and it mediated the RNA binding by a direct interaction with RxRE. The second region is located near the RNA-binding domain and is necessary for the assembly of multimeric Rex complex onto RxRE. Finally, the third action domain presents a short leucine-rich sequence, which interacts with Rab and additional nucleoporin-like factors to mediate the nuclear export [51, 52].

The molecular mechanism by which Rex/RxRE interaction leads to the expression of structural and enzymatic viral proteins is still unclear; however, it has been reported that Rex-1 enhances the transport of incompletely spliced HTLV-1 and HIV RNA. An additional study found that Rex-1 induced the significant increase of unspliced viral RNA by reducing intron excision and degradation in the nucleus. Experimental evidence shows that Rex-2 promotes the stability of the unsliced Gag/Pol RNA and the transport of spliced RNAs from the nucleus to the cytoplasm. The increased rate of incomplete spliced RNA was found to be associated with a decrease of complete sliced Tax/Rex RNA. Moreover, evidence suggests that Rex -1 improves the stability of Interleukin 2 receptor IL-2R alpha chain (IL-2R) mRNA and might be consequently involved in the cellular transformation of infected cells. Based on Rex’s essential role in HTLV-1 replication, it is hard to investigate its contribution to cellular transformation. In HTLV-1 and -2, between the termination of the Env genes and the third exon of the Tax and Rex genes, a region of approximately 600 nucleotides has been described. This sequence was named pX, and it was initially referred to as an untranslated region because the ORF within this region was not found in infected cells. The pX region is conserved in the HTLV family, although the homology in HTLV-1 and -2 is low compared to the rest of the viral genome [53, 54].
HBZ

The oncoprotein Tax has an essential role in HTLV replication and T-cell immortalization. While the transcripts of the Tax gene are detected in nearly 40% of ATL cells, HBZ RNA is ubiquitously expressed in ATL cells. The distinct expression of HBZ and Tax suggests that these proteins have a different role in HTLV-1 pathogenesis.

The HTLV-1 genome encodes for an antisense transcript termed the HTLV-1 bZIP factor, HBZ. Recent evidence has shown that the HBZ gene presents two transcription isoforms: the unspliced form, termed usHBZ, and a spliced form, named sHBZ. These transcripts display different 5′ UTRs; however, the usHBZ and sHBZ forms have high similar sequences except for the first amino acids - MVNFVSA for usHBZ and MAAS for sHBZ form - and they have similar functions [55-60].

HBZ is a nuclear protein, which presents an activation domain, called AD, located at the N-terminus, a central domain, CD, and a basic leucine zipper, bZIP, domain localized in the C-terminus. The N-terminus of HBZ has been reported to have a transactivating potential when it is fused with the DNA-binding domain of GAL4 and for this reason is named AD. This HBZ domain has two LXXLL-like motifs that bind to the KIX domain of CBP/p300, a transcription coactivator involved in a broad range of cellular functions. These motifs also participate in the activation of TGF-β/Smad signaling, which is essential for HBZ-induced Foxp3 expression. The bZIP domain of HBZ undergoes heterodimerization with cellular bZIP proteins of the AP1 superfamily, including CREB2, c-Jun, JunB, JunD, CREB, MafB, and ATF3. Mainly, HBZ/AP1 Heterodimerization leads to an impaired association of AP1 with their responsive DNA elements, however, in some cases, it promotes the DNA binding, for example for JunD.
The HBZ viral protein can undergo phosphorylation, acetylation or methylation; however, recent evidence has shown that none of these modifications affects its function [60].

The HBZ viral protein possesses cell proliferative function by interacting with CREB, CREB-2, CREM-Ia, ATF-1, c-Jun, JunB, and JunD. Importantly, thanks to its bZIP domain, HBZ suppresses Tax-mediated viral transcription and inhibits the canonical NF-κB pathway by compromising the DNA binding of p65 and promoting its degradation. Tax, in contrast, activates both the canonical and non-canonical NF-κB pathway, which has an antiapoptotic function in lymphoma cell lines [60, 61].

A further study suggested that the HBZ transcripts are also essential for the proliferation of HTLV-1-infected cells. Consistently, the HTLV-1 molecular clone with a mutation in the leucine zipper domain of HBZ displays reduced proviral load compared to wild-type when inoculated into rabbits. Importantly, HBZ enhances the activity of the human telomerase reverse transcriptase (hTERT) gene. Altogether this evidence supports the idea that HBZ is involved in viral replication and cellular proliferation. A possible interpretation of the 'antagonist' activity of Tax and HBZ is that the first is required to initiate transformation while the latter is needed to maintain the transformed phenotype in the late stage of the disease. It is still unclear if HBZ is also involved in the pathogenesis of HTLV-1-associated HAM/TSP [58, 62-66].

**pX region ORFs I and II**

Different studies were performed to investigate the splice into ORFs located in the pX region in HTLV-1 and -2. Based on these studies three alternative splicings were described in HTLV-1 that potentially encode for proteins from the pX sequence called ORF I and II (ORF III and IV...
encode for Rex and Tax). By using mRNA from ORF I and II, scientists demonstrated that ORF I preferentially produced 12 kD protein and p12I, however, p27 kD products can be encoded. p12I presents four minimal SH3 binding motifs (PXXP) and is highly hydrophobic and localized in the cellular endomembranes. Evidence suggests that p12I can bind the beta and gamma chain of the IL-2 receptor. In contrast, ORF II in HTLV-1 encodes for two proteins: a full-length p 30 II and truncated p13 II, which derived from the initiation of the first methionine codon in ORF II (that correspond at the carboxyl terminal of 87 residues of p30 II). Moreover, ORF II in alternative reading frame extends into the last exon of Tax and Rex. P30 II is localized in the nucleus and p13 II in the mitochondria. Segregation of ORF II suggests that p30 II and p13 II might have regulatory properties. Interestingly, p30 II has high homology with POU-M1, Pit1, Oct1 and -2. T cells transformed with the HTLV-1 virus that displays a mutation in p13 II present a different pattern of phosphorylation Vav, a signal transduction adaptor protein. Moreover, it has been reported that ATL patients frequently show early stop codons, suggesting that ORF II is probably not involved in the maintenance of the leukemia status. Based on what is known, the pX seems not to be involved either in the virus replication or cellular transformation in an HTLV-1 context [67-69]. However, additional studies are needed to better investigate the role of this region in HTLV pathogenesis.

**LTR**

The structure of the LTR promoter region is conserved in retroviruses. As in another retrovirus, three different parts, called U3, R and U5, characterize HTLV-1 LTR. The U3 region is responsible for the control of the transcription of the provirus genome. More specifically, three
21-nucleotides repeat, named Tax-responsive elements 1, TRE1, and are required for Tax transcriptional activation. Importantly, the U3 region presents a sequence involved in the termination of the polyadenylation of the messenger RNA. The polyadenylation site in the U3 region is far away from the polyadenylation signal of R, at nearly 250 nucleotides. The 5’ termination encodes for the end of the carboxyl terminus of Tax protein. Comparison analysis of LTR elements in HTLV-1 and -2 clearly show that these regions are essential for the regulation of viral gene expression, as they are consistently conserved in the HTLV family. The R and U5 sequences are found to be longer when compared with other retroviruses [70-73].

Multiple factors bind different sites of the HTLV LTR region to regulate viral gene transcription. Tax induces the transcription indirectly, without binding the LTR element. The most significant interaction occurs at the 21-nucleotides repeats, or TRE-1, of the LTR with the cyclic AMP response element protein/ activating transcription factor family, CREB/ATF. Several technical methods have been used to identify the proteins that interact with TRE-1 elements at the LTR promoter, which includes CREB homodimer, CREB/ATF-1 heterodimer, ATF-1 homodimer and others reported in Figure 2. The viral protein Tax does not interact directly with LTR. However, it stabilizes the binding of these factors. The Tax responding element, TRE-2, also called Ets response region, ERR-1, is localized between the two proximal TRE-1 domains. This element presents binding sites for Tif-1, Myb, Sp1, and Ets-1. Moreover, an additional Ets binding element has been reported, named ERR-2. Tax was found to interact with other transcription factors at the promoter region, such as CBP, TFIIA, and TFIID [74-78].
Genetic Variation in HTLV family

HTLV-1 and HTLV-2 display high homology, nearly 65% of the nucleotides sequence. The similarity is greater in the Rex and Tax genes and lower in the 5’ pX region and LTR. The variability among the genome isolated from different HTLV-1 viruses was found to be limited. The sequence of HTLV-1 isolated in Japan show from 97 to 99% homology; a similar percentage was found in isolated virus from other geographic areas, such as the Caribbean and Africa. Interestingly, phylogenetic studies identify four different HTLV-1 subtypes, named A, B, C, and D. The subtype A, also called Cosmopolitan, is the most diffuse and isolated in many different populations. This subtype comprises four groups: Transcontinental, North African, West African and Japanese. This distribution is not surprising and is consistent with the large-scale migration of the infected population, which includes the slave trade from Africa to the Americas. Subgroups are also described in subclone B, the Central African clade. Subclone C, also named Australo-Melanesia clade, was shown to be present in the Solomon Islands and Papua New Guinea. In contrast, the subtype D, termed Central African Pygmies clade, was isolated in subjects in Gabon, Cameroon, and the Central African Republic.

The origin of the HTLV-1 virus in not fully understood, however evidence suggests that it arises from the related simian virus, STLV-1, which has been present in Africa and Asia for thousands of years. The interaction between STLV-1-infected monkeys and humans and consequent evolution in the new host might be responsible for the origin of HTLV-1.

The absence of genetic variability in the HTLV family is probably associated with the low level of virus replication. Genome replication occurs by clonal expansion of infected cells rather that reverse transcription. The frequency of mutations is significantly lower when the HTLV genome
is replicated during cell division compared to during reverse transcription, which introduces a high level of mutations. Importantly, cell-associated replication allows immune escape from the antibody response. Consistently, HTLV Env gene does not tolerate a high rate of mutation without becoming un-functional. This evidence might be involved in the conservation of the HTLV genome through the evolutionary process.

**HTLV-1 infectivity**

Efficient transmission of HTLV-1 requires cell-to-cell contact. However, infection with the cell-free virus might occur. Usually, in vitro infection of target cells with HTLV is achieved by co-culturing them with gamma-irradiated and mitomycin-C-treated HTLV-producing cells. Infection of HTLV has a slow course and is considered inefficient compared to other retroviruses. A possible explanation might be connected to the positive and adverse regulation mediated by Tax and Rex, respectively. Only T cells are reported to be transformed by HTLV-1 infection, but it is well established that HTLV-1 can infect but not transform a wide range of cell types. For example, B cells and macrophages can be infected by HTLV-1 in vitro [79-81].

Cases of ATL patients that present a central nervous system (CNS) involvement are rare, but a few cases are reported with a direct infection in the CNS. In vivo studies show that infection of HTLV-1 in dendritic cells can occur and might play a role in antigen presentation, immune activation, and neurological diseases. In ATL patients the HTLV-1-positive cells are CD4+/CD25+ T lymphocytes. Consistently, in vitro study shows that HTLV-1 transcription is enhanced in CD4+ T cells. In contrast, CD8+ T lymphocytes are predominantly infected by HTLV-2 [82-84].
Figure 4. HTLV-1 transmission requires the formation of a virological synapse

Unlike many other viruses, cell-free HTLV-1 viral particles are poorly contagious. The figure illustrates HTLV-1 infection, which requires cell–cell contact between infected and uninfected T cells and the formation of a virological synapse. The viral genome is transmitted from one cell to another thanks to the synapse. In this molecular event, the lymphocyte function-associated antigen 1, LFA1, and intercellular adhesion molecule 1, ICAM1, are involved in the cell–cell contact, as shown in the figure. Importantly, the viral protein Tax contributes to the formation of a microtubule organizing center, MTOC.
**HTLV-1 receptor**

As previously discussed, the HTLV-1 virus can infect different cell types. Accordingly, glucose transporter one protein, GLUT1, has been reported to be the receptor, mediating the entry of the virus. Moreover, additional evidence has shown that surface heparin proteoglycan serves as a receptor for HTLV-1 [85]. Heparin proteoglycan and GLUT1 were found to be ubiquitously expressed on the surface of a wide range of cell types. Both CD4+ and CD8+ T cells can be infected by HTLV-1, however, in vivo studies demonstrated that the infection occurs in CD4+ T cells. A possible explanation might be that HTLV-1 is capable of infecting different cell types; however, the infection selectively increases the abundance of a particular subset of cell types, possibly because of the action of virus accessory proteins [80, 85, 86].

**Role of Tax in T-cell immortalization**

In vitro, both HTLV-1 and -2 can transform and immortalize primary peripheral blood T cells. Immortalization is achieved when cells proliferate in the presence of IL-2 in the culture media (IL-2-dependent). After a period, which ranges from 2 months to years, the cells became fully transformed and can grow in the absence of IL-2 in the culture media.

HTLV-1-transformed cells constitutively expressed a high proportion of proliferating cell nuclear antigens, such as erg-1 and erg-2, and the oncogenes c-myc, c-fos, c-jun and c-sis. Moreover, it is reported that the viral protein Tax repressed the transcription of β-polymerase and Bax, which accelerate the apoptosis. Tax indirectly activates different promoters and leads to the activation of several cellular pathways that promote the proliferation and survival of the infected cells, such as CREB/ATF, NF-KB/Rel, serum response factor (SRF), NF-AT, and basic helix-loop-helix
proteins (bHLH). Tax activates HTLV-1 gene transcription through the interaction with CREB/ATF proteins and the transcriptional coactivators CREB binding protein p300, CBP, which leads to the binding of those factors to the three 21-bp repeats present in the LTR [87-91].

In addition, Tax activates NF-KB/Rel target genes by interacting with the member of this family and their inhibitors, like p50, RelA, p100/NF-KB2, and p105/NF-KB2. The NF-KB family members are sequestered in the cytoplasm by the high-affinity binding inhibitors, IKBa and IKBb, which, following their ubiquitination degradation, result in the translocation in the nucleus of the NF-KB family members. Tax is involved in specific sites of phosphorylation of IKBa and IKBb, which leads to the degradation and nuclear translocation of the active NF-KB subunit. The phosphorylation involved different complexes containing the viral protein Tax, such as IkB kinases (IKK); IKKg/NEMO, IKKa, and IKKb; mitogen-activated kinases kinase 1 (MEKK1); NF-kB-inducing kinase (NIK); NF-kB/Rel, and IkBa or IkBb [92, 93].

Mutants of Tax1 and Tax2 have been identified that specifically abrogate the ability of Tax to activate the NF-kB pathway or CREB/ATF pathway. These mutants are of particular importance because they can be used to understand which cellular pathway is involved in the interplay between Tax and transcription factors and cellular transformation [91, 94-100].

Positive and negative regulators control cell cycle progression. HTLV-1 viral proteins affect this regulation by promoting cellular growth. Tax is reported to influence the p53 pathway and Bax. More specifically, Tax stabilizes p53 and inhibits its transactivation function, leading to the suppression of Bax expression. Tax enhances the transition between G0/G1 and S phase by interfering with the activity of Cyclin D, Cyclin-dependent kinases, cdk4 and cdk6, p21WAF and p16INK4A. Deregulation of the cell cycle progression might be involved in HTLV-1-mediated
cellular transformation. Moreover, it has been shown that Tax viral protein can interact with MAD1, mitotic checkpoint protein, leading to multinucleated cells and chromosomal abnormality [47, 101-113].

**Adult T-cell Leukemia**

Several stages can be identified in an ATL context: (a) asymptomatic carriers, (b) pre-leukemia stage, pre-ATL, (c) chronic/smoldering ATL, (d) lymphoma type, and (e) acute ATL. The vast majority of HTLV-1-infected individuals are asymptomatic carriers; only 3-5% develop ATL after 20-30 years. Asymptomatic carriers can transmit the virus because the viral HTLV-1 genome is integrated into the host cells. Infected individuals have a 1% possibility of developing ATL during their lifetime; however, the cases of HTLV-1-associated conditions range from 5-10% [13, 114-116].

Adult T-cell leukemia is frequently diagnosed during adulthood, at least 20-30 years after infection. Some patients display pre-leukemia ATL, which is asymptomatic in most cases. HTLV-1 can be identified in infected individuals by using PCR amplification; the provirus can be detected in abnormal T cells by using Southern blot. These alterations indicate the early stage of the disease, which might progress to malignancy. Approximately 50% of patients with pre-ATL undergo spontaneous repression of lymphocytosis. In the remaining 50% the lymphocytosis persists and some will develop acute ATL. Nearly 30% of patients with clinical symptoms will develop a chronic/smoldering ATL condition, which is less aggressive than the acute form of the disease, and is characterized by skin lesions, a low level of leukemic cells in the blood stream, and absence of visceral involvement [117-119].
Cell–cell transmission of HTLV-1 requires early expression of the viral protein, which is essential for the cellular transformation. On the other hand, HBZ is continuously expressed in infected cells. In the figure cells that contain '+' in the nucleus represent the HTLV-1-infected cells. The uninfected T cells are represented with an empty nucleus. The formation of a virological synapse is created by cell-to-cell contact with an infected cell. The clonal expansion of infected cells is enhanced by Tax and other HTLV-1 accessory proteins. After 20 to 40 years
of an asymptomatic period, ATL cells, also called flower cells for the characteristic shape of the nucleus, emerge in 5% of infected individuals.

Patients with smoldering ATL display skin lesions and bone marrow involvement; on the other hand, patients with the chronic form of the disease present a high level of circulating ATL cells. Patients affected by smoldering/chronic ATL might progress into the acute form within a few months. Pre-ATL and smoldering/chronic ATL represent a transitional phase in the development of the more lethal and aggressive stage of the disease: acute ATL. In acute ATL a malignant and dominant clone of ATL cells is present. Consistently, single rearrangement of the T-cell antigen receptor and rearrangement of one or a few proviruses is found in the ATL cell populations. Typically, acute ATL patients show an elevated white blood cell count and abnormal T cells. Moreover, hepatosplenomegaly and skin lesions are frequent in acute ATL patients and might depend upon ATL cell infiltration. These patients manifest alteration of lactate dehydrogenase (LDH), hyperbilirubinemia, and hypercalcemia. Hypercalcemia is the result of increased lytic bone lesions in the skull and long bones. The hypercalcemia conditions depend upon the production of IL-1a and a parathyroid hormone by HTLV-1-infected cells. ATL patients are immunocompromised and display opportunistic infection. The only significant prognostic factor reported in ATL patients is the presence of ascites, which is associated with a poor prognosis. Median survival with acute ATL is six months, even with chemotherapy treatment [120, 121].

ATL cells present a lobulated nucleus, also called flower shape nucleus, however the degree of this irregularity might vary. The characteristic shape of the nuclei of HTLV-2-transformed cells is consistent with the pleiotropic effect of the viral protein Tax, which includes chromosomal
abnormality and formation of micronuclei. ATL patients might present infiltration of lymph nodes, but nodal histopathology does not correlate with the prognosis or the response to treatment. Immunological study of ATL cells derived from patients shows that these cells are CD4+ and CD25+, showing that ATL cells are activated T cells. ATL rarely expressed a CD8 marker; however, another specific marker of ATL cells is the increased expression of the IL-2Ra chain, which is attributed to the Tax pleiotropic effect [122-124].

When HTLV-1-infected T cells are present at high levels in the blood stream, Southern Blot analysis can be used to detect the viral sequence. This type of analysis highlights the integration of the HTLV-1 sequence in the host genome, and typically the integration is mono or oligoclonal. Rarely in healthy carriers and smoldering ATL is the integration polyclonal. Interestingly, cases of subjects that present similar ATL pathogenesis in the absence of HTLV-1 infection are reported. The phenomenon can be explained by the loss of the HTLV-1 genome or by alternative factors that lead to development of a similar disease. It is well established in the field that HTLV-1 infects a polyclonal population of T lymphocytes, which undergo selective pressures leading to the evolution of a clone responsible for the malignancies. The chromosomal abnormalities are documented in ATL cells and correlate significantly with the severity of the malignancies. Consistently, the acute form of the disease displays a high rate of chromosomal abnormalities, which are less frequent in the smoldering/chronic form of ATL. As explained below, HTLV-1 transformed a pool of T cells that are oncogenic, but they might result in the evolution of a malignant clone responsible for the disease. This phenomenon requires an extended period, which goes from the infection to the manifestation of the disease. Tax, thanks to its pleiotropic effect, might contribute to this event [125, 126].
In acute ATL patients, when the number of ATL cells are elevated in the blood stream, the HTLV-1 genome can be detected and is monoclonal based on the integration side. However, some patients display a polyclonal characteristic based on the integration side, which was found to be in different chromosomes. The integration of HTLV-1 is not in specific regions of the host genome, which often occurs in animal retroviruses wherein they randomly integrate into particular cellular oncogenes.

One of the most typical characteristics of ATL cells is that even if all ATL cells present an integrated copy of the HTLV-1 genome, most of them display absent expression of HTLV-1 genes. If ATL cells are cultured in vitro, they shortly express the HTLV-1 genes, suggesting that the lack of activation is not related to intrinsic defects of the viral genome. The absence of expression of the HTLV-1 genes might depend upon virus latency or an immune selection that avoids recognition of the viral antigens on the cellular surface of the infected cells [127-129].
Matsuoka M, Retrovirology 2005
Figure 6. Adult T-cell leukemia

(a) Typical morphology of ATL cells, showing multilobed nuclei, flower cells. (B) Elevated number of osteoclasts are noted in the bone of a hypercalcemic ATL patient.

HTLV-1-associated myelopathy/tropical spastic paraparesis

In 1985 a group of West Indian patients affected by tropical spastic paraparesis was found to be positive for HTLV-1. Consistently, in Japan patients with myelopathy and pyramidal disorder were found to be positive for HTLV-1. These pieces of evidence lead to the identification of another HTLV-1-associated disease, named HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). The risk to develop HAM/TSP in HTLV-1-infected individuals is around 1%, similar to ATL. Neurological symptoms of HAM/TSP include weakness and spasticity of the extremities, hyperreflexia, Babinski sign, urinary/fecal incontinence and peripheral sensory loss. The cerebrospinal fluid, CSF, for the patients typically contained increased levels of protein and lymphocytic pleocytosis. The number of lymphocytes is frequently normal in HAM/TSP patients. However, some cases display morphological alteration of T cells, similar to ATL patients, in the peripheral blood or CSF. Magnetic resonance shows that HAM/TSP patients present lesions in the white matter and periventricular regions. Interestingly, autopsy studies highlighted the presence of abnormalities in the thoracic spinal cord, including demyelination, capillarity proliferation, and perivascular cuffing related to lymphocytic infiltration [53, 125, 130-132].
Figure 7. HTLV-1-associated myelopathy/tropical spastic paraparesis

Schematic representation of the natural history of HTLV-1-associated myelopathy/tropical spastic paraparesis, which is a progressive disease of the Central Nervous System characterized by weakness and/or paralysis of the legs, urinary symptoms, and lower back pain. HTLV-1-associated myelopathy/tropical spastic paraparesis induces chronic disability. HAM/TSP has limited therapeutic strategies because antiretroviral drugs have a limited effect on the infection.
Other HTLV-associated diseases

Several studies have indicated a possible association between HTLV-1 infection and T-cell non-Hodgkin’s lymphoma, T-prolymphocytic leukemia, Sezary’s syndrome and mycosis fungoides, small cell carcinoma and large granular lymphocytic leukemia. However, the limited data present in the studies make it difficult to determine a real association between the infection and these clinical conditions. HTLV-1 might have a role in some chronic lymphocytic leukemia, CLL, with B-cell origins. Consistently, in Jamaica where the virus is endemic the rate of HTLV-1 infection in patients with B-cell CLL is significantly increased compared to the general population, suggesting that HTLV-1 is a risk for this type of hematopoietic malignancy.

Clinical studies show that rare complications of HTLV-1 infection are uveitis and arthropathy. Moreover, lines of transgenic mice for HTLV develop chronic arthritis, showing that the virus might be one of the etiological agents of chronic arthritis in patients.

ATL patients and some HTLV-1 carriers display defects in cellular immune response, which leads to parasitic infection. Consistently, immunosuppression is commonly associated with opportunistic infections [133-139].

Interestingly, epidemiological studies show that a small group of subjects are positive for HTLV-1 and HIV. This phenomenon typically happens in the intravenous drug abuser, IVDA, community, wherein contaminated needles are shared leading to infection with both viruses. These studies show that coinfection leads to acceleration of AIDS progression. It is probable that HTLV-1 can generate envelope pseudotyped with HIV particles, which promote the tropism of the virus and diffusion of the infection; however, this was not confirmed in vivo [138, 139].
HTLV-2 related diseases

HTLV-2 infection has been reported worldwide, but primarily among IVDA in Amerindian populations in North, Central, and South America and in Africa. The role of HTLV-2 in the pathogenesis of leukemia and neurodegenerative disorder is still unclear since the number of clinical cases is not sufficient to clear epidemiological analysis. A better understanding of the role of HTLV-2 in human hematopoietic malignancies is needed, but more recent evidence shows that HTLV-2 might play a role in human diseases. Comparison studies between the different HTLV viruses might be useful to investigate the similarity and difference among this family [25, 140].

Immune response in ATL

All ATL patients produce antibodies against different HTLV-1 antigens, Gag, Env and Tax. Sera derived from infected subjects recognized Gag proteins, p15, p19, and p24. Moreover, antibodies against envelope glycoprotein gp46 and transmembrane proteins, such as p21, have been detected in infected individuals. Many subjects also present antibody against non-structural proteins, for example, against the regulatory protein Tax. This is likely because the nuclear Tax is released from the nucleus after the lysis of infected cells, exposing this antigen to the immune system. Time course evaluation of seroconversion of infected individuals after transfusion with infected blood shows that antibodies against Gag proteins are produced in these subjects in the post-transfusion period. It is interesting to note that anti-p14 appears before anti-p19. The first antibody detected against Env proteins is anti-p21, while anti-gp46 and anti-gp61 are produced
later. Consistently, anti-Tax antibodies are the last to appear, several months after the transfusion [141, 142].

**Epidemiology of HTLV infection**

HTLV-1 virus infection was identified for the first time in Japan and has now been found to be worldwide. Several pieces of evidence have shown HTLV-1 to be endemic in particular geographic areas in southwestern Japan, the Caribbean, Central Africa, parts of South America, the Middle East and Melanesia. The number of infected individuals around the world is found to be between 15 and 20 million. Initially, the association between HTLV-1 infection and ATL was discovered in southwestern Japan, which provides the first evidence of the etiologic relationship. Since then several clinical and epidemiological studies were performed to estimate the number of infected individuals in that country better. More recent evidence shows that the number of HTLV-1-infected subjects in Japan is over a million in a population of 121 million. A variety of the rate of HTLV-1 infection is reported in different regions: 35% in Okinawa, which is the area with the highest percentage, 8-18% in Kyushu Province, and 0-1.2% in the non-endemic area. Infection with HTLV-1 commonly occurs early in life. However, it was estimated that 30,000-50,000 Japanese individuals had been infected through blood transfusion [143, 144].

Recent studies show that the incidence of HTLV-1 infection is increasing worldwide, especially in the United States and Western Europe. The estimated percentage of HTLV-1 infection is 9% in Ghana, 8% in Kyushu district in Japan, 8% in Uganda, 3.2% in Zaire, 3% in Mashhad, Iran, 2% in the Dominican Republic, and 0.003-0.04% in the United States of America. However, a serological study shows a prevalence of 9%, 18% and 41% of HTLV-1, HTLV-2, and HIV,
respectively, among intravenous drug abusers in New York City [145-150]. These data show that in populations exposed to both HTLV and HIV, HTLV-1 spreads less efficiently compared to HIV.

**Transmission of HTLV-1**

There are three routes of HTLV-1 transmission: from mother to child, through sexual contact, and from exposure to contaminated blood. Transmission of the HTLV-1 virus occurs similarly to HIV, with the only exception being that cell-free body fluids cannot transmit HTLV-1 because free viral particles are poorly infected. Consistently, co-cultivation of HTLV-1-positive cells with uninfected cells is required to infect target cells. An HTLV-1-infected mother can transmit the virus to the unborn or newborn child. The transmission can occur through transplacental passage or T cells present in breast milk. The percentage of HTLV-1 in children born from infected mothers is 16%. However, the prevalence in newborn children breastfed for over three months is significantly higher at 27%. And the percentage is 5% in children breastfed for less than three months. Several studies show that HTLV-1 is present in peripheral blood and breast milk, but rarely in cord blood. For this reason, transmission through the transplacental route is rare; postpartum transmission through breastfeeding is the primary transmission mode. For this reason, the HTLV-1-positive mother should refrain from breastfeeding to reduce the incidence of transmission [151-154].

The second mode of HTLV-1 transmission occurs by sexual contact from an infected man to sexual partner, as HTLV-1-infected cells are found in semen. This was confirmed in Japanese
macaques infected by STLV-1, a retrovirus genetically similar to HTLV-1. It is possible that the transmission happens from women to men, however the efficiency is very low [155].

The transmission of HTLV-1 can also occur through exposure to contaminated blood or transfusion, however, the transmission requires the presence of lymphocytes. The HTLV-1 screening of blood donors started in 1988 in the United States of America. Before that, the incidence of HTLV-1 was higher that HIV, 0.043% compared to 0.013%, respectively [156]. This evidence shows the importance of a routine screening of blood donors for HTLV-1. However, only a few European countries perform the testing at the present day.

**Diagnosis of HTLV infection**

Several methods can be used for the diagnosis of HTLV-1, such as detection of antibodies and nucleic acid. HTLV-1 antibodies are relatively low compared to HIV-infected individuals. However, the level is sufficient for detection with ELISA assay or particle agglutination. In primary screening, ELISA/agglutination test is performed and then the results are confirmed using Western blot, radioimmunoprecipitation or PCR. To avoid the risk of a false positive a second validation is required. These depend upon the variability of the serological response to the virus and limit number of circulating antibodies in some subjects.

In infected individuals, the development of antibodies against HTLV-1 can require some time; in these cases detection of the HTLV-1 genome might represent a good alternative. In ATL patients the large majority of T cells have an integrated copy of the HTLV-1 genome and in these cases the virus can be detected by using Southern Blot. However, asymptomatic carriers display a small percentage of cells infected by the HTLV-1 virus. In this case, the cells can be cultured for
3 to 5 weeks, leading to virus replication and amplification of the HTLV-1 genetic materials. Then the virus can be detected by using Southern hybridization or in situ hybridization to HTLV RNA. However, this method is not suitable for clinical screening because it is time-consuming and expensive [157-159].

PCR amplification of specific sequences represents the most efficient way for the diagnosis of HTLV-1. The PCR technique can be used to detect a single provirus of HTLV-1 or HTLV-2. The false-positive results represent the negative aspect of this methodology, related to sample contamination. Target inactivate protocol are used to reduce the risk of a false positive. The PCR method is commonly used nowadays for the diagnosis of HTLV-1 and HTLV-2 [158].

**Differential diagnosis of HTLV viruses**

Initially, the serological analysis of HTLV-1 antibodies does not allow for discrimination between HTLV-1 and HTLV-2. To avoid that problem, radioimmunoassay and ELISA have been developed. The vast majority of monoclonal antibodies against the Gag protein p19 and p24 react against both viruses. However, few of them can distinguish between HTLV-1 and HTLV-2. Western Blot analysis can be used to distinguish between HTLV-1 and HTLV-2, however the best method for the differential diagnosis is PCR amplification of specific sequence. PCR is commonly used in routine screening because it provides a rapid, inexpensive and sensitive tool for the differential diagnosis of HTLV viruses [158].
Treatment of HTLV diseases

Only a small percentage of HTLV-1-infected individuals develop HTLV-1-related diseases; for this reason exclusively subacute and acute ATL patients undergo treatment. ATL is aggressive with limited therapeutic options. The acute form of ATL displays an average survival of less than one year. The standard combination of chemotherapy frequently used in aggressive non-Hodgkin’s lymphoma or acute lymphoblastic leukemia (ALL) has a limited effect on ATL patients. It has been reported that deoxycoformycin might be effective, however clinical trials show a variable efficacy.

Moreover, administration of β-interferon and γ-interferon has been used to treat ATL patients. Interestingly, anti-Tac antibodies have been used because of the elevated level of IL-2R in ATL tumor cells. These therapeutic options have been shown to lead to remission in some ATL cases. Treatment with zidovudine and α-interferon used independently or in combination has also proven to be promising in patients [160].

HAM/TSP patients are treated with corticosteroids, but efficiency was found to be limited. Administration of a high dose of intravenous gamma globulin has a beneficial effect for a limited period. Interestingly, the therapeutic effect of α-interferon on HAM/TSP patients has been investigated and found to be useful in a reduced number of cases. That study demonstrated that the treatment induces immunomodulation and consequent reduction of HTLV-1 proviral load. Consistently, clinical benefits were noted in HAM/TSP patients treated with anti-Tac antibodies. However, additional studies are needed to find better therapeutic strategies to cure HTLV-1-associated diseases [161].
**Vaccine development**

Among HTLV-1-infected individuals, the percentage of HTLV-1-associated diseases is 5%, including HAM/TSP and ATL, which leads to consideration of the development of a suitable vaccine. There are several advantages to establishing a vaccine for HTLV-1: (i) HTLV-1 presents limited antigen variability, (ii) natural immunity occurs in humans, and (iii) vaccination with envelope antigens has been shown to be successful in animal models. Recombinant DNA is used to develop potential new vaccines for HTLV-1, and envelop proteins synthesized in bacteria culture were used to immunize cynomolgus monkeys. In the study, several animals display sufficient antibody titers and were capable of withstanding an HTLV-1 infection [162].

Studies with subunit vaccinia have shown to induce immunization for STLV in monkeys. Marmosets can be infected with the virus by feeding with breast milk derived from HTLV-1-infected women. Moreover, rabbits can be infected by HTLV-1 when the transmission of the virus is obtained by using rabbit cells. Interestingly, in some cases the HTLV-1-infected rabbits develop tumors. A rabbit animal model has demonstrated that passive immunization can protect rabbits from the virus. Few studies have found that exposure to synthetic proteins corresponding to HTLV-1 1/-2 Env neutralization regions induces protection in rabbits. Other have shown that humoral immunity to HTLV-1 Env is direct against conformation epitopes. The rabbit model appears to be an ideal model for testing HTLV-1 vaccine prior to human trials. A long-term immunization for HTLV-1 probably requires induction of a neutralizing antibody and specific cell-mediated immunity [62, 162-167].
References


82. Lepoutre V, Jain P, Quann K, Wigdahl B, Khan ZK: Role of resident CNS cell populations in HTLV-1-associated neuroinflammatory disease. Front Biosci (Landmark Ed) 2009, 14:1152-1168.


106. Fraedrich K, Muller B, Grassmann R: The HTLV-1 Tax protein binding domain of cyclin-dependent kinase 4 (CDK4) includes the regulatory PSTAIRE helix. Retrovirology 2005, 2:54.


108. Li J, Li H, Tsai MD: Direct binding of the N-terminus of HTLV-1 tax oncoprotein to cyclin-dependent kinase 4 is a dominant path to stimulate the kinase activity. Biochemistry 2003, 42(22):6921-6928.


123. Yamada Y, Sugawara K, Hata T, Tsuruta K, Moriuchi R, Maeda T, Atogami S, Murata K, Fujimoto K, Kohno T et al: Interleukin-15 (IL-15) can replace the IL-2 signal in IL-2-


167. Iakovleva LS, Bogomolova NN: [A mixed infection of lymphoid cell lines by the human T-lymphotropic retrovirus type I (HTLV-I) and the measles vaccine virus]. Vopr Virusol 1990, 35(3):209-211.

Chapter II "The Emerging Role of miRNAs in HTLV-1 Infection and ATLL Pathogenesis"

Abstract

Human T-cell leukemia virus (HTLV)-1 is a human retrovirus and the etiological agent of adult T-cell leukemia/lymphoma (ATLL), a fatal malignancy of CD4/CD25+ T lymphocytes. In recent years, cellular as well as virus-encoded microRNA (miRNA) have been shown to deregulate signaling pathways to favor virus life cycle. HTLV-1 does not encode miRNA, but several studies have demonstrated that cellular miRNA expression is affected in infected cells. Distinct mechanisms such as transcriptional, epigenetic or interference with miRNA processing machinery have been involved. This article reviews the current knowledge of the role of cellular microRNAs in virus infection, replication, immune escape and pathogenesis of HTLV-1.

Keywords: human, HTLV-I infections, T-lymphotrophic virus 1, leukemia-lymphoma, adult T-cell, microRNAs, virus replication, cell line, cell transformation, gene expression regulation.

Introduction

The transmission of the human T-cell leukemia virus (HTLV-1) retrovirus requires close contact with infected T cells, and occurs from mother to child, predominantly through breastfeeding as well as through sexual contact and blood transfusion [1,2]. The HTLV-1 infection is also associated with other diseases, such as: a chronic and progressive neurologic disorder named HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP), polymyositis, infective dermatitis, HTLV-1-associated arthropathy, and HTLV-1-associated uveitis [2,3]. According to the Shimoyama classification, adult T-cell leukemia/lymphoma (ATLL) can be distinguished into four subtypes: smoldering, chronic and acute leukemic forms and ATLL lymphoma [4]. The overall survival of ATLL with different regimens of chemotherapy is poor, ranging between 5.5
and 13 months in patients presenting acute leukemia or lymphoma [5]. HTLV-1 mediates T lymphocyte transformation using a multistep process in which the virus promotes genomic instability, accumulation of genetic defects, and chronic proliferation of infected cells [6]. The genome of HTLV-1 encodes common retrovirus structural and enzymatic proteins, Gag, Pro, Pol, and Env, and additional accessory and regulatory proteins such as Tax, Rex, P30, p12, p13, and HTLV-1 basic leucine zipper factor protein (HBZ). Tax and HBZ regulatory proteins have been reported to play a central role in regulation of viral and cellular genes that lead to proliferation of infected cells [7]. Tax is a transcriptional trans-activator that promotes the expression of genes linked to the 5′ long terminal repeat promoter (LTR) element of the HTLV-1 genome [8]. Tax induces genomic instability [9] and promotes cell-cycle progression, survival and growth of HTLV-1-positive T cells [10]. HBZ is involved in the proliferation of infected cells in vitro and in vivo and plays an essential role in oncogenesis mediated by HTLV-1 in late stages of the disease when Tax is not expressed [11]. Consistently, HBZ was found to be expressed in ATLL cells through the whole period of ATLL development, suggesting that it might be involved in maintenance of HTLV-1-transformed cells [12]. Rex is a post-transcriptional regulator of viral expression, which activates viral replication in the early phase of HTLV-1 infection by promoting the nuclear export of HTLV-1 mRNA [13]. Several studies have shown altered expression of microRNAs (miRNAs) in HTLV-1/ATLL cell lines and primary peripheral blood mononuclear cells (PBMCs) from ATLL patients, suggesting that miRNA deregulation is involved in HTLV-1 infection and adult T-cell leukemia/lymphoma pathogenesis. MicroRNAs play an essential role in a wide range of biological processes, including development, differentiation, cell cycle, apoptosis and oncogenesis [14,15,16].
MiRNA Biogenesis

MicroRNAs (miRNAs) are small, non-coding RNA molecules that transcriptionally regulate gene expression. The first miRNA identified in animals is Lin-4, discovered in 1993 by Ambros and colleagues. Lin-4 was identified as heterochronic genes in Caenorhabditis elegans involved in cell fate [17,18]. Subsequent studies have shown the involvement of miRNAs in different biological processes, including tumorigenesis by targeting oncogenes or tumor suppressor genes [16]. MiRNA sequences are localized in different genomic contexts. Some miRNAs are encoded by exon; however, the majority are encoded by the intronic region of non-coding and coding transcripts [19]. MiRNAs are transcribed by the RNA polymerase II or III into the nucleus as primary miRNAs (pri-miRNAs). Pri-miRNAs are normally over 1 kilobase and contain a local steam-loop structure in which mature miRNA sequences are included. The nuclear RNase III Drosha recognized and processed pri-miRNAs into a hairpin-shaped RNA of nearly 65 nucleotides in length, named precursor miRNAs (pre-miRNAs). After transport to the cytoplasm by the RanGTP-dependent dsRNA-binding protein Exportin 5, pre-miRNAs are processed by the cytoplasmic RNase III Dicer, liberating a mature 20–24 nucleotide long duplex. Argonaute family proteins, AGO, and Trans-Activation Responsive RNA-Binding Protein (TARBP2), together with the duplex form a complex named RNA-Induced Silencing Complex (RISC) [19,20]. One strand of the duplex, called guide strand, is incorporated into the RISC complex while the other strand, named passenger strand, is targeted for degradation [21]. Apart from the canonical miRNA biogenesis described above, different alternative mechanisms, which bypass Drosha processing, were described [22]. MiRNAs can be generated through non-canonical pathways, wherein the precursor miRNAs are cleaved by Dicer. Mirtrons represent an example of miRNA processed by a non-canonical pathway. They are generated from intron lariats serving
as pri-miRNAs, which is processed by Spliceosome that function as Drosha, to release pre- 
miRNAs [22,23]. MiRNAs bind complementary sequences usually localized at 3′UTR of 
messenger RNA and guide RISC to target mRNA. MiRNAs used different mechanisms to 
regulate post-transcriptional gene expression: inhibition of translation and/or messenger RNA 
degradation. The repression of many miRNA targets is frequently associated with their 
destabilization. Degradation of target mRNA is characterized by gradual shortening of the 
mRNA poly-Adenine tail, which is catalyzed by the exosome or exonuclease XRN1. MiRNAs 
might also induce gene silencing by interfering with protein translation [24]. Several pieces of 
evidence show that miRNA silencing is observed with either no change in the mRNA level or 
with a significantly smaller decrease of mRNA compared to the protein level [25,26]. 
Deregulated MiRNAs in HTLV-1 context will be discussing in the next section of the review.
MiRNAs are transcribed by the RNA polymerase II or III into the nucleus as primary miRNAs (pri-miRNAs) from coding or non-coding part of genes. The nuclear RNase III Drosha recognized and processed pri-miRNAs into a hairpin-shaped RNA, named precursor miRNAs. Pre-miRNAs are transported to the cytoplasm by Exportin 5, and processed by the cytoplasmic RNase III Dicer in the mature miRNA duplex. The duplex forms a complex named RNA-Induced Silencing Complex (RISC). MiRNAs bind complementary sequences usually localized at 3’UTR of messenger RNA and this binding results in the inhibition of translation and/or messenger RNA degradation. HTLV-1 deregulates the cellular miRNA pathway by suppressing the function of Drosha and Dicer. Tax directly interacts with Drosha and the binding leads to
Drosha degradation mediated by proteasome complex. The regulatory protein, Rex, is reported to directly interact with Dicer. Rex suppresses the ribonuclease-directed processing activity of Dicer, protecting against the cleavage Rex-mRNA.

**MiRNA Profile in HTLV-1-Transformed Cell Lines and ATLL Patients**

Four studies have characterized miRNA expression profiles in HTLV-1/ATLL cell lines and ATLL patients. Pichler [27] and colleagues chose the phenotype of regulatory T cells (Treg) as a starting point to study miRNA expression in HTLV-1-transformed cells. The authors have selected and analyzed the expression of a set of miRNAs characteristic of murine Treg and downregulated in different tumors. The analysis identified five deregulated miRNAs: miR-21, miR-24, miR-146a, and miR-155 were found upregulated, whereas miR-223 was downregulated. Bellon [28] and colleagues analyzed miRNA profiles from ATLL patients compared to HTLV-1-negative donors by using microarray. The results were confirmed by Real Time (RT)-PCR of mature miRNAs in uncultured ATLL cells and HTLV-1-transformed cell lines. Microarray analysis and RT-PCR demonstrated downregulation of miR-181a, miR-132 and miR-125a and upregulation of miR-155 and miR-142-3p. This study identifies two miRNAs differently expressed in vitro and in vivo. MiR-150 and miR-223 were both upregulated in uncultured ATLL cells and downregulated in HTLV-1-transformed cell lines. Yeung [29] and colleagues examined miRNA profiles in several ATLL-derived cell lines and primary peripheral blood mononuclear cells (PBMCs) from acute ATLL patients using miRNA microarray. Several HTLV-1/ATLL cell lines and four ATLL patients were studied. Thirteen miRNAs were found to be upregulated and thirty downregulated among the different cell lines. In parallel, 22
upregulated and 22 downregulated miRNAs were identified in acute ATLL patients. Among those, miR-9, miR-17-3p, miR-20b, miR-93, miR-130b and miR-18a were found to be induced; in contrast, miR-1, miR-144, miR-126, miR-130a, miR-199a, miR-338, miR-432, miR-335 and miR-337 were found to be downregulated. Yamagishi and colleagues [30] studied the miRNA expression signature in primary ATL cells by using microarray analysis compared to CD4+ T cells from healthy donors. The results show that 59 of the miRNAs tested were found with a decrease in ATL primary cells. Among them, miR-31 was the one most profoundly repressed.
Figure 2. MiR-28-3p targets the HTLV-1 genome.

The figure illustrates a natural feedback loop that regulated cellular miRNA expression in response to virus infection. MiR-28-3p suppresses HTLV-1 expression by targeting a sequence localized within the viral gag/pol HTLV-1 sequence. MiR-28-3p expression leads to abortive infection by inhibiting HTLV-1 reverse transcription and preventing the formation of the pre-integration complex.
HTLV-1 Interferes with Cellular miRNA Machinery

The dysregulation of miRNA pathways has been reported across several viruses, including HIV, Ebola, Epstein–Barr, Influenza, HBV, HCV, Adenovirus, and HTLV-1 [31,32,33,34,35,36,37,38]. Drosha was reported to be downregulated in HTLV-1-infected cell lines, HTLV-1-transfected cells, and infected primary cells [38]. Van Duyne [38] and colleagues proposed that HTLV-1 deregulates the cellular RNAi pathway, including miRNAs, by suppressing the function and degrading Drosha (Figure 1).

The authors have demonstrated a direct interaction between the Tax oncoprotein and Drosha, which is responsible for its downregulation. The N-terminal region of Tax presents two putative motifs, the Zinc finger motif and leucine-zipper-like region, which interact with Drosha. The Tax N-terminal region is reported to interact with the proteasome complex. Van Duyne and colleagues demonstrated that the binding between Tax and Drosha leads to its degradation mediated by proteasome complex. In addition, Drosha increases HTLV-1 replication and is not efficient in processing miRNAs when Tax is expressed, suggesting that the dysregulation of miRNA machinery might be involved in the rate of HTLV-1 infection [38]. The HTLV-1 regulatory protein, Rex, is reported to directly interact with Dicer. Abe [39] and colleagues have demonstrated that Rex suppresses the ribonuclease-directed processing activity of Dicer, protecting against the cleavage Rex-mRNA (Figure 1). Inhibition of Dicer activation might represent an additional mechanism used by HTLV-1 to deregulate cellular miRNA expression.
Figure 3. MiRNAs promote cell proliferation

MiR-155 and miR-146a were found elevated in HTLV-1-infected cells in vitro. Tax induces the transcription factors NF-κB and AP-1, which promote miR-155 expression by binding the miRNA promoter. This binding resulted in an increased expression of the B-cell integration cluster (BIC) gene whose transcript is processed into miR-155. The interferon regulatory factor-4, IRF4, which is induced in HTLV-1-infected cells, promotes BIC/miR-155 expression. NF-κB also mediates miR-146a transactivation; both miRNAs enhance cellular growth in HTLV-1-infected cells. MiR-150 and miR-223 are differentially regulated in ATLL samples and in HTLV-1-transformed cells. MiR-150 and miR-223 were found upregulated in acute ATLL patients and downregulated in HTLV-1-transformed cell lines. MiR-150 and miR-223 target the
STAT1 3'UTR. Inhibition of STAT1 expression, through miR-150, miR-223 reduced proliferation of HTLV-1-transformed and ATLL-derived cell lines. MiR-150 and miR-223, by decreasing STAT1 expression and dampening STAT1-dependent signaling in human T cells, regulated proliferation in an HTLV-1 context.

**MiRNAs Target the HTLV-1 Genome**

The cellular environment has an essential role in virus infection and replication. Many cellular genes prevent replication and virus dissemination by acting as innate immunity factors. However, viruses have evolved strategies to avoid activation of an antiviral state: virus-derived miRNAs can enhance viral gene expression, replication, and infectivity [40] or suppress the IFN response [41]. The genome of the Epstein–Barr virus (EBV), Kaposi sarcoma-associated herpesvirus (KSHV), human cytomegalovirus (hCMV) and bovine leukemia virus (BLV) encodes for virus-derived miRNAs [42,43,44,45]. BLV shares many characteristics in disease pathogenesis with HTLV-1 and is associated with the development of B-cell tumors. Kincaid and colleagues [45] show that BLV is capable of producing miRNAs in vitro. A subsequent study demonstrated that BLV encodes a conserved cluster of miRNAs located in a specific BLV proviral region, which is essential for in vivo infectivity [46]. BLV has different common features in genomic organization with HTLV-1, however HTLV-1-encoded miRNAs have not been reported. Cellular miRNAs can promote virus replication or negatively regulate virus expression and infectivity [47]. MiR-28, miR-125b, together with miR-150, miR-223 and miR-382 target 3’ ends of HIV-1 messenger, promoting viral latency [47]. Bai and colleagues identified a binding site in the HTLV-1 genome for miR-28-3p and demonstrated a mechanism
used by a cellular miRNA to prevent HTLV-1 gene expression and viral transmission (Figure 2) [48].

MiR-28-3p was found to target a sequence localized within the viral gag/pol HTLV-1 mRNA and reduced viral replication and gene expression. MiR-28-3p-expressing cells are characterized by reduced levels of HTLV-1 gag p19 and p24 products and they are resistant to infection. MiR-28-3p expression leads to abortive infection by inhibiting HTLV-1 reverse transcription and preventing the formation of the pre-integration complex. MiR-28-3p suppresses HTLV-1 expression and infection; this is consistent with the high levels of miR-28-3p reported in resting T cells and their inability to be infected by HTLV-1 without prior activation. Bai and colleagues [48] demonstrated a natural feedback loop that regulated miR-28-3p expression in response to virus infection. It is well established that de novo infection in T cells activates the interferon antiviral response. MiR-28-3p expression was found to be induced after IFN-α or -γ stimulation, suggesting that miR-28-3p might contribute to restricting virus expansion to neighboring cells by reducing local inflammation and the initial establishment of latent infection. The miR-28-3p site is highly conserved in HTLV-1 subtypes B and C, at nearly 90%. However, the subtype 1A, Japanese ATK-1, presents a natural polymorphism (T to C substitution) within the miR-28-3p target site. The mutation is silent and more resistant to miR-28-3p inhibition of viral replication. Bai and colleagues [48] proposed a model where the modulation of miR-28-3p expression affected HTLV-1 virus spreading. Virus particles can transiently activate resting T cells by reducing miR-28-3p expression and favoring infection. Because IFN response is a potent inducer of miR-28-3p expression, the initial antiviral response might backfire, helping to protect newly infected cells from being eliminated by the immune system.
Figure 4. MiRNAs induce resistance to apoptosis

MiR-31 is one of the most profoundly repressed miRNAs in primary ATLL cells. The Polycomb protein complex is overexpressed in ATLL cells and suppresses miR-31 expression. MiR-31 negatively regulates NF-κB-inducing kinase (NIK) and leads to apoptosis resistance. MiR-130b and miR-93 are upregulated in HTLV-1 cell lines and ATLL patients and both target Tumor protein p53-inducible nuclear protein (TP53INP1). TP53INP1 is a tumor suppressor gene that has anti-proliferative and pro-apoptotic activities via both p53-dependent and p53-independent means. TP53INP1 has in its 3’ UTR two binding sites for miR-93 and two sites for miR-130b.
MiRNAs Promote Cell Proliferation

MiR-146a

MiR-146a has a central role in the regulation of immune response and its expression is induced by NF-κB signaling. MiR-146a is deregulated in different cancers. A high level of expression was reported in papillary thyroid carcinoma, anaplastic thyroid cancer, breast cancer, glioblastoma and cervical cancer [49,50,51,52,53]. In contrast, low-expressing levels were described in pancreatic carcinoma, gastric cancer, prostate cancer, acute myeloid leukemia (AML), myeloblastic syndrome and chronic myeloid leukemia (CML) [54,55,56,57,58,59]. MiR-146a was found to be upregulated in HTLV-1-transformed cell lines [27]. Ectopic expression of Tax in HTLV-1-negative T cells, Jurkat, induced miR-146a expression. Promotor analysis showed a 15-fold activation of miR-146a by Tax [60], suggesting that HTLV-1 infection might be involved in the regulation of miR-146a expression. Pichler [27] and colleagues used a mutated form of Tax and dominant active NF-κB inhibitor to show that miR-146a transactivation is mediated by NF-κB (Figure 3).

Tomita et al. [60] also reported an NF-κB binding site on the miR-146a gene. In addition, it has been described as having a suppressive effect of miR-146a on NF-κB signaling [51]. This might represent a negative feedback loop, which seems to be ineffective in HTLV-1-infected cells. MiR-146a has also been shown to induce proliferation in several human cancers, including cervical cancer [53], breast cancer cells [61], gastric cancer cells [62] and mesenchymal stem cells (MSCs) [63]. Consistent with this report, treatment with an anti-miR-146a inhibitor suppressed the proliferation of HTLV-1-transformed cell lines but not uninfected T-cell lines. In addition, overexpression of miR-146a increased the growth of HTLV-1-transformed cell lines.
Because overexpression of miR-146a has also been described in an EBV context, Tomita and colleagues suggested that miR-146a up-regulation might represent a common mechanism in the pathogenesis of persistent viruses. Wang et al. [64] identified 622 putative target genes of miR-146a that are predicted by using different prediction programs. Gene ontology analysis shows that these genes are involved in the inhibition of cell growth and promotion of apoptosis, and this partially explains the role of miR-146a in the proliferation of HTLV-1-transformed cells.

MiR-155

MiR-155 has been implicated in normal hematopoiesis [65], immune response [66], and in the carcinogenesis of different human tumors [67,68]. Mouse studies have reported that transgenic overexpression of miR-155 results in the increased frequency of tumor formation [69]. Overexpression of miR-155 was found in breast cancer [70], pancreatic cancer [71], lung cancer [72], B-cell lymphoma [67], MALT lymphoma [73] and acute myeloid leukemia (AML) [74]. MiR-155 was found elevated in HTLV-1-infected cells in vitro and in vivo [28,75], suggesting that this miRNA might play an important role in the biology and pathogenesis of HTLV-1. Babar and colleagues used an inducible knock-in mouse model to show that miR-155 induction in the lymphoid tissue led to disseminated lymphoma. In contrast, reduction of miR-155 resulted in the decrease of tumor size. In humans, Calin and colleagues identified a miRNA signature associated with progression and prognosis in chronic lymphocytic leukemia (CLL) and showed an association between miR-155 upregulation and poor prognosis. Several lymphoma-associated viruses, including the Epstein-Barr virus, Kaposi sarcoma-associated herpesvirus and Marek’s
disease virus, are characterized by overexpression of miR-155 [73,78], suggesting that HTLV-1 infection might be responsible for the induction of miR-155 in infected T cells. MiR-155 upregulation has been reported in HTLV-1 cell lines and ATLL patients [28,75]. Tomita [75] and colleagues demonstrated that transcription factors NF-κB and AP-1 induced miR-155 expression by binding the miRNA promoter in an HTLV-1 context (Figure 3). This binding resulted in an increased expression of the B-cell integration cluster (BIC) gene whose transcript is processed into miR-155 (Figure 3). Tomita and colleagues demonstrated that miR-155 overexpression enhanced the growth in HTLV-1-transformed cells. Consistently, treatment with anti-miR-155 reduced the proliferation of these cells and had no effect on HTLV-1-negative T cells. Wang [79] and colleagues demonstrated that interferon regulatory factor-4, IRF4, which is reported to be oncogenic [80], induces BIC/miR-155 expression in HTLV-1-transformed cells (Figure 3). In normal lymphocytes, IRF4 is involved in cellular proliferation and differentiation [80]. In mature human CD4+ T cells, IRF4 is essential for cytokine production and survival [81,82]. Several studies show that IRF4 is overexpressed in HTLV-1-transformed and primary ATLL/L cells and associated with poor prognosis [81,82,83], suggesting that IRF4 might be involved in HTLV-1 pathogenesis. Wang and colleagues show that depletion of IRF4 drastically reduced cell proliferation of HTLV-1-transformed cell lines, suggesting that the IRF4/miR-155 pathway might play a central role in the malignant proliferation of HTLV-1-infected cells [80]. In addition, miR-155 is reported to target Tumor Protein 53-Induced Nuclear Protein 1 (TP53INP1) in liver cancer stem cells [84], which promotes cell cycle arrest and apoptosis, suggesting a possible mechanism that could enhance cellular proliferation in an HTLV-1 context.
MiR-150 and MiR-223

MiR-150 and miR-223 were reported to be differentially regulated in HTLV-1-transformed cells and in ATLL samples. MiR-150 and miR-223 were found upregulated in acute ATLL patients and downregulated in HTLV-1-transformed cell lines, suggesting that different selective pressure in vitro and in vivo might regulate the expression of those miRNAs. MiR-150 can have either oncogenic or tumor suppressor activity in different human tumors. It is overexpressed in chronic lymphocytic leukemia (CLL) [85,86] and downregulated in chronic myeloid leukemia (CML) [87,88], acute lymphoblastic leukemia (ALL) [89] and mantle cell lymphoma (MCL) [90]. Additional studies show that miR-150 promotes the proliferation and migration in lung cancer by targeting SRC kinase signaling inhibitor 1 (SRCIN1) and SRC activity [91]. In contrast, miR-150 expression was reported to inhibit cell migration and invasion in breast cancer [92,93]. C-MYB, NOTCH3, CBL, EGR2, AKT2 and DKC1 are established targets of miR-150 [94,95,96,97,98]. MiR-223 was reported to be differentially regulated in human cancers; it is downregulated in hepatocellular carcinoma, B-cell chronic lymphocytic leukemia (B-CLL), acute myeloid leukemia (AML), gastric MALT lymphoma and recurrent ovarian cancer [99,100,101,102,103]. In contrast, miR-223 is upregulated in T-cell acute lymphocytic leukemia (T-ALL), EBV-positive diffuse large B-cell lymphoma, and metastatic gastric cancer [104,105,106,107,108]. FBXW7/Cdc4, RhoB, STMN1, E2F1, STAT3, C/EBPβ, FOXO1 and NFI-A are validated targets of miR-223 [106,107,108,109,110,111]. It has previously been shown that E2F1 represses the miR-223 promoter [110,111,112]. Interestingly, viral HBZ mRNA increases the expression and transcriptional activity of E2F1. HBZ expression is consistently increased in ATLL cells in vivo [11]. These observations can partially explain the differential regulation of miR-223 in vitro and in vivo. MiR-150 and miR-223 target the STAT1
3′UTR in an HTLV-1 context (Figure 3). STAT1 plays an essential role in immune modulatory functions, anti-viral responses, apoptosis and anti-proliferative responses [113]. In addition, several studies have shown that STAT1 can also act as a potent tumor promoter for leukemia development [114] and that many T-ALL leukemic cells are dependent upon the TYK2-STAT1-BCL2 pathway for continued survival [115]. Inverse correlation between STAT1 expression and miR-150 and miR-223 was identified in HTLV-1-transformed and IL-2-independent ATLL-derived cells [116]. IL-2-dependent ATLL cells display a high level of miR-150 expression, but low miR-223, suggesting that miR-150 might be regulated through the IL-2 signaling pathway. Absence of IL-2 signaling results in miR-150 downregulation in IL-2-dependent ATLL cells. In contrast, IL-2 stimulation in IL-2-independent ATLL-derived cells leads to miR-150 induction. This evidence suggests that miR-150 is regulated by the IL-2 signaling pathway. It was reported that ATLL tumor cells in vivo produce IL-2 or IL-15 and express IL-2 receptor alpha chain, CD25. These observations partially explain the higher levels of miR-150 in ATLL patients compared with HTLV-1 cell lines. Despite the miR-150 and miR-223 overexpression in freshly isolated ATLL samples, STAT1 was found to be induced in a majority of ATLL samples, suggesting that miR-150 and miR-223 cannot efficiently suppress STAT1 expression in ATLL patient cells. STAT1 has been reported to have tumor promoting activities. Inhibition of STAT1 expression, through miR-150, miR-223 or directly by shRNA targeting, reduced proliferation of HTLV-1-transformed and ATLL-derived cell lines. MiR-150 and miR-223, by decreasing STAT1 expression and dampening STAT1-dependent signaling in human T cells, regulated proliferation in an HTLV-1 context.
Figure 5. MiR-149 and miR-873 promote chromatin remodeling

The Tax protein promotes HTLV-1 gene expression by its interaction with the long terminal repeat (LTR) or U3 region of the viral promoter. To activate the transcription, Tax recruits the p300/CREB-binding protein (p300/CBP) and p300/CBP-associated factor (P/CAF), which bind two different regions of Tax, resulting in histone acetylation and chromatin remodeling. MiR-149 and miR-873 are downregulated in HTLV-1-transformed cell lines and target the chromatin remodeling factors p300 and p/CAF.
MiRNAs Induce Resistance to Apoptosis

MiR-31

Yamagishi [30] and colleagues identified miR-31 as one of the most profoundly repressed miRNAs in primary ATLL cells. MiR-31 is reported as a tumor suppressor and correlates inversely with metastasis in breast cancer [117]. MiR-31 in vivo targets several genes, such as Fzd3, ITGA5, MMP16, RDX, RhoA, WAVE3 and integrin α5 subunit, that contribute to cell migration and metastatic invasion [117,118]. The Polycomb protein complex has been reported to be a strong suppressor of miR-31 in breast cancer and adult T-cell leukemia [30,117]. Polycomb group proteins are overexpressed in ATLL cells [119] and have an important role in cellular development and regeneration by controlling histone methylation, especially at histone H3 Lys27 (H3K27), which induces chromatin compaction. The Polycomb family is associated with cancer phenotypes and malignancy in breast cancer, prostate cancer, bladder tumors, and other neoplasms [120,121]. MiR-31 negatively regulates NF-κB-inducing kinase (NIK) expression and activity in adult T-cell leukemia and other cancers [30]. NIK has an important role in tumor progression and the aggressive phenotypes of various cancers. It is well established that the NIK level directly regulates NF-κB activity in various cell types [122]. Constitutive activation of the nuclear factor NF-κB is observed in the ATLL cell lines and primary isolated tumor cells from ATLL patients [123]. NF-κB activation contributes to cell propagation and anti-apoptotic responses in ATLL [124]. An inverse correlation has been reported between the expression level of miR-31 and NIK in ATLL patients. Rescue of miR-31 represses NF-κB expression and leads to increased proliferation and apoptosis resistance. The inhibition of NF-κB promotes tumor cell death in HTLV-1-transformed cells and primary ATLL cells. The model proposed by Yamagishi and colleagues show that the Polycomb group regulates miR-31
expression and leads to NF-κB activation via NIK-miR-31 regulation and apoptosis resistance in HTLV-1 context (Figure 4). The downregulation of miR-31 might play an important role in ATLL pathogenesis.

**MiR-130b and MiR-93**

Microarray analyses demonstrated that miR-130b and miR-93 were consistently upregulated in HTLV-1 cell lines and ATLL patients and both target Tumor protein p53-inducible nuclear protein, TP53INP1 [29]. MiR-130b was found to be deregulated in several human cancers. Overexpression of miR-130b has been reported in colorectal cancer, gastric cancer, bladder cancer, cutaneous malignant melanoma, and head and neck squamous cell carcinoma [125,126,127,128]. In contrast, miR-130b is downregulated in papillary thyroid carcinoma, ovarian cancer and endometrial cancer [129,130,131,132]. Identified targets of miR-130b are STAT3, PTEN and TGF-b1 [133,134,135]. MiR-93 belongs to the miR-106b-25 cluster, which also includes miR-106b and miR-25 [136]. The miR-106b-25 cluster is overexpressed in neuroblastoma, multiple myeloma, and lung, prostate and gastric tumors [136,137,138]. Reported targets of miR-93 are PTEN, VEGF, ITGB8, DAB2 and LATS2 [139,140,141,142,143]. TP53INP1 is a tumor suppressor gene that has anti-proliferative and pro-apoptotic activities via both p53-dependent [144] and p53-independent means [145]. TP53INP1 has in its 3’ UTR two binding sites for miR-93 and two sites for miR-130b. Yeung [29] and colleagues have shown that transfection of antagomirs against miR-93 and miR-130b into an HTLV-1-transformed cell line increased the expression of TP53INP1 and decreased cellular viability by promoting apoptosis (Figure 4). These results show that TP53INP1 has anti-
proliferative properties and can be regulated by miR-130b and miR-93. Transfection of miR-93 or miR-130b in HTLV-1-negative T-cell lines reduced TP53INP1 expression and increased cellular proliferation. It has been reported that loss of TP53INP1 correlates with the development of cancers [146,147] and its induction promotes G1 cell cycle arrest and apoptosis [144,145,148]. This evidence suggests that up-regulation of miR-130b and miR-93 reduces TP53INP1 levels in ATLL cells and promotes cellular proliferation. TP53INP1 is also able to reduce cell migration in pancreatic cancer cells [149] and this might be significant because it is well established that HTLV-1 infection promotes T-lymphocyte migration [150].

MiRNAs Promote Chromatin Remodeling

The Tax protein promotes HTLV-1 gene expression by its interaction with the long terminal repeat (LTR) or U3 region of the viral promoter [151,152]. To activate the transcription, Tax recruits the p300/CREB-binding protein (p300/CBP) and p300/CBP-associated factor (P/CAF), which bind two different regions of Tax, resulting in histone acetylation and chromatin remodeling (Figure 5) [153,154,155,156,157,158]. Rahman [157] and colleagues identified the chromatin remodeling factors, p300 and p/CAF, as a target of miR-149 and miR-873. MiR-149 has been reported to have a role as an oncogene and tumor suppressor in different human cancers [158,159]. Downregulation of miR-149 has been described in prostatic cancer, astrocytomas and renal carcinoma [160,161,162]. In contrast, miR-873 was found to be suppressed in colorectal cancer, glioblastoma and breast cancer [163,164,165]. Recent evidence has established the role of miR-873 in cell proliferation, tumor growth and tamoxifen resistance in breast cancer [165]. MiR-149 and miR-873 were found to be profoundly downregulated in HTLV-1-transformed cell
lines, MT-2, compared to an uninfected control, Jurkat [157]. To verify that miR-149 and miR-873 could target p/CAF and p300, the authors over-expressed these miRNAs in HTLV-1-transformed cells and observed a significant reduction in the expression of chromatin-remodeling enzymes. In addition, the cell culture supernatant was analyzed for viral protein p19 before and after transfection. The results show a decrease in the levels of viral progeny production in cells transfected with miR-149 and miR-873, suggesting that these miRNAs, by targeting chromatin remodeling factors p/CAF and p300, might play a role in HTLV-1 infection and pathogenesis (Figure 5).
Figure 6. MiRNAs induce genetic instability

MiR-17 and miR-21 are upregulated in an HTLV-1 context. HBZ inactivates OBFC2A via miR-17 and miR-21, promoting genetic instability and cell proliferation. OBFC2A encodes for hSSB2, which is involved in the ATM signaling pathway, the activation of the cell cycle checkpoint and promotes DNA repair.
MiRNAs Induce Genetic Instability

MiRNA expression analysis in CD4+ lymphocytes, derived from HAM/TSP patients, has identified a high expression level of miR-17 and miR-21 [166]. Spry 1, Spry 2, PTEN, TPM1 and Pdcd4 have been reported to be miR-21 targets, suggesting its central role in cell proliferation, apoptosis, and invasion [167,168,169,170,171]. MiR-17, instead, is the main effector of the miR-17-92 cluster component, which has been identified as a member of the miRNA signature in solid tumors [172]. MiR-17 regulates E2F1 and c-Myc, p21, PTEN and BIM expression [173,174,175,176], suggesting its potential functions in cell migration, invasion and proliferation. Vernin [166] and colleagues identified OBFC2A as a potential target of miR-17 and miR-21 in an HTLV-1 context. OBFC2A encodes for hSSB2, which is involved in the ATM signaling pathway, the activation of the cell cycle checkpoint and promotes DNA repair. The down-regulation of OBFC2A and a positive correlation between miR-17, miR-21 and HBZ expression has been reported in HTLV-1-infected cells [166]. Vernin and colleagues suggested that HBZ inactivates OBFC2A via miR-17 and miR-21, promoting genetic instability and cell proliferation (Figure 6).

The authors have shown that ectopic expression of HBZ does not decrease cellular growth in DNA-damaged cells. HBZ-expressing cells continued to proliferate when treated with a DNA-damaging agent, neocarzinostatin. This phenotype can be reversed by ectopic expression of OBFC2A, which leads to a decrease of proliferation rates and restores the DNA damage response. This evidence suggested a potential role of miR-17 and miR-21 in genetic instability and cell proliferation in HTLV-1-infected cells.
Conclusions and Prospective

The role of miRNAs in HTLV-1 infection and ATLL pathogenesis is beginning to emerge. Available evidence shows a complex interplay between cellular miRNA machinery and virus infection. HTLV-1 inhibits proteins involved in biogenesis and maturation of cellular miRNAs, resulting in a perturbation of the expression profile of host miRNAs. In this review, we focused on miRNAs, which are involved in virus production, establishment of latency, tumor cell transformation and proliferation. A potential role of MiRNA modulation could represent a therapeutic approach for ATLL patients. The combination delivery of miRNAs with chemotherapy drugs might provide a promising strategy to overcome chemo-resistance. Different studies have shown that co-delivery of miRNA and chemotherapeutic agents are effective to inhibit tumor growth by targeting genes, which are involved in tumor cell proliferation and/or survival [177,178,179]. In addition, in combination with antitumor drugs, miRNAs might have an important role by targeting genes involved in drug resistance, thus overcoming the chemo-resistance in ATLL patients.
References


60. Tomita M, Tanaka Y, Mori N: MicroRNA miR-146a is induced by HTLV-1 tax and increases the growth of HTLV-1-infected T-cells. Int J Cancer 2012, 130(10):2300-2309.


Chapter III "Clinical significance of microRNAs in chronic and acute human leukemia."

Yeh CH *, Moles R *, Nicot C [* equal contribution].

Abstract

Small non-coding microRNAs (miRNAs) are epigenetic regulators that target specific cellular mRNA to modulate gene expression patterns and cellular signaling pathways. miRNAs are involved in a wide range of biological processes and are frequently deregulated in human cancers. Numerous miRNAs promote tumorigenesis and cancer progression by enhancing tumor growth, angiogenesis, invasion and immune evasion, while others have tumor suppressive effects [1, 2]. The expression profile of cancer miRNAs can be used to predict patient prognosis and clinical response to treatment [3]. The majority of miRNAs are intracellular localized, however circulating miRNAs have been detected in various body fluids and represent new biomarkers of solid and hematologic cancers [4, 5]. This review describes the clinical relevance of miRNAs, IncRNAs and snoRNAs in the diagnosis, prognosis and treatment response in patients with chronic lymphocytic leukemia (CLL), chronic myeloid leukemia (CML), acute lymphocytic leukemia (ALL), acute myeloid leukemia (AML) and acute adult T-cell leukemia (ATL).

Chronic lymphocytic leukemia (CLL)

Chronic lymphocytic leukemia (CLL) is characterized by slow growth and the accumulation of incompetent CD5+, CD19+ and CD23+ B lymphocytes in blood, marrow, and other lymphoid tissues. This disease can be distinguished into aggressive and indolent subtypes with deletion of chromosome 13q14 being the most common genetic alteration found at diagnosis.
miRNA signature in CLL

The miR-15/16 cluster, miR-34b/c, miR-29, miR-181b, miR-17/92, miR-150, and miR-155 represent the most frequently deregulated miRNAs reported in CLL and these microRNAs have been associated with disease progression, prognosis, and drug resistance [6]. Nearly two-thirds of CLL cases presented a down-regulation of miR-15a/16-1 expression. In fact, miR-15a and miR-16-1 are located in the locus 13q14.3, a genomic region frequently deleted in CLL patient samples [7]. However, additional mechanisms, such as overexpression of histone deacetylases (HDACs), also down-regulate expression of miR-15 and miR-16 [8]. Another point to highlight is that there is an inverse correlation between miR-15a/16-1 and BCL2 expression reported in CLL, as inhibition of this microRNA expression in leukemic cell lines leads to increased BCL2 expression and resistance to apoptotic signals. In addition, comparative microarray analysis in CLL patients with high or low levels of miR-15a/16-1 identified a gene signature that contains the anti-apoptotic BCL2 family member MCL-1, which is associated with B-CLL cell survival and chemotherapy resistance [9-11]. Down-regulated miR-15a and miR-16-1 in CLL patients is associated with a good prognosis, consistent with previous reports that correlate 13q14.3 deletions with a favorable course of CLL [12]. On the other hand, the miR-29 family, which includes miR-29a, miR-29b and miR-29c, is significantly down-regulated in a subset of CLL patients and is associated with an unfavorable prognosis. miR-29b targets DNA methyltransferase (DNMT) isoforms and inhibition of miR-29b expression may lead to hypermethylation and epigenetic silencing of several tumor suppressors [12]. In addition, evidence shows that miR-29 targets the oncogene T-cell leukemia 1 gene, TCL1A. This TCL1 oncogene is involved in translocations and inversions characteristic of mature T-cell prolymphocytic leukemia (PLL) and is overexpressed in patients with unmuted
immunoglobulin heavy chain variable regions (IgVH). Transgenic mice that overexpressed TCL1 in B cells display a similar phenotype to aggressive forms of human CLL [12]. Another genomic region frequently deleted in CLL patients is found in the 11q region where a miR-34 cluster is located. In fact, down-regulation of miR-34a in CLL is associated with p53 inactivation, impaired DNA damage response, and apoptosis resistance [13-15]. Since miR-34a also inhibits E2F1 and B-Myb [16], loss of miR-34a expression may increase tumor cell proliferation. Alternatively, the miR-17/92 polycistronic microRNA cluster is overexpressed in several lymphoid malignancies and studies have shown that miR-17/92 inhibited the expression of the pro-apoptotic factor Bim and the tumor suppressor PTEN [17]. In addition, STAT3-induced IL-6 stimulates the expression of miR-17 and miR-19a, resulting in lower expression of TLR7 and TNFα. Also of interest, CLL patient cells expressing zeta-chain-associated protein 70 kDa (ZAP-70) demonstrate significantly lower levels of miR-150 when compared with ZAP-70-negative CLL cells. In CLL cells miR-150 targets forkhead box P1 (FOXP1) and GRB2-associated binding protein 1 (GAB1), thereby reducing B-cell receptor signaling [18].

Another STAT3-activated microRNA, miR-155[19], is overexpressed in cells and in circulating microvesicles in CLL samples [19]. Induction of the onco-miR-155 in the plasma of CLL patients correlates with poor response to treatment and disease progression. Consistently, patients who achieved complete remission presented low levels of miR-155 in the plasma [6]. In addition, the expression of miR-155 is increased with disease progression from monoclonal B-cell lymphocytosis (MBL) to CLL and is higher in MBL and CLL than normal controls [20]. High expression of miR-155 is associated with a more aggressive disease and a poor clinical prognosis in CLL [21, 22]. Another miRNA, miR-181b, is frequently down-regulated in CLL patients with disease progression [23, 24]. Important targets of miR-181b include MCL-1 and
BCL2 [23], which, as stated before, are important for cancer cell survival, and low expression of miR-181b is associated with poor prognosis as indicated by treatment-free survival [23, 24].

**miRNA expression and drug response in CLL patients**

Higher expression of miR-650 and miR-708 is associated with a favorable CLL prognosis [25] and affects B-cell proliferation [26]. On the other hand, overexpression of miR-21, miR-148a, miR-155 and miR-222 in CLL patients is associated with poor therapeutic response and prognosis [20-22, 27, 28]. For example, the expression of miR-155 is higher in CLL patients that failed to achieve a complete response to a chemo-immunotherapy combination of fludarabine [28, 29], cyclophosphamide, and rituximab (FCR) [20] and, as stated in the previous section, it is associated with poor clinical prognosis in CLL [21, 22]. The relapsed patients have higher miR-155 expression compared to baseline despite reduced expression at the beginning with response. Importantly, when the gene expression profile is analyzed, p53 down-stream genes are only detected in fludarabine-responsive patients, but not resistant patients [30]. The mutation of TP53 in CLL is associated with unfavorable treatment response and clinical outcome [31], and in some CLL patients inactivation of TP53 correlates with reduced miR-34 expression, which is associated with an unfavorable prognosis [32]. In addition, miR-132 and miR-212 expression is lower in progressive CLL patients compared with stable CLL patients [33]. Gene expression profiling showed that the miRNAs miR-132 and miR-212 affect the Rb or TP53 signaling pathway, which may explain the clinical observation [33].

The expression of miRNAs can also be used as a biomarker to monitor CLL progression. For example, there is reduced expression of miR-181b, miR-29c and miR-223 with disease
progression in CLL patients and this correlates with unfavorable prognosis, such as shorter progression-free survival and overall survival [27, 34-36]. miR-150 is highly expressed in both cellular and serum samples of CLL patients [37]. It is interesting to note, though, that low cellular but high serum expression of miR-150 is associated with poor prognosis as indicated by tumor burden, treatment-free survival (TFS) and overall survival (OS) [37]. This could be because lower expression of cellular miR-150 may enhance cell survival and proliferation in response to BCR signaling stimulation, which worsens the patient prognosis [37]. Furthermore, high serum miR-150 is correlated with high lymphocytosis, which contribute to high tumor burden and poor clinical outcome [37].

**Chronic myeloid leukemia (CML)**

Chronic myeloid leukemia (CML) is a hematopoietic malignancy characterized by abnormal expansion of immature hematopoietic progenitor cells in the bone marrow and increased levels of myeloid cells in the peripheral blood. The genetic hallmark of CML is a t(9;22)(q34;q11) reciprocal translocation, also called 'Philadelphia chromosome'. This translocation results in a BCR-ABL fusion gene that leads to constitutive tyrosine kinase activation [38].

**miRNA signature in CML**

The most frequently down-regulated miRNAs in chronic myeloid leukemia include miR-10a, miR17–92, miR-150, miR-203, and miR-318 [39]. Several oncogenic miRNAs such as the miR17–92 cluster have been found to facilitate cellular transformation and to be up-regulated by
the BCR-ABL fusion protein in CML [40]. Induction of this miRNA cluster is usually observed in the chronic phase of CML, but not in the blast crisis phase [41]. Similarly, loss of miR-328 is observed in blast crisis CML in a BCR/ABL dose- and kinase-dependent manner. Ectopic expression of miR-328 in cell lines restores differentiation of leukemic blasts by induction of the survival factor PIM1 and inhibition of the hnRNP E2 interaction with the hematopoietic transcription factor CEBPA. Down-regulation of miR-328 is essential in CML blast crisis [39]. The differentiation arrest observed during the blastic phase of CML requires the activity of hnRNP E2, a poly(rC)-binding protein, which behaves as a translational regulator. Finally, expression of miR-130a and miR-130b controlled by BCR-ABL down-regulates the expression of CCN3, a growth inhibitory protein [42], whereas some miRNAs inhibit BCR-ABL expression. For instance, miR-203 and miR-451 act as tumor suppressors in CML and suppress ABL and BCR-ABL protein expression [43]. Consistent with this notion, miR-203 is frequently silenced by monoallelic loss and hypermethylation of the remaining allele [44]. Another consistency found in CML patients is the reduction of miR-150 and miR-10a expression [45, 46]. CML patients display inverse expression levels of miR-150 and the transcriptional activator MYB, which correlates with BCR-ABL (fusion gene) transcript levels [47], while miR-10a is associated with increased proliferation by targeting the upstream stimulatory factor 2 (USF2) [45]. Finally, recently miR-362-5p was found to act as an oncomiR by down-regulating GADD45α, which in turn activated the JNK1/2 and P38 signaling in CML patient samples [48].
miRNA expression and drug response in CML patients

As discussed with CLL, the expression of miRNAs can be used as a biomarker to monitor CML progression as well. For example, the expression of miR-15a and miR-145 is higher in CML patients at chronic phase than in normal controls, but lower at acute phase than chronic phase [49]. The expression profile of some miRNAs can predict the Imatinib therapy response in CML patients[50]. For example, expression of miR-26a, miR-29c, miR-130b and miR-146a is lower in patients with an Imatinib response than in patients with Imatinib-resistant treatment [49]. A loss of or reduced expression of miR-23a, miR-30a, miR-30e, miR-203, miR-320 and miR424 have been associated with resistance to Imatinib in CML, and re-expression of these miRNAs targets BCR-ABL and restores sensitivity to Imatinib treatment [51-55]. In contrast, loss of miR-217 and miR-199b correlates with resistance to ABL tyrosine kinase inhibitors [56, 57]. It should be noted that the tyrosine kinase inhibitor (TKI) Dasatinib affected miR-let-7d, miR-let-7e, miR-15a, miR-16, miR-21, miR-130a and miR-142-3p expressions while Imitanib affected miR-15a and miR-130a levels [49]. Consistent with the notion that miR-130a can act as a tumor suppressor by targeting BCL2 and MCL-1 expression, lower expression of miR-130a is associated with poor prognosis as indicated by shorter overall survival and treatment-free survival in CML patients [58]. Importantly, a low expression of miR-148b is found in a subset of CML patients with stable complete molecular responses after stopping Imatinib treatment[59]. These studies suggest that expression or lack thereof of some miRNA may predict the ability of some CML patients to be safely removed from TKI treatments.
### Table 1. microRNAs deregulated and associated with clinical outcome in human Leukemia

The figure represents a summary of miRNAs associated with a poor or a favorable prognosis in CLL, CML, ALL, AML and ATL. Highlighted in red and green are the miRNAs that are found most frequently associated with an unfavorable or favorable outcome, respectively, across different human leukemias.
Acute lymphoblastic leukemia (ALL)

ALL is a lymphoid malignancy affecting the B or T lineages. Distinct microRNA signatures are reported in different ALL subtypes and can be used for the diagnosis and classification of ALL. ALL can be divided into T-cell, MLL-rearranged, TEL-AML1-positive, E2A-PBX1-positive, hyperdiploid ALL, BCR-ABL-positive, and “B-other” ALLs. Studies of the distinct microRNA signatures of ALL subtypes can be used for the diagnosis and classification of the disease [60].

miRNA signature in ALL

The B and T lineages of ALL can be distinguished by miR-148, miR-151, miR-424, miRNA-425-5p, miRNA-191, miRNA-146b, miRNA-128, miRNA-629, and miRNA-126. In addition, miRNA-708 was found highly expressed in TEL-AML1, BCR-ABL, E2A-PBX1, hyperdiploid, and B-other cases[60, 61]. The miRNA signature in hyperdiploid and TEL-AML1-positive patients partly overlap, suggesting a common underlying biology. Mavrakis et al. identified five miRNAs – miRNA-19b, miRNA-20a, miRNA-26a, miRNA-92, and miRNA-223 involved in T-ALL development in a mouse model. These five miRNAs have been shown to target T-ALL tumor suppressors such as IKAROS, PTEN, BIM, PHF6, NF1 and FBXW7 [62]. The expression pattern of these miRNAs can be used as a biomarker to distinguish the B and T lineages of ALL. Higher expression of miR-128b and lower expression of miR-223 has independently been reported for human ALL cell lines and ALL cells isolated from pediatric patients [63]. In a different study, single nucleotide polymorphisms (SNPs) analyses of precursor miRNAs (pre-miRNA) and miRNA-processing genes revealed eleven SNPs associated with ALL susceptibility [64]. Among them, eight are located at miRNA biogenesis pathway genes (TNRC6B, DROSHA,
DGCR8, EIF2C1, CNOT1, and CNOT6) and three at miRNA genes (miR-612, miR-499, and miR-449b). Interestingly, miRNA-612 and miRNA-499 have significant correlations with ALL susceptibility [60]. In addition, miRNA profiles can be useful to distinguish myeloid or lymphoid lineages of leukemia. De Leeuw et al. identified miRNA-23a, miRNA-27a, miRNA-199b, miRNA-221, and miRNA-223 as the most lineage-discriminative miRNAs between AML and ALL [65]. AML patients present down-regulation of let-7b and miRNA-223 and overexpression of miRNA-128a and -128b compared to ALL. Consistently, Wang et al. [66] identified miR-23a, miR-27a, miR-27b, miR-150, miR-199a, miR-199b, miR-221 and miR-340 as miRNAs differentially expressed in patients with ALL compared to AML.

**miRNA expression and drug response in ALL patients**

Epigenetic deregulation is one of the mechanisms that accelerates ALL progression. miR-124a is methylated in 59% of ALL patients and down-regulation of miR-124a increased the expression of its target CDK6 resulting in phosphorylation of retinoblastoma (Rb), which is involved in cell proliferation. As a result, hypermethylation of miR-124a in ALL patients is correlated with a higher relapse and mortality rate and can be used as an independent prognostic factor for disease-free survival (DFS) and overall survival (OS) in the multivariate analysis [67]. microRNA analysis shows that expression of miR-10a, miR-134, miR-214, miR-221, miR-128b, miR-484, miR-572, miR-580, miR-624 and miR-627 are significantly correlated with a favorable clinical outcome [63, 66, 68]. In contrast, deregulation of the expression of miR-9, miR-33, miR-92a, miR-142-3p, miR-146a, miR-181a/c, miR-210, miR-215, miR-369-5p, miR-335, miR-454, miR-496, miR-518d, and miR-599 are associated with an unfavorable long-term clinical outcome in
ALL patients [66, 68-74]. Low expression of miR-151-5p and miR-451, and high expression of miR-1290 or a combination of all three, predicted inferior relapse-free survival (RFS) in pediatric B-ALL [75]. Importantly, activation of NOTCH intracellular domain (NCID) signaling leads to transcriptional repression of miR-451 and miR-709, two tumor suppressor microRNAs in T-ALL [76]. Furthermore, different independent analyses identified relapse-related miRNAs. When globally analyzed the relapse-related miRNAs – miR-7, miR-100, miR-216 and let-7i – were up-regulated, and miR-486, miR-191, miR-150, miR-487 and miR-342 were down-regulated in early relapse ALL patients[77]. In addition, overexpression of miR-708, miR-223 and miR-27a is associated with lower relapse-free survival in patients [78], possibly through regulation of FOXO3, BMI1 and E2F1. Expression of miR-126, miR-345, miR-222, and miR-551a are reduced in ALL patients with central nervous system (CNS) relapse compared to non-CNS-relapsed ALL patients [77]. Furthermore, higher expression of miR-7, miR-198 and miR-633 was found in patients with CNS relapse compared with non-CNS-relapsed ALL [77].

Glucocorticoids can be used to treat ALL because they induce apoptosis in lymphoid lineage cells [79]. In ALL patients, sensitivity to prednisone treatment is an important indicator for treatment outcome [77]. While miR-16 is lower in ALL patients with low leukocytes and good cytogenetic characteristics [80], higher expression of miR-16 is found in patients with corticosteroid resistance [80] and is correlated with shorter disease-free survival and overall survival. The expression of miR-223 and the miR-15/16 family is increased in ALL patients treated with systemic glucocorticoid monotherapy [63, 79]. In contrast, the expression of miR-548d-1 and miR-106b–93 is reduced after ALL patients are treated with glucocorticoids [79]. Differential expression of miR-18a, miR-532, miR-218, miR-625, miR-193a, miR-638, miR-550 and miR-633 can be used as a marker to predict prednisone response in pediatric ALL.
patients[77]. For example, high miR-18a but low miR-193a expression is associated with good prednisone response. Although up-regulation of miR-128a [81, 82] and miR-128b [63] is frequently found in childhood ALL patients, patients with poor prednisolone response are often associated with lower miR-128b expression, with higher expression of miR-128b correlated with a longer disease-free period [63]. miR-128b expression is higher in the bone marrow of relapsed ALL patients compared with the values detected at diagnosis [63]. Increased miR-708 expression is detected in childhood ALL with a good response to prednisone and with better remission status after 15-day and 33-day chemotherapy protocol [78]. The expression of let-7e is generally reduced in pediatric ALL patients [81, 82], but higher expression of let-7e is associated with positive minimal residual disease (MRD) at day 14 after treatment [82].

Acute myeloid leukemia

Adult acute myeloid leukemia (AML) presents abnormal miRNA expression diversely expressed in the different subtypes. Both the t(8;21) and inv(16) chromosomal aberration is associated with the formation of novel chimeric fusion genes that involve the core-binding factor (CBF) complex, an important regulator of hematopoiesis, providing the designation CBF-AML[83].

miRNA signature in AML

A distinct miRNA signature characterized by an alteration of miR-29, miR-125, miR-142, miR-146 and miR-155 expression has been reported to play a role in AML progression and pathogenesis [84]. miR-29 family members miR-29a, miR-29b, and miR-29c act as oncogenes
and tumor suppressors in myeloid malignancies [85]. miR-29b targets DNA methyltransferase DNMT3A, DNMT3B, and Sp1 (a transcriptional regulator of DNMT1) [86]. Inhibition of miR-29b promotes DNA hyper-methylation in AML and contributes to methylation status in AML cells, suggesting its potential role as a therapeutic target in AML. In addition, miR-29a and miR-29b affect the expression of genes involved in apoptosis, cell cycle progression, and cellular proliferation. Consistently, altered expression of MCL-1 and CDK6 was reported in primary AML blasts following ectopic expression of miR-29b [85]. Interestingly, injection of precursor miR-29b oligonucleotides in mice engrafted with K562 cells reduce their tumor sizes [84]. The miR-125 family includes miR-125a/miR-99b/let-7e, miR-125b-2/miR-99a/let-7c-1, and miR-125b-1/miR-100/let-7a-2 located on human chromosomes 19, 21, and 11, respectively. The miR-125 family is involved in self-renewal, both in hematopoietic stem cells (HSC) and Megakaryocyte-Erythroid Progenitor Cells (MEC) [87]. Overexpression of miR-125 enhances the development of an MPN-like phenotype, which progresses to AML. Based on the cellular context, miR-125b can act as a tumor suppressor or an oncogene[88]. In acute myeloid leukemia, miR-125b is significantly overexpressed in patient blasts and can promote the transformation of normal hematopoietic cells into malignant cells in an in vitro and in vivo model. miR-125b is located on chromosome 21 and involved in the development of a rare subtype of AML, acute megakaryocytic leukemia (AMKL), especially in patients with Down’s syndrome (DS). The trisomy chromosome 21, typical of DS, is associated with overexpression of miR-125b in both DS- and non-DS-related AMKL patients [89]. Down-regulation of miR-146a promotes AML disease progression by TRAF6-mediated induction of NF-kB [90]. miR-142 promotes the development of lymphoid and myeloid leukemia and is found recurrently mutated in AML [91]. miR-155 is located on human chromosome 21 in the B-cell integration cluster (BIC) gene[92].
BIC cooperates with c-Myc to induce lymphomas[92]. In addition, miR-155 inhibits the cell-cycle regulators WEE1 and the mismatch repair genes hMLH1, hMSH2, and hMSH6, resulting in an increase in spontaneous mutation rates in hematopoietic stem and progenitor cells (HSPC) and solid tumor cell lines [84, 93, 94]. In contrast, other studies suggest that in FLT3-wildtype AML cells, miR-155 induces myelomonocytic differentiation and apoptosis[95].

**MicroRNAs in the diagnosis of AML**

Up-regulated let-7a-2-3p is associated with a favorable prognosis, longer overall survival and event-free survival in cytogenetically normal AML [96]. The effects of let-7a-2-3p are possibly through regulating miRNAs (miR-135a, miR-335 and miR-125b and all members of the miR-181 family) and genes (FOSB, IGJ, SNORD50A and ZNF502, and FOSB) that are favorable signatures in AML. The role of miR-181 in AML is controversial. High miR-181 expression is associated with a better clinical outcome in cytogenetically normal acute myeloid leukemia patients [97, 98] through reverse regulation of toll-like receptors and interleukin-1β. In addition, miR-181 contributes to a better clinical outcome in cytogenetically abnormal AML patients [99] by regulation of HOXA7, HOXA9, HOXA11, and PBX3. Drug resistance is the main reason for AML relapse and poor prognosis. miR-181b can increase AML drug sensitivity through down-regulation of HMGB1 and MCL-1. Therefore, miR-181b is found to be down-regulated in relapsed and refractory AML patients [100].
MicroRNA expression associated with favorable prognosis in AML

In analysis of the expression of the meningioma 1 (MN1) gene and MN1-associated microRNA in Chinese adult de novo acute myeloid leukemia (AML) patients, Xiang found that increased expression of MN1 is associated with reduced miR-20a expression and increased miR-181b expression. In further analyzing the clinical outcome, miR-20a up-regulation is associated with a higher complete remission rate and longer overall survival [101]. In contrast, high miR-181b expression is found in patients with a lower complete remission rate, shorter relapse-free survival and shorter overall survival [101].

Cytogenetic risk factors and molecular markers are important factors for AML prognosis [102]. Expression signatures of a minimum of two (miR-126/126*), three (miR-224, miR-368, and miR-382), and seven (miR-17-5p and miR-20a, along with the aforementioned five) miRNAs could correctly distinguish CBF, t(15;17), and MLL-rearrangement AMLs, suggesting that these microRNAs may cooperate with specific translocation in leukemogenesis [103]. In fact, KIT-mediated upregulation of miR-17, which targets RUNX1-3'UTR, mimic the effects of CBF-AML fusion protein [104]. The expression of miR-29a is lower in the bone marrow of pediatric AML patients compared with normal controls [105], and reduced miR-29a expression is associated with unfavorable karyotypes and shorter relapse-free and overall survival in pediatric AML patients [105]. Importantly, the association of miR-29a and prognosis is more apparent in intermediate-risk cytogenetic AML patients [105]. The same is true for miR-29b in that AML patients with low miR-29b expression have an unfavorable overall survival [106]. Analyses of 238 intermediate-risk cytogenetic AML patients, reduced expression of miR-135a and miR-409-3p is associated with a higher risk of relapse [102]. Higher miR-142 expression was associated with a better overall survival in AML patients with intermediate cytogenetic risk [107]. In AML
patients with complex karyotype, p53 status plays a role in determining miR-34a’s role in clinical prognosis. Up-regulation of miR-34a expression is correlated with unfavorable overall survival in TP53 (unaltered)-AML with complex karyotype, but is correlated with favorable overall survival and chemotherapy sensitivity in TP53 (biallelic altered)-AML with complex karyotype [108]. miR-96 is down-regulated in newly diagnosed AML patients and is associated with a higher white blood cell (WBC) count, bone marrow blast count, and lower hemoglobin and platelet count. Importantly, the expression of miR-96 increased after patients were treated with standard cytarabine plus daunorubicin induction chemotherapy [109]. When analyzing the relapse-free survival (RFS) and overall survival (OS), low expression of miR-96 is associated with shorter RFS and OS [109]. miR-204 expression is reduced in AML patients and low miR-204 expression is correlated with short patient survival [110]. After patients received induction chemotherapy (daunorubicin plus cytarabine), high expression of miR-204 is associated with complete remission [110]. In addition, increased expression of miR-212, miR-25 and/or miR-203 has been associated with a favorable overall survival, event-free and relapse-free survival in AML patients independent of cytogenetic subtypes [66, 111-113].

**miRNA expression associated with unfavorable prognosis in AML**

miR-378 is increased in 31% of AML patients and is associated with lower hemoglobin levels and shorter relapse-free survival [114]. There is a positive correlation between miR-155 expression and white blood cell (WBC) count, serum lactate dehydrogenase (LDH), C-reaction protein (CRP) value in peripheral blood (PB), and miR25 and miR-196b expression in AML [115]. miR-126 is highly expressed in hematopoietic stem cells and leukemic stem-like cells. In
AML patients high miR-126 expression is correlated with poor survival, higher chance of relapse and expression of stem cell related genes [116, 117]. In vitro, overexpression of miR-126-5p increased the phosphorylation of Akt and caused cytarabin resistance. Increased miR-124, miR-128–1, miR-194, miR-219–5p, miR-220a and miR-320 expression are associated with increased risk in AML [97]. The expression of miR-320d is increased in AML patients [118] and higher expression of miR-124-1 is associated with shorter overall survival and relapse-free survival [119]. AML patients with worse overall and event-free survival are known to have higher expression of miR-191 and miR-199a [120]. In de novo AML patients, miR-9-5p and miR-155-5p are independent unfavorable prognostic factors [112]. miR-155 is up-regulated in AML patients compared to normal controls [115, 118]. Consistent with this finding, high miR-155 expression is associated with an unfavorable prognosis, including lower complete remission rate and shorter disease-free survival and overall survival in AML [112, 115, 121]. The deregulation of miR-155 is associated with a gene expression profile enriched for genes involved in apoptosis, nuclear factor-kappaB activation, and inflammation [121]. Analyzing 53 AML patients, increased expression of miR-26a, miR-29b, miR-146a, and miR-196b is associated with an unfavorable overall survival [66]. The role of miR-196b was further supported by analyzing 238 intermediate-risk cytogenetic AML patients, whereby high miR-196b and miR-644 expression is associated with shorter overall survival [102]. In 40 non-M3 AML patients, high expression of miR-26a, miR-29b and miR-146a is associated with short overall survival [66]. It is worth noting that miR-146a expression is reversely correlated with prognosis in both ALL and AML patients [66]. The opposite role of miR-29b in AML prognosis has been reported. miR-29b expression is inversely associated with MLLT11 expression, which is a poor prognostic biomarker for AML patients. Low miR-29b and elevated MLLT11 expression are found in patients with poor overall
survival [106]. Whether the cooperation between miR-29b and MLLT11 caused poor prognosis needs to be further confirmed. Reduced miR-188-5p expression is associated with favorable prognosis as indicated by longer overall survival (OS) and event-free survival (EFS) in cytogenetically normal AML patients [96]. The effects of miR-188-5p are possibly through regulating miRNAs (miR-135a, miR-335 and miR-125b and all members of miR-181 family) and genes (FOSB, IGJ, SNORD50A and ZNF502, and FOSB) that are a favorable signature in AML. Up-regulated miR-3151 is found in AML patients with an unfavorable prognosis, such as short overall survival and leukemia-free survival, and higher relapse risk [122, 123]. High expression of miR-3151 is associated with low expression of genes involved in transcriptional regulation, posttranslational modification, and cancer pathways, such as FBXL20 and USP40 [123]. High miR-3151 expression is associated with high miR-501-5p and low miR-590, miR-135a, miR-100*, miR-186* and let-7a* expression [122]. The expression of let-7a-3 is increased in 25% of de novo AML patients and is associated with shorter overall survival and relapse-free survival [124] in AML patients with complete remission. Further studies are needed to confirm the opposite role of let-7a-3 and let-7a-2-3p.

**miRNA expression and drug response in AML patients**

Higher expression of miR-29b is found in older AML patients with single-agent decitabine response compared with non-response patients [125]. The ability of miR-29b to target DNA methyltransferases may explain the role of miR-26b in decitabine response. miR-29c expression is higher in AML patients compared with healthy controls and is associated with poor survival [126]. Reduced miR-29c expression is associated with complete remission after initial treatment
(intensive chemotherapy: daunorubicin plus cytarabine or low dose chemotherapy (low dose cytarabine or azacitidine)). Higher miR-29c expression was associated with relapse after patients achieved complete remission. Importantly, low miR-29c expression is associated with better response to azacitidine treatment and remission achievement in elderly AML patients who are not suitable for intensive chemotherapy [126]. The increased expression of miR-181a is associated with a higher complete remission rate, longer overall survival and disease-free survival [98, 99] in AML patients treated similarly with intensive induction chemotherapy and consolidation with autologous peripheral blood stem-cell transplantation on Cancer and Leukemia Group B (CALGB) protocols 9621 and 19808. In addition, after AML patients received double induction and one consolidation therapy, increased miR-181b expression was associated with worse complete remission rates, relapse-free survival and overall survival in adult patients with de novo AML [101]. In contrast, up-regulation of miR-20 is associated with better complete remission rates and overall survival [101]. The following drugs are included in the induction therapy and consolidation therapy: daunorubicin, cytarabine, idarubicin, and fludarabine [101]. miR-204 expression is reduced in AML patients and low miR-204 expression is correlated with short patient survival [110]. After patients received induction chemotherapy (daunorubicin plus cytarabine), high expression of miR-204 is associated with complete remission [110]. miR-331 is up-regulated in AML patients. AML patients with longer complete remission after induction chemotherapy have reduced miR-331 expression [127]. miR-335 is up-regulated in pediatric AML patients both in bone marrow and serum [128]. High serum miR-335 is associated with poor relapse-free and overall survival after patients received 10 days of induction chemotherapy [128]. However, a separate study reported no association between serum miR-335 expression and Ara-C-based chemotherapy response. However, high expression of miR-335 in the bone
marrow was indicative of poor Ara-C-based chemotherapy response, lower relapse-free survival and overall survival in AML patients [129]. High expression of the miR-10 family is associated with complete remission after AML patients received induction chemotherapy [130].

**Adult T-cell Leukemia (ATL): signature and prognosis**

Adult T-cell Leukemia is a fatal malignancy of mature CD4+, CD25+ T lymphocytes induced by the retrovirus Human T-cell leukemia virus (HTLV)-1[131, 132]. Several studies have reported deregulated microRNAs in ATL patient samples and HTLV-1-transformed cells, among them miR-155, miR-146a, miR-150, miR-223 were found up-regulated and miR-31 and miR124a down-regulated [133-136]. Interestingly, a recent study demonstrated that virus-encoded protein HBZ targets the expression of DICER thereby modulating the expression of a subset of microRNAs [137]. Deregulation of miR-146a, miR-155, miR-150 and miR-223 is reported to affect cellular proliferation [138-140] and alteration of miR-31, miR-130b and miR-93 are involved in apoptosis resistance [141], suggesting a possible role of miRNA expression in ATL progression and pathogenesis. Differential analyses of microRNA expression in non-infected healthy individuals, chronic ATL patients and acute ATL patients revealed an increased number of up-regulated miRNAs in acute ATL patients when compared with chronic ATL patients [142]. Among these, increased miR-155 expression correlates with disease progression from HTLV-1 carrier to chronic ATL and then to acute ATL [142]. Both STAT3 and Myb, which transcriptionally up-regulate miR-155, are activated in HTLV-1-transformed cells and ATL samples [136, 143, 144]. On the other hand, let-7g is highest in healthy donors and its expression is significantly reduced in an HTLV-1 carrier, chronic and acute ATL patient samples [142]. For
clinical outcomes, high miR-155 and low miR-126 is associated with a poor prognosis [142]. High miR-130b and low miR-145 and miR-223 expression in aggressive-type ATL are associated with shorter overall survival. Among miR-130b, miR-145 and miR-223, only miR-145 can act as an independent risk factor for ATL prognosis by a multivariate prognostic analysis. An in vitro study showed that overexpression of miR-145 in ATL cells reduced cell proliferation [145]. A recent study demonstrated that epigenetic silencing of miR-124-1 resulted in STAT3-mediated Pim1 kinase activation and increased tumorigenic potential [136].

<table>
<thead>
<tr>
<th>microRNAs</th>
<th>Predicted Target Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-148</td>
<td>PXR, DNMT1, CAND1, p27, HLA-C, WNT10B, PTEN, MK51, CDC26B, IGFR-IR, IRS1, CCBR, DNMT1, DNMT3, NR112, PPARA, RPS6KA</td>
</tr>
<tr>
<td>miR-155</td>
<td>FADD, IKKα, MyD88, Ripk, SHIP1, SOCS1, TAB2, AID, Bach1, Bcl-6, C/EBPδ, Ets1, HDAC4, Jarid2, PU1, Smad2, Smad5, CTLA-4, MEIS1, GF1, c-MYC, c-JUN, FOS, CTNNB1, TRIB2, WEE1, hMLH1, hMSH2, hMSH6</td>
</tr>
<tr>
<td>miR-130b</td>
<td>PTEN, CVLD, NR1P1, PPAR-γ, MMP2, Dicer1, CSF-1, CCN3, TP53INP1, RUNX3, c-IAP1, MCL1</td>
</tr>
<tr>
<td>miR-146a</td>
<td>IRAK1, NUMB, IL-8, FADD, EGFR, CXCR4, c-IAP1, MCL1, IRAK8, TRAF</td>
</tr>
<tr>
<td>miR-210</td>
<td>MNT, Casp8ap2, PTBP3/ROD1, E2F3, BNIP3, AIFM3, EFNA3, VMP1, RAD52, PTNP1, HOXA1, TP53I1</td>
</tr>
<tr>
<td>miR-335</td>
<td>RASA1, MT1-MMP, SIAH2, PAR6, Rb, p53, MAPK, TGF-β, Wnt, ERBB, mTOR, Toll-like receptor</td>
</tr>
<tr>
<td>miR-34</td>
<td>Notch, c-Myc, c-Met, Bcl-2, SIRT1, Survivin, AR, MDM4, Src, YY1, HDAC1, SNAIL, E2F1, Myb, TCL1, BCR-ABL</td>
</tr>
<tr>
<td>miR-150</td>
<td>MYB, FLT3, CBL, EGR2, AKT2, DKC1, Notch3, FOXP1, GAB1</td>
</tr>
<tr>
<td>miR-212</td>
<td>PXN, hRNPH1, SIRT1, SOX4, SMAD2, HBEGF, RBP2, MnSOD, PED, MeCP2</td>
</tr>
<tr>
<td>miR-223</td>
<td>NF-1A, IKKα, E2F1, STMN1, FBXW7, KRAS, EGFR, EGFR2, MMP9, SEPTING6, GLUT-4, IGFR-IR, RhoB, HSP90B1, IKAROS, PTEN, BIM, PHF6, NF1, FoxO3, BMI1, IGF2</td>
</tr>
<tr>
<td>miR-10</td>
<td>MIB1, BDNF, KLF4, FLT1, HOXD10, HoxB1α, HoxB3a, USF2</td>
</tr>
<tr>
<td>miR-203</td>
<td>NUAK1, Survivin, Bmi-1, LASP-1, Bcl-w, TBK1, IL-8, Slug, ADAM9, Yes-1, FGF2, SNAI2, Rap1A, BCR-ABL</td>
</tr>
</tbody>
</table>

Table 2. microRNAs deregulated in human leukemia and their predicted target genes

microRNAs most frequently deregulated in human leukemia (CLL, CML, ALL, AML and ATL) and their characterized target genes
Role of circulating RNA

The majority of miRNAs are cellular miRNAs, however an emerging class of circulating miRNAs has been described. Circulating miRNAs have been observed in various body fluids[146]. They are involved in proliferation and differentiation. Recent evidence shows elevated expression of the miR-29 family (miR-29a, miR-29b and miR-29c), miR-150 and miR-155 in CLL-derived exosomes compared to healthy donors [147]. The plasma expression of miR-29a and miR-150 is associated with absolute lymphocyte count in the blood [148]. The miR-29 family is significantly down-regulated in a subset of CLL patients and is associated with an unfavorable prognosis [12]. miR-150 is highly expressed in cellular and serum samples of CLL patients [37]. Interestingly, low cellular expression of miR-150 but high serum expression of the same is associated with poor prognosis as indicated by tumor burden, treatment-free survival (TFS) and overall survival (OS) [37]. The expression of miR-155 is increased with disease progression from monoclonal B-cell lymphocytosis (MBL) to CLL and is higher in MBL and CLL than normal controls [20]. In addition, high plasma miR-155 expression is associated with CLL patients poorly responding to fludarabine, cyclophosphamide, and rituximab (FCR) chemotherapy [20]. Therefore, high expression of miR-155 is associated with more aggressive disease and poorer clinical prognosis in CLL [21, 22].

Consistent with the finding in CLL patients, the expression of miR-150 and miR-155 is higher in AML-derived exosomes and can act as a biomarker to distinguish AML patients from normal controls [130]. There is a positive correlation between miR-155 expression and white blood cell count, serum lactate dehydrogenase (LDH) and C-reaction protein (CRP) value in peripheral blood in AML patients [115]. High miR-155 expression is associated with an unfavorable prognosis, such as lower complete remission rate and shorter disease-free survival and overall
survival in AML patients [112, 115, 121]. However, the role of miR-150 in AML is controversial. The expression of miR-150 has been reported to be lower in AML-derived plasma compared to healthy donors [149]. Up-regulation of miR-150 after treatment is associated with AML patients with complete remission [149]. In addition, circulating miR-155-5p and miR-181b-5p are up-regulated in AML patients when compared with normal controls [118]. Up-regulated circulating miR-181b-5p is associated with shorter overall survival [118] and is found in patients with a lower complete remission rate, shorter relapse-free survival and shorter overall survival [101].

Other circulating miRNAs can also act as biomarkers for AML prognosis. For instance, miR-210 is up-regulated in the bone marrow and serum of AML patients compared with normal controls. Reduced serum miR-210 expression is found in patients with complete remission, while high miR-210 expression is correlated with poor relapse-free survival and overall survival in AML patients [150]. Similarly, the expression of miR-375 is higher in the serum and bone marrow of pediatric AML patients and is associated with unfavorable karyotypes and poor prognosis as indicated by shorter relapse-free survival and overall survival [151]. Like miR-29a [105], the association of miR-375 and prognosis is more apparent in intermediate-risk cytogenetic AML patients [151]. Plasma miR-511, miR-222, and miR-34a are up-regulated in B-ALL patients compared with normal controls, whereas plasma miR-199a-3p, miR-223, miR-221, and miR-26a are lower in B-ALL patients [152]. Moreover, the low expression of miRNA-100 and miRNA-146a is associated with poor clinical outcome in ALL patients [63, 66, 68, 126]. Together these studies clearly demonstrate that detection of circulating miRNAs has significant value for detection of disease progression and can also serve as indicators of therapeutic response.
Clinical significance of other non-coding RNAs: lncRNA and snoRNAs

In addition to microRNA, other non-coding RNAs have been reported to play a role in human leukemias. Long non-coding RNAs (lncRNAs) are RNA molecules longer than 200 nucleotides with undefined open reading frames involved in gene expression regulation. A small subset of lncRNAs have been reported in leukemia and an lncRNA expression profile correlated with treatment response and survival in AML patients [153]. The X-inactive specific transcript Xist lncRNA, involved in epigenetic regulation of transcriptionally inactive chromatin, is overexpressed in some leukemias [154]. NOTCH-regulated lncRNA LUNAR1 (leukemia-induced non-coding activator RNA) has been shown to have oncogenic effects in T-ALL [155]. LUNAR1 has been demonstrated to increase IGF1R mRNA expression and IGF1 signaling [155]. Another NOTCH-related lncRNA, RP11-611D20.2 (NOTCH-associated lncRNA in T-ALL (NALT)), has been found to be overexpressed in pediatric ALL and may play a role in the leukemia stem cell compartment [156]. In CML patients with BCR-ABL translocation, deregulation of two lncRNAs has been described: the Beta Globin Locus 3 (BGL3) lncRNA [157] and the imprinted H19 lncRNA [158]. Little is known about the function of these lncRNAs in CML. BGL3 lncRNA has been shown to increase the expression levels of the tumor suppressor PTEN by acting as a competing endogenous RNA (ceRNA) [159]. In contrast, lncRNA H19, which is transcriptionally activated by the oncogene c-Myc [158], has been shown to inhibit the expression of the onco-suppressor let-7 microRNA family [160]. In AML patients, lncRNA IRAIN [161], which is transcribed from the IGF1R imprinted locus, is down-regulated in patients with high-risk AML, while urothelial carcinoma-associated 1 (UCA1) lncRNA is specifically up-regulated in AML [162], although its role in the pathogenesis is unclear. Finally, the lncRNA B-ALL-associated long RNAs-2 (BARL-2) was found to affect B-ALL patient...
response to corticosteroid treatment [163]. Another class of non-coding ncRNAs, the small nucleolar snoRNAs, is also affected in cancers and leukemia. Elevated levels of SNORD112–114 snoRNAs has been found in acute promyelocytic leukemia (APL) [164]. In a different study, Affymetrix GeneArray screening identified snoRNA SNORA70F as significantly down-regulated in poor prognostic subgroups of CLL patients. In addition, high expression of SNORA74A and SNORD116-18, and low expression of SNORD56 were associated with shorter progression-free survival (PFS) in these patients [165]. Although lncRNA and snoRNA are not as greatly studied as miRNA, they are likely to play an increasing role in the future and eventually become a part of patients’ genetic signatures for individualized targeted medicine.
References


with disease progression and low miR-34a expression in previously treated chronic lymphocytic leukemia patients. Blood 2013, 121(18):3650-3657.


65. de Leeuw DC, van den Ancker W Fau - Denkers F, Denkers F Fau - de Menezes RX, de Menezes Rx Fau - Westers TM, Westers Tm Fau - Ossenkoppele GJ, Ossenkoppele Gj Fau - van de Loosdrecht AA, van de Loosdrecht Aa Fau - Smit L, Smit L: MicroRNA profiling can classify acute leukemias of ambiguous lineage as either acute myeloid leukemia or acute lymphoid leukemia. (1078-0432 (Print)).


134. Pichler K, Schneider G, Grassmann R: MicroRNA miR-146a and further oncogenesis-related cellular microRNAs are dysregulated in HTLV-1-transformed T lymphocytes. Retrovirology 2008, 5(1742-4690 (Electronic)).


152. Luna-Aguirre CM, de la Luz Martinez-Fierro M, Mar-Aguilar F, Garza-Veloz I, Trevino-Alvarado V, Rojas-Martinez A, Jaime-Perez JC, Malagon-Santiago GI, Gutierrez-Aguirre CH,


Chapter IV: "STAT1: A Novel Target of miR-150 and miR-223 Is Involved in the Proliferation of HTLV-I-Transformed and ATL Cells."

Abstract

We have previously reported on the deregulation of cellular microRNAs involved in hematopoiesis and inflammation in human T-cell lymphotropic virus type 1 (HTLV-I)–transformed cells. In this study, we demonstrate that miR-150 and miR-223 specifically target the signal transducer and activator of transcription 1 (STAT1) 3’ untranslated region, reducing STAT1 expression and dampening STAT1-dependent signaling in human T cells. The effects of miR-150 and miR-223 on endogenous STAT1 were confirmed using inducible cell lines. Our studies also showed that miR-150 expression is upregulated by interleukin-2 signaling in adult T cell leukemia/lymphoma (ATL) cells. HTLV-I–transformed and ATL-derived cells have reduced levels of miR150 and miR223 expression, which coincide with increased STAT1 expression and STAT1-dependent signaling. Knockdown of STAT1 by short hairpin RNA demonstrated that the constitutive activation of STAT1 is required for the continuous proliferation of HTLV-I–transformed cells. Our studies further demonstrate that increased expression of STAT1 in ATL cells is associated with higher levels of major histocompatibility complex class I expression. Previous studies have demonstrated that the pressure exerted by natural killer (NK) cells in vivo can edit leukemic tumor cells by forcing an increased expression of major histocompatibility complex class I to escape immune clearance. STAT1-expressing tumor cells produce more aggressive tumors because they cannot be eliminated by NK cells. Our results suggest that therapeutic approaches using combined targeting of STAT1 and MHC class I may be an effective approach to activate NK cell–mediated clearance of ATL tumor cells.
Introduction

MicroRNAs (miRNAs) are involved in a wide range of biologic processes, including cellular survival, differentiation, immune response, and oncogenesis. miRNAs are short non-coding RNAs that target genes through imperfect base pairing with mRNAs, thereby affecting their stability and/or their translation. An individual miRNA has numerous cellular gene targets and the manner in which to accomplish a coordinated regulation of biologic processes is unclear. The role of miR-150 in human cancer is context-dependent as this miRNA can have either oncogenic or tumor suppressor activity in cells that originate from different tissues. This is best illustrated by upregulated expression of miR-150 in CD19 + B cells from chronic lymphocytic leukemia (CLL) [1,2] but downregulated expression in chronic myeloid leukemia [3,4], acute lymphoblastic leukemia [5], and mantle cell lymphoma [6]. Additional studies have further demonstrated that miR-150 stimulates the proliferation and migration of lung cancer cells by targeting SRC kinase signaling inhibitor 1 (SRCIN1) and SRC activity [7]. In contrast, in situ hybridization revealed that miR-150 expression levels are reduced in both estrogen receptors positive and triple-negative breast cancer samples compared to adjacent normal cells, and miR-150 expression was shown to inhibit breast cancer cell migration and invasion [8,9]. Some of the known validated cellular gene targets of miR-150 include c-MYB, NOTCH3, CBL, EGR2, AKT2, and DKC1 [10–14]. Similar to miR-150, miR-223 is also differentially regulated. Studies showed that it is frequently repressed in hepatocellular carcinoma [15], B-CLL [16], acute myeloid leukemia (AML) [17], gastric mucosa-associated lymphoid tissue lymphoma [18], and recurrent ovarian cancer [19]. In contrast, miR-223 is upregulated in T cell acute lymphocytic leukemia (T-ALL) [20], EBV-positive diffuse large B-cell lymphoma [21], and metastatic gastric
cancer [22,23]. Among validated cellular gene targets of miR-223 are FBXW7/Cdc4, RhoB, stathmin 1, E2F transcription factor 1 (E2F1), signal transducer and activator of transcription 3 (STAT3), CCAAT/enhancer binding protein beta, forkhead box O1, and nuclear factor I/A [22–27].

Human T-cell lymphotropic virus type 1 (HTLV-I) is a human retrovirus present in 20 million people worldwide [28]. Infection with HTLV-I is the etiological agent of adult T cell leukemia/lymphoma (ATL) [29] and a neurodegenerative disease called tropical spastic paraparesis/HTLV-I–associated myelopathy [30,31]. Only a few studies have investigated miRNA expression in HTLV-I–mediated T cell transformation and pathogenesis [32–37]. HTLV-I–associated disease pathogenesis is still poorly understood [38–40]. Both diseases originate from deregulated proliferation of infected CD4/CD25 + T cells. While it is unclear how the virus induces cellular transformation, the viral oncoprotein Tax plays an essential role and is sufficient to immortalize human primary T cells [41]. Tax expression leads to accumulation of DNA double-strand breaks during cellular replication and simultaneously targets DNA repair pathways to increase genetic instability [42,43]. In addition, Tax targets many tumor suppressors, cell cycle regulators, and survival factors and affects chromosome stability and segregation [44–48]. The molecular events linked to the switch from immortalization [interleukin-2 (IL-2)–dependent growth] to transformation (IL-2–independent growth) are largely unknown. A common characteristic found in HTLV-I–transformed cells in vivo and in vitro is the constitutive activation of the Janus activated kinase (JAK)/STAT signaling [49,50]. In fact, pharmacological targeting of the JAK/STAT axis has shown that activation of this pathway is required for continuous proliferation and survival of HTLV-I–transformed cells [51–53]. STAT1 plays a role in immune modulatory functions, anti-viral responses, apoptosis, and anti-proliferative
responses [54]. In contrast, several studies have shown that STAT1 can also act as a potent tumor promoter for leukemia development [55] and that many T-ALL leukemic cells are dependent on the TYK2-STAT1-BCL2 pathway for continued survival [56]. However, a potential role played by STAT1 in HTLV-I pathogenesis has not yet been investigated.

In this study, we demonstrate for the first time that miRNAs miR-150 and miR-223 directly target the STAT1 3’ untranslated region (UTR) gene. Expression of both miR-150 and miR-223 was significantly reduced in HTLV-I–transformed cell lines and ATL-derived cells, while STAT1 protein expression was elevated. Restoring miR-150 and miR-223 expression inhibited endogenous STAT1 expression and the activation of STAT1 transcription–dependent genes and significantly reduced the proliferation of HTLV-I–transformed cells and ATL cells. A direct role of STAT1 on cell cycle and expansion of HTLV-I cells was further confirmed by using short hairpin RNA (shRNA) targeting STAT1. We also found that IL-2–dependent ATL cell lines express higher levels of miR-150, suggesting that IL-2 signaling may be involved in the transcriptional up-regulation of miR-150 expression. Finally, we found that a majority of freshly isolated uncultured ATL samples have high expression of STAT1, which correlated with higher major histocompatibility complex class I (MHC-I) expression and may help to conceal ATL cells against immune clearance by natural killer (NK) cells.
Experimental Procedure

miRNA Target Predictions

STAT1 sequences were obtained from the National Center for Biotechnology Information Nucleotide database (http://www.ncbi.nlm.nih.gov/). Sequences of mature human miRNAs were obtained from the miRNA Registry at miRBase (http://www.mirbase.org). The prediction algorithms Targetscan and PicTar were used to search for miRNA target sites in the human STAT1 3′UTR.

Cell Culture and Patient Samples

A 293T cell line was cultured in complete Dulbecco's modified Eagle's medium with 10% FBS, penicillin, and streptomycin. The HTLV-I cell lines, MT-4, MT-2, C8166, C91PL, and human T cell lymphoblast cell line, Jurkat, were maintained in RPMI-1640 (Invitrogen, Carlsbad, CA) supplemented with 10% FBS, penicillin, and streptomycin. The ATL-like cell lines, MT-1, ATL-T, ED-40515(−), ALT-25, ATL-43T, LMY1, and ATL-55T, were maintained in RPMI-1640 supplemented with 10% FBS, penicillin, and streptomycin and IL-2 (50 U/ml) as needed [57–59]. Patient samples were obtained after informed consent was provided and in agreement with regulations for the protection of human subjects according to the National Institutes of Health guidelines. RNA was extracted from peripheral blood mononuclear cells (PBMCs) of patients with acute ATL and healthy donors (HD). RNA was extracted from PBMCs of patients with ATL.
Plasmids

Precursors of miR-150 and miR-223 were cloned in pTRIPZ. pTRIPZ lentivirus is an inducible vector engineered to be Tet-On. pTRIPZ contains puromycin resistance that was used to select a stable cell line. The stable cell lines derived were incubated in the absence or presence of doxycycline and subjected to reverse transcription–polymerase chain reaction (RT-PCR) to analyze miR-150 and miR-223 expression. Precursors of miR-150 and miR-223 were also cloned in the pSIH1-green fluorescent protein (GFP) lentivirus and pcDNA vector. shRNA against STAT1 was cloned in the pSIH1-GFP vector.

3’UTR and Luciferase Reporter Assay

The full-length 3’UTR of the human STAT1 gene was cloned into the luciferase vector, pGL3 (Promega). STAT1 3’UTR containing the mutated miR-150 target sequence and the mutated miR-223 target sequence were generated by QuikChange Site-Directed Mutagenesis kit (Stratagene). miR-150 and miR-223 were cloned into the pcDNA expression vector. 293T cells were cotransfected using Polyfect Transfection reagent (Qiagen), with 300 ng of the indicated luciferase reporter plasmid, 200 ng of pRL-TK–Renilla luciferase plasmid (Promega, for normalization), and the indicated miRNAs (125, 250, 500, or 750 ng). Cell extracts were prepared 48 hours after transfection. Cell extracts were lysed in 1 × passive lysis buffer (Promega) and analyzed using the Luciferase Reporter Assay System (Promega), according to the manufacturer’s instructions. Luciferase assays were performed twice from independent experiments, and data were normalized for transfection efficiency using Renilla luciferase activity. 293T cells were cotransfected with 300 ng of the STAT1 luciferase reporter plasmid,
200 ng of pRL-TK–Renilla luciferase, and the indicated miRNAs. Cell extracts were prepared 48 hours after transfection according to the manufacturer’s instructions.

293T cells were stimulated with 500 pg/ml human interferon-γ (IFN-γ; PBL Assay Science, Piscataway, NJ) or 100 U/ml human IFN-β (ProSpec, East Brunswick, NJ) and cotransfected with 300 ng of pISRE-TA-Luc Vector, 200 ng of pRL-TK–Renilla luciferase plasmid, and 500 ng of the indicated miRNAs by using Polyfect. Cell extracts were prepared 48 hours after transfection and luciferase assays were performed as described above.

Transfection, Viruses, and Generation of Stable miR-150 and miR-223 Inducible Cell Lines

293T cells were infected with lentiviral particles expressing miR-150 and miR-223. Lentiviruses were prepared by transfection of 293T cells with 10 μg of pSIH1-miR-150, pSIH1-miR-223, pTRIPz/miR-150, or pTRIPz/miR-223, 5 μg of vesicular stomatitis virus G proteins, and 5 μg of pDNL6 using calcium phosphate (Invitrogen). The virus was collected in the supernatant for 4 days, concentrated by ultracentrifugation, and resuspended in phosphate-buffered saline. For inducible miR-150 and miR-223 expression assays, MT-4 or Jurkat cells were infected with lentiviruses encoding vector control (pTRIPz), miR-150 (pTRIPz/miR-150), or miR-223 (pTRIPz/miR-223). Cells were selected with 10 μg/ml puromycin for 12 days. Stable cell lines derived were incubated in the absence or presence of doxycycline and subjected to RT-PCR to analyze miR-150 and miR-223 expression.
RNA Extraction and Real-Time Quantitative PCR

RNA was extracted using TRIzol (Invitrogen) and treated with DNaseI (Roche Applied Science, Indianapolis, IN). RNA was reverse-transcribed using the RNA-to-cDNA synthesis kit (Applied Biosystems, Carlsbad, CA). Quantitative real-time PCR was performed using Real-Time SYBR Green PCR Master Mix (SABiosciences, Valencia, CA) on the StepOnePlus Real-Time PCR System (Applied Biosystems), with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression as an internal control. The miScript II RT Kit was used for mature miRNA quantification. cDNA generated with the miScript II RT Kit was used as a template for real-time PCR to detect the expressive level of mature miR-150 and miR-223 in HTLV-I and ATL cell lines. Real-time PCR was performed in duplicate, and samples were normalized to RNU6b (U6) expression.

Western Blot Analysis

Cells were washed with phosphate-buffered saline and lysed in RIPA buffer with phosphatase and protease inhibitors (Complete Cocktail; Roche). Protein concentration was determined with the Bio-Rad protein assay (Bio-Rad, Hercules, CA). Total protein extracts were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to Immobilon polyvinylidene difluoride membranes (Millipore, Darmstadt, Germany), and probed with STAT1 (Cell Signaling Technology, Danvers, MA) and Bcl2, p21, p27, or actin (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies. Western blot quantification was performed by using the Java-based image processing program ImageJ (http://imagej.nih.gov/ij/).
<table>
<thead>
<tr>
<th>miRNAs</th>
<th>Total sites</th>
<th>8mer</th>
<th>7mer-m8</th>
<th>7mer-1A</th>
<th>Aggregate PCT</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-223</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0.27</td>
</tr>
<tr>
<td>miR-150</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0.14</td>
</tr>
<tr>
<td>miR-144</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>miR-130ac</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>miR-155</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>miR-140</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>miR-203</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>miR-27abc</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>miR-200bc</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>miR-101</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>miR-146ac</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>miR-132</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>miR-26ab</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>&lt; 0.1</td>
</tr>
</tbody>
</table>

Table 1. Predicted miRNA Target Sites of Human STAT1
Results

miRNAs miR-150 and miR-223 Target the STAT1 3'UTR

The prediction algorithms Targetscan and PicTar were used to identify common gene targets to miRNAs downregulated in HTLV-I–transformed cells. Among potential targets, STAT1 appeared as a possible target and was selected for further analyses (Table 1). We identified a 7-mer-1A binding site for miR-150 in position 426-432nt of the STAT1 3'UTR (Figure 1A). To demonstrate a direct effect of miR-150 on STAT1 mRNA, the full-length (1667 bp) STAT1 3'UTR was cloned into a pGL-3 reporter luciferase construct and miR-150 was cloned into the pcDNA expression vector. First, we verified the transfection of the mature miR-150 by using RT-PCR (Figure 1B). Expression of the STAT1 3'UTR vector along with a different amount of miR-150 demonstrated a 50% inhibition of luciferase activity (Figure 1C). To further demonstrate specificity, we performed site-directed mutagenesis to modify the seed sequence and generate a mutated STAT1 3'UTR vector (Figure 1A). As expected, miR-150 had no significant effect on the mutated STAT1 3'UTR construct (Figure 1C). These results demonstrated a specific and direct effect of miR-150 on the STAT1 3'UTR. We next wanted to validate these results on the endogenous STAT1 gene. To this end, we expressed in 293T cells the pSIH1-miR-150 or pSIH1-GFP control vector. Our results confirmed the effect of miR-150 on the endogenous STAT1 gene at both the RNA and the protein levels (Figure 1, D and E). The down-regulation was not as efficient as seen with the 3'UTR luciferase experiment because in the latter experiment the transfection efficiency limits the percentage of cells in which an effect can be observed. We then investigated the effect of miR-223. The STAT1 3'UTR sequence displays two miR-223 target sites positioned at 970-977nt and 1194-1200nt (Figure 2A). We verified the transfection of the mature miR-223 by using RT-PCR (Figure 2B). To investigate if one site was
more important than the other, we mutagenized each site independently (mutant-UTR 970 and mutant-UTR 1194; Figure 2A). These mutants were tested along with wild-type STAT1 3'UTR in transient assays. Results indicated that mutation of a single site had no effect and miR-223 was still able to repress activity from the STAT1 3'UTR (Figure 2C). However, when both sites were simultaneously mutated, miR-223 was no longer able to suppress the activity of the STAT1 3'UTR, suggesting that the effect of miR-223 is specific and requires only one functional binding site (Figure 2D). We next validated these results on the endogenous STAT1 gene. We expressed in 293T cells the pSIH1-miR-223 or pSIH1-GFP control vector and analyzed by RT-PCR and Western blot. Results confirmed the effect of miR-223 on the endogenous STAT1 gene, which was observed at the protein level (Figure 2E). RT-PCR analysis shows no significant change in STAT1 expression (Figure 2E). That result suggested that miR-223 regulated STAT1 expression at a post-transcriptional level.
FIG. 1A

426-432 STAT1 3' UTR 5'-AACGGUAAAUUUCUGUGGGAGAA
miR-150 3' - GUGACCAUGUUCCCA-ACCCUCU
Mutated STAT1 UTR 5'-AACGGUAaAUUUCUGcTAGAGAA
miR-150 3' - GUGACCAUGUUCCCA-ACCCUCU

FIG. 1B

< .05

miR-150 relative expression

0 100 200 300 400
pcDNA miR-150

FIG. 1C

Wild-type UTR + + + - - -
Mutated UTR + - - - + + +

Relative fold change

FIG. 1D

< .05

STAT1 relative expression

0 20 40 60 80 100
pcDNA miR-150

FIG. 1E

pSH1-GFP pSH1-miR-150

STAT1

Actin
Figure 1. STAT1 is a direct target of miRNA miR-150

(A) A diagram representing the sequence alignment of miR-150 and its target site within STAT1 3'UTR and the relative mutated version. The mutations made in the STAT1 3'UTR are highlighted. (B) Real-time PCR was performed on mature miR-150 from cDNA derived from 293T cells transfected with pcDNA or miR-150. Real-time PCR was performed in duplicate, and samples were normalized to RNU6b (U6) expression. (C) 293T cells were cotransfected with wild-type (or mutated) STAT1 3'UTR luciferase reporter plasmids and pTK–Renilla luciferase plasmids, together with a control plasmid (pcDNA) or a different amount of miR-150 (0.125, 0.25, 0.75 μg). After 48 hours, firefly luciferase activity was measured and normalized by Renilla luciferase activity. The data are representative from two independent experiments. (D) Real-time PCR of STAT1 expression was performed from total cDNA from 293T cells transfected with pcDNA or miR-150 (2 μg). Extracts were analyzed 48 hours after transfection and normalized to GAPDH expression. P value was < .05. (E) Western blot analysis of STAT1 expression from total cellular extracts of 293T cells infected with lentivirus expressing miR-150 or empty lentivirus used as a control. Extracts were normalized to actin expression. Semiquantitative analysis of Western blot data demonstrated 24.9% inhibition of STAT1 expression by miR-150. Western blot quantification was performed by using the Java-based image processing program ImageJ.
FIG. 2A

970-977 STAT1 UTR 5’-AGUAGAAAAAGCAGUAACUGACAG
miR-223 3’-ACCCCAUAACUGUUUGACUGU

1194-1200 STAT1 UTR 5’-UUUUCUUCCUAUAUCACUGACAC
miR-223 3’-ACCACCAAUCUGUUUGACUGU

Mutated 970 STAT1 UTR 5’-AGUAGAAAAAGCAGUUGCUGA
miR-223 3’-ACCCCAUAACUGUUUGACUGU

Mutated 1194 STAT1 UTR 5’-UUUUCUUCCUAUACCCAGACAC
miR-223 3’-ACCACCAAUCUGUUUGACUGU

FIG. 2B

<.05

miR-223 relative expression

pcDNA miR-223

FIG. 2C

<.05  <.05  <.05

Relative fold Change

miR-223

Wild-type-UTR + + - - - -
Mutant-UTR 970 - - + + - -
Mutant-UTR 1194 - - - + + +

FIG. 2D

<.05

Relative fold Change

miR-223

Wild-type-UTR + + - -
Mutant-UTR 970+1194 - - + +

FIG. 2E

Relative STAT1 expression

pcDNA miR-223

pSH1-GFP pSH1-miR-223

STAT1
Actin
miR-150 and miR-223 Decrease STAT1 Protein Expression and STAT1 Signaling

STAT1 is a transcription factor involved in inflammation and cancer. STAT1 is a central mediator of the IFN responses involved in antiviral and immune defense [60]. Since miR-150 and miR-223 target STAT1, we hypothesized that these miRNAs may hamper the IFN-mediated
response by dampening STAT1 activity. To test this hypothesis, we first used the ISRE reporter construct plasmid containing four tandem repeats of the IFN-inducible response element. As expected, IFN-γ and IFN-β treatment resulted in the induction of ISRE-luciferase reporter gene activity. Expression of either miR-150 or miR-223 resulted in significant inhibition, which was efficiently rescued by co-expression of a STAT1 cDNA expression vector lacking the 3'UTR sequence (Figure 3, A and B). IFN-independent, STAT1-dependent signaling was also inhibited by miR-150 and miR-223 as demonstrated by a reduction in STAT1-luciferase reporter construct activity when transfected along with miR-150 or miR-223, which was efficiently rescued by co-expression of a STAT1 cDNA expression vector lacking the 3'UTR sequence (Figure 3C). An additive effect of miR-150 and miR-223 was demonstrated in transient transfection inhibition of the STAT1-3'UTR construct (Figure 3D). Finally, Western blot analysis demonstrated that STAT1 expression is reduced more efficiently by the co-transfection of miR-150 and miR-223 (Figure 3E).
Figure 3. miR-150 and miR-223 hamper the IFN-mediated response by inhibition of STAT1 activity

(A) 293T cells stimulated with 500 pg/ml human IFN-γ and co-transfected with the pISRE firefly luciferase reporter plasmid and pTK–Renilla luciferase plasmid, together with a control plasmid (pcDNA), miR-150, miR-223, and/or STAT1 cDNA expressing vector lacking the 3’UTR sequence. After 48 hours, firefly luciferase activity was measured and normalized by Renilla luciferase activity. The data are representative from two independent experiments. (B) 293T cells stimulated with 100U/ml human IFN-β and co-transfected with the pISRE firefly luciferase reporter plasmid and pTK–Renilla luciferase plasmid, together with a control plasmid (pcDNA), miR-150, miR-223, and/or STAT1 cDNA expressing vector lacking the 3’UTR sequence. After 48 hours, firefly luciferase activity was measured and normalized by Renilla luciferase activity. The data are representative from two independent experiments. (C) 293T cells were cotransfected with STAT1-luciferase reporter plasmid and pTK–Renilla luciferase plasmid, together with a control plasmid (pcDNA), miR-150, miR-223, or STAT1 cDNA expressing vector. (D) 293T cells were cotransfected with wild-type STAT1 3’UTR firefly luciferase reporter plasmid and pTK–Renilla luciferase plasmids, together with a control plasmid (pcDNA), miR-150, and/or miR-223. After 48 hours, firefly luciferase activity was measured and normalized by Renilla luciferase activity. (E) 293T cells transfected with pcDNA, mir-150, and/or mir-223 (1 μg) and 50 ng of pSIH1-puromycin. Forty-eight hours after transfection, the cells were treated with puromycin (1 μg/ml) for 7 days. Western blot analyses of STAT1 expression from total cellular extracts were performed. Extracts were normalized to actin expression. Semiquantitative analysis of Western blot data shows 9.7% and 9% inhibition of STAT1 expression by mir-150 and mir-223, respectively, compared to the control. Co-
miR-150 and miR-223 Expression Is Inversely Correlated With STAT1 Expression in HTLV-I–Transformed and ATL Cell Lines

We next investigated the expression of miR-150 and miR-223 in relation to STAT1 in HTLV-I–transformed cell lines (MT-4, MT-2, and C8166). Jurkat cells were used as a negative control since Jurkat cells are human T cells not transformed with HTLV-I. In HTLV-I–transformed cell lines, the expression of both miR-150 and miR-223 was significantly reduced when compared to Jurkat cells (Figure 4A). In contrast, the expression of STAT1 was increased at both the RNA and the protein levels (Figure 4, B and C). We then investigated if such a correlation could also be found in ATL-derived cell lines (MT-1, ATL-T, ED-40515(−), ATL-25). Results from these studies confirmed that both miRNAs, miR-150 and miR-223, were significantly downregulated when compared to Jurkat cells (Figure 4D). Consistent with the above data, STAT1 RNA and protein expression was also increased in ATL cells (Figure 4, E and F). These results suggest an inverse correlation between miR-150 or miR-223 and STAT1 expression in HTLV-I–transformed and ATL-derived cells in vitro. Surprisingly, we found that IL-2–dependent ATL-derived cells (ATL-43T and ATL-55T) displayed elevated levels of miR-150 but not miR-223 (Figure 4G), suggesting that the former may be regulated through the IL-2 signaling pathway. To further investigate this possibility, we withdrew the IL-2 from the culture media of ATL–IL-2–dependent cells and analyzed the expression of miR-150 after 48 hours. Our results suggest that the absence of IL-2 signaling in ATL cells may be associated with decreased expression of miR-
150 (Figure 4J). To validate the results, we cultured ATL–IL-2–independent cell lines (MT-1, ATL-T) in the presence of IL-2 and analyzed the expression of miR-150 after 48 hours. Our results show that IL-2 signaling is associated with a significant increase in miR-150 (Figure 4J). Next, we analyzed STAT1 expression. The absence of IL-2 signaling in ATL–IL-2–dependent cells is not correlated with an increase in STAT1 expression (data not shown). Interestingly, IL-2 stimulation in MT-1 and ATL-T cells resulted in STAT1 down-regulation (Figure 4K). These results may be significant because several studies have reported that in vivo ATL tumor cells can produce IL-2 or IL-15 and express the high affinity IL-2 receptor α chain, CD25. We think that these observations may, in part, explain the higher levels of miR-150 found in freshly isolated uncultured ATL cells compared to in vitro cell lines. Despite higher expression of miR-150 in IL-2–dependent cell lines, STAT1 RNA and protein were expressed at higher levels compared to Jurkat cells (Figure 4, H and I).
Figure 4. miR-150 and miR-223 expression correlates with STAT1 expression in HTLV-I and ATL cell lines.

(A, D) Real-time PCR was performed on mature miR-150 and miR-223 from cDNA derived from HTLV-I–transformed cells (MT-4, MT-2, and C8166) and ATL-derived IL-2–independent cell lines (MT-1, ATL-T, ED-40515(−), and ATL-25). The non–HTLV-I Jurkat T cell line was used as a control. Real-time PCR was performed in duplicate, and samples were normalized to RNU6b (U6) expression. Fold change was calculated by comparing values with Jurkat normalized miRNA expression. (B, E) Real-time PCR was performed on STAT1 from cDNA derived from HTLV-I cell lines and ATL cell lines. Real-time PCR was performed in duplicate,
and samples were normalized to GAPDH expression. Fold change was calculated by comparing
values with Jurkat-normalized STAT1 expression. P values were < .05. (C, F) Western blot
analysis of STAT1 expression was performed from total cellular extracts of Jurkat, HTLV-I, and
ATL cell lines. Extracts were normalized to actin expression. Semiquantitative analysis of
Western blot data shows 165%, 158%, and 131% increase in STAT1 expression in MT-4, MT-2,
and C8166, respectively, and 178%, 195%, 166%, and 148% activation of STAT1 in MT-1,
ATL-T, ED-40515(−), and ATL-25, respectively, compared to Jurkat. Western blot
quantification was performed by using the Java-based image processing program ImageJ. (G)
Real-time PCR was performed on mature miR-150 and miR-223 from cDNA derived from ATL-
derived, IL-2–dependent cell lines (LMY1, 55-T, 43T). The non–HTLV-I Jurkat T cell line was
used as a control. Real-time PCR was performed in duplicate, and samples were normalized to
RNU6b (U6) expression. (H) Real-time PCR was performed on STAT1 from cDNA derived
from ATL-derived, IL-2–dependent cell lines (LMY1, 55-T, 43T). Real-time PCR was
performed in duplicate, and data were normalized to GAPDH expression. (I) Western blot
analysis of STAT1 expression was performed from total cellular extracts of Jurkat and ATL–IL-
2–dependent cell lines. Extracts were normalized to actin expression. Semiquantitative analysis
of Western blot data shows 225%, 188%, and 162% increase in STAT1 expression in LMY1,
ATL-55T, and ATL-43T, respectively, compared to Jurkat. Western blot quantification was
performed by using the Java-based image processing program ImageJ. (J) Real-time PCR was
performed on mature miR-150 from ATL-derived, IL-2–dependent cell lines (LMY1, 55-T, 43T)
cultured with and without IL-2 for 48 hours. Fold change was calculated by comparing values
with LMY1, 55-T, and 43T cell lines cultured with IL-2. (J) Real-time PCR was performed on
mature miR-150 from ATL-derived, IL-2–independent cell lines (MT-1, ATL-T) cultured with
and without IL-2 for 48 hours. Fold change was calculated by comparing values with MT-1, ATL-T cell lines cultured without IL-2. (K) Western blot analysis of STAT1 expression was performed from total cellular extracts of MT-1, ATL-T cell lines cultured with and without IL-2 for 48 hours. Extracts were normalized to actin expression. Semiquantitative analysis of Western blot data shows 50.8% and 67.7% inhibition of STAT1 expression in MT-1 and ATL-T cell lines, respectively, cultured with IL-2.

**Enforced Expression of miR-150 and miR-223 in Human T Cells Decreases STAT1 Expression and STAT1-Dependent Transcription**

To further demonstrate the effect of miR-150 and miR-223 expression on STAT1, we constructed miR-150 and miR-223 Tet-inducible expression vectors. These vectors were used to generate stable cell lines expressing pTRIPZ (control line), pTRIPZ-miR-150, or pTRIPZ-miR-223 in HTLV-I–transformed MT-4 cells. Treatment with doxycycline for 3 days efficiently restored expression of miR-150 or miR-223 in MT-4 cells (Figure 5A). In Jurkat T cells, induction of miR-223 was more limited. We believe that this is the result of saturation because Jurkat cells naturally express high levels of endogenous miR-223 (Figure 6A). Induction of miR-150 and miR-223 expression was associated with down-regulation of endogenous STAT1 expression (Figures 5B and 56B). Notably, we also confirmed that expression of several STAT1-dependent genes, MAP/microtubule affinity-regulating kinase 3 (MARK3), inositol monophosphatase domain containing 1 (IMPAD1), and SET binding protein 1 (SETBP1) [61], in the absence of IFN stimulation, was suppressed after induction of miR-150 and miR-223 (Figures 5, C–E, and 6, C–E). Similar results were obtained in both MT-4 and Jurkat cells. The
effects were generally more pronounced in Jurkat and we believe that this may reflect the fact that these genes are also transcriptionally regulated by nuclear factor of kappa light polypeptide gene enhancer in B-cells, which is constitutively activated in MT-4 cells. SETBP1 was not studied after miR-223 overexpression because the 3'UTR for SETBP1 contains a putative miR-223 target site (data not shown). Overall, these results demonstrate that under physiological conditions miR-150 and miR-223 can reduce STAT1 expression and STAT1-dependent signaling in human leukemic T cell lines.
Figure 5. miR-150 and miR-223 inhibit STAT1 in an HTLV-I line

(A) MT-4 miR-150 and MT-4 miR-223 stable cell lines were induced with doxycycline (2 μg/ml) for 72 or 96 hours. Real-time PCR was performed on mature miR-150 and miR-223 to verify miRNA induction. Real-time PCR was performed in duplicate, and samples were normalized to U6 expression. (B) Western blot analysis of STAT1 expression from total cellular extracts of stable cell lines MT-4 miR-150 and MT-4 miR-223 treated with and without doxycycline. MT-4 pTRIPZ cells treated with and without doxycycline were used as a control. Extracts were normalized to actin expression. Semiquantitative analysis of Western blot data shows 73.1% and 71.5% inhibition of STAT1 expression in stable cell lines MT-4 miR-150 and MT-4 miR-223, respectively, treated with doxycycline compared to cells treated without doxycycline. Western blot quantification was performed by using the Java-based image processing program ImageJ. (C–E) Real-time PCR was performed on STAT1 target genes SETBP1, MARK3, and IMPAD1 from cDNA derived from MT-4 miR-150 and MT-4 miR-223. Real-time PCR was performed in duplicate, and samples were normalized to GAPDH expression.
Figure 6. miR-150 and miR-223 inhibit STAT1 in the non–HTLV-I cell line, Jurkat

(A) Jurkat miR-150 and Jurkat miR-223 stable cell lines were induced by doxycycline (2 μg/ml) for 72 hours. Real-time PCR was performed on mature miR-150 and miR-223 to verify miRNA induction. Real-time PCR was performed in duplicate, and samples were normalized to U6 expression. (B) Western blot analysis of STAT1 expression from total cellular extracts of Jurkat miR-150 and Jurkat miR-223 stable cell lines treated with and without doxycycline. Jurkat cell lines treated with and without doxycycline were used as a control. Extracts were normalized to actin expression. Semiquantitative analysis of Western blot data shows 78.1% and 79.4%
inhibition of STAT1 expression in stable cell lines Jurkat miR-150 and Jurkat miR-223, respectively, treated with doxycycline compared to cells treated without doxycycline. Western blot quantification was performed by using the Java-based image processing program ImageJ.

(C–E) Real-time PCR was performed on STAT1 target genes SETBP1, MARK3, and IMPAD1 from Jurkat miR-150 and Jurkat miR-223 cDNA treated with and without doxycycline. Real-time PCR was performed in duplicate, and samples were normalized to GAPDH expression.

**STAT1 Is Required for the Proliferation of HTLV-I–Transformed and ATL Cells**

STAT1 expression has been frequently associated with the increased expression of genes with anti-proliferative properties, such as p21WAF or p27KIP [62]. We find no correlation between STAT1, p21WAF, or p27KIP expression in HTLV-I and ATL cell lines (Figure 9E). However, some reports also suggest that increased expression of STAT1 can contribute to rather than inhibit transformation and proliferation of breast cancer, head and neck cancer, melanoma, lymphoma, and leukemia cells [63]. Hence, the role of STAT1 in different cancer types may depend on other genetic alterations and other signaling pathways activated or not in these cells. To evaluate the biologic significance of STAT1 in HTLV-I–transformed cells, we first used MT-4 and MT-4 miR-150 and miR-223 inducible cell lines. Induction of either miR-150 or miR-223 expression was associated with reduced cell proliferation as determined by cell count and 2,3-bis-(2-methoxy-4-nitro- 5-sulfophenyl)-2H-tetrazolium- 5-carboxanilide (XTT) assays (Figure 7, A and B). In T-ALL, STAT1 has been shown to activate Bcl2 and promote proliferation and survival of transformed cells [64]. Consistent with these reports, we found that
STAT1 was involved in Bcl2 expression in MT-4 and Jurkat inducible cell lines (Figure 7D). Treatment with doxycycline for 3 days significantly increased Bcl2 expression compared to untreated cells (Figure 7C). For this reason, we analyzed Bcl2 expression in treated MT-4 miR-150, MT-4 miR-223, Jurkat-miR-150, and Jurkat-miR-223 cells compared to the treated MT-4 and Jurkat control. As expected, the induction significantly decreased Bcl2 expression in MT-4 and Jurkat cell lines (Figure 7D). To verify the specificity of these results, we included Western blot analysis of untreated MT-4 and Jurkat inducible cell lines. The results show no change or an increased level of Bcl2 expression compared to the control (Figure 7D). Because many T-ALL leukemic cells are dependent on the TYK2-STAT1-BCL2 pathway for continued survival, we analyzed the sequence of Tyk2 in several ATL samples. However, no mutation was found in the kinase domain of Tyk2 in ATL samples, but a high frequency of Tyk2 V362F single nucleotide polymorphism was found. The possible significance of this mutation remains to be established.
Figure 7. Exogenous expression of miR-150 and miR-223 inhibits proliferation of HTLV-I cells

(A, B) MT-4 pTRIPZ, miR-150, and miR-223 inducible cell lines were induced with doxycycline (2 μg/ml). After 72 hours, cell count and XTT assays were performed; P value was < .05. MT-4 pTRIPZ cells induced with doxycycline were used as a control. (C) Western blot analysis of Bcl2 expression from total cellular extracts of Jurkat cell lines treated with and without doxycycline. Semiquantitative analysis of Western blot data shows a 229% increase in Bcl2 expression. Western blot quantification was performed by using the Java-based image processing program ImageJ. (D) Western blot analysis of Bcl2 expression from total cellular
extracts of MT-4 and Jurkat inducible cell lines treated with and without doxycycline. Extracts were normalized to actin expression. Semiquantitative analysis of Western blot data shows 45.8% and 67.7% inhibition of Bcl2 expression in stable cell lines MT-4 miR-150 and MT-4 miR-223, respectively, treated with doxycycline compared to MT-4 pTRIPZ cell lines and 50% and 62% inhibition in Jurkat miR-150 and Jurkat miR-223, respectively, compared to Jurkat. Western blot quantification was performed by using the Java-based image processing program ImageJ.

We are aware that miR-150 and miR-223 likely exert some of their effect through STAT1-independent pathways. To demonstrate a direct function of STAT1 in HTLV-I–transformed cells, we next generated a lentiviral vector expressing shRNA to knock down STAT1 expression. Functionality was first verified following infection of 293T cells (Figure 8, A and B). shRNA STAT1 efficiently reduced STAT1 and Bcl2 expression (Figure 8B). We confirmed a reduction in STAT1 expression in the HTLV-I–transformed MT-2 transduced cells by more than 50% (Figure 8C), which was associated with a disruption in cell cycle progression, a 24.3% reduction in the number of cells in S phase and an accumulation of cells in G2/M (Figure 8D). To confirm that these observations were not specific to MT-2 and could be generalized to other HTLV-I–transformed cells, we transduced MT-2, C91PL, and MT-4 HTLV-I–transformed cells and an MT-1 ATL cell line. Transduction of STAT1 shRNA was first confirmed by using RT-PCR (Figure 9A). STAT1 down-regulation was associated with reduced cell proliferation as determined by cell count, XTT assays, and bromodeoxyuridine incorporation (Figure 9, B–D). Together our results demonstrate that STAT1 is required for the proliferation of HTLV-I–transformed and ATL cells.
Figure 8. Inhibition of STAT1 expression in the HTLV-I cell, MT-2, results in cell cycle disruption

(A) Real-time PCR analysis of STAT1 expression was performed from total cDNA from 293T cells infected with pSIH1-GFP and pSIH1-GFP-shSTAT1. Extracts were analyzed 48 hours after transfection and normalized to GAPDH expression. P value was < .05. (B) Western blot analysis of Bcl2 and STAT1 expression from total cellular extracts of 293T cells infected with lentiviruses expressing shSTAT1 or empty lentivirus (pSIH1-GFP) used as a control. Extracts
were normalized to actin expression. Semiquantitative analysis of Western blot data shows 70.9% of Bcl2 expression and 70.4% inhibition of STAT1 expression in 293T cells infected with shSTAT1, compared to the control. Western blot quantification was performed by using the Java-based image processing program ImageJ. (C) Real-time PCR analysis of STAT1 expression was performed from total cDNA from MT-2 cells transfected with pSIH1-GFP or pSIH1-GFP-shSTAT1. Extracts were analyzed 48 hours after transfection and normalized to GAPDH expression. P value was < .05. (D) Cell-cycle analysis of MT-2 cells infected with lentiviruses encoding vector control (pSIH1-GFP) and shSTAT1. Cell-cycle analysis demonstrated a 24.3% reduction in the number of cells in S phase compared to GFP control.

**Increased STAT1 and MHC-I Expression in ATL Cells**

Since our results demonstrate an important role of STAT1 in proliferation of HTLV-I–transformed cells, we then wanted to investigate expression of STAT1 in freshly isolated uncultured ATL samples. We next analyzed RNA samples from 35 patients with acute ATL and 9 HDs by RT-PCR. Our results showed that STAT1 was significantly elevated in patients with ATL compared with HD (almost three-fold higher; Figure 10A). Early studies suggest that ATL cells originate within the helper-inducer T cell subtype and have a common cytokine production profile including IL-1α, IL-1β, tumour necrosis factor alpha, IFN-γ, and granulocyte-macrophage colony-stimulating-factor [65]. Among these factors, IFN-γ is a well-known potent inducer of MHC class I through activation of the JAK/STAT1/interferon regulatory factor 1 signal transduction pathway [66]. Tumor cells with low or no MHC-I cell surface expression may escape recognition by anti-tumor T cells but are highly sensitive to NK-mediated killing. In fact,
increased expression of MHC-I is one important mechanism that allows for immune escape [67–70]. We then investigated the expression of MHC-I in ATL and HD samples. Overall, ATL samples have significantly higher expression of MHC-I when compared to HD samples (Figure 10B).
Figure 9. Inhibition of STAT1 expression results in a decrease in cellular proliferation in HTLV-I cells

(A) Real-time PCR analysis of STAT1 expression was performed from total cDNA from MT-2, C91PL, MT-1, and MT-4 cells infected with pSIH1-GFP or pSIH1-GFP-shSTAT1. Extracts were analyzed 48 hours after infection and normalized to GAPDH expression. P values were < .05. (B) MT-2, C91PL, MT-1, and MT-4 cells were infected with lentivirus encoding vector control (pSIH1-GFP) or shSTAT1. After 48 hours, cells were counted for proliferation (B) and XTT proliferation (C) assays, and bromodeoxyuridine assays were performed (D). P values were < .05 (B–D). (E) Western blot analysis of p21, p27, and STAT1 expression from total cellular extracts of LMY1, MT-2, C91PL, MT-1, and MT-4. Extracts were normalized to actin expression.
Figure 10. STAT1 expression correlates with increased MHC-I expression in ATL patient samples

(A) Real-time PCR analysis of STAT1 expression from cDNA derived from PBMCs of patients with ATL. Samples were normalized to GAPDH expression, and the fold change was calculated by comparing values to HD's normalized gene expression. (B) Real-time PCR analysis of MHC-I expression from cDNA derived from PBMCs of patients with ATL. Samples were normalized to GAPDH expression, and the fold change was calculated by comparing values to HD's
normalized gene expression. Statistical analysis was performed by using unpaired two-sided Student's t test.

**Discussion**

In this study, we demonstrate for the first time that miR-150 and miR-223 target the STAT1 3'UTR, reduce STAT1 expression, and reduce both IFN-dependent and IFN-independent STAT1-mediated signaling. We have previously reported that miR-150 and miR-223 are differentially regulated in HTLV-I cells transformed in vitro and in vivo ATL samples, suggesting that ATL cells and in vitro established HTLV-I cell lines may derive from distinct cellular populations. In this study, we have shown that IL-2R–mediated signaling can increase miR-150 expression in ATL cells. Previous studies have demonstrated that ATL cells overexpress an IL-2Rα chain (CD25). Additional studies have shown that ATL cells may produce or respond to IL-2 or other IL-2Rγ chain user cytokines in the microenvironment [71–75]. In contrast, in vitro transformed cells do express CD25 but do not produce IL-2. With regard to miR-223, it has been previously shown that E2F1 represses the miR-223 promoter [26,76]. Interestingly, viral HTLV-I bZIP factor mRNA increases the expression and transcriptional activity of E2F1 [77]. In contrast to other viral genes, HTLV-I bZIP factor expression is consistently increased in ATL cells in vivo [77]. These observations can, in part, explain the differential regulation of miR-223 expression between in vitro and in vivo HTLV-I–transformed cells. Others have shown that overexpression of miR-150 in bone marrow progenitor cells resulted in modest inhibition of cell proliferation [3], suggesting low side effects. Therefore, reinstating miR-150 expression may provide an effective therapeutic strategy to treat HTLV-I
leukemia. Our studies also revealed that STAT1 expression is inversely correlated to that of miR-150 and miR-223 in HTLV-I–transformed and ATL cells and is generally increased in HTLV-I–transformed cells. Several studies have shown that an increase in STAT1 expression is associated with radio-protection of cancer cells. Since HTLV-I–transformed cells are notoriously resistant to high dose radiotherapy, it is possible that increased STAT1 expression plays a role in the resistance to high dose radiotherapy and warrants additional studies. STAT1 has been associated with both tumor suppressor and tumor promoting activities [78,79]. Our studies suggest that in HTLV-I–infected cells STAT1 has a tumor promoting effect since inhibition of STAT1 expression and STAT1-dependent transcription through miR-150 or miR-223 or directly by shRNA targeting resulted in reduced proliferation of HTLV-I–transformed and ATL cells. In addition, STAT1 may play an important role in the immune-suppressive status observed in patients with ATL and allow HTLV-I–transformed cells to evade NK-mediated killing. Along these lines, knockout mice studies showed that the IFN-α receptor 1 and STAT1 are both required for normal tumor Treg frequencies and the release of IL-10, a potent inhibitor of NK activity, in the tumor microenvironment. Indeed, high levels of IL-10 in the serum of patients with acute ATL have been reported. Furthermore, increased STAT1 as observed in patients with ATL may be involved in higher levels of MHC-I expression. Although MHC-I is not a direct target for STAT1, previous studies have demonstrated that STAT1 plays an essential role in chromatin decondensation of the MHC locus through interactions with the remodeling brahma protein-like 1 factor [80]. As indicated above, increased expression of MHC-I on tumor cells has been associated with escape from NK-mediated killing of tumor cells and more aggressive tumor phenotypes [81,82]. Our results showed that patients with ATL with a higher expression of STAT1 displayed higher levels of MHC-I as well. Since STAT1 plays an important role in
regulation of inducible nitric oxide synthase activity, it is possible that it participates in upregulated expression of inducible nitric oxide synthase in ATL cells.

There are also some unforeseen observations in our study. First, suppressor of cytokine signaling 1, a terminator of STAT1-dependent signaling, has been reported to be activated in HTLV-I–transformed cells through a mechanism involving the Tax oncoprotein [83]. However, it should be remembered here that in vivo about half of ATL samples have no detectable Tax expression [84], and therefore, the ability of suppressor of cytokine signaling 1 to block STAT1 may vary greatly among patients and is currently under investigation. In addition, several kinases, including cyclin-dependent kinase 8, can phosphorylate STAT1 at serine 727 to restrain NK cell cytotoxicity by reducing expression of perforin and granzyme B [85]. Targeted inhibition of cyclin-dependent kinase 8-mediated STAT1 phosphorylation at serine 727 may represent a possible approach to stimulate NK cell–mediated tumor surveillance in patients with ATL.

Despite expression of both miR-150 and miR-223 in freshly isolated ATL samples, STAT1 did not seem to be affected, as both STAT1 RNA and protein were increased in a majority of ATL samples. These data suggest that in ATL patient cells miR-150 and miR-223 may not be able to efficiently target the STAT1 3'UTR. Although the reason for this deficiency is unclear, it is possible that other higher affinity gene targets expressed at higher levels act as competitors and sequester miR-150 and miR-223 or that the sequence or the spatial structure/folding of the STAT1 3'UTR is altered in ATL cells in vivo and warrants additional future studies.

Targeted inhibition of STAT1 has been investigated in various types of leukemia. For instance, treatment of acute myeloid cells (AML) with retinoic acid resulted in an increased STAT1
expression and reduced proliferation [80]. In addition, STAT1 has been shown to play a key role in dasatinib-induced differentiation of acute myeloid cells (AML) and in Bryostatin-1–induced differentiation of CLL cells [61,86]. The results presented here suggest that STAT1 may represent a target for therapeutic intervention in some patients with ATL.
References


35. Tomita M, Tanaka Y, Mori N: MicroRNA miR-146a is induced by HTLV-1 tax and increases the growth of HTLV-1-infected T-cells. Int J Cancer 2012, 130(10):2300-2309.


60. Rauch I, Muller M, Decker T: The regulation of inflammation by interferons and their STATs. JAKSTAT 2013, 2(1):e23820.


82. Ko NL, Taylor JM, Bellon M, Bai XT, Shevtsov SP, Dundr M, Nicot C: PA28gamma is a novel corepressor of HTLV-1 replication and controls viral latency. Blood 2013, 121(5):791-800.


Chapter V: "Small PARP inhibitor PJ-34 induces cell cycle arrest and apoptosis of adult T-cell leukemia cells”

Abstract

HTLV-I is associated with the development of an aggressive form of lymphocytic leukemia known as adult T-cell leukemia/lymphoma (ATLL). A major obstacle for effective treatment of ATLL resides in the genetic diversity of tumor cells and their ability to acquire resistance to chemotherapy regimens. As a result, most patients relapse and current therapeutic approaches still have limited long-term survival benefits. Hence, the development of novel approaches is greatly needed.

In this study, we found that a small molecule inhibitor of poly (ADP-ribose) polymerase (PARP), PJ-34, is very effective in activating S/G2M cell cycle checkpoints, resulting in permanent cell cycle arrest and reactivation of p53 transcription functions and caspase-3-dependent apoptosis of HTLV-I-transformed and patient-derived ATLL tumor cells. We also found that HTLV-I-transformed MT-2 cells are resistant to PJ-34 therapy associated with reduced cleaved caspase-3 activation and increased expression of RelA/p65.

Since PJ-34 has been tested in clinical trials for the treatment of solid tumors, our results suggest that some ATLL patients may be good candidates to benefit from PJ-34 therapy.

Introduction

Human T-cell leukemia virus type I (HTLV-I) is etiologically linked to the development of an aggressive type of peripheral T-cell leukemia known as ATLL [1]. The clinical course varies among infected patients and the disease has been classified into four distinct entities: smoldering, chronic, acute, or lymphoma [2]. Although many features of HTLV-I biology have been
discovered [3], the treatment of the disease remains unsatisfactory, with minimal improvements in the overall survival of patients [4]. Overall, the current therapies used for the treatment of ATLL patients in the acute phase have limited impact and the overall projected 4-year survival rate of acute ATLL is around 5% [5]. The mechanism by which HTLV-I causes ATLL is still not fully understood, but a latency period of several decades before the onset of the disease suggests that long-term survival and expansion of virus-infected cells are required. Along these lines, we have previously shown that reactivation of telomerase activity is one of the essential steps in the transformation process of HTLV-I-infected cells [6]. HTLV-I transformed CD4/CD25+ T cells in vivo and in vitro. In early stages, infected cells may rely on an autocrine/paracrine IL-2/IL-2R or IL-15/IL-15R cytokine loop for active proliferation [7]. During that stage, HTLV-I-infected cells accumulate genetic and epigenetic mutations and are prone to genomic instability. At the basis of this phenomenon is the viral oncoprotein Tax, which has been shown to inactivate tumor suppressors such as p16ink, p53, RB, and p21WAF [8], affect genome stability [9], and activate oncogenic signaling pathways such as NF-κB, Notch, and JAK/STAT [10–12]. In addition, Tax also induces DNA breaks during cellular replication and inhibits DNA repair pathways, leading to accumulation of genetic alterations [13, 14]. Eventually, an infected IL-2-independent transformed cell emerges with a selective growth advantage resulting in clonal expansion. The molecular basis for IL-2 independence is still unknown although a majority of HTLV-I-transformed cells simultaneously acquire constitutive JAK/STAT activation. The transition from IL-2 dependent to IL-2 independent is believed to mimic the disease progression from smoldering or chronic to the acute type of ATLL. Recently, we showed that Tax can induce genomic DNA double-strand breaks (DDSB) by targeting the fork of replication during cell division [13].
homologous recombination repair (HR) pathway [14], we hypothesized that HTLV-I-transformed and ATLL cells might be particularly sensitive to small drug inhibitors targeting DNA replication. Although poly (ADP-ribose) polymerase (PARP) is a single-strand break sensing protein, PARP inhibitors (PARPi) have been shown to be selectively effective in cells with an HR-defective pathway [15]. Numerous PARPi (PJ-34, MK4827, ABT-888, AZD2281, and BSI-201) are in clinical trials for breast cancer, ovarian cancer, and prostate cancer [16, 17]. The PARPi PJ-34 has been shown to cause cell cycle arrest in various human cancers, including myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML) [18, 19].

In this study, we investigated the efficacy of the PARPi PJ-34 in targeting HTLV-I-transformed cells and a panel of patient-derived ATLL cell lines. Our results demonstrate that PJ-34 used as a single agent is a potent inhibitor of cellular growth in IL-2-dependent as well as IL-2-independent transformed ATLL cells. We also found that another PARPi (olaparib/AZD2281) is also effective against HTLV-I-transformed cells. We further show that cells treated with PJ-34 reactivated p53 functions and accumulated in G2/M. Tumor cells died from apoptosis as shown by annexin V staining but this process appears to be largely p53-independent since ATLL-derived cells not expressing p53 (MT-1 and ED) were still efficiently killed by PJ-34. We found that HTLV-I-transformed MT-2 and C91PL cell lines were resistant to PJ-34 treatment. We found that PJ-34-resistant cells expressed higher basal levels of Bax and were unable to engage the cleavage of pro-caspase-3. In addition, resistance of MT-2 cells was independent from p53BP1 and PARP1 but coincides with activation of NF-κB.
Materials and methods

Cell lines and reagents

HTLV-I-transformed cell lines (MT-4, MT-2, C8166, C91PL) and ATL-like cell lines, IL-2 independent (MT-1, ATL-T, ED-40515(−), ALT-25), were maintained in RPMI-1640 media supplemented with 10 % FBS, penicillin, and streptomycin. ATL-like cell lines, IL-2 dependent (ATL-43T, KOB, and ATL-55T), were maintained in RPMI-1640 supplemented with 10 % FBS, penicillin, and streptomycin and IL-2 (50 U/mL). PARP-1 inhibitor, PJ-34 (N-(5, 6-Dihydro-6-oxo-2-phenanthridinyl)-2-(dimethylamino) acetamide, ab120981), was purchased from Abcam. Stock solutions (25 mM) were made with dimethyl sulfoxide (DMSO). In all experiments, cells were treated with different concentrations of PJ-34 (5, 10, 25, and 50 μM) or DMSO for 3 or 5 days, as indicated. Olaparib was used at 25 μM for 3 days. Cell Proliferation Kit II (XTT) was purchased from Roche Life Science.

Cell cycle and cell proliferation

HTLV-I-transformed and ATL cells were treated with DMSO or PJ-34 for 3 days. The cells were collected and washed with 1× PBS and fixed with 75 % EtOH overnight at −20 °C. The next day, cells were washed with ice-cold 1× PBS, followed by treatment with RNase for 15 min at 37 °C, stained with 100 μg/mL propidium iodine (PI) for 15 min at room temperature, and analyzed by flow cytometry. Cell proliferation was measured by the XTT assay. For the XTT assay, 100 μL (10,000) cells were seeded in 96-well plates and treated with different concentrations of PJ-34 (5, 10, 25, and 50 μM) or DMSO for 5 days. A 50-μL of XTT labeling mixture was added to each well and incubated for 6 h. The absorbance was measured at 450 and
620 nm by spectrophotometry. Results were plotted as mean ± SD from at least two independent experiments.

**Cell tracking assay**

CFDA-SE (Molecular Probes, Eugene, OR) was used to label cells according to the manufacturer’s protocol. After labeling, 1 × 10^5 cells were analyzed by FACS assay for 0 h control. The rest of the labeled cells were divided into two groups: one treated with DMSO, the other one with PJ-34. Every 24 h, 1 × 10^5 cells were taken out for FACS assay until 72 h.

**Apoptosis assays and mitochondrial membrane potential**

The cells were collected and washed with 1× PBS then stained with annexin V/propidium iodide using the Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit (Molecular Probes, Eugene, OR) according to the manufacturer’s instructions. MT-4, C91PL, and MT-2 cells were treated with DMSO or PJ-34. Then, the cell mitochondrial membrane potential (ΔΨm) was measured using the JC-1 Assay Kit (Invitrogen) according to the manufacturer’s instructions.

**Western blotting**

Total cell extracts were prepared with RIPA buffer (50 mM Tris HCl pH 8.0, 150 mM NaCl, 1 % NP-40, 0.5 % sodium deoxycholate, 0.1 % SDS). Samples were separated on SDS-PAGE followed by electroblotting to polyvinylidene difluoride membranes. The following antibodies
were used: cyclin E (C-19), cyclin A (h432), cyclin B1 (H-20), p21 (C-19), actin (C-11) and p53 (FL-393), Bcl-2 (100), Bax (N-20), Bcl-xS/L (S-18), caspase-8 (H-134), and caspase-3 (H-60) are all from Santa Cruz Biotechnology; Phospho-Histone H2A.X (Ser139) (p-H2AX, 2577) and Phospho-p53 (Ser15) (pS15-p53, D4S1H) are from Cell Signaling Biotechnology.

**Real-time RT-PCR**

Total RNA was prepared with TRIzol reagent (Invitrogen, Carlsbad, CA). After DNase I treatment, the RNA was reverse transcribed and the cDNA was used for PCR and real-time PCR. The real-time PCR assay was performed using the iTaq™ Universal SYBR® Green Supermix. Real-time PCR was used to detect the expression of Bax, MDM2, GADD45α, XIAP, FLIP, p53BP1, BRCA1, p65/RELA, and PARP1. Real-time PCR was performed in duplicate, and data were normalized to GAPDH expression.

**Results**

**PJ-34 induces cell cycle arrest in HTLV-I-transformed and ATLL-derived cell lines**

HTLV-I-transformed and ATLL cells are characterized by excessive chromosomal instability associated with defects in HR DNA repair pathways. We, therefore, hypothesized that these cells might be particularly sensitive to PARPi. HTLV-I-transformed T-cell lines MT-2, MT-4, C8166, and patient-derived ATLL cell lines ED and ATL-25 were treated with PJ-34 for 3 days and cell cycle was analyzed by propidium iodide staining and DNA content analyses on live gated cells by FACS. Our results revealed a marked increase of cells in the G2/M phase of cell cycle.
following treatment (Fig. 1a, b). These results are consistent with previous findings that the mitotic spindle checkpoint is functional in HTLV-I-transformed and Tax-expressing cells, which arrest in G2/M following treatment with NU7026, Taxol, or Nocodazole [20, 21]. We next used carboxyfluorescein succinimidyl ester (CFSE) incorporation and confirmed that HTLV-I-transformed MT-2 cells treated with PJ-34 are no longer dividing (Fig. 1c), suggesting that these cells are arrested in all the different phases of the cell cycle. Consistent with these results, we observed a significant increase in cyclin-dependent kinase inhibitor (CDK) p21WAF expression in MT-2, C91PL, and MT-4 cells after treatment with PJ-34 (Fig. 1d). Cyclin A associates with CDK2 and is involved in the initiation and completion of DNA replication during S phase [22]. Our experiments revealed a significant decrease in cyclin A expression after treatment with PJ-34. Since cyclin A is also important for centrosome replication and G2/M transition [23], these data may provide an explanation for G2/M arrest observed in most cells after treatment with PJ-34. In contrast, cyclin E, involved in G1 exit and initiation of S phase [24], was downregulated by PJ-34 in C91PL cells only (Fig. 1d). We next investigated the expression of Cyclin B1 because of its role in mitosis [25]. We found that cyclin B1 expression was significantly reduced in MT-2 and C91PL but not in MT-4 transformed cells (Fig. 1d). Overall, our results suggest that PARPi PJ-34 affects multiple cyclins and is effective in preventing proliferation of HTLV-I-transformed cells.

Next, we measured quantitative inhibition of cellular proliferation using XTT assays, a colorimetric assay for the quantification of cellular proliferation. These assays were performed in four HTLV-I-transformed cells and seven ATLL-derived cell lines and a dose increase of PJ-34 (5–50 μM) or DMSO vehicle as a control (Fig. 2). To demonstrate specificity of PJ-34, we used human PBMCs from a HTLV-I-negative healthy donor. These studies demonstrate that IC50 for
most HTLV-I-transformed or ATL cells is around 10 μM (MT-1 and C91PL 20 μM) (Fig. 2). Even at 50 μM of PJ-34, PBMCs present less than 25 % inhibition when most HTLV-I-transformed cells had more than 80 % inhibition (Fig. 2).

Figure 1. PJ-34 induces S/G2M cell cycle arrest

a Cell cycle analysis of HTLV-I-transformed and ATL cells after DMSO or PJ-34 treatment. b Cell cycle profile of DMSO- or PJ-34-treated cells. c Cell tracking of MT-2 with CFDA-SE after DMSO or PJ-34 treatment. Representative results are shown here. d Western
blot of cyclin E, cyclin A, cyclin B1, and p21 in MT-2, MT-4, and C91PL cells after DMSO or 25 μM PJ-34 treatment. Actin was used to confirm equal loading.

Figure 2. Antiproliferative effect of PJ-34

a PBMCs, HTLV-1-transformed (C8166, MT-2, C91PL, MT4), and (b) ATL cells (ATL-MT-1, ATL-ED, ATL-KOB, ATL-T, ATL-43T, ATL-25, ATL-55T) were treated with DMSO or
different concentrations of PJ-34 (5, 10, 25, 50 μM) for 5 days; cell proliferation was measured using XTT assay. Results were plotted as mean ± SD from at least two independent experiments. PJ-34 induces p53-dependent and p53-independent apoptosis in HTLV-I-transformed and ATLL-derived cell lines.

PARPi have been used to induce apoptosis in breast cancer and ovarian cancer cells [26–28]. The lack of effective therapy for ATLL prompted us to investigate the cytotoxicity of PJ-34 in four HTLV-I-transformed and seven ATLL-derived cell lines. Cells were treated for 5 days and analyzed by annexin V staining. Our results demonstrated a significant level of apoptosis in all patient-derived ATLL cell lines and most HTLV-I-transformed cell lines, with the exception of C91PL and MT-2 which appear to be resistant to PJ-34-induced apoptosis (Fig. 3a, b). These results were confirmed using a dose-dependent increase of PJ-34 on HTLV-I-transformed MT-4 cells (Fig. 3c). To confirm specific effects of PJ-34 on HTLV-I-transformed cells, we used a dose-dependent increase of PJ-34 and normal human PBMCs as a negative control (Fig. 4). Overall, these studies confirmed our previous observations and showed that HTLV-I-transformed cells are more sensitive than PBMC controls (Fig. 4). Also in agreement with above-mentioned data, C91PL had kinetics similar to PBMC and MT-2 was resistant to PJ-34 even at a dose of 50 μM (Fig. 4). Interestingly, another PARPi, olaparib (AZD2281), also induced apoptosis in HTLV-I-transformed MT-4 and ATL-ED resulting in only 34 and 10% live cells, respectively, after 3 days of treatment. In contrast, similar to PJ-34, MT-2 cells were more resistant, with 67% live cells after 3 days of olaparib (Fig. 5).
Figure 3. PJ-34 induces cell apoptosis in most HTLV-I-transformed and ATL cells except for C91PL and MT-2 cells.

(a) HTLV-1-transformed and (b) ATL cells were treated with 25 μM PJ-34 for 3 days. Cells were subsequently stained for annexin V and PI and analyzed for apoptosis by FACS analysis. c MT-4
cell line was treated with DMSO or different amounts of PJ-34 (5, 10, 25 μM). Cells were subsequently stained for annexin V and PI and analyzed for apoptosis by FACS analysis.

**PJ-34 treatment leads to reactivation of p53 functions in HTLV-I-transformed cells**

As a result of PARP inhibition by PJ-34, unrepaired single-strand DNA breaks (SSB) are converted into double-strand breaks (DDSB) at fork replication and result in accumulation of DDSB and p-H2AX foci. Consistent with this notion, we found an increased p-H2AX expression in cells treated with PJ-34 (Fig. 6a). Accumulation of p-H2AX is known to result in ATM/ATR activation and downstream Chk1-p53 pathway. Indeed, treatment of HTLV-I-transformed cells with PJ-34 caused an increased p-p53 at serine 15 and p21WAF (Fig. 6a), suggesting that p53 may be active in these cells. Drug cytotoxicity is often associated with activation of p53-dependent apoptosis, and tumors with mutated inactive p53 are frequently resistant to radio- and chemotherapy. HTLV-I-transformed cells in vitro and in vivo have been shown to express high levels of p53 that is genetically or functionally inactivated [29–31] but can be reactivated leading to either apoptosis or senescence of HTLV-I-transformed cells [32]. We compared activation of p53-mediated transcription in MT-4 (PJ-34 sensitive) and C91PL or MT-2 (PJ-34 resistant). Our results suggested that p53 was functionally reactivated in all HTLV-I-transformed cells following treatment with PJ-34, as demonstrated by an increase in p53 (Fig. 6b) and increased mRNA expression of the p53 target genes Bax, MDM2, p21, and GADD45α (Fig. 6c–e). One common target of both p53-dependent G1 arrest and p53-independent G2 arrest is p21WAF, whose expression was consistently increased after PJ-34 treatment of HTLV-I-transformed cells. However, our studies suggest that the sensitivity of HTLV-I-transformed cells to PJ-34-induced
apoptosis may not be dependent upon p53 signaling since both MT-2 and C91PL cells were resistant to PJ-34-mediated apoptosis despite reactivation of p53 in these cells. In addition, some ATLL-derived HTLV-I-transformed cells such as ED or MT-1 have no detectable expression of functional p53 and yet these cells were sensitive to PJ-34 treatment (Figs. 2 and 4).
Figure 4. PJ-34 induces cell apoptosis in most of HTLV-1-transformed and ATL cells except for C91PL and MT-2 cells.

a PBMCs, HTLV-1-transformed (C8166, MT-2, C91PL, MT4), and (b) ATL cells (ATL-MT-1, ATL-ED, ATL-KOB, ATL-T, ATL-43T, ATL-25, ATL-55T) were treated with DMSO or different concentrations of PJ-34 (5, 10, 25, 50 μM) for 5 days. Cells were subsequently stained for annexin V and PI and analyzed for apoptosis by FACS analysis. Percentage of increased apoptotic and necrotic cells in HTLV-1-transformed cells and ATL cell treatment with different amounts of PJ-34 were graphed.

Resistance of HTLV-I-transformed cells to PJ-34-induced apoptosis is associated with defective caspase-3 activation

Disruption of the mitochondrial membrane potential, ΔΨm, represents a critical step in the activation process of apoptosis cell death. Therefore, we measured ΔΨm in MT-4, MT-2, and C91PL cells treated with PJ-34. Our results suggested that the ΔΨm collapse was pronounced when HTLV-I-transformed cells were treated with PJ-34, and MT-4 cells were significantly more affected than MT-2 (Fig. 7a). The collapse of the ΔΨm is usually associated with activation of caspases. Caspase-9 is activated immediately downstream of the mitochondria following cytochrome C release and apoptosome formation. In contrast, caspase-8 is traditionally activated by death receptor signaling. These are referred to as canonical and non-canonical apoptosis pathways [33]. Caspase-3 is a central mediator in the caspase pathway and poly (ADP-ribose) polymerase (PARP) is a substrate of caspase-3 protease activity and its processing is usually associated with apoptotic cell death. To gain further insight into the mechanism used by MT-2
and C91PL-transformed cells to resist PJ-34 toxicity, we analyzed different markers associated
with pro-apoptotic and anti-apoptotic activities. Although Bcl-xL is overexpressed and an
important anti-apoptotic factor in HTLV-I cells [34], the surge in levels of Bax expression
cannot be counteracted by high levels of Bcl-xL, since the latter does not prevent Bax-mediated
apoptosis [35]. Expression of anti-apoptotic Bcl-2 was largely unchanged after treatment with
PJ-34 (Fig. 7b). We then evaluated whether the observed apoptotic phenotype of HTLV-I-
transformed cells treated with PJ-34 was dependent upon the mitochondrial pathway by
assessing Bax activation and activation of caspase-3. Surprisingly, active caspase 3 cleaved
products were readily detected in MT-4-treated cells but not detected in MT-2 and C91PL cells
after exposure to PJ-34 (Fig. 7c). Therefore, we investigated levels of FLIP and XIAP since these
are known to affect activation of caspase-3, but no difference was detected for MT-2 and C91PL
cells (Fig. 7d). We amplified and sequenced the cDNA of the caspase-3 gene, but it was wild
type in both MT-2 and C91PL. The reason for the lack of active caspase-3 in MT-2 and C91PL
after PJ-34 treatment is still unclear at the moment and under further investigation.
PARPi olaparib (AZD2281) was used at 25 μM for 3 days on HTLV-I-transformed MT-4, C91PL and MT-2 cells and apoptosis was measured by annexin V staining and FACS.
Figure 6. PJ-34 causes DNA damage and p53 pathway activation even in MT-2 and C91PL cells

a Western blot of p-H2AX, pS15-p53 and p21 in MT-4 cells treated with DMSO or PJ-34. Actin was used to confirm equal loading. b Western blot of p53 in MT-2, MT-4 and C91PL cells after treatment with DMSO or PJ-34. c–e Cells were treated with DMSO or PJ-34 and the changes of p53 target genes Bax (c), MDM2 (d), and GADD45a (e) were analyzed using real-time RT-PCR. Real-time PCR was performed in duplicate, and data were normalized to GAPDH expression. Results were presented as mean ± SD from two independent experiments.
Figure 7. The defect of caspase-3 activation in MT-2 and C91PL contributes to the resistance of PJ-34 in MT-2 and C91PL cells

a MT-4 and MT-2 were treated with DMSO or PJ-34. Cells were subsequently stained with JC-1, and mitochondrial membrane potential (ΔΨm) was measured by FACS analysis. Representative results of two experiments are shown here. b Western blot of analysis of Bcl-2, Bax, and Bcl-xL in MT-2, C91PL, and MT-4 after treatment with DMSO or PJ-34. c MT-2, MT-4, and C91PL were treated with DMSO or PJ-34 for 3 days. Then, total cell extracts were probed for caspase-8, caspase-3, and PARP-1 expression. d Relative expression of FLIP and XIAP were compared using real-time RT-PCR among resistant cell lines MT-2, C91PL, and other sensitive cell lines. Results were presented as mean ± SD from two independent experiments.
Resistance to PJ-34 in HTLV-I-transformed MT-2 cells coincides with increased expression of RelA/p65

We next wanted to understand the molecular mechanism involved in the resistance of HTLV-I-transformed cells MT-2 and C91PL. This has direct and important implications for treatment of ATLL patients with PJ-34, and identification of resistance biomarkers could also provide new insights for PARPi treatment of breast, ovarian, and prostate cancer patients. Acquisition of resistance to PARPi can be achieved by genetic alterations that restore HR functions [36]. Frequently, increased expression of BRCA1 or p53BP1 has been shown to correlate with resistance to PARPi [37, 38]. However, expression levels of p53BP1 or BRCA1 were not affected in HTLV-I-transformed MT-2 or C91PL before or after PJ-34 treatment (Fig. 8a, b). Increased expression of miR-107 was associated with PARPi sensitivity [39]. However, analyses of mature miR-107 expression revealed a significant increased expression in both resistant and sensitive HTLV-I-transformed cells (except for ED) when compared with HTLV-I-negative Jurkat T cells (data not shown). Similarly, the expression of PARP1 was not affected in MT-2-treated cells (Fig. 8c). In contrast, a significant increase in RelA/p65 was detected specifically in MT-2 but not MT-4 or C8166 cells following treatment with PJ-34, suggesting that canonical NF-κB activation may play an important role in resistance to PARPi in these cells.
Figure 8. The expressions of known resistant markers in MT-2 and sensitive cell lines

a, b Relative expression of p53BP1 (a) and BRCA1 (b) was compared using real-time RT-PCR among resistant cell lines MT-2, C91PL, and other sensitive cell lines. Real-time PCR was performed in duplicate, and data were normalized to GAPDH expression. Results were presented as mean ± SD from two independent experiments. c Relative expression of p53BP1, p65/RELA, BRCA1, and PARP-1 was compared using real-time RT-PCR among MT-4, C8166, and MT-2 treated with DMSO or 25 μM of PJ-34 for 5 days. Real-time PCR was performed in duplicate, and data were normalized to GAPDH expression.
Discussion

ATLL has a poor clinical outcome and current therapies have limited long-term benefits, in addition to most patients with acute or lymphoma ATLL having a short relapse-free survival. Patients diagnosed with chronic or smoldering forms of ATLL have a longer life expectancy but current treatments are ineffective and patients eventually progress to the acute type. Numerous studies have established that cancers with a dysfunction of the HR DNA repair pathway, also referred to as “BRCAness,” share characteristics with BRCA1- or BRCA2-mutated cancer cells and are very sensitive to PARPi [40, 41]. We have previously reported that HTLV-I-transformed cells have pronounced defects in the homologous recombination (HR) DNA repair pathway. Additional studies demonstrated that tumor cells with a defective base excision repair (BER) pathway are prone to DSB accumulation and hypersensitive to PARP targeting [42]. Notably, HTLV-I-transformed cells expressing the viral oncoprotein Tax have a defective BER pathway [43, 44]. These observations prompted us to investigate the potential benefits of PARPi therapy for ATLL.

In this study, we found a potent anti-proliferative effect of PARP small inhibitor PJ-34 against HTLV-I-transformed cells and patient-derived ATLL cells. Reduced tumor cell proliferation was demonstrated using XTT and CFSE staining assays. We showed that HTLV-I-transformed cells arrested in the G2/M phase of the cell cycle following treatment with PJ-34 was consistent with alterations in cyclins and cyclin-dependent kinase inhibitor (CDKI) expression. The effect of PJ-34 was time- and dose- dependent and affected both IL-2-dependent and IL-2-independent HTLV-I-transformed cells. We further demonstrated that PJ-34 is very effective against a panel of patient-derived ATLL cell lines, suggesting therapeutic potential. In general, anti-proliferative effects were associated with apoptotic cell death as shown by annexin V staining and the
activation of caspase-3. We detected a loss of the mitochondrial membrane potential in HTLV-I cells following PJ-34 treatment, supporting the notion that PJ-34 induces cell death via the intrinsic mitochondrial pathway. Interestingly, treatment with PARPi PJ-34 resulted in accumulation of DNA breaks and reactivation of the tumor suppressor p53 transcriptional functions, as demonstrated by an increased expression of target genes p21WAF, MDM2, BAX, and GADD45. However, reactivation of p53 was not critical for PJ-34’s effects since ATLL ED cells do not express p53 and were still sensitive to PJ-34. Interestingly, our studies identified two HTLV-I-transformed cell lines that were resistant to PJ-34 treatment: MT-2 and C91PL. We then investigated differences between MT-4 (PJ-34 sensitive) and MT-2 and C91PL (PJ-34 resistant). We found no specific pattern of expression for p53BP1 or BRCA1 in PJ-34-resistant versus sensitive cells. We then investigated expression of pro- and anti-apoptotic factors. Overall, anti-apoptotic proteins were not differentially affected but only MT-4 cells showed an increase in pro-apoptotic Bax expression. Bcl-xL, a pro-survival member of the Bcl-2 family, cannot directly inhibit Bax and specifically inhibits certain upstream BH3-only proteins. This supports our observation that the upregulation of Bcl-xL seen in MT-4 cells is unable to inhibit PJ-34-induced apoptosis and Bax activation, leading to apoptosis. We found that PJ-34-resistant cells were unable to activate caspase-3. This was unexpected because we and others previously demonstrated that MT-2 cells can activate caspase-3-dependent apoptosis [45–47].

Recently, PARP1 and PARP2 were found to play a role in sensing stalled or collapsed replication forks and to recruit the Mre11-Rad50-NBS1 (MRN) complex for resection and single-stranded DNA (ssDNA) formation, which allows RAD51 binding onto resected DNA to initiate HR [48, 49]. Thus, PARP is also involved in HR repair at replication forks and inhibition of PARP leads to increased DNA lesions that can cause stalling and collapse of the DNA
replication machinery. These observations suggest that targeting PARPi may be a good approach for ATLL patients. Combination therapy using PJ-34 and DNA-damaging agents such as cisplatin has shown promising results in other cancers and may be considered for a phase I trial of HTLV-I-associated ATLL. The reproducible growth arrest in cells from different genetic backgrounds highlights the potential value of PARPi as therapeutic agents for the treatment of ATLL.

**Footnotes**

Xue Tao Bai and Ramona Moles contributed equally to this work.
References


Chapter VI: "WRN-targeted therapy using inhibitors NSC 19630 and NSC 617145 induce apoptosis in HTLV-1-transformed adult T-cell leukemia cells"

Abstract

Human T-cell leukemia virus type 1 (HTLV-1) infection is associated with adult T-cell leukemia/lymphoma (ATLL), a lymphoproliferative malignancy with a dismal prognosis and limited therapeutic options. Recent evidence shows that HTLV-1-transformed cells present defects in both DNA replication and DNA repair, suggesting that these cells might be particularly sensitive to treatment with a small helicase inhibitor. Because the "Werner syndrome ATP-dependent helicase" encoded by the WRN gene plays important roles in both cellular proliferation and DNA repair, we hypothesized that inhibition of WRN activity could be used as a new strategy to target ATLL cells.

Our analysis demonstrates an apoptotic effect induced by the WRN helicase inhibitor in HTLV-1-transformed cells in vitro and ATL-derived cell lines. Inhibition of cellular proliferation and induction of apoptosis were demonstrated with cell cycle analysis, XTT proliferation assay, clonogenic assay, annexin V staining, and measurement of mitochondrial transmembrane potential.

Targeted inhibition of the WRN helicase induced cell cycle arrest and apoptosis in HTLV-1-transformed leukemia cells. Treatment with NSC 19630 (WRN inhibitor) induces S-phase cell cycle arrest, disruption of the mitochondrial membrane potential, and decreased expression of anti-apoptotic factor Bcl-2. These events were associated with activation of caspase-3-dependent apoptosis in ATL cells. We identified some ATL cells, ATL-55T and LMY1, less sensitive to NSC 19630 but sensitive to another WRN inhibitor, NSC 617145.

WRN is essential for survival of ATL cells. Our studies suggest that targeting the WRN helicase with small inhibitors is a novel promising strategy to target HTLV-1-transformed ATL cells.
Background

Human T-cell leukemia virus type 1 (HTLV-1) is a retrovirus that infects over 20 million people worldwide and is the etiological agent of adult T-cell leukemia/lymphoma (ATLL) [1, 2], an aggressive malignancy of mature activated T cells. HTLV-1 is associated with transformation of T lymphocytes and the development of ATLL in approximately 1–4% of infected individuals following a long latency period [3]. The diversity in clinical presentation and response to therapy of ATLL patients leads to classification into four subtypes: acute, lymphoma, chronic, and smoldering, based on organ involvement, lactate dehydrogenase (LDH), and calcium values [4]. Patients with aggressive forms of ATLL, the acute and lymphoma types, display a poor prognosis, with a median survival of approximately 1 year [5], and resistance to aggressive combined chemotherapy. Several clinical trials show that first-generation polychemotherapy containing doxorubicin (CHOP) has a limited effect on ATLL patients [6], while other approaches have yielded limited long-term benefits to ATL patients with acute or lymphoma type. In general, HTLV-1-associated disease has a poor clinical outcome, with 4-year survival rates for acute and lymphoma subtypes at 5 and 5.7%, respectively [7, 8]. The mechanism by which HTLV-1 induces T-cell transformation remains unclear; however, the viral Tax protein plays an essential role in the immortalization of human T lymphocytes [9, 10]. HTLV-1 activates an oncogenic signaling pathway, such as NF-kB and Jak/STAT [11, 12], and affects the expression of cellular miRNAs [13–16]. In addition, HTLV-1 infection leads to inactivation of several tumor suppressors and epigenetic regulators, including p16ink, Rb, p53 and p21waf, TET2, and MLL3 [17, 18]. HTLV-1-transformed cells are characterized by an increase of phospho-ATM and accumulation of γ-H2AX, suggesting a high level of DNA damage in those cells [19, 20]. Consistently, Tax was found to induce DNA double-strand breaks (DDSB), in an
NF-kB-dependent manner, and is responsible for alteration of DNA repair machinery. Tax induces DSB during the S-phase of the cell cycle, which are normally repaired through error-free Homologous Recombination repair (HR); however, in HTLV-1-transformed cells, the DNA damage is preferentially repaired by the error-prone non-homologous end joining (NHEJ) pathway [21, 22]. Overall, this evidence shows that Tax, by inducing DSB and altering the DNA repair, promotes genetic instability that might be involved in the initiating events leading to transformation. Furthermore, our recent study shows that Tax-expressing cells display DNA replication issues. The replication fork progression was found to be slower and stalls more frequently in the presence of the viral protein Tax [23], suggesting that these cells might be sensitive to a DNA replication inhibitor.

Targeting DNA replication and repair machinery has been proposed as a promising strategy to combat cancer [24, 25]. Helicases are highly conserved enzymes that unwind nucleic acid duplexes during DNA replication and repair [26]. WRN mutations of the gene lead to Werner syndrome, which is characterized by genetic instability and hematological disease [27]. WRN helicases are generally highly expressed in human leukemia [28] and depletion of the gene results in mitotic catastrophe, leading to cancer cell death [29]. Interestingly, evidence shows that treatment with a WRN inhibitor (NSC 19630) significantly affects cellular proliferation of leukemia cell lines [30]. WRN helicases are involved in replication fork progression and participate in DNA double-strand break repair through homologous repair and the non-homologous end joining pathway [21]. Defects in DNA replication and DNA damage response in the HTLV-1 context lead us to hypothesize that ATL cells may be sensitive to treatment with a WRN inhibitor. Here, we demonstrate that two small WRN inhibitors, NSC 19630 and NSC 617145, induce cell cycle arrest and apoptosis in HTLV-1-transformed ATL cells.
Methods

Cell lines and reagent

HTLV-1-transformed cell lines (MT-4, C8166, C91PL, and 1186.94 [31–33]) and ATL-derived cell lines, IL-2-independent (ED-40515(−), TL-0m1, and ATL-25 [34, 35]), were maintained in RPMI-1640 media supplemented with penicillin, streptomycin, and 10% fetal bovine serum (FBS). ATL-derived cell lines, IL-2-dependent (LMY1, ATL-55T, ATL-43T SO4, KK1 [36–42]), were maintained in RPMI-1640 media supplemented with IL-2 (50 U/mL), penicillin, streptomycin, and 10% FBS. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors by using Ficoll-Paque PLUS reagent (GE Healthcare Life Sciences). PBMCs were maintained in RPMI-1640 media supplemented with penicillin, streptomycin, and 20% fetal bovine serum (FBS). WRN inhibitor NSC 19630 was purchased from EMD Millipore’s Calbiochem® and NSC 617145 was purchased from Tocris Bioscience. Cells were treated with different concentrations of WRN inhibitors, and cells exposed to DMSO were used as a control, as indicated in figure legends.

Cell cycle and proliferation

Cells were treated with the WRN inhibitor or DMSO as a control. After 72 h, cells were collected and washed twice with phosphate-buffered saline (PBS) and then were fixed with 80% EtOH overnight at −20 °C. The following day, cells were washed twice with PBS, incubated with RNase for 15 min at 37 °C, stained with 100 μg/mL propidium iodide (PI) for 20 min, and analyzed on an LSR II flow cytometer. Cell proliferation was measured by microscopic cell count, Cell Proliferation Kit II (XTT) (Roche), the XTT assay, according to the manufacturer’s
instructions. Clonogenic assay was used to study cell proliferation. Briefly, cells were washed twice with PBS, fixed with cold Methanol (MeOH), and then stained with crystal violet dye (0.5% MeOH) for 20 min at room temperature.

**Apoptosis assay and mitochondrial membrane potential**

Inhibitor-treated and control cells were collected and washed twice with PBS then stained with annexin V/propidium iodide using the Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit (Molecular Probes, Eugene, OR) according to the manufacturer’s instructions. Mitochondrial membrane potential (ΔΨm) was measured using the JC-1 Assay Kit (Invitrogen) according to the manufacturer’s instructions.

**Western blotting**

Cell lysates were separated on SDS-PAGE followed by electroblotting to polyvinylidene difluoride membranes and probed with cyclin D1 (M-20), cyclin E (C-19), cyclin A (h432), cyclin B1 (H-20), actin (C-11), Bcl-2 (100), Mcl-1 and caspase-3 (H-60), Tax mouse monoclonal antibody (NIH AIDS Reagent Program, HTLV-I Tax Hybridoma (168B17)), and appropriate secondary antibodies purchased from Santa Cruz Biotechnology.
**Immunofluorescence**

Cells were centrifuged on slides at 800 rpm for 10 min. Slides were fixed in 4% paraformaldehyde (PFA) for 15 min at room temperature and then permeabilized with 0.5% Triton X-100 for 5 min on ice for 5 min. Slides were blocked for 1 h in PBS with 0.5% gelatin and 0.25% bovine serum albumin at room temperature. For γ-H2AX and PCNA staining, slides were incubated with anti-γ-H2AX (Ser139) antibody FITC conjugated (Millipore Sigma) and PCNA antibody (DAKO, Agilent Technologies) 1/200 in PBS for 2 h, washed three times in PBS-0.2% gelatin for 10 min each time, and incubated with the appropriate Alexa Fluor secondary antibody (Molecular Probes, Invitrogen) in PBS-0.2% gelatin for 1 h at room temperature. Cells were washed three times in PBS-0.2% gelatin for 10 min each time and mounted by using DABCO mounting medium (2.5% DABCO from Sigma, 200 mM Tris-HCl pH 8.6 and 90% glycerol). Fluorescent images were captured with Nikon TE2000E epifluorescence microscope by using the ×100 objective.

**Statistical analysis**

Experiments in Figs. 1, 2, 3, and 4 were performed multiple times in duplicate. Representative results were shown in the final figures. P values were calculated by using paired and two-tailed Student’s t test. P values are reported in the figures and in the legends.
Results

**NSC 19630 inhibitor induces S-phase cell cycle arrest**

HTLV-1-derived cell lines and Tax-expressing cells display impaired DNA replication and repair, leading us to hypothesize that these cells may be sensitive to treatment with a small helicase inhibitor. In order to determine if the small inhibitor NSC 19630 affects cellular proliferation, we exposed in vitro HTLV-1-transformed cell lines (MT4, C8166, and C91PL) and patient-derived ATLL cell lines (ED) to 3 μM of NSC 19630 or DMSO control for 48 h. Cells were stained with propidium iodide and DNA content was analyzed by FACS. Consistent with the fact that WRN helicases are required to unwind double-stranded DNA to single-stranded DNA during DNA replication [43], NSC 19630 treatment showed significant accumulation of cells in the S-phase when compared with DMSO-exposed cells (Fig. 1a, ,b). Previous studies demonstrated that cells expressing a WRN-specific shRNA displayed a reduction in cellular growth [44]. In fact, WRN-depleted human fibroblasts show a marked delay in completing the cell cycle by spending more time in late S- and/or G2-phases of the cell cycle [45]. Consistent with these observations, perturbation of cell cycle progression was noted in HTLV-1-transformed and ATL-derived cell lines (Fig. 1a, ,b). We included Western blot of the Tax viral protein in cellular lysates derived from MT4, C8166, C91PL, and ED (Fig. 1c). As previously reported, our analysis identified ED as Tax-negative and MT4, C8166, and C91PL as Tax-positive cell lines. [23, 46]. Our analysis shows that NSC 19630 induces perturbation of cell cycle progression in both Tax-negative and Tax-positive cells.
Figure 1. NSC 19630 inhibitor induces S-phase cell cycle arrest

a HTLV-1-transformed cell lines (C8166, C91PL, and MT4) and patient-derived cell lines (ED) were treated with 3 μM of NSC 19630 and DMSO vehicle has a control. After 48 h, cells were stained with propidium iodide (PI) and DNA content was analyzed by FACS to distinguish the different phases of the cell cycle (G0/G1, S, G2/M). The cell cycle analysis indicated an accumulation of the percentage of cells in S-phase, suggesting that exposure to the helicase inhibitor induced accumulation of cells in the S-phase in HTLV-1-transformed and ATL-derived cell lines. Experiment was performed multiple times in duplicate. Representative results are
shown in the final figures. b Graphic representation of the different percentages of G0/G1-, S-, and G2/M-phase cells treated with 3 μM of NSC 19630 compared to DMSO control. c Western blots of Tax viral protein in ED, C8166, C91PL, and MT-4 cell lines. d Western blots of cyclin D1, cyclin A, cyclin E, and cyclin B1 in ED and C8166 cells following 72 h of treatment with DMSO or 3 μM of NSC 19630. Actin was used to confirm equal loading.

The expression of cell cycle progression regulatory proteins was studied by Western blot in ED cells exposed to 3 μM of WRN inhibitor for 72 h. We compared the protein level of cyclins D1, E, A, and B1 in ED cells treated with NSC 19630 versus DMSO-treated controls (Fig. 1d). Cyclin E has a critical role in the control of the G1- and S-phase transitions and in the initiation of DNA replication [47]. Cyclin D1 levels vary during the cell cycle, with an elevated level of cyclin D1 maintained through G1-phase and required for the initiation of S-phase, while levels are reduced to allow DNA synthesis in S-phase [48]. However, increased levels of cyclin D1 are required to exit S-phase [48]. Similarly, cyclin A is accumulated during S-phase and is degraded before metaphase, while cyclin B1 is accumulated during the G2/M-phase [49]. Treatment of ATL cells with the WRN inhibitor NSC 19630 was associated with a decrease in the expression of cyclin D1, which may prevent treated cells from S-phase exit and result in accumulation of cells in S-phase (Fig. 1d). While there was no significant change in cyclin A expression in NSC 19630-treated cells, expression of cyclin E and cyclin B1 was significantly reduced (Fig. 1d). We believe that a relative decreased population in G2/M as a result of S-phase arrest accounts for the decrease in cyclin B1.
In addition, we decided to include Western blot of cyclins D1, E, A, and B1 in Tax-expressing cell line lysate C8166 extracted from cells exposed to NSC 19630 compared to DMSO-treated controls. Our analysis shows a reduction of cyclins E, D1, and B1 in Tax-positive cells (Fig. 1d), suggesting that cells arrested during the S-phase of the cell cycle. Overall, our data revealed a profound alteration of the cyclin expression profile in ATL cells exposed to the WRN helicase inhibitor consistent with S-phase arrest.
Figure 2. NSC 19630 inhibits cellular proliferation in patient-derived cells

a C91PL cells were exposed to increasing amounts of the WRN helicase inhibitor NSC 19630 (0, 0.2, 2, and 20 μM). After 72 h, cells were stained with annexin V to determine the percentage of apoptosis. The figures include the percentage of cells in the four quarters: Q1, Q2, Q3, and Q4. Q3 included the live cells that are annexin V and PI negative. Q4 included early apoptotic cells, which are annexin V positive and PI negative. Q2 included cells in late apoptosis, which are both annexin V and PI positive. Finally, Q1 included necrotic cells, which are PI positive and annexin V negative. A dose-dependent effect was noted. Experiment was performed multiple times in duplicate. Representative results are shown in the final figures. b Normal resting PBMCs and C91PL were exposed to increasing amounts of the WRN helicase inhibitor NSC 19630 (0, 0.2, 2, and 20 μM). After 72 h, cells were stained with annexin V and survival cells were graphed.
Experiment was performed in duplicate. P values were calculated comparing NSC-treated cells to DMSO control by using paired and two-tailed Student’s t test and indicated in the figure. c HTLV-1-transformed (MT-4, C8166, C91PL, 1186.94) and ATL-derived (ED, TL, ATL-25, ATL-43T, ATL-55T, LMY1, KK1, SO4, KOB) cell lines and normal resting PBMCs with increasing doses of NSC 19630 (0.2, 2, and 20 μM) show inhibition of cellular growth as measured by using cell count. Experiment was performed multiple times in duplicate. Representative results are shown in the final figures. d, e Patient-derived cell lines ATL-25 and LMY1 were treated with DMSO or 3 μM of NSC 19630 for 72 h and stained with crystal violet to test the anti-proliferative property of the WRN helicase inhibitor. The results were confirmed with XTT assay and cell counts. Experiment was performed multiple times in duplicate. Representative results were shown in the final figures. P values were calculated by using paired and two-tailed Student’s t test and indicated in the figure.

NSC 19630 inhibits cellular proliferation and induces apoptosis in HTLV-1-transformed and patient-derived cells

Recent evidence shows that treatment with the WRN inhibitor NSC 19630 significantly affects the cellular growth of different leukemia cell lines [30]. We next exposed HTLV-1-transformed C91PL to increasing logarithmic doses (0.2, 2, and 20 μM) or DMSO vehicle as a control (Fig. 2a, ,b).b). Induction of cell death was measured by using annexin V/PI staining. Apoptotic cells were scored as annexin V+, necrotic dead cells as PI+, versus live cells, which were annexin V-/PI-. We calculated the IC50 by using logarithmic transformation and we compared it to normal PBMCs isolated from healthy donors. Resting PBMCs were treated with the same
concentrations of NSC 19630, 0.2, 2, and 20 μM, and induction of apoptosis by using annexin V/PI staining was measured (Fig. 2b). The IC50 in normal cells is higher compared to the HTLV-1-transformed cell line, C91PL (9.28 ± 0.23 and 2.76 ± 0.29, respectively), showing that resting PBMCs isolated from a healthy donor are less sensitive to the drug.

We expanded our analysis by testing the HTLV-1-transformed (MT-4, C8166, C91PL, 1186.94) and ATL-derived (ED, TL, ATL-25, ATL-43T, ATL-55T, LMY1, KK1, SO4, and KOB) cell lines with increasing doses of NSC 19630, 0.2, 2, and 20 μM. Inhibition of cellular growth was measured by using cell count and reported in Fig. 2c. We calculated the IC50 for every cell line by using logarithmic transformation, and the values are indicated in Table 1. Interestingly, LMY1 and ATL-55T displayed a limited reduction of cellular growth when cells were treated with 2 μM of WRN inhibitor (Fig. 3c). Consistently, the IC50 was found to be higher in those lines compared to the other cell lines tested in our study, suggesting that LMY1 and ATL-55T are less sensitive to the drug (Table 1). We included normal PBMCs isolated from healthy donors in our analysis as a negative control. As expected, limited inhibition of proliferation was noted in resting PBMCs treated with WRN inhibitor (Fig. 2c) even at high concentrations.

Our analysis shows a dose-dependent inhibition of cellular proliferation, suggesting that targeting WRN activity represents a promising strategy to kill ATL cells. These results were further confirmed by clonogenic assay. Two patient-derived ATL cell lines with adherent characteristics were exposed to 3 μM of NSC 19630 or DMSO for 72 h, washed, and then stained with crystal violet. A significant reduction in the number of cells was noted in ATL-25 (Fig. 2d); however, no significant changes were observed in LMY1, suggesting that these cells are resistant to the WRN inhibitor (Fig. 2e). To confirm these results, the anti-proliferative effect of NSC 19630 was quantified by measuring cleavage of XTT to an orange formazan dye using
an ELISA reader at 450 nm and confirmed by microscopic cell count. Consistently, our analysis identified LMY1 as less sensitive to NSC 19630 (Fig. 2e). In contrast, ATL-25 was sensitive to the anti-proliferative effect of the WRN inhibitor (Fig. 2d).

The lack of effective therapeutic treatment for ATL patients led us to investigate the cytotoxicity of NSC 19630. WRN inhibitor is reported to induce cell death through accumulation of DNA double-strand breaks (DDSB) [30]. A previous article published from our laboratory shows that Tax affects the DNA repair machinery, more specifically by inhibiting Homologous Recombination (HR) repair [21]. That evidence leads us to speculate that NSC 19630 might induce cell death more prominently in Tax-expressing cells. To verify our hypothesis, we tested NSC 19630’s apoptotic effect on a Tax-expressing cell line (MT-4) versus a Tax-negative cell line (ED). Evident induction of cell death was noted in both Tax-positive and Tax-negative cells (Fig. 3a). Then we investigated if the compound affects Tax expression in three Tax-positive cell lines (C8166, MT-4, and C91PL); an ED-negative cell line was included in our analysis. Tax expression was found to be unchanged in NSC-treated cells compared to a DMSO control (Fig. 3b), suggesting that Tax expression is not a marker of drug sensitivity. A possible explanation of our finding is that Tax inhibits Homologous Recombination repair in an NF-κB-dependent manner [21]; however, constitutive activation is described in ATL cells that do not express detectable Tax [50].

Our preliminary data show that a WRN helicase inhibitor induces apoptosis in vitro (Fig. 3a), so we decided to include additional cell lines (MT-4, C8166, C91PL, and 1186.94) and nine ATL patient-derived cell lines (Fig. 3c). Previous studies suggest that IL-2-dependent ATL cell lines represent a model of smoldering and chronic forms of ATL, while IL-2-independent lines may better relate to the acute form of ATL. To investigate the potential use of WRN in various stages of ATL, we...
of ATL disease, we selected ATL-IL-2-independent (ED, TL, and ATL-25) and ATL-IL-2-dependent (ATL-43T, ATL-55T, LMY1, KK1, SO4, and KOB) cell lines for study. These cells were exposed for 72 h to the WRN helicase inhibitor and analyzed by annexin V/PI staining to measure the percentage of apoptosis induced by the compound (Fig. 3c). Significant levels of apoptosis were detected in IL-2-dependent and IL-2-independent ATL cell lines (Fig. 3c). These results suggest that HTLV-1-transformed ATL cells are highly sensitive to the WRN inhibitor NSC 19630 and that patients with the chronic, smoldering, or acute form of ATL are potential candidates for this therapeutic agent.

To gain some insights into the molecular mechanisms involved in NSC 19630’s effects on ATL cells, we next investigated disruption of the mitochondrial transmembrane potential (ΔΨm) (Fig. 3f). Activation of mitochondrial pathway cell death leads to the opening of the mitochondrial permeability transition (MPT) pore. The major consequences of this event are the disruption of ΔΨm and the release of pro-apoptotic proteins. Our analyses show that treatment with the NSC 19630 inhibitor resulted in the collapse of mitochondrial transmembrane potential in ED cells (Fig. 3f). Since the loss of mitochondrial transmembrane potential is associated with the activation of the caspase pathway [51], we investigated the activation of caspase-3, an essential mediator of apoptosis activated by proteolytic cleavage. Our data indicate that cleaved caspase-3 products were readily detected in ED-treated cells when compared to DMSO-treated control cells (Fig. 3d). We then analyzed the expression of B-cell lymphoma 2 (Bcl-2) (Fig. 3d), a protein that prevents apoptosis either by sequestering caspases or by preventing the release of mitochondrial apoptogenic factors, such as cytochrome c, and an apoptosis-inducing factor, AIF, into the cytoplasm [52]. Consistent with results from annexin V/PI, the treatment with the WRN helicase inhibitor led to decreased Bcl-2 expression in ED-treated cells (Fig. 3d). Previous
studies showed that an increased expression of Mcl-1 significantly inhibits progression through the S-phase of the cell cycle [53]. Consistent with our cell cycle results demonstrating that exposure to the WRN helicase inhibitor results in S-phase arrest (Fig. 1a), increased levels of Mcl-1 were detected in ED-treated cells (Fig. 3d). Moreover, we performed Western blot of caspase-3, Bcl-2, and Mcl-1 on protein lysates of ATL-55T and LMY1 treated with DMSO or NSC 19630. As expected, no significant change of expression was noted (Fig. 3d), confirming that these lines are less sensitive to the drug.

The WRN helicase stabilizes and maintains the replication fork during DNA replication. Failure to stabilize the fork induces DNA double-strand breaks (DDSB); in fact, treatment with replication inhibitors induces fork collapse, leading to serious DNA damage and cell death [54]. Consistent with this concept, M. Aggarwal et al. demonstrated that a WRN inhibitor, NSC 19630, induces DDSB and accumulation of PCNA foci, which is associated with stalled replication forks [30]. In order to investigate if apoptosis is WRN-dependent in an HTLV-1 context, we dual-stained γ-H2AX (a specific marker of DDSB) and PCNA in cells exposed to NSC 19630 compared to DMSO-treated cells. Our analysis shows accumulation of PCNA and γ-H2AX foci, suggesting that the treatment induces DNA replication issues and, consequentially, DNA damage (Fig. 3e).
Figure 3. NSC 19630 induces apoptosis in HTLV-1-transformed and patient-derived cells

a ED and MT-4 cells were exposed to WRN helicase inhibitor NSC 19630 (3 μM) or DMSO. After 72 h, cells were stained with annexin V. The figures include the percentage of cells in the four quarters: Q1, Q2, Q3, and Q4. Q3 included the live cells that are annexin V and PI negative. Q4 included early apoptotic cells, which are annexin V positive and PI negative. Q2 included cells in late apoptosis, which are both annexin V and PI positive. Finally, Q1 included necrotic cells, which are PI positive and annexin V negative. b Western blots of Tax viral protein in ED, C8166, C91PL, and MT-4 cells exposed to NSC 19630 compared to DMSO-treated controls. c HTLV-1-transformed cell lines (MT-4, C8166, C91PL, and 1186.94) and patient-derived cell lines (ED, TL, ATL-25, ATL-43T, ATL-55T, LMY1, KK1, SO4, and KOB) were treated for 72 h with 3 μM of NSC 19630. Cells were stained with annexin V to measure the apoptotic effect of the WRN helicase inhibitor. The percentage of apoptosis and necrosis was graphed. HTLV-1-transformed, ATL-derived IL-2-dependent cell lines and IL-2-independent cell lines are represented in blue, yellow, and red, respectively. Tax viral protein is expressed in MT-4, C8166, C91PL, 1186.94, and ATL-25 cell lines [23]. Experiments were performed multiple times in duplicate. Representative results are shown in the final figures. d Western blot of Caspase-3 and apoptotic markers Bcl-2 and Mcl-1 was performed in ED, ATL-55T, and LMY1 cells exposed to DMSO or 3 μM of NSC 19630. Our analysis shows the activation of caspase-3 after treatment with the WRN helicase inhibitor. e Immunofluorescence of γ-H2AX and PCNA in ED cells exposed for 72 h with 3 μM of NSC 19630 compared to DMSO control. Fluorescent images were captured by using the ×100 objective f Distraction of mitochondrial transmembrane potential in ED cells treated with NSC 19630 compared to DMSO control.
NSC 19630-resistant ATL-55T and LMY1 cell lines are sensitive to NSC 617145

Data presented above suggest that NSC 19630 is a promising agent for the treatment of ATL. However, our analyses show that the apoptotic effect of the WRN helicase inhibitor was very limited in two ATL lines, namely LMY1 and ATL-55T (Fig.3c), suggesting potential resistance mechanisms that warrant further investigations. We studied the endogenous expression of WRN helicases in HTLV-1-transformed and ATL-derived cell lines. Consistent with previously published studies, our analyses show no direct correlation between levels of WRN protein expression and sensitivity to the WRN inhibitor (data not shown).

We then investigated NSC 617145, a WRN inhibitor identified as a close structural analog of NSC 19630 but with more potent inhibition of WRN helicase activity. We next exposed HTLV-1-transformed, ATL-derived cell lines (MT-4, C8166, C91PL, 1186.94, ED, TL, ATL-25, ATL-43T, ATL-55T, LMY1, KK1, SO4, KOB) and resting PBMCs isolated from healthy donors to increasing doses of NSC 617145 (0.02, 0.2, 2, and 20 μM), and inhibition of cellular growth was measured by cell count (Fig. 4a). We calculated the IC50 for every cell line by using logarithmic transformation and the values are reported in Table 2. Our analysis clearly shows that NSC 617145 was found to be more potent in inhibiting cellular growth compared to NSC 19630. In fact, ATL-55T cells were sensitive when exposed to increasing concentrations of NSC 617145, which inhibited cellular growth, as shown by cell count and XTT proliferation assay (Fig. 4b, c). Next, we exposed LMY1 cells to 0.25 μM for 4 days then stained with crystal violet. Consistently, again, a significant reduction in the number of cells was observed in LMY1, which was confirmed by cell count and XTT assay (Fig. 4d). Finally, we performed apoptosis assay and found that low concentrations of NSC 617145 induced high levels of cell death in both ATL-55T and LMY1 cell lines (Fig. 4e). Interestingly, limited inhibition of proliferation was noted in
normal PBMCs (Fig. 4a). To confirm the limited effect of WRN inhibitor on normal cells, we estimated the IC50 in an HTLV-1-transformed cell line, C91PL, and in resting PBMCs based on induction of apoptosis. C91PL and normal resting PBMCs were exposed to increasing logarithmic doses (0.02, 0.2, and 2 μM) or DMSO vehicle as a control. Induction of cell death was measured by using annexin V/PI staining (data not show). Our analysis shows that the IC50 in normal cells is higher compared to the HTLV-1-transformed cell line C91PL (0.32 ± 0.013 and 0.13 ± 0.047, respectively), suggesting that NSC 617145 might be suitable for treating ATL patients.

Overall, our data suggest that HTLV-1-transformed ATL cells are very sensitive to the anti-proliferative and apoptotic effects of WRN helicase inhibitors.
Figure 4. ATL-55T and LMY1 cell lines are sensitive to NSC 617145

a HTLV-1-transformed (MT-4, C8166, C91PL, 1186.94) and ATL-derived (ED, TL, ATL-25, ATL-43T, ATL-55T, LMY1, KK1, SO4, KOB) cell lines and normal resting PBMCs were treated with increasing doses of NSC 617145 (0.02, 0.2, 2, and 20 μM). Inhibition of cellular growth was measured by using cell count. Experiment was performed multiple times in duplicate. Representative results are shown in the final figures. b Patient-derived cell line, ATL-55T, was exposed to increasing amounts of NSC 617145 for 4 days, and the anti-proliferative effect was evaluated by cell count and XTT assay. Representative results are shown in the final figures. P values were calculated using paired two-sided Student’s t test. c LMY1-ATL-derived cells were treated with 0.25 μM of NSC 617145 for 96 h and stained with crystal violet. Cell count and XTT assay were used to measure the cellular growth of NSC 617145-treated cells compared to DMSO control cells. Experiment was performed multiple times in duplicate. Representative results are shown in the final figures. P values were calculated by using paired and two-tailed Student’s t test and indicated in the figure. d ATL-55T and LMY1 cells were stained with annexin V to measure the apoptotic effect of the WRN helicase inhibitor NSC 617145. Cells were treated for 92 h with 0.25 μM of NSC 617145. e The figures include the percentage of cells in the four quarters: Q1, Q2, Q3, and Q4. Q3 included the live cells that are annexin V and PI negative. Q4 included early apoptotic cells, which are annexin V positive and PI negative. Q2 included cells in late apoptosis, which are both annexin V and PI positive. Finally, Q1 included necrotic cells, which are PI positive and annexin V negative. Experiment was performed multiple times in duplicate. Representative results are shown in the final figures.
Table 1. Estimated IC50 of NSC 19630 in HTLV-1-transformed and patient-derived cells

<table>
<thead>
<tr>
<th>HTLV-1–transformed cell lines</th>
<th>IC50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MT-4</td>
<td>1.99 +/- 0.065</td>
</tr>
<tr>
<td>C8166</td>
<td>2.84 +/- 0.19</td>
</tr>
<tr>
<td>C91PL</td>
<td>2.76 +/- 0.28</td>
</tr>
<tr>
<td>1186.94</td>
<td>2.23 +/- 0.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ATL-derived cell lines</th>
<th>IC50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ED</td>
<td>0.75 +/- 0.053</td>
</tr>
<tr>
<td>TL</td>
<td>1.73 +/- 0.29</td>
</tr>
<tr>
<td>ATL-25</td>
<td>1.79 +/- 0.22</td>
</tr>
<tr>
<td>ATL-43T</td>
<td>1.69 +/- 0.23</td>
</tr>
<tr>
<td>ATL-55T</td>
<td>6.1 +/- 0.15</td>
</tr>
<tr>
<td>LMY1</td>
<td>4.35 +/- 0.21</td>
</tr>
<tr>
<td>KK1</td>
<td>1.64 +/- 0.038</td>
</tr>
<tr>
<td>SO4</td>
<td>1.45 +/- 0.12</td>
</tr>
<tr>
<td>KOB</td>
<td>1.73 +/- 0.086</td>
</tr>
</tbody>
</table>

We tested the HTLV-1-transformed, ATL-derived cell lines (MT-4, C8166, C91PL, 1186.94, ED, TL, ATL-25, ATL-43T, ATL-55T, LMY1, KK1, SO4, KOB) with increasing doses of NSC 19630 (0.2, 2, and 20 μM), and inhibition of cellular growth was measured by using cell count. We calculated the IC50 for every cell line by using logarithmic transformation and the values are reported in the table. Our analysis shows that ATL-55T and LMY1 were found to be less sensitive compared to the HTLV-1- and ATL-derived cell lines included in the study.
<table>
<thead>
<tr>
<th>HTLV-1–transformed cell lines</th>
<th>IC50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MT-4</td>
<td>0.19 +/- 0.042</td>
</tr>
<tr>
<td>C8166</td>
<td>0.22 +/- 0.023</td>
</tr>
<tr>
<td>C91PL</td>
<td>0.21 +/- 0.0091</td>
</tr>
<tr>
<td>1186.94</td>
<td>0.22 +/- 0.063</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ATL-derived cell lines</th>
<th>IC50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ED</td>
<td>0.15 +/- 0.033</td>
</tr>
<tr>
<td>TL</td>
<td>0.17 +/- 0.039</td>
</tr>
<tr>
<td>ATL-25</td>
<td>0.16 +/- 0.007</td>
</tr>
<tr>
<td>ATL-43T</td>
<td>0.099 +/- 0.013</td>
</tr>
<tr>
<td>ATL-55T</td>
<td>0.22 +/- 0.0012</td>
</tr>
<tr>
<td>LMY-1</td>
<td>0.29 +/- 0.0087</td>
</tr>
<tr>
<td>KK1</td>
<td>0.28 +/- 0.032</td>
</tr>
<tr>
<td>SO4</td>
<td>0.14 +/- 0.061</td>
</tr>
<tr>
<td>KOB</td>
<td>0.17 +/- 0.031</td>
</tr>
</tbody>
</table>

Table 2. Estimated IC50 of NSC 617145 in HTLV-1-transformed and patient-derived cells

We tested the HTLV-1-transformed, ATL-derived cell lines (MT-4, C8166, C91PL, 1186.94, ED, TL, ATL-25, ATL-43T, ATL-55T, LMY1, KK1, SO4, KOB) with increasing doses of NSC 617145 (0.02, 0.2, and 2 μM), and cell count was used to measure the inhibition of cellular growth. We calculated the IC50 for every cell line by using logarithmic transformation and the
values are reported in the table. Our analysis clearly shows that NSC 617145 is more potent in inhibiting cellular proliferation compared to NSC 19630.

**Discussion**

In the absence of effective chemotherapy treatments, most patients with aggressive forms of the disease have a poor clinical outcome. Patients with the lymphoma type also have an unfavorable prognosis, with a median survival of 10.2 months, while patients with acute ATLL present a median survival of 6.2 months [55]. The projected 4-year survival rates of patients with the acute and lymphoma forms are only 1–5% [56]. DNA repair inhibitors induced cell death in different human leukemias [57, 58], and in the absence of an effective treatment for ATLL, we decided to test the cytotoxicity of a WRN helicase inhibitor. WRN helicases are involved in HR DNA repair and helicase activity is required during DNA replication. Previous studies showed that both DNA repair and DNA replication are impaired in HTLV-1-transformed cells.

In this study, we investigate small inhibitors of WRN as a potential therapeutic agent for ATLL. NSC 19630 targets WRN helicase activity but does not affect other DNA helicases (Bloom syndrome (BLM), Fanconi anemia group J (FANCJ), RECQ1, RecQ, UvrD, or DnaB) [30]. Our results demonstrate an apoptotic effect induced by the WRN helicase inhibitor in HTLV-1-transformed cells in vitro and in a majority of ATL-derived cell lines tested. Inhibition of cellular proliferation and induction of apoptosis were demonstrated with XTT proliferation assay, clonogenic assay, and annexin V staining. Consistent with previous studies, we observed an S-phase delay in ATL cells following treatment with NSC 19630. All the cyclins tested (cyclins E, B1, and D1) were found to be downregulated after the treatment, except for cyclin A, which is
specific to the S-phase of the cell cycle. The effect of the WRN inhibitor was found to be dose- and time-dependent. Exposure to 3 μM of NSC 19630 shows significant caspase-dependent apoptosis in HTLV-1-transformed and patient-derived cells, both IL-2-dependent and IL-2-independent. We found a disruption of the mitochondrial potential, suggesting that the WRN inhibitor induces cell death through an intrinsic apoptotic pathway. Consistently, expression of Bcl-2 intrinsic anti-apoptotic factor was reduced in ED cells exposed to the WRN inhibitor. Our study shows that the WRN inhibitor efficiently kills HTLV-1-transformed and patient-derived cells. In addition, non-cancerous cells are resistant to the anti-proliferative and apoptotic effects of NSC 19630 [30], suggesting that NSC 19630 may represent a suitable strategy for initiation of phase I clinical trials. Our study also identified two cell lines less sensitive to NSC 19630, ATL-55T and LMY1. Nonetheless, these cells were efficiently killed by an NSC 19630 analog, NSC 617145, with more potent inhibitory effects on WRN helicase activity. In addition, a possible explanation of the different sensitivities to WRN helicase inhibitors in different ATL cells is that the compounds act on different domains of the enzyme. NSC 19630 is reported to inhibit the helicase domain and mildly reduce the ATPase and exonuclease activities of WRN. On the other hand, NSC 617145 reduces mainly the ATPase domain in a dose-dependent manner [59].

Conclusions

The WRN inhibitors NSC 19630 and NSC 617145 efficiently kill HTLV-1-transformed and patient-derived cells, suggesting that WRN helicases represent a novel therapeutic target for ATLL patients.
References


Chapter VII: “Mutation of epigenetic regulators TET2 and MLL3 in patients with HTLV-I-induced acute adult T-cell leukemia.”

Abstract

Epigenetic regulators play a critical role in the maintenance of specific chromatin domains in an active or repressed state. Disruption of epigenetic regulatory mechanisms is widespread in cancer cells and largely contributes to the transformation process through active repression of tumor suppressor genes. While mutations of epigenetic regulators have been reported in various lymphoid malignancies and solid cancers, mutation of these genes in HTLV-I-associated T-cell leukemia has not been investigated.

Here we used whole genome next generation sequencing (NGS) of uncultured freshly isolated ATL samples and identified the presence of mutations in SUZ12, DNMT1, DNMT3A, DNMT3B, TET1, TET2, IDH1, IDH2, MLL, MLL2, MLL3 and MLL4.

TET2 was the most frequently mutated gene, occurring in 32 % (10/31) of ATL samples analyzed. Interestingly, NGS revealed nonsense mutations accompanied by loss of heterozygosity (LOH) in TET2 and MLL3, which was further confirmed by cloning and direct sequencing of DNA from uncultured cells. Finally, direct sequencing of matched control and tumor samples revealed that TET2 mutation was present only in ATL tumor cells.

Our results suggest that inactivation of MLL3 and TET2 may play an important role in the tumorigenesis process of HTLV-I-induced ATL.
**Background**

Human T-cell leukemia virus type I (HTLV-I) is associated with fatal lymphoproliferative disorders known as adult T-cell leukemia/lymphoma (ATL) [1, 2]. The disease is classified into distinct subtypes - smoldering, chronic, acute and lymphoma - that differ in their clinical presentation and in their response to treatment [3]. Since the clinical subtypes of ATL have distinct genomic alterations and different clinical progression, these diseases require a different approach for treatment [4]. However, current therapies for ATL do not result in long-term remission and even the clinically less aggressive forms ultimately evolve to the acute. The 4 year survival rate for acute-, lymphoma-, chronic- and smoldering-type ATL is 11, 16, 36, and 52 %, respectively [5, 6]. The viral oncoprotein Tax plays an important role in initiation of events leading to cellular transformation [7, 8]. However, the fact that the disease has a low penetrance and is observed after a long latency period of several decades has led to the hypothesis that the virus initiates oncogenic events but is not sufficient for cellular transformation [9, 10]. In support of this notion epigenetic alterations are required for the development of ATL. Promoter hypermethylation associated with loss of SHP1 expression coincides with the IL-2-independent transformation of T cells by HTLV-I in vitro [11]. SHP1 is one of the most frequently altered genes in ATL patients, with an overall hyper-methylation rate of 90 % [12]; other tumor suppressor genes inactivated by methylation in ATL include p53-related p73, CDKN2A and p21CIP1/WAF1 [13]. The fact that histone methyl-transferase EZH2 has been demonstrated to repress p57KIP2 expression through histone H3 lysine 27 trimethylation (H3K27me3) [14], and that p57KIP2 is methylated in nearly 50 % of newly diagnosed ALL patients [15], prompted us to analyze the status of cellular genes involved in chromatin silencing. In this study we use next
generation sequencing (NGS) to characterize the genetic mutations in EZH1, EZH2, EED, SUZ12, DNMT1, DNMT3A, DNMT3B, TET1, TET2, TET3, IDH1/2, MLL, MLL2, MLL3, MLL4 and ASXL1. Our study revealed a high frequency of mutation in epigenetic regulators in ATL samples, suggesting that chromatin remodeling by some of these genes may play a role in the pathogenesis of ATL.

Figure 1. HTLV-1 proviral load in cell lines and patients

a A set of standard samples was prepared through dilution of HTLV-I transformed cell line DNA (TL) containing a single copy of integrated HTLV-I with HTLV-I negative 293 T cell DNA. Real time PCR was performed with 100 ng of mixed DNA. Both GAPDH and gag were detected and ΔCt was calculated by Ct gag - Ct GAPDH. The standard curve was created with ΔCt (X) and the percentage of HTLV-I viral load relative to TL cell (Y). b Proviral load was calculated in DNA samples isolated from high grade ATL lymphoma and matched-control B cells by real time PCR. Relative proviral loads were calculated using the standard curve established above.
Methods

ATL patient samples

All patient samples were obtained after informed consent was provided and in agreement with regulations for the protection of human subjects according to the National Institutes of Health (NIH) guidelines. As for the samples from the Japanese material bank, they were provided from the biomaterial bank of the Japanese nationwide cohort study (Joint Study of Predisposing Factors for ATL Development, JSPFAD) that is approved by the ethical committee of the University of Tokyo (No. 14-15, No. 07-07 and No. 10-50). Genomic DNA was extracted using DNAZol (Invitrogen) from uncultured acute and lymphoma ATL samples. DNA samples 1–7, 10 and 11 were isolated from patients diagnosed with acute ATL. DNA samples 8, 9, 12 and 13 were isolated from patients with lymphoma ATL. HTLV-I proviral load was calculated by TaqMan real time PCR and compared with a standard curve established using C91PL HTLV-I transformed cell line harboring one proviral copy (Fig.1a). High tumor grade lymph node biopsy was used for ATL lymphoma patients as confirmed by real time PCR compared with B cells isolated from matched patient (Fig.1b).

Next generation sequencing (NGS)

Exome Sequencing was performed by Perkin Elmer. DNA samples were evaluated using an e-gel and PicoGreen fluorometry to measure quality and quantity, respectively. DNA samples were then physically sheared to the desired size using a Covaris E220 Focused-ultrasonicator. Library
preparation and enrichment were carried out using an Agilent SureSelectXT All Exon V3 kit and an automated sample preparation method derived from the manufacturer’s protocol. All subsequent steps were based on sequencing by Next Generation Sequencing methods on the Illumina Hiseq 2000 platform. Basecall files (*.bcl) were generated by the Illumina instruments and de-multiplexed and converted to fastq.gz format using CASAVA v1.8.2. Each pair of fastq.gz files was then aligned against human reference build 37 using BWA, v0.6.2. The resulting SAM files were converted to BAM format, sorted and indexed using SamTools v0.1.18. Duplicate reads in the sorted BAM file were marked using PicardTools v1.86. The duplicate marked BAM files were processed using GATK v1.6–13, following their “Best-Practices V3”. Each BAM was realigned around known INDELs and base quality scores were recalibrated, resulting in a recalibrated BAM file. Variants for each recalibrated BAM file were called using GATK Unified Genotyper, with SNPs and INDELs saved to separate files. These files were then hard filtered.
Figure 2. Schematic representation of the MLL3 protein and distribution of mutations found in ATL patients

a Schematic representation of the MLL3 protein. Nonsense and missense mutations found in ATL patients are shown in red and blue, respectively. b Chromatogram of the sequence of MLL3 from normal PBMC DNA (top) and MLL3 from ATL DNA with amino acid 904 nonsense mutation (bottom). c Alignment of ATL patient DNA KOE with MLL3 demonstrates the presence of a stop codon TGA in position 904. SNPs identifying the MLL3 sequence (different from Chr13) are labeled in red
**Direct PCR-sequencing and TA cloning for analyses**

Direct sequencing of ATL DNA was performed after PCR amplification using specific primers described below. In the case of MLL3 primers were located in introns surrounding exon 16. Primers amplify genome sequence from 152235498 to 152236241 of chromosome seven which encompass the MLL3 Exon16 nucleotide 2653 to 2769 (amino acid 884 to 923). MLL3 F: CAGGCTATAGTTGTTGTCGTCACCAAG; MLL3 R: CATAACATGATAGTAAGCAAATATCTATC. TET2-414 primers amplify nucleotide 842 to 1379 exon1 of TET2, which correspond to TET2 amino acid 281 to 459. TET2 Q414-F: ACTCTGAGCTGCTCCTCAAAG; TET2 Q414-R: GAAGGTGGTGCTCCTCAGGTTT. TET2-876 primers amplify nucleotide 2403 to 2866 exon1 of TET2, which correspond to amino acid 801 to 955. TET2 Q876-F: TGTCCAAATGGGACTGGAGG; TET2 Q876-R: GATGCCACCTTAGAGCAGCA. Individual clones were obtained by TA-cloning (Invitrogen) and five clones were sequenced.
Figure 3. Schematic representation of TET2 protein and distribution of mutations found in ATL patients

(a) Schematic representation of TET2 protein. (b and c) Somatic LOH Q414* and LOH Q876* were confirmed by direct sequencing and analyses of TA clones from high grade tumor and matched non-tumor of two ATL samples
Results

Despite profound epigenetic alterations in the genome of ATL cells, the genetic status of chromatin modifiers has not been investigated. In this study we performed next generation exome sequencing (NGS) to identify novel mutations in epigenetic regulators in ATL samples. The polycomb repressive complex 2 (PRC2) has histone methyltransferase activity and primarily trimethylates histone H3 on lysine 27 (H3K27me3), a mark of transcriptionally silent chromatin [16]. The PRC2 complex has four subunits: SUZ12, EED, EZH1 and EZH2. LOH mutations of EZH2 or SUZ12 have been reported in 25 % of T-ALL [17]. In addition, the loss of PRC2 activity cooperates with mutated Notch1 by allowing recruitment of the intracellular domain of Notch onto the promoter of target genes [17]. Along these lines, activated Notch is also required for ATL cell growth and tumor formation in an ATL mouse model [18]. While the EZH2 gene was not mutated in our study, 1/13 ATL sample had a mutation in SUZ12 (Additional file 1: Table S1). It will be interesting in future studies to investigate if there is any cooperation of EZH2 and/or SUZ12 with activated Notch in a larger cohort of acute ATL patients. The coding sequence of the other two subunits, EED and EZH1, was not mutated in any of the ATL samples tested. The possibility that some members of PRC2 may be regulated post-transcriptionally by microRNA or LncRNA in ATL cells is under investigation. Similarly, miR101, miR-26 and miR208b have been shown to target EZH2, miR-323-3p to target EED, and miR-200b to target SUZ12. An earlier study demonstrated that decreased expression of miR-101, but not MiR-26b, in acute ATL is in part responsible for elevated expression of EZH2 in these cells [19]. Consequently, increased expression of the EZH2 protein induced the silencing of miR-31, resulting in NIK-mediated activation of NF-kB in ATL cells [20]. Additional sex combs like transcriptional regulator 1 (ASXL1) interact with PRC2 and are likely involved in a cross-talk
between chromatin silencing systems, PRC1/PRC2, the HP1α/CBX5 heterochromatin repressive complex and the polycomb repressive deubiquitinase (PR-DUB) complex. Mutation of ASXL1 has been reported in AML and chronic myelomonocytic leukemia (CMML) patients [21]. Our study revealed mutations of ASXL1 in 2/13 ATL samples (Additional file 1: Table S1). Interestingly, an ASXL1 somatic mutation, V1092M, detected in one ATL patient has also been reported in myeloproliferative neoplasms (MPN) and myelodysplastic syndromes (MDS) [22].

We next analyzed DNA (cytosine-5)-methyltransferases (DNMT1, DNMT3A and DNMT3B), which catalyze the transfer of methyl groups to specific CpG islands in DNA and are involved in maintenance or de novo methylation. Somatic mutations in DNMT3A have been reported as nonsense, frameshift, and missense mutations throughout the open-reading frame in 5–20 % of AML and MDS [23]. These studies suggested a potential gain-of-function that did not require the presence of a wild type copy of DNMT3A for altered function. Our analyses identified mutations in 7.5 % (1/13) of DNMT1 (isoform a) and DNMT3A (isoform b) and 15 % (2/13) of DNMT3B (isoform 1) of ATL samples. Interestingly, the same mutation at position N442K of DNMT3B was identified in two different unrelated ATL patients and has been reported in prostate cancer cells and the Cosmic Database.

The Mixed Lineage Leukemia (MLL) family of genes (also known as lysine (K)-specific methyltransferases (KMT2)) plays an important role in histone methylation and transcriptional activation and is involved as a regulator of growth of hematopoietic precursor cells. Mutation of MLL and MLL2 was observed in 7.5 % (1/13) of ATL patients. The MLL3 gene, which encodes a component of a histone H3 lysine 4 methyltransferase complex named the ASC-2- and Mll3-containing complex (ASCOM), has been implicated as a tumor suppressor gene due to its frequent mutations in multiple types of human tumors [24]. Exome sequencing has recently been
used to identify an MLL3 germ line mutation in a pedigree of colorectal cancer and acute myeloid leukemia [25]. Mutations and LOH in MLL3 has been reported in various human cancers [26]. Our initial NGS analyses identified a high rate of nonsense mutations in MLL3 at position R904* of ATL samples (Fig. 2a). This was interesting because early termination of MLL3 is predicted to produce a dominant negative form with oncogenic activities [27]. The presence of R904* on a highly conserved sequence homologous to MLL3 present on chromosome 13 likely contributed to the wrong assignment of a SNP (rs200662726) in position R904* of the MLL3 gene in the NCBI database (Additional file 2: Table S2). Nevertheless, direct sequencing for all ATL DNA samples confirmed LOH for MLL3 in one ATL patient (Fig. 2b and c).

Ten-eleven translocation methylcytosine dioxygenase genes (TET1-3) are involved in DNA demethylation. Our investigations reveal mutation in the coding sequence of TET1 in 15% (2/13). The mutation I1229M has been reported in the cosmic database. We also noticed the presence of TET1 single nucleotide polymorphism (SNP rs3998860) I1123M. This SNP has a global minor allele frequency (MAF) of 0.3067/1536 but was detected in all ATL samples tested (Additional file 1: Table S1). A larger cohort study is needed to confirm these data. No mutations were detected for TET3 (Additional file 1: Table S1). Interestingly, TET2 was mutated at a high frequency of 38% (5/13) in ATL patients. These results are in line with the high rate of somatic TET2 inactivation observed in MDS, MPN, chronic myelomonocytic leukemia (CMML) and AML [28], and they suggest that TET2 may play an important role in ATL pathogenesis. TET2 LOH was found in two ATL patients with nonsense mutations at positions Q876* and Q414* (Fig. 3a), two mutations previously reported in CML patients. For these two ATL
patients, we PCR amplified the TET2 region overlapping these mutations and cloned and sequenced five clones for high tumor grade and matched samples. ATL12 DNA was extracted from a high grade lymph node biopsy from megakaryocytes as a tumor negative control. For ATL11 DNA was extracted from high proviral load (high grade tumor sample) samples before therapy and control sample DNA obtained after complete remission. Proviral loads were confirmed by quantitative real time RT-PCR for all samples (Fig. 1). Both mutations, Q876* and Q414*, were somatic mutations found in TET2 of ATL cells and not detected in control samples (Fig.3b and c). We then analyzed an additional 18 acute ATL patients by direct PCR, cloning and sequencing and found 6/18 (30 %) with the mutation (Fig. 4). Among missense ATL mutations only a mutation in position Q414R has previously been reported, although it was Q414L (COSM1618223). Of note, we found another unrelated ATL patient with a Q876* mutation suggesting this may represent a frequently mutated region for ATL (Fig. 4).
Discussion

In this study we report a high frequency of TET2 missense mutations (8/31 (25 %)) and LOH of TET2 (3/31 (10 %)) in acute ATL patients. These data suggest that TET2 may be involved in HTLV-I pathogenesis and warrant additional studies. Studies have shown that the TET2 mutation results in global low levels of 5hmC compared with normal controls, supporting a
functional relevance of TET2 mutations in leukemogenesis. Activating mutations of IDH1/2 have been shown to be mutually exclusive with mutations of TET2 [29]. Although mutations of IDH1/IDH2 have the same final epigenetic effect as TET2 inactivation, mainly a global promoter hypermethylation, mutation in IDH1/2 was not observed in any ATL samples tested here. Consistent with this notion, increased methylation of CDKN2A promoter has been associated with the progression of ATL disease [30]. Wilms tumor (WT1) mutant AML patients have reduced 5hmC levels similar to the TET2/IDH1/IDH2 mutant in AML, suggesting that WT1 may also play an important role in control of the epigenome [31]. WT1 and TET2 interact with one another. Although there are no reports regarding the genetic status of WT1 in ATL cells, we have previously shown PI3K-dependent cytoplasmic retention and inactivation of WT1 in HTLV-I transformed cells [32]. It will be interesting to evaluate the role of cytoplasmic WT1 in the regulation of TET2 functions in HTLV-I transformed T cells. Although a number of genes have been shown to be hypo- or hypermethylated in ATL cells, a direct implication of these genes in cellular transformation and/or ATL pathogenesis is lacking. In this study, we also found LOH in 1/13 ATL patient for MLL3. ASCOM-MLL3 has a redundant but crucial role in transactivation of p53 and participates in DNA damage-induced expression of p53-targeted genes [33]. Notably, p53 transcriptional functions are impaired in ATL patients in the absence of genetic mutations in p53 [34] and the possibility that loss of MLL3 participates in this process for some ATL patients warrants future studies.
Conclusions

In summary, this report used both next generation sequencing and classic direct sequencing methods to identify mutations of epigenetic regulators in freshly isolated uncultured ATL samples. This study identifies for the first time mutations in multiple genes involved in the maintenance of the epigenome. Notably, a high frequency of mutation was detected in the TET2 gene with the presence of nonsense mutations leading to LOH in ATL patients. Our data suggest that TET2 and possibly MLL3 LOH may be involved in ATL pathogenesis and larger clinical correlative studies will now be needed to assess the effect on prognosis, diagnosis, and treatment of ATL.
<table>
<thead>
<tr>
<th></th>
<th>ATL 1</th>
<th>ATL 2</th>
<th>ATL 3</th>
<th>ATL 4</th>
<th>ATL 5</th>
<th>ATL 6</th>
<th>ATL 7</th>
<th>ATL 8</th>
<th>ATL 9</th>
<th>ATL10</th>
<th>ATL11</th>
<th>ATL12</th>
<th>ATL13</th>
</tr>
</thead>
<tbody>
<tr>
<td>EZH2/2</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
</tr>
<tr>
<td>EED</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
</tr>
<tr>
<td>SUZ12</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>V68A</td>
<td>P745</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
</tr>
<tr>
<td>DNMT1 iso-a</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>T361A</td>
<td>P553E</td>
<td>R1315</td>
<td>M122K</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
</tr>
<tr>
<td>DNMT3A iso-b</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>I130S</td>
<td>V107G</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
</tr>
<tr>
<td>DNMT3B iso-1</td>
<td>wt</td>
<td>wt</td>
<td>N442K</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>N442K</td>
</tr>
<tr>
<td>TET1</td>
<td>I1123M</td>
<td>I1123M</td>
<td>I1123M</td>
<td>K931M</td>
<td>I1123M</td>
<td>I1123M</td>
<td>I1123M</td>
<td>I1123M</td>
<td>I1123M</td>
<td>I1123M</td>
<td>I1123M</td>
<td>I1123M</td>
<td>I1123M</td>
</tr>
<tr>
<td>TET2</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>del R1543</td>
<td>P1545</td>
<td>wt</td>
<td>K1924E</td>
<td>del R1543</td>
<td>P1545</td>
<td>wt</td>
<td>Q876*</td>
</tr>
<tr>
<td>TET3</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
</tr>
<tr>
<td>IDH1/2</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
</tr>
<tr>
<td>MLL</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>52511F</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
</tr>
<tr>
<td>MLL2</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>T598I</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
</tr>
<tr>
<td>MLL3</td>
<td>wt</td>
<td>wt</td>
<td>R284Q</td>
<td>wt</td>
<td>R504C</td>
<td>I590K</td>
<td>wt</td>
<td>V1088L</td>
<td>K433T</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
</tr>
<tr>
<td>MLL4</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>P635R</td>
<td>G378E</td>
<td>E382K</td>
<td>wt</td>
<td>wt</td>
<td>G491S</td>
<td>P635R</td>
<td>wt</td>
<td>wt</td>
</tr>
<tr>
<td>ASK1</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>V1092M</td>
<td>51166R</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
</tr>
</tbody>
</table>
Table 1. Novel mutations identified in epigenetic regulators EZH1, EZH2, EED, SUZ12, DNMT1, DNMT3A, DNMT3B, TET1, TET2, TET3, IDH1/2, MLL, MLL2, MLL3, MLL4 and ASXL1 in ATL patients

The mutations previously reported as SNPs are not included. Novel mutations are labeled in blue (missense) or in red (nonsense). Samples 1–7, 10 and 11 were isolated from patients diagnosed with acute ATL. Samples 8, 9, 12 and 13 were isolated from high proviral load tissues (Fig. 1) from patients diagnosed with lymphoma ATL. (PDF 231 kb)
304


Table 2. Sequence alignment of MLL3 gene and genome regions of very high homology found in chromosome 1, 2, 3, 13 and 21

Nucleotide differences specific to MLL3 are highlighted in red. TGA snp rs200662726 correspond to chromosome 13.
References


Chapter VIII: MiRNA-based therapy: future directions
MicroRNAs are non-coding RNAs reported to be involved in critical biological processes. In past years, deregulation of miRNAs has been documented in several pathological conditions, including cancer. Overexpression and suppression of miRNA expression affect cellular pathways and contribute to carcinogenesis and tumor progression. This evidence makes miRNAs a suitable candidate for therapeutic applications and results in the development of strategies to manipulate their expression. There are different approaches that can be used to restore or reduce miRNA expression [1, 2].

Restoring miRNA expression could represent a successful strategy for cancer treatment. The reintroduction of miRNA function can be obtained by using double-stranded RNA mimics. MiRNA-mimics present a strand that is identical to the miRNA of interest, with the other strand conjugated with a molecule, promoting cellular uptake. Synthetic RNA duplexes are currently modified to improve stability and cellular uptake. [3, 4]. Moreover, chemical modifications can be used to prevent the processing of miRNA-mimics RISC and nucleases to avoid degradation and increase the stability. [5]. Importantly, potential issues can occur by using synthetic RNA duplexes, for example, activation of non-specific interferon response through Toll-like receptors and overexpression of the miRNA of interest over the ‘normal’ level of expression [6].

Moreover, enforced delivery of miRNA mimics might have off-target effects due to the expression of the miRNAs of interest not only in target cells but also other cell types. Consistently, alteration of miRNA expression in normal tissues can generate potential side effects. To avoid this possibility, lenti-, adeno- or adeno- associated viruses can be used. [3].

Alternatively, suppression of miRNA expression can be achieved by antisense miRNAs, which present a complementary binding site of the miRNA of interest to repress its function. Several
chemical modifications have been developed to promote the stability and the pharmacokinetics of anti-miRNA oligonucleotides, such as 2’-O-Methyl (2’-O-Me) and 2’-O-Methoxyethyl (2’-MOE) and bicyclic locked nucleic acid (LNA) sugar modifications. These changes improve the resistance of anti-miRNA oligonucleotides to nuclease and increase the melting temperature (Tm) [7, 8].

The first strategy used to suppress miRNA function in vivo was to link the cholesterol to antagonirs or chemically modified antimiRs [3]. However, the effect was found to be limited. More recent studies show that a nanoparticle encapsulated with antimiRs efficiently inhibits miRNA function both in vitro and in vivo, indicating that they might represent the most efficient methodology to suppress miRNA expression [9-11].

In cancer therapy, miRNA-based therapeutics are finalized to restore or reintroduce miRNA expression. Based on preclinical and clinical studies, miRNA therapeutics appear to be a novel promising strategy for human diseases. Importantly, the first phase II clinical trial testing anti-miR-122 in chronic HCV patients showed it to be a promising and well-tolerated therapy, supporting the notion that pharmacological modulation of miRNA activity might represent a new efficient treatment for cancer.
References


Chapter IX: DNA Repair inhibitions: future directions
HTLV-I-associated diseases have limited therapeutic options. The four-year survival average for acute and lymphoma types of ATL is 5 and 5.7%, respectively [1-14], because HTLV-1-transformed cells are resistant to conventional chemotherapy. Therefore effective therapy for ATL is lacking and development of more efficient treatments is essential.

The genomic instability induced by the viral protein Tax is reported to be involved in cellular transformation [15-25]. In addition, Tax has been shown to constitutively activate NF-kB [26-32], which promotes the proliferation and survival of the infected cells [33-45]. The mechanisms that the oncoprotein Tax used to lead to cellular transformation are different. Tax prematurely activates the anaphase promoting complex [46-49], inhibits nucleotide excision repair [50-52] and affects the topoisomerase activity [53;54] and beta-polymerases [55]. Tax has been reported to interfere with the DNA replication fork and induces DNA double-strand breaks (DDSB).

As discussed in the previous section, Tax inhibits homologous recombination repair (HR) in HTLV-I-transformed cells. Based on these observations we hypothesized that HTLV-I-transformed and ATL cells might be particularly sensitive to small PARP inhibitors, which have been shown to induce selective apoptosis in HR-deficient cell lines, such BRCAness cells. Consistently, our studies have shown that HTLV-1-transformed and ATL-derived cell lines are sensitive to the treatment with PARP inhibitor PJ-34. In the absence of a curative therapy for ATL, our preclinical study has validated the PARP inhibitor PJ-34 as a potential novel therapeutic approach for patients. The future prospective of this project is to study the efficacy of several PARP inhibitors as monotherapy or in combination therapy in vitro and in vivo.

In order to investigate the therapeutic efficiency of PARPi we want to investigate the apoptotic effect of additional PARP inhibitors currently in phase II/III clinical trials, such as Olaparib,
Veliparib, ABT-888, Talazoparib, BMN-673, and Niraparib, MK-4827. For every inhibitor we want to test dose-dependent and time-dependent toxicity in HTLV-1 and ATL-derived cell lines. Interestingly, our study has shown that PJ-34 has a superior apoptotic effect on ATL cells compared to Olaparib. We believe these types of experiments are important to individuate among the different PARP inhibitors which is more efficient for ATL patients. In addition, we will include activated isolated and unstimulated PBMCs isolated form HTLV-1 negative donors to confirm absence of PARPi toxicity at the drug concentration selected above for ATL cells.

Combination therapy using a DNA damaging agent along with PARPi might represent a promising approach because it would lead to the accumulation of DDSB in ATL cells, which might enhance the apoptotic effect of PARPi. The DNA damaging agents Paclitaxel, Doxorubicin or Vincristine have been shown to induce apoptosis in HTLV-I-transformed cells [64-67]. We want to investigate the potential synergistic effects of PARPi in combination with Paclitaxel, Doxorubicin or Vincristine.

Alternatively, we will investigate the effect of small inhibitors of signaling pathways involved in DNA repair or typically activated in cancer cells. Activation of the PI3K pathway is reported in a wide range of tumors and promotes cellular growth and the survival. More importantly, the PI3K pathway plays an essential role in the sensing of DDSB during the S-phase of the cell cycle [68]. Consistently, activation of the PI3K was noted in ATL cells and is involved in cellular proliferation [69-71]. Moreover, the treatment with BKM120, a PI3K inhibitor, has significantly reduced the expression of BRCA1, BRCA2 and, consequently, the efficiency of HR DNA repair.

In addition, an interesting study published by our laboratory shows that Ku80 and DNA-PK specifically co-localized with γ-H2AX foci on DNA breaks in HTLV-I-transformed cells,
suggesting that these cells preferentially repair the DDSB by using Non-homologous End Joining repair (NHEJ). Consistently, when these cells are exposed to NU7026, a specific DNA-PK inhibitor, a significant inhibition of proliferation and survival of HTLV-I-transformed cells were noted [72]. Based on this evidence we would like to test BKM120 or NU7026 alone and in combination with each PARPi on HTLV-I-transformed and ATL-derived cells to evaluate the synergistic effects. Alternatively, since drug synergies can be manifested through concomitant blockade of parallel signaling pathways or dual inhibition of the same pathway at distinct nodes, combination therapies can be better understood (and predicted) by monitoring gene and protein expression in response to a specific pharmacological pressure.
References


22. Hall AP, Irvine J, Blyth K et al. Tumours derived from HTLV-I tax transgenic mice are characterized by enhanced levels of apoptosis and oncogene expression. J.Pathol. 1998;186:209-214.


46. Merling R, Chen C, Hong S et al. HTLV-1 Tax mutants that do not induce G1 arrest are disabled in activating the anaphase promoting complex. Retrovirology. 2007;4:35.

47. Kuo YL, Giam CZ. Activation of the anaphase promoting complex by HTLV-1 tax leads to senescence. EMBO J. 2006;25:1741-1752.


69. Saito K, Saito M, Taniura N, Okuwa T, Ohara Y. Activation of the PI3K-Akt pathway by human T cell leukemia virus type 1 (HTLV-1) oncoprotein Tax increases Bcl3 expression, which is associated with enhanced growth of HTLV-1-infected T cells. Virology 2010;403:173-180.


