TELOMERASE REGULATION AND TELOMERE MAINTENANCE DURING HUMAN T-CELL LEUKEMIA/LYMPHOMA VIRUS (HTLV-I) TRANSFORMATION

By

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Introduction

INTRODUCTION:

Discovery of the first Human Retrovirus:

The closing of the 1970s ushered a new direction for tumor biology when Robert C. Gallo published the first report of a virus capable of causing a human leukemia (1). Prior to this discovery, belief in a human tumor retrovirus was diminishing, as little evidence could be found bringing credence to the theory. Retroviruses had been found in non-human primates capable of causing tumors, but evidence was still lacking in humans. This changed when Gallo and colleagues, who were working on animal retroviruses that caused leukemia, were able to isolate a T-cell specific growth hormone (now known as interleukin-2, IL-2) which allowed growth of normal lymphocytes beyond the one week limit which was achieved when growing cells under phytohemagglutinin (PHA) stimulation (2, 3). In addition, the discovery, and subsequent purification, of a reverse transcriptase (RT) in 1970 (4,5) provided researchers with a powerful tool to identify retroviruses. With these tools, in 1979, Gallo and colleagues isolated human T-cell leukemia virus-I (HTLV-I) from a T-cell line established from a patient, with, what was then described as a cutaneous T-cell lymphoma (6). Around the same time, in 1976, a group of Japanese scientists, led by Kiyoshi Takatsuki recognized a distinct geographical distribution of leukemia, which they termed adult T-cell leukemia (ATL), found in the southwestern region of Japan (7). These patients later tested positive to antibodies to HTLV-I. In 1981, it was agreed that HTLV-I was etiologically linked to ATL, and subsequently found to be endemic to particular regions of Japan, the Caribbean, South America, and Africa. Confirmation for a direct causative role for HTLV-I in ATLL, was

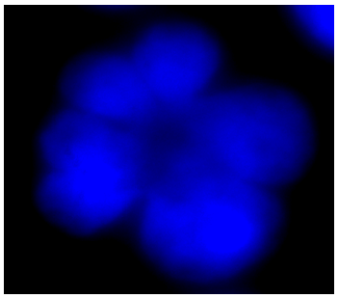
confirmed when it was discovered that proviral integration was nearly all due to clonal infection of leukemic cells, supporting the idea that HTLV-I was the cause of ATL (8). Subsequent studies found HTLV-I to be the etiological agent of tropical spastic paraparesis (TSP) in Jamaica and HTLV-1-associated myelopathy (HAM) in Japan, later established as identical neurodegenerative diseases of the spinal cord (9,10). Since these initial discoveries, HTLV-I has been implicated in some forms of HTLV-I-associated-uveitis (HAU), HTLV-I-assoicated-arthropathy (HAAP), infective dermatitis, and polymyositis (11).

Today it is estimated that 20-30 million people worldwide are infected with HTLV-I, with 1-5% afflicted with adult T-cell leukemia/lymphoma (ATLL) (12). It is estimated that each year, over 700 cases of ATLL are diagnosed, and antibodies against HTLV-I are detected in over 1 million individuals, from Japan alone (13). HTLV-I is endemic to Japan, Africa, South America, the Caribbean basin, southwestern portions of North America and Eastern Europe (14).

ATLL is classified into several forms, including acute, chronic, smoldering, or lymphoma. Approximately 75% of individuals with ATLL manifest with leukemia, the great majority of which are acute. The remaining 25% have a pure lymphoma form, with no blood involvement (15). The clinical forms of ATLL are based largely on the extent of systemic leukemia, organ involvement, and the extent of hypercalcaemia, with the acute and lymphoma forms generally having a worse prognosis. The most common diagnostic parameters include the morphology of circulating lymphocytes, which take on a characteristic “flower cell” appearance due to the presence of polylobulated nuclei (Figure 1), along with

positive tests to HTLV-I serum antibodies and a distinct immunophenotype (15).

Figure 1: "Flower cell" nuclei in HTLV-I infected cell:



DAPI stain of an HTLV-I infected cell, demonstrating the characteristic, polylobutlated nuclei.

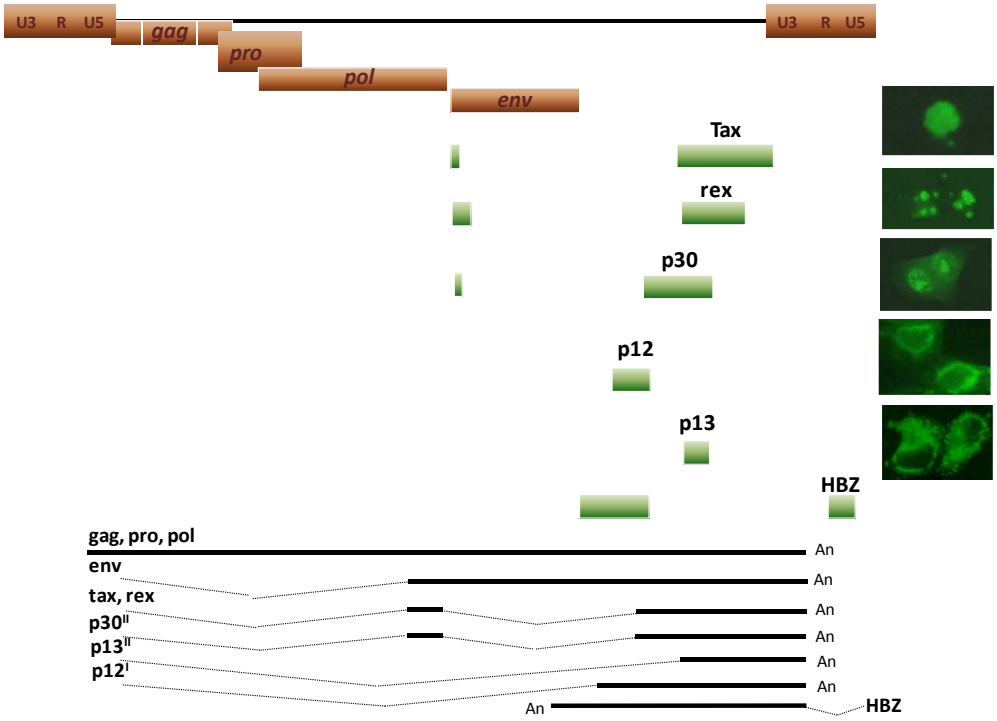
The clinical prognosis of patients diagnosed with acute and lymphoma ATLL is extremely poor. Acute ATLL is rapidly fatal, with a median survival time of 6 months with therapy. Survival times for chronic and lymphoma type ATLL, are 24 and 10 months, respectively (16). Survival times for smoldering ATLL are quite long, but can progress to an acute or lymphoma type ATLL. Fatality is often ascribed to pulmonary complications, opportunistic infections, sepsis, and uncontrolled hypercalcaemia (14).

HTLV-I is primarily spread by vertical transmission through breast feeding, but also occurs by parenteral, sexual, or blood transmission, whereby live infected T-lymphocytes are exchanged via milk, semen, or blood. HTLV-I predominately infects CD4+ T-lymphocytes, with minor infections seen in CD8+ T-lymphocytes, epithelial and endothelial cells, and astrocytes (in the case of TSP/HAM) (17). Viral spread usually occurs by cell-to-cell contact, via viralogical synapses, and infection by fee virions is poor. Upon entering the cell, the diploid RNA genome of HTLV-I is reverse transcribed into DNA, whereupon the provirus integrates randomly into the host genome. HTLV-I multiplication, however, depends

upon the enhancement of proviral-infected cells, and the processes of reverse transcription/integration are usually brief, occurring only during initial infection by new virions (14). Clonal expansion of infected cells is mainly attributed to viral spread.

The HTLV-I genome consists of the *gag*, *pol*, and *env* genes, along with the 5' and 3' long terminal repeats (LTRs) (Figure 2). *gag* encodes for the viral matrix, capsid and nucleocapsid, *pol* encodes for the viral reverse transcriptase, integrase, and protease and *env* encodes for the envelope protein (7)**.** Between the *env* gene and the 5'-LTR, is a unique pX region. This region encodes for the viral regulatory and accessory proteins, tax, rex, p12, p13, p30, and p21, which are encoded through alternatively splicing (Figure 2).

Figure 2: HTLV-I genome and the expression of unique proteins (Adapted from (8)): The unique accessory proteins (green) encoded in the pX region of HTLV, include tax, rex, p30, p12, p13, and HBZ. The cellular localization of tax, rex, p30, p12, and p13 is represented with green fluorescence on the left. The splicing patterns for the unique proteins are indicated below.



Expression of the main viral transactivator protein, Tax, increases viral and cellular transcription by enhancing transcription from the viral 5'-LTR and increasing the activities of key cellular transcriptional regulators, such as NF-κB, serum response factor (SRF), activated protein 1 (AP-1), cyclic AMP response element-binding protein (CREB) and CREB binding protein (CBP) (19). Rex suppresses splicing of HTLV-I transcripts, decreasing viral gene expression by negatively regulating Tax, and instead favoring expression of viral structural proteins (20). p12 increases viral infectivity by increasing nuclear factor of activated T-cells (NFAT) expression and affecting janus-associated kinases (JAKs) signaling, leading to signal transducer and activator of transcription-5 (STAT5) transcriptional activities. p12 also has a role in HTLV-I avoidance of host cellular immunity by retranslocting major histocombatability

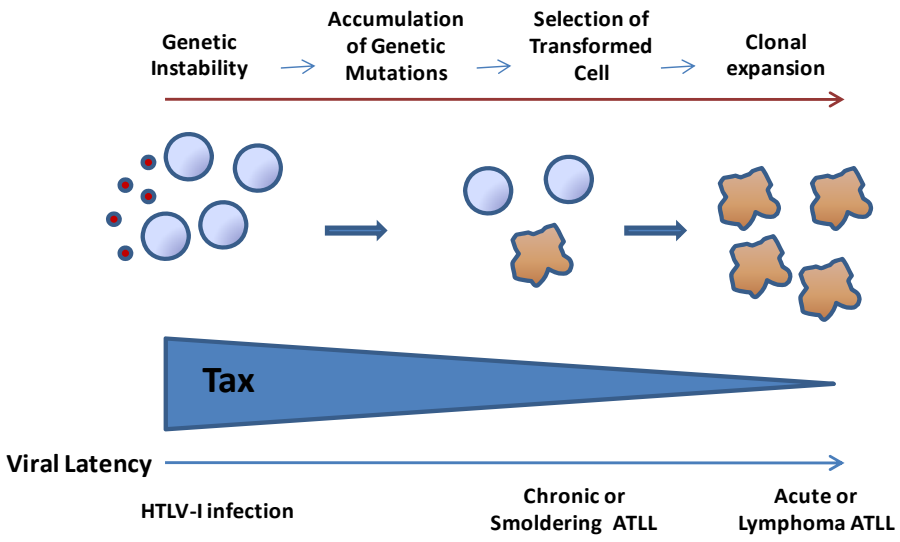
complex I (MHC I) to the cytosol where it is degraded (21). p30 allows for establishment and long-term maintenance of proviral-infected cells by selectively retaining the *tax/rex* message in the nucleus (22). By inhibiting Tax/Rex protein expression, p30 effectively "silences" HTLV-I, allowing for the persistence of cells containing the proviral genome. Recently, the minus strand of HTLV-I has been found to encode for the HTLV-I b-ZIP factor (HBZ), which has dual roles in enhancing or suppressing HTLV-I proliferation (23).

HTLV-I Viral Transformation:

HTLV-I infection is characterized by enhanced viral expression during early stages of disease, with little, to no viral gene expression as the disease progresses. In fact, over 95% of ATLL cell *in vivo* are negative for HTLV-I expression (24). Despite this fact, integrated proviral DNA can readily be detected and culturing of ATLL cells *ex vivo* leads to immediate expression of viral genes. Studies have shown that a single HTLV-I clone can persist in an infected individual for over 7 years (25,26). This suggests that the proviral genome remains intact and is suppressed during latency. Current models of the HTLV-I life cycle, propose that the initial infection of T-cells leads to proviral integration, followed by expression of key viral proteins (ie. Tax), which promote cellular proliferation in absence of apoptosis and cellular senescence (Figure 3). Tax initiates viral transformation; yet viral expression is tempered by the expression of HTLV-I encoded negative regulators, allowing the proviral-harboring cell to persist. With barely detectable levels of viral gene expression, and without the need for

the production of free virions to replicate, HTLV-I provides an environment whereby cells can expand in absence of detection by host immune surveillance mechanisms. Enhanced proliferation of infected cells over a long latency period leads to genetic alterations and mutations, which drive the creation of a malignant phenotype. This model is supported by ATLL disease progression, in which ATLL requires 20-30 years after initial HTLV-I infection to develop.

Figure 3: Model of HTLV-I-mediated cellular transformation. Initial HTLV-I infection is characterized by high expression of the tax protein, which may promote genetic instability during early infection. Over a long latency period, of approximately 20-30 years, ATLL develops in absence of viral protein expression.



HTLV-I-mediated T-cell transformation is largely carried out by over-expressing cellular genes involved in normal T-cell growth and survival and deregulating cellular genes involved in cell cycle

control and DNA damage repair. As stated above, viral transformation, at least in the early stages of disease, is mainly carried out by Tax (27). Tax has been shown to immortalize T-cells and transform fibroblasts *in vitro,* and produce tumors in transgenic mice (28-32).

Expression of Tax leads to up-regulation of several cytokine receptors. Some of their targets, such as, phosphoinositide kinase-3(PI3K)/AKT, and extracellular signal-regulated kinase (ERK)/Jun N-terminal kinase (JNK), are activated in HTLV-I infected cells and contribute to transformation (33,34). Also, transforming growth factor-β (TGF-β) is inactivated in HTLV-I infected cells (35). Arguably the most vital of cytokine receptors in Tax-mediated cellular transformation is the IL-2Rα chain (36, 37). Expression of the IL-2Rα chain allows signaling at low concentrations of IL-2, through a complex consisting of IL-2Rβ and γ. Independence of IL-2 is an established characteristic of HTLV-I transformed cells, whereas HTLV-I infected cells that require exogenous IL-2 are considered immortalized. Though expression of Tax can lead to activation of the IL-2 promoter, IL-2 mRNA and protein are not detected in HTLV-I infected cells. This, along with the fact that HTLV-I immortalized cell lines are IL-2 dependent, suggests that an IL-2 autocrine loop does not contribute to HTLV-I-mediated transformation. Instead, HTLV-I infected cell lines and ATLL cells demonstrate constitutive activation of the JAK/STAT pathways, with constitutive tyrosine phosphorylation of JAK3 and STAT5 (38-41). In addition to Tax, p12 can also stimulate IL-2 production and activation of the JAK/STAT pathway (42).

Another characteristic of HTLV-I transformed cells is their constitutive activation of the NF-κB pathway (36, 37, 43-45). NF-

κB promotes cellular growth and survival, and suppresses proteins involved in cell cycle regulation and DNA repair (46). Studies have shown that NF-κB is essential for IL-2 independent, Tax-induced growth of T-cells (47). Though the exact mechanism of NF-κB activation is not known in ATLL patients, in HTLV-I cells lines, it likely involves deregulation of several aspects of the NF-κB pathway by Tax (46). Expression of Tax leads to phosphorylation and degradation of IκBα and β, negative regulators of NF-κB (48-52). Degradation of IκB is due in part, to Tax stimulation of IκB kinase complexes (IKKs) (53-55). Tax has also been shown to bind IKKγ, the docking subunit of the IKK complex, thus allowing IKK to become constitutively active.

HTLV-I enhances proliferation in infected cells by altering cell cycle regulation and inhibiting apoptosis. Expression of the anti-apoptotic regulators, Bcl-xL and Bcl-2 are enhanced in HTLV-I infected cells, as well as the Bcl-2 related protein A1 (Bfl-1) and human inhibitor of apoptosis (HIAP-1)/cellular inhibitor of apoptosis 2 (CIAP-2) (56-58**).** In addition, activation of AKT, in HTLV-I infected cells, leads to phophorylation of Bad, an inhibitor of Bcl-xL and activation of NF-κB, leading to increased expression of Bcl-xL (59). Expression of Tax has also lead to inhibition of the caspase cascade (60), increases in the expression of survivin in ATLL cells and HTLV-I cell lines (61,62), inhibition Fas-mediated apoptosis, and aiding in the resistance to Apo2ligand/TRAIL-mediated apoptosis (63-65).

p53 is a tumor suppressor which guards the genome by arresting cells in the G_1/S phase of the cell cycle, in order to repair damaged DNA. Around 50% of all cancers show mutations in p53; however, the majority of HTLV-I infected cells have wild-type p53,

with only 25% carrying p53 mutations (66, 67). Despite stable p53 expression, p53 is transcriptionally inactive in the majority of HTLV-I infected cells. It is currently unclear whether p53 activity is inhibited by competition with CBP/p300, perturbations in the NF-κB pathway, and/or a novel, undefined mechanism (68). In addition to inactivating p53, HTLV-I infected cells also inactivate the tumor suppressor, retinoblastoma protein (Rb), which negatively regulates cell cycle progression (69). Tax expression leads to hyperphosphorylation of Rb - thereby releasing the transcription factor, E2F, which can further activate various cyclins, cyclin-dependent kinases (cdks), and proliferating cell nuclear antigen (PCNA), which promote cell cycle progression. Tax has also been shown to bind Rb directly and cause its degradation (69). Tax has pleitropic effects on cell cycle regulators, with the general effect to promote cellular proliferation. Tax increases the expression of cyclins (ie. cyclin D2), cdks (ie. cdk4 and 6) (70) and the anti-apoptotic cyclin kinase inhibitor (CKI), $p21^{WAF/CIP1}$ (71-73). Tax can also negatively regulate other CKIs ($p15^{INK4b}$, $p16^{INK4a}$, $p18^{INK4c}$, $p19^{INK4d}$, $p27^{KIP1}$) (74-77). In addition to these Tax-mediated effects, acute ATLL patient's samples often demonstrate deletions in $p16^{INK4a}$ and $p15^{INK4b}$ (78,79). In contrast, HTLV-I cell lines demonstrate hypermethylation of the $p16^{INK4a}$ promoter (79).

HTLV-I infected cells are predisposed to somatic mutations and chromosome instability, and often have severe cytogentic defects (16,80). In fact, 96% of ATLL cases demonstrate clonal chromosomal abnormalities (Kamada A, 1992). Aneuploidy and chromosome breaks are frequent in ATLL patients, with no specific karyotype abnormality (80,11). Faulty chromosome transmission and failures in cytokinesis occur. This suggests severe impairments

in mitosis. Tax leads to early activation of the anaphase promoting complex (APC), prior to initiation of mitosis that leads to deficiencies in the functions of securin and cyclin B1 (81,82). HTLV-I infected cells also target the functions of centrosomes, and demonstrate centrosome amplification and micronuclei formation (83). HTLV-I infection may also lead to genetic instability by repressing DNA repair mechanisms. Studies have shown that the most affected DNA repair pathway by Tax overexpresion is the base excision repair pathway (BER) (84). HTLV-I infected cells repress DNA polymerase β, which leads to impairments in BER of DNA (84, 85). Nucleotide excision repair (NER) is also repressed in HTLV-I infected cells through Tax activation of PCNA and p53 dysfunction (86-88). In addition, interferon regulatory factor 4 (IRF-4) is constitutively actived in HTLV-I infected cells, and causes a reduction in mitotic and DNA repair genes, including cyclin B1 and Rad51 (89, 90).

Despite Tax's pleitropic roles in promoting cellular transformation, Tax is barely detectable in leukemic cells (24, 91), implying that Tax expression is not required to maintain the transformed phenotype. However, Tax can be detected in seropositive carriers, suggesting it has a more important role in initiating cellular events. During the long latency period that follows infection, the accumulation of genetic mutations and the appearance of a "tax phenotype", in absence of Tax expression, are expected to drive *in vivo* cellular transformation, yet little is known regarding the transition from a Tax-dependent phenotype to a Tax-independent phenotype (16). The process is believed to involve multiple genetic and epigenetic events that promote leukemogenesis and ATLL. According to the cancer theory, a characteristic set of molecular and

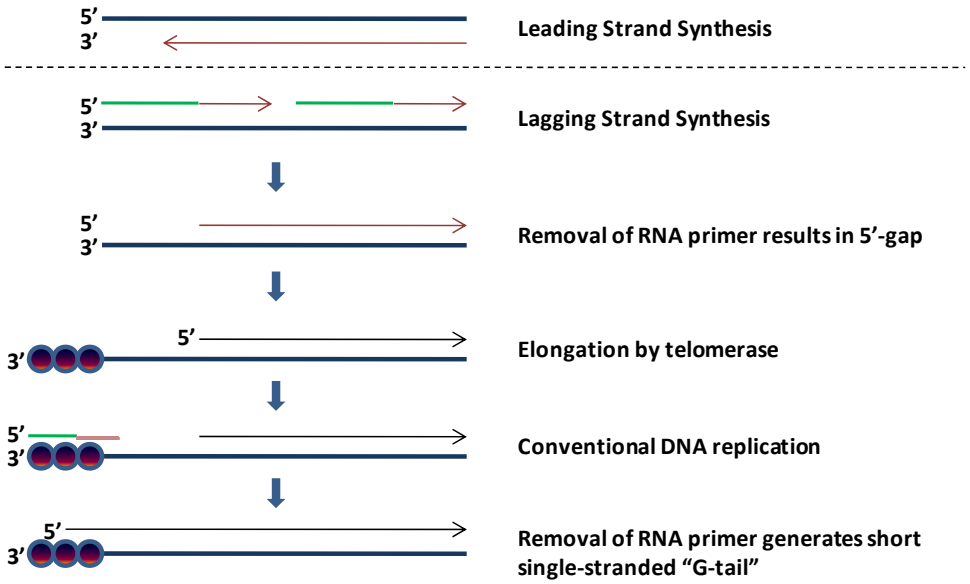
cellular alterations are required in order to obtain and maintain a malignant phenotype. Some studies report at least 5 genetic or epigenetic events must be acquired for leukemogenesis (92). In absence of Tax, somatic mutations and chromosomal instability may provide genetic "hits" that allow an infected cell to proliferate indefinitely. We have found that one of these genetic hits is the reactivation of telomerase.

Telomeres and Telomerase:

Prior to the discovery of the structure of DNA, Barbara McClintock and Hermann Muller realized that chromosomes had unique ends, which they termed telomeres (93). Subsequently, researchers discovered that somatic cells had a finite proliferative capacity due to the "end replication problem": DNA synthesis leads to a progressive shortening of DNA termini after successive cellular divisions. This is due to the fact that DNA can only be synthesized in the 5'-3' direction. To overcome this problem, lagging strand DNA synthesis requires the addition of small RNA oligonucleotides that are used as primers for DNA polymerase. However, removal of the RNA primer from the terminal end of chromosomal DNA, leads to incomplete DNA replication, with a loss of 50-200 nucleotides, which cannot be replicated (94). Through successive replication cycles, this would eventually lead to shortening of DNA, with the possibility of degradation of vital, gene-encoding sequences (Figure 4).

Figure 4: <u>The End Replication Problem (Adapted from(95)).</u> The end replication problem occurs during normal DNA replication, in which there is incomplete DNA synthesis of the lagging strand. This

leads to a loss of DNA after every replication cycle. Telomerase can extend the ends of chromosomes by synthesizing telomere repeats (circles) onto the DNA termini.

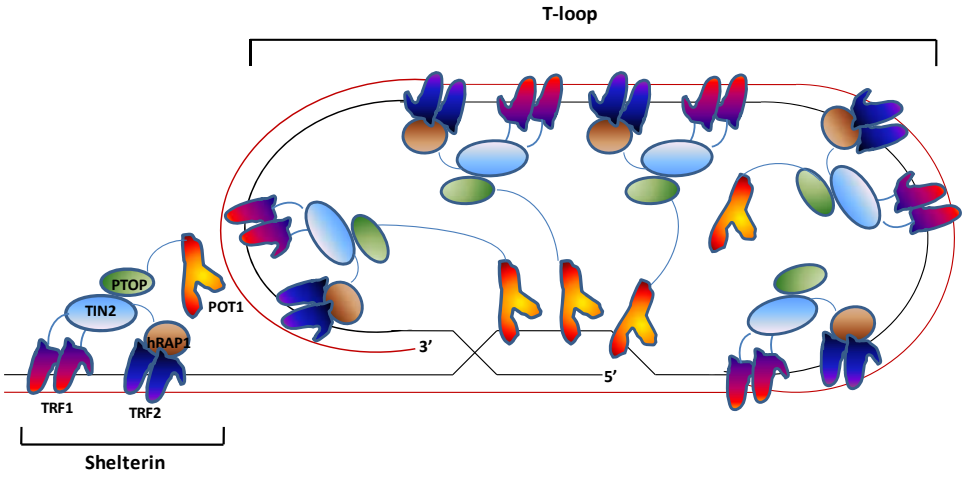


In 1985, Elizabeth Blackburn and colleges discovered that a cellular enzyme, telomerase, could extend the ends of chromosomal DNA by synthesizing repetitive tracts (TTAGGG) of DNA (96). These tandem arrays, or telomeres, which reached an average length of 12 kb in somatic cells, could solve the "end replication" problem.

Telomerase is a ribonucleotide complex, composed of an RNA component (hTR) that partially serves as a template for the synthesis of the six base-pair repeat that is added onto chromosome ends (97). This action requires a reverse transcriptase component, hTERT that uses hTR as a template for synthesizing DNA (96, 98, 99). Additional proteins have been discovered that aid in telomerase activity, along with the assembly and maturation of the telomerase complex (100). In addition, telomeric DNA is bound by various

components of the shelterin complex, which can alter the state of the telomere and protect chromosome ends from fusion and recognition as DNA double-stranded breaks (101) (Figure 5). Shelterin is composed of the TTAGG-repeat binding factors – 1 and -2 (TRF1 and TRF2), their binding partners, TRF1-interacting nuclear protein 2 (TIN2) and hRAP1, along with protection of telomeres (POT1) and its regulator, POT1- and TIN2-interacting protein (PTOP). Shelterin determines the structure of the telomere ends, generates the t-loop (a possible protection mechanism against non-homologous end joining (NHEJ) repair, whereby the single-stranded 3'-end of telomeres invades the double-stranded telomeric DNA), and controls telomerase synthesis of telomeres, by blocking telomerase access to telomere ends (101, 102). Telomere attrition or inhibition of TRF2, TIN2, or POT1 results in unprotected telomeric sequences and activation of DNA damage response factors (101, 103, 104). ATM or ATR kinases are activated leading to p53-dependent G1/S cell cycle arrest, eventually ending in apoptosis or senescence. In p53-deficient cells, telomere dysfunction leads to p16 induction and proliferation inhibition (105).

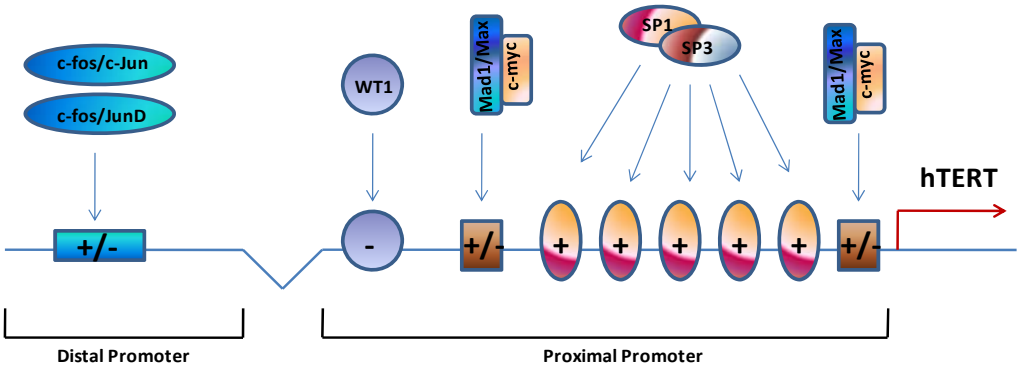
Figure 5: Shelterin bound to Telomeres (Adapted from (106)): A model of telomere ends. Telomeres are bound by various complexes of shelterin, composed of the DNA binding proteins, TRF1 (purple), TRF2 (Blue), and POT-1 (Yellow), and their binding partners, TIN2 (light blue), hRAP1 (Brown), and PTOP (Green). Shelterin can aid in the formation of the t-loop, which may protect telomere ends.



Telomerase is regulated through alternative splicing, epigenetic modifications, and both transcriptional and post-transcriptional control. Alternative splicing leads to the presence of multiple hTERT transcripts carrying deletions of critical motifs required for reverse transcription (107). Post-transcriptional control occurs through several mechanisms. AKT and RelA/p65 can lead to nuclear translocation of hTERT, the former, by phosphorylation of hTERT, the latter by direct binding (108,109). PKC can also phosphorylate hTERT resulting in interactions with hsp90 that have a role in hTERT holoenzyme integrity (110). Alternatively, phosphatase 2A (PP2A) can negatively regulate hTERT through dephosphorylation (111). Despite these regulatory mechanisms, telomerase is primarily regulated at the level of transcription (Figure 6).

Figure 6: Select positive and negative regulators of hTERT transcription. The hTERT promoter consists of the proximal and distal regions, which are bound by transcription factors. Sp1/Sp3 and c-Myc are positive regulators, whereas, WT1 is a negative regulator.

Members of the AP-1 transcription family (c-fos, c-Jun, and JunD) bind to the distal promoter and are positive and negative regulators of hTERT expression.



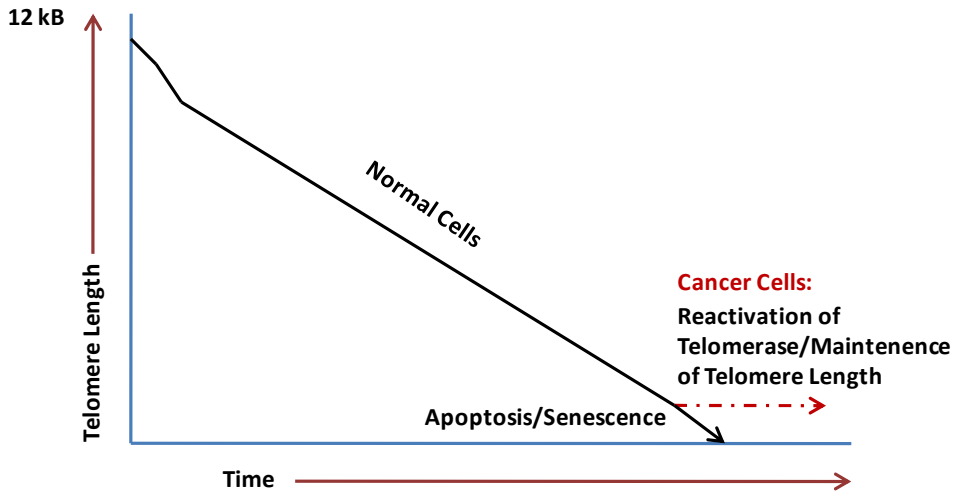
Several E-box and GC-rich elements are present in the hTERT promoter that bind c-Myc and Sp1, respectively (112). These transcription factors have been shown to be strong positive regulators of *hTERT* expression. Other positive regulators include survivin and estrogen (113, 114). Known negative regulators are Wilm's tumor protein (WT1), p53, $p16^{INK4A}$, smad3, and $p27^{KIP1}$ (115-118). A positive and negative role on *hTERT* transcription for the AP-1 transcription factors, E2F-transcription factor (E2F-1), and upstream stimulatory factor (USF-1/2) has also been shown (119, 120).

Telomerase and Cancer:

Telomere length is either maintained at a steady-state length or is lengthened or shortened depending on the level of telomerase and/or the state of the telomere (100). The effects of either a loss or

gain of telomerase function is implicated in the processes of aging and cancer, respectively (Figure 7).

Figure 7: Change overtime in Telomere Length in Normal and Carcinogenic Cells (121)). Normal human cells start with approximately 12kb of telomeres, which are gradually lost during normal DNA replication over the life of the individual. Eventually this leads to either apoptosis or senescence when the telomeres reach a critical length. Cancer cells circumvent this problem, by increasing the activity of telomerase, which can extend the lifetime of a cell.



hTERT is expressed in transformed cells, but not in primary somatic cells in culture (100). In fact, 90% of all cancers over express telomerase, with the additional 10% relying on an alternative lengthening of telomeres (ALT) pathway to maintain telomere length (122, 123). Despite the strong association between telomerase and carcinogenesis, a direct role for telomerase in causing cellular

transformation is unlikely. Telomerase is able to enhance the replication span in somatic cells; however, the cells do not form tumors in mice, nor colonies in soft agar assays (124). When telomerase is overexpressed with either the large T-antigen of simian virus 40 (SV40) and Ras, or when Rb and p53 are inactivated along with expression of Ras and c-Myc, normal human cells can become transformed (125, 126). A direct correlation between telomere length and replicative senescence is clear. Normal cells expressing telomerase, maintain telomere lengths and show indefinite cellular proliferation (127).

The majority of cancer cells demonstrate high telomerase activity with short telomeres (128, 129, 120). A study has shown that the level of telomerase activity mainly depends on the number of tumor cells present, whereby hTERT is detected in nearly all cells in cancers with high telomerase activity, and in fewer cells where telomerase activity is low (130).

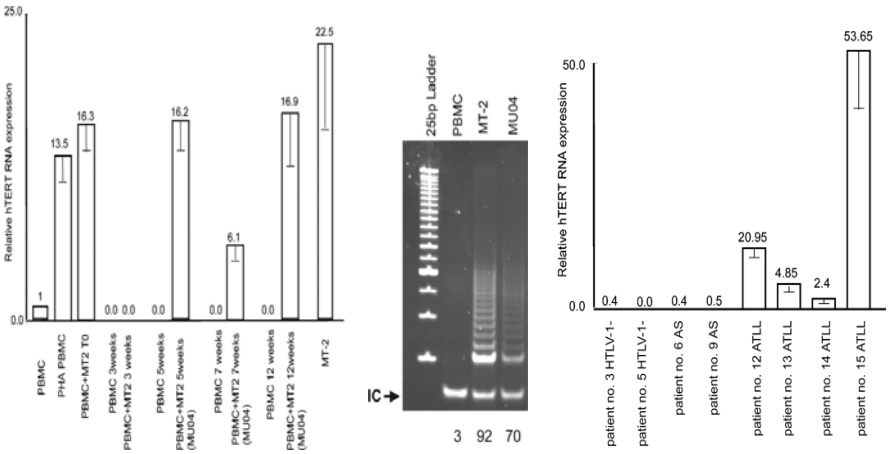
HTLV-I and Telomerase:

Elevated telomerase activity is seen in all ATLL patients, including smoldering, chronic, and acute ATLL (131). Initial studies found that telomerase activity, correlated with disease status, in that acute and chronic ATLL patients demonstrated higher telomerase activity than asymptomatic carriers.

We were the first lab to demonstrate a direct role for HTLV-I in increasing telomerase expression and activity (Figure 8) (132). Co-culturing of PBMCs with a gamma-irradiated HTLV-I producing cell line (MT2), led to increases in telomerase expression and activity. These *in vitro* results were confirmed *in vivo*, as real-time

PCR analysis demonstrated that ATLL patient samples had elevated *hTERT* expression compared to normal PBMCs. We further showed that Tax-mediated NF-κB activation was able to increase *hTERT* expression by increasing the binding of c-Myc and Sp1 to the hTERT promoter.

Figure 8: Increased telomerase expression and activity in HTLV-I cells and ATLL patients. Real-Time quantitative PCR of hTERT expression in normal PBMCs co-cultivated with gamma-irradiated MT2 (HTLV-I producing cell line) cells over time. After 12 weeks of cultivation, the cells became immortalized (MU04). TRAP assay of telomerase activity in normal PBMCs, MT2, and MU04 cells. TPGs are indicated below. Real-Time quantitative PCR of hTERT expression in HTLV-I, negative donors, asymptomatic patients, and ATLL patients.



The following studies continue the evaluation of telomerase and telomeres in HTLV-I infected cells. We demonstrate that telomerase activity is increased in HTLV-I infected cells and ATLL

patient samples, and that this increase is accompanied by a progressive shortening of telomere lengths (133). However, telomere lengths are stabilized, and HTLV-I infected cells are prevented from entering cellular senescence and apoptosis, by an increase in shelterin components, including TRF1, TRF2, and TIN2. Continuing the study of telomerase regulation in HTLV-I cells, we find that the IL-2R signaling pathway is vital in promoting strong telomerase activity (Bellon M and Nicot C, Submitted). IL-2R signaling leads to PI3K activation, which allows for cytoplasmic retention of the telomerase inhibitor, WT1.

Finally, we demonstrate that inhibition of telomerase activity leads to cellular senescence in HTLV-I infected cells and ATLL patient samples (134). We find that treatment with AZT (zidovudine), can lead to complete or partial remission of ATLL patients that carry a functional p53.

The data presented herein, clearly demonstrate a strong association between HTLV-I infection and telomerase activity. Similar to all oncogenic viruses, telomerase is required to maintain long-term proliferation and tumorgenesis during HTLV-I disease progression.

CHAPTER I

Increased Expression of Telomere Length Regulating Factors TRF1, TRF2 and TIN2 in Patients with Adult T-Cell Leukemia

This work was published in *International Journal of Cancer*: 2006; 119, 2090-2097.

Increased Expression of Telomere Length Regulating Factors TRF1, TRF2 and TIN2 in Patients with Adult T-Cell Leukemia.

ABSTRACT:

Here, we report that freshly isolated unstimulated adult T-cell leukemia (ATL) cells present high telomerase activity compared to asymptomatic carriers or normal donors. In spite of this high telomerase activity, ATL cells retained shorter telomeres compared to those of uninfected cells isolated from the same patients. Because the safeguarding of telomere length is critical to the unlimited proliferation of tumor cells, we investigated the underlying mechanism for short telomere maintenance in ATL cells. Transcriptional and post transcriptional expression of telomere binding proteins TRF1, TRF2, TIN2 and POT1, known to regulate telomere homeostasis and protection, were evaluated. We found that TRF1 and TRF2 are over-expressed in *in vivo* patient's samples from ATL but not asymptomatic carriers while levels of POT1 expression did not specifically increase in ATL. To gain insights into the regulation of *TRF* genes in HTLV-I infected cells we investigated the expression of *TIN2*, a regulator of these genes, and found an increase

in *TIN2* expression in ATL patients. Together our results underscore the importance of telomerase and telomere length regulating factors as novel markers for ATL disease progression and as potential therapeutic targets for the treatment of HTLV-I-associated malignancies.

INTRODUCTION:

Human T-cell leukemia virus type 1 (HTLV-I) is the etiological agent of adult T-cell leukemia/ lymphoma (ATL), an aggressive and fatal lymphoproliferative disorder (1,2). The poor prognosis of ATL patients is associated with the resistance of neoplastic cells to the conventional combination of high-dose chemotherapy and radiotherapy (3). The mechanism by which HTLV-I engenders ATL is not clear, but the long latency period of several decades preceding the disease suggests it relies upon long term survival and proliferation of virus-infected cells (4-6). In fact, previous studies have shown that proliferation of ATL cells occurs mainly by replication of infected cells leading to oligoclonal or monoclonal expansion (4-6). HTLV-I has evolved regulatory mechanisms to usurp cell cycle checkpoints (7-12) and apoptosis regulators (13-17), and infected cells may escape from immune defenses using a

combinatorial effect of the regulatory proteins p30 and p12 that reduce viral expression and down-regulate major histocompatibility complex (MHC) expression, respectively (18-19).

The DNA polymerase is unable to replicate telomeric structures, and thus each cellular division results in a progressive shortening of the telomere length. While telomere shortening may be associated with increased chromosomal instability and may facilitate carcinogenesis, it can also lead to irreversible senescence. Although in most cancer cells avoidance of telomere shortening beyond a critical size requires reactivation of telomerase expression, the sole expression of telomerase may not be sufficient for telomere elongation due to the "open or closed" nature of the telomeric ends, which controls accessibility of telomerase to the telomeres(20). The nature of the telomeric ends is governed by the binding of TTAGGG repeat binding factors, TRF1 and TRF2, which act as negative regulators of telomere length (21-23), and of the protection of telomere, POT1, which binds to the single-stranded TTAGGG repeats of telomeres, protecting the ends of chromosomes and relaying signals of telomere length through its binding with TRF1 (24,25). Studies have shown that enforced over-expression of TRF1 or TRF2 leads to rapid telomere shortening while prolonged over-expression of TRF1 is associated with the maintenance of stable,

short telomeres. Many other factors involved in the regulation of telomere length have been identified, including the TRF1-interacting nuclear protein 2, TIN2, which can interact with both TRF1 and TRF2, and indirectly influence telomere length (26). In tumor cells, a dynamic equilibrium exists in the relative expression of these factors in order to maintain the telomere length and endure unlimited cellular proliferation.

Here we report that *in vivo* HTLV-I-infected ATL patient's samples have high telomerase activity as evidenced by TRAP assay. Despite this finding we found that telomere lengths were significantly shorter in ATL cells when compared to non-infected cells isolated from the same patients. Analysis of telomere binding factors showed no specific alteration in the expression of POT1. However, we found a significant increase in *TRF1*, *TRF2* and *TIN2* gene expression in ATL patients compared to asymptomatic carriers. These results were further confirmed at the protein level, suggesting that these factors represent novel makers for ATL and may play a role in disease progression. TRF1, TRF2, and TIN2 have been shown to inhibit apoptosis and DNA damage response cell cycle arrest and may contribute to the proliferation of ATL cells despite telomere attrition. The presence of shorter telomeres in ATL cells compared to normal cells and the elevated expression of TRF1 and TIN2 support

the concept that telomeric binding proteins may be used as therapeutic targets and warrant future investigations.

RESULTS:

We and others have reported that HTLV-I infection increased hTERT mRNA expression 27-(27-31), in part through activation of the NF-kB pathway and stimulation of c-Myc and Sp1 binding to the promoter region of the hTERT promoter (27). Telomerase activity was assessed by the telomeric amplification protocol assay (TRAP) using the TRAPeze telomerase detection kit (Intergen, NY), in IL-2-dependent or independent, immortalized or transformed, HTLV-I cell lines established *in vitro*. Our results indicated high telomerase enzymatic activity in all HTLV-I expressing cells independent of their IL-2 requirement status (Fig.1A).

Figure 1: Increased Telomerase activity in HTLV-I infected cells in *in vitro* and *in vivo* ATL samples. (A) TRAP assay in human leukemic T-cells lines not infected with HTLV-I (Jurkat and CEM), HTLV-I transformed cell lines (MT-2, HUT-102, C91-PL, C10MJ, MT-4, and C8166), and IL-2-dependent HTLV-I cell lines (1185, SP, and LAF). (B) TRAP assay for detection of telomerase activity in AS: asymptomatic and ATL: adult T-cell leukemia patients. PBMC:

Resting Peripheral Blood Mononuclear Cells served as a control. CHAPS buffer and TSR8, served as negative and positive controls for telomerase activity, respectively. The 36 bp internal amplification control band (IC) is indicated by an arrow. (C) Detection of *PinX-1* expression by RT-PCR in ATL and asymptomatic patient's samples. Amplification of *GAPDH* in non-saturating conditions was used to normalize RNA samples. Primers are described in Table1.

FIGURE 1A

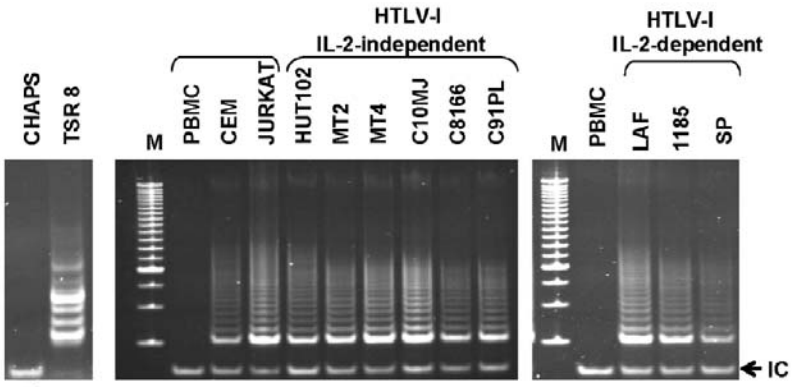


FIGURE 1B

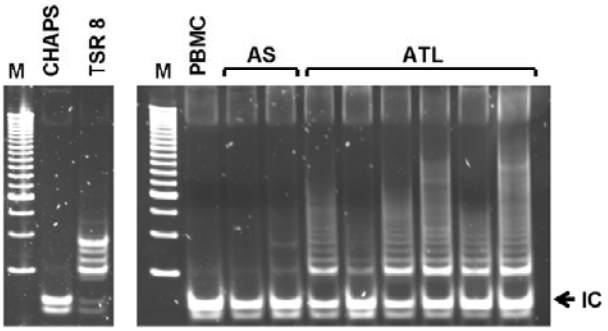
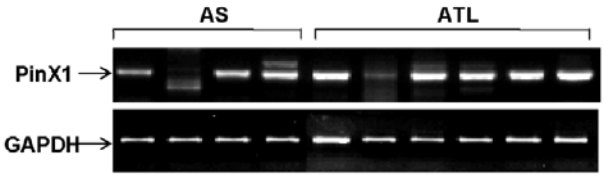


FIGURE 1C



We then investigated telomerase activity in *in vivo* ATL patient's samples. Freshly isolated, uncultured, samples from HTLV-I-infected asymptomatic (AS) carriers and adult T-cell leukemia (ATL) patients were tested for telomerase activity. Resting peripheral blood mononuclear cells (PBMC) isolated from a healthy donor not infected with HTLV-I were used as a negative control. Telomerase activity was much higher in all ATL patients when compared to HTLV-I infected asymptomatic carriers and, as expected, control resting PBMCs showed no activity (Figure 1B). These findings are in agreement with our previous results showing an increased expression of the *hTERT* catalytic subunit mRNA in ATL patients as compared to asymptomatic carriers as well as a recent study showing high levels of telomerase in ATL patients (32). Recent studies showed that the tumor suppressor, PinX1, acts as a potent inhibitor of telomerase activity *in vitro* and *in vivo* (33). Expression of many tumor suppressors is prevented in ATL cells through either gene deletion or promoter hypermethylation (34-36), raising the possibility that high telomerase activity detected in ATL as compared to asymptomatic carriers (Fig.1B) resulted from the inactivation of *PinX1* expression. Analysis of PinX1 mRNA expression in ATL and asymptomatic samples showed that this was not the case as expression of *PinX1* mRNA was readily detected in all ATL samples tested (Fig.1C).

Although we can not rule out a posttranscriptional inactivation mechanism, in absence of a PinX1 specific antibody, expression of this protein can not be investigated in ATL samples at the moment and warrants further studies.

To further investigate whether the high telomerase activity present in HTLV-I infected cells may be associated with longer telomeres, the average telomere size of HTLV-I cell lines was determined by Southern blot analysis (Fig.2A).

Figure 2: HTLV-I infected cells have short telomeres. (A). Southern blot analysis of the telomere lengths in HTLV-I transformed (MT2, MT4, C8166, C91PL and C10MJ) and IL-2-dependent HTLV-I-infected cell lines (LAF and 1185). (B) TRF (telomere restriction fragments) average telomere lengths are reported.

Figure 2A

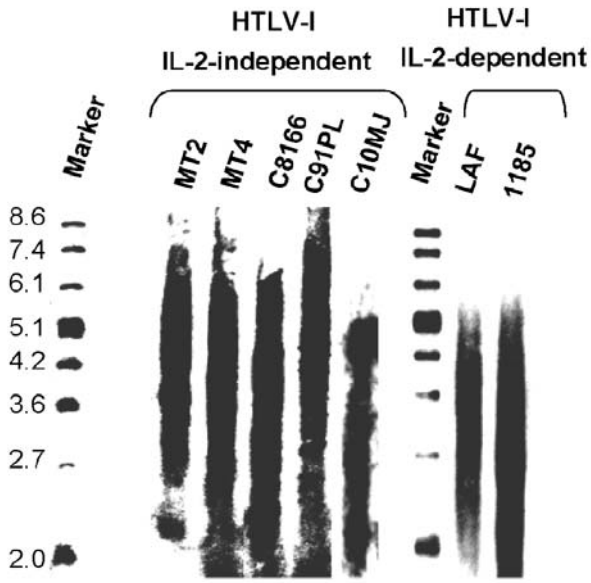


Figure 2B

TRF	MT2	MT4	C8166	C91PL	C10MJ	LAF	1185
Kbp	5.5	4.6	4.5	5.2	3.5	3.7	3.6

While normal human T-cells have an average telomere size of 15 Kbp, our results indicated that all HTLV-I established cell lines have shorter telomeres, 3.5 to 7 Kbp (Fig.2B). As observed in many solid (37) and hematologic cancers (38,39), ATL cells retained shorter telomere lengths comparable to the average telomere sizes of other human leukemic cell lines not infected with HTLV-I, such as Jurkat and CEM (data not shown). Due to the limited amount of samples available, the average telomere size of ATL cells cannot be measured by Southern blot and was determined by quantitative fluorescence *in situ* hybridization (Q-FISH) using the Telomere PNA /FITC kit. To

avoid potential variations in telomere size between different patient's samples, we cell sorted HTLV-I-infected from non-infected cells from five uncultured ATL patient samples and compared their relative telomere size. As HTLV-I infected ATL cells overexpress the CD25 activation marker on their surface, we used anti-CD25-coupled magnetic beads to sort HTLV-I infected from their non-infected counterparts. Although regulatory T-cells share the same phenotype as ATL cells, CD4+/CD25+, their relative abundance, about 5% compared to 50% or more in ATL cells in the peripheral blood of acute leukemia ATL patients, is negligible and does not affect our assay. Successful sorting was confirmed by staining each sorted cell population with an anti-CD25 fluorescein-isothiocyanate (FITC)-conjugated antibody, and fluorescence-activated cell sorter scanner (FACS) analysis showed at least 95% purity (Fig.3A).

Figure 3: HTLV-I cell lines and ATL cells have short telomeres.

(A) CD25 positive and CD25 negative sorted cells analyzed by FACS after staining with an anti CD25-FITC conjugated antibody. PBMCs were incubated with or without a FITC-conjugated PNA telomere probe and analyzed by FACS. (B) Three ATL patient samples were sorted into CD25 positive and CD25 negative cell populations and each were subsequently analyzed for integrated

HTLV provirus by PCR. PBMC and MT-2 were used as negative and positive controls, respectively. PCR amplification of GAPDH served as a control. (C) Five ATL patient samples were used for CD25 cell sorting and both CD25 positive and CD25 negative cell population were analyzed by Q-FISH and telomere sizes calculated from two independent measurements. Telomere sizes in PBMC and MT-2 were measured by Q-FISH for comparison.

Figure 3A

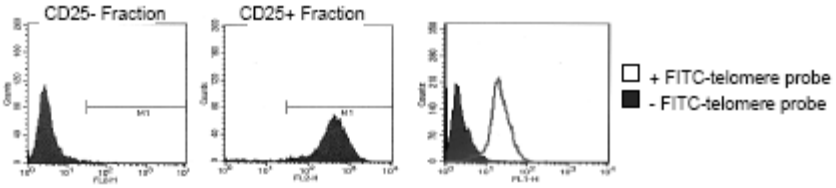


Figure 3B

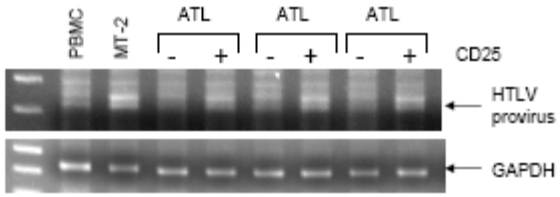
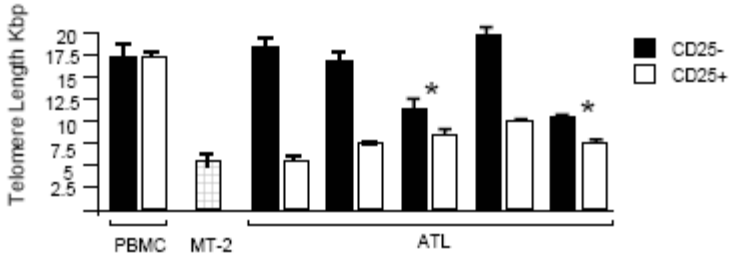


Figure 3C



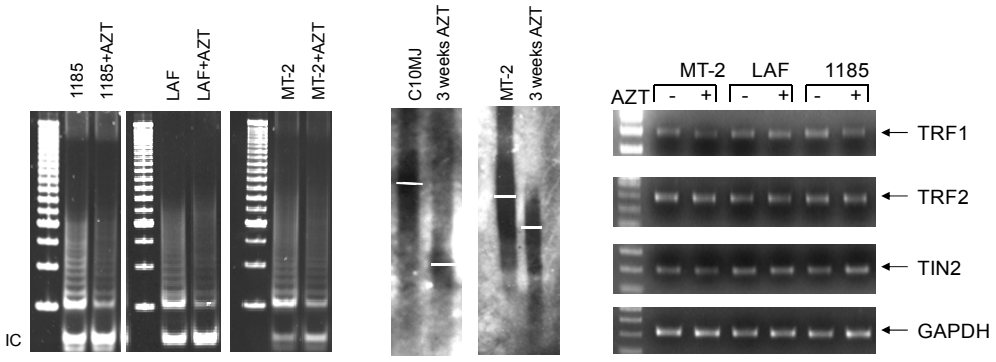
To further ensure that CD25+ sorted cells correspond to HTLV-I infected cells and not uninfected CD25+ regulatory T-cells (Tregs), CD25+ and CD25- cells isolated from PBMCs of a healthy

individual and those of an ATL patient were tested by TRAP assay. Telomerase activity was only detected in the CD25+ population isolated from the ATL patient and no telomerase activity was detectable in either population isolated from the non-HTLV-I infected donor (data not shown), suggesting that Tregs have barely detectable telomerase activity and that the CD25+ cell fraction isolated from the ATL patients indeed corresponds to HTLV-I infected cells. In fact, integrated HTLV-I provirus was detected by PCR in the CD25+, but not CD25- cells isolated from ATL patients (Fig.3B). Due to the limited amount of ATL samples available, telomere sizes were measured in five patients. Strikingly, our results demonstrated that despite the high telomerase activity in HTLV-I-infected cells from ATL patients, these cells maintained significantly shorter telomeres compared to their non-infected PBMC counterparts isolated from the same patient (Fig. 3C). For three of the five ATL patients, the difference in telomere size, between HTLV-I-infected and non-infected cells, averaged a remarkable 10 Kbp shorter in ATL cells and reached a significant 3 Kbp for the other two patients tested (Fig. 3C). We next investigated whether the maintenance of short telomeres in HTLV-I infected cells was dependent on telomerase activity. The use of the reverse transcriptase inhibitor,

azidothymidine (AZT), efficiently reduced telomerase activity in various HTLV-I-infected cell lines (Fig.4A).

Figure 4: Telomerase dependent telomere length maintenance in HTLV-I infected cells.

(A) TRAP assay in HTLV-I transformed (MT-2) and IL-2-dependent HTLV-I-infected cell lines (LAF and 1185), as described in Figure 1. (B) Cells were grown continuously with AZT or DMSO control for three weeks. Southern blot analysis of telomere lengths in HTLV-I cell lines untreated or treated with AZT as described in Figure 2. (C) RT-PCR detection of *TRF1, TRF2,* and *TIN2* mRNA expression in HTLV-I cell lines untreated or treated with AZT. RNA samples were normalized to *GAPDH* amplification in non-saturating conditions. Primers are described in Table1.



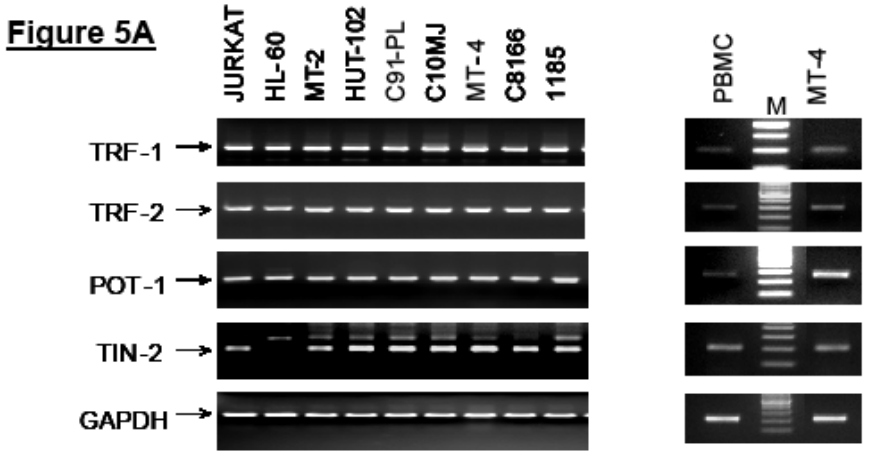
After 3 weeks of culture in the presence of AZT, HTLV-I infected cells had significant reductions in telomere size, suggesting that the maintenance of short telomeres requires telomerase, which could be used as a potential therapeutic target (Fig.4B). Telomere shortening was independent from variations in expression levels of shelterin proteins TRF1, TRF2 and TIN2 (Fig.4C).

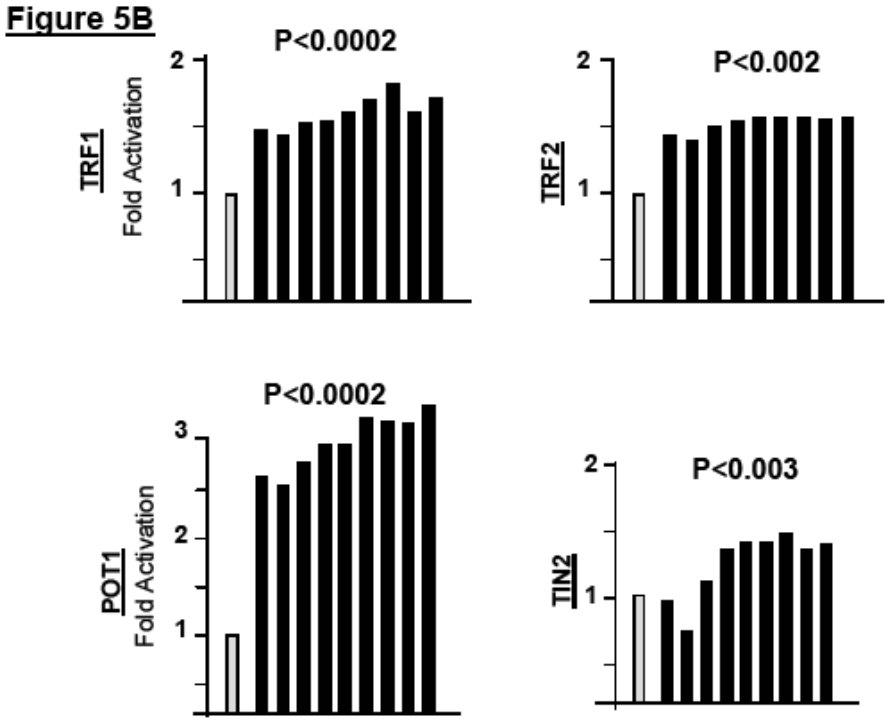
The fact that in ATL patients telomere lengths were significantly shorter in virus-infected cells, suggests that despite an increased expression of telomerase and telomerase activity, the latter may not gain access to the telomeric ends in ATL cells. To investigate this hypothesis further, we analyzed mRNA and protein expression of telomere-binding proteins, TRF1, TRF2 and POT1, known to regulate access of telomerase to the telomere and promote

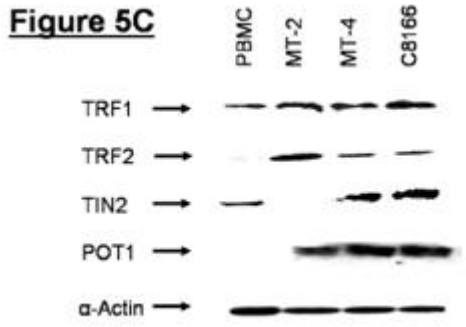
telomere length shortening. Results indicated similar levels of expression for *TRF1*, *TRF2 and POT1* in the HTLV-I negative human leukemic cell lines Jurkat or HL60, as well as in HTLV-I transformed or immortalized cell lines (Figure 5A). We found no correlation between HTLV-I transformation status (IL-2 independent), and the *in vitro* expression of *TRF1*, *TRF2* and *POT1* genes (Fig.5A and 5B). However, comparison of mRNA extracted from HTLV-I transformed MT-4 to PBMCs, indicated the overexpression of *TRF1*, *TRF2* and *POT1* in human leukemic cells and HTLV-I expressing cells (Figure 5A).

Figure 5: *In vitro* expression of telomere binding proteins TRF1, TRF2, TIN2 and POT-1. (A) Detection of *TRF1*, *TRF2*, *TIN2* and *POT-1* mRNA expression by RT-PCR in HTLV-I cell lines and leukemic cell lines Jurkat and HL-60. RNA samples were normalized to *GAPDH* amplification in non-saturating conditions. Primers are described in Table1. Comparison of *TRF1*, *TRF2*, *TIN2* and *POT-1* mRNA expression in MT-4 and PBMC was evaluated by RT-PCR. (B) Spot densitometry quantification of mRNA expression PBMC (gray bar), HTLV-I negative and positive leukemic cell lines in the order shown in A (black bars). Statistical analysis by the student *t*-tests against PBMC control was performed and values are

presented. Increased TRF1, TRF2, POT-1 and TIN2 were found to be highly significant. (C) TRF1, TRF2, TIN2 and POT-1 protein expression was detected by western blot analysis. Equal amounts of protein loaded were confirmed by α-actin detection.







These results were further confirmed at the protein level by western blot analysis of TRF1, TRF2 and POT1 in three HTLV-I cell lines (MT-2, MT-4 and C8166) compared to the PBMCs of a HTLV-I negative donor (Figure 5C). Previous studies have demonstrated that TRF1 and TRF2 interact with double-stranded telomere repeats, and that the amount of DNA-bound TRF proteins influences telomere length (40). In addition, TRF1 and TRF2 have been shown to interact with TIN2, which in turn regulates their activity to modulate telomere length. To gain insights into the possible functions mediated by the up-regulated expression of *TRF* genes in HTLV-I infected cells, we also investigated the expression of *TIN2*. As shown in figure 5A and 5B, with the exception of MT-2, HTLV-I infected cells expressed comparable levels of TIN2 compared to Jurkat or normal PBMC. In agreement with previous studies, levels of TIN2 were low in HL60.

Finally, we analyzed samples isolated from HTLV-I-infected asymptomatic and ATL patients. We also included PHA-stimulated and resting PBMCs, from an HTLV-I negative donor as an additional comparison. No difference was found in the levels of expression of *TRF1*, *TRF2* or *TIN2* between CD25+ and CD25- purified T-cells from resting PBMC of non infected patient (data not shown). However we found a significantly higher expression of *TRF1* and, to a lesser extent, *TRF2* in ATL patients as compared to HTLV-I-infected asymptomatic carriers or PBMC (Figure 6A and B).

Figure 6: Expression of telomere binding proteins TRF1, TRF2, TIN2 and POT-1 in uncultured AS and ATL samples.

(A) Detection of *TRF1*, *TRF2*, *TIN2* and *POT-1* mRNA expression by RT-PCR in AS and ATL samples. RNA samples were normalized to *GAPDH* amplification in non-saturating conditions. Primers are described in Table1. (B) Spot densitometry quantification of mRNA expression PBMC (gray bar), asymptomatic carriers (white bars) and acute ATL (black bars). Statistical analysis by the student *t*-tests between ATL and asymptomatic was performed and values are presented. Increased TRF1, TRF2 and TIN2 were found to be highly significant. (C) TRF1, TRF2 and TIN2 protein expression was detected by

western blot analysis. Equal amounts of protein loaded were confirmed by α-actin detection.

Figure 6A

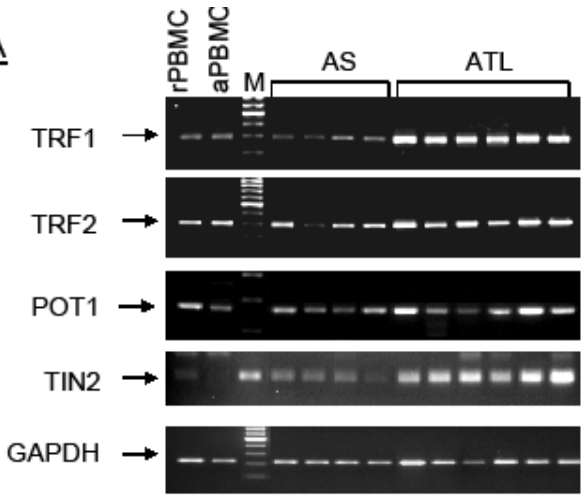
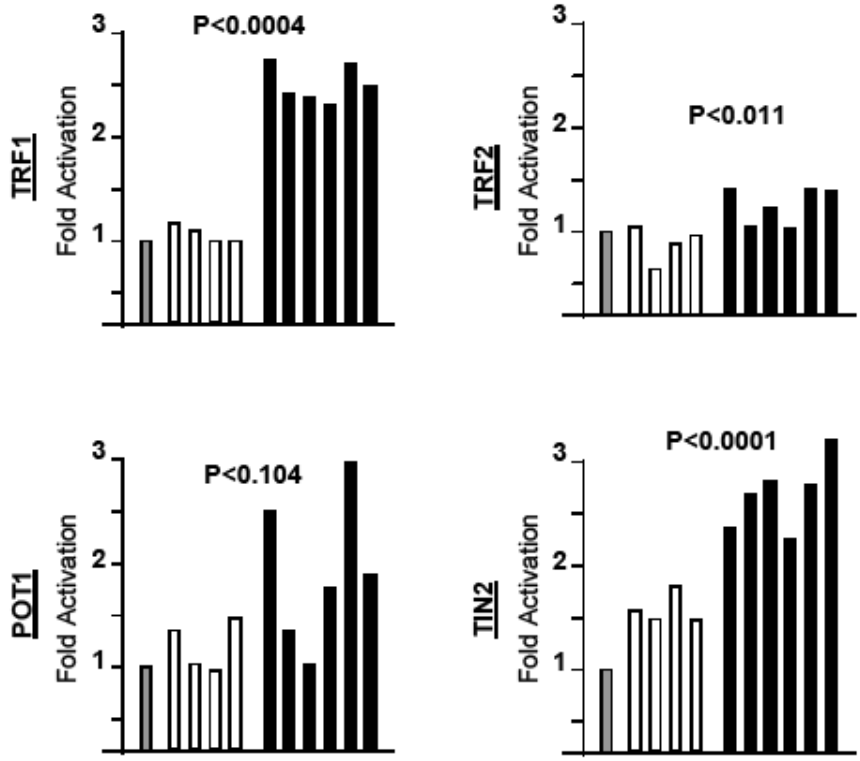
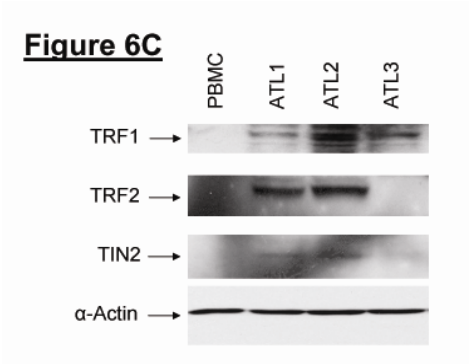


Figure 6B





In contrast, transcriptional levels of *POT1* expression did not correlate with HTLV-I infection or the disease status (Fig.6A and B). The upregulation of TRF1 and TRF2 was confirmed in immunoblots, in which an increase in expression of TRF1 and TRF2 was seen in comparison to PBMCs (Figure 6C). In clear contrast to the *in vitro* established HTLV-I cell lines, analysis of patient's samples revealed a specific increase in *TIN2* expression in ATL, when compared to asymptomatic carriers or an HTLV-I negative donor, both at the transcriptional and post transcriptional levels (Figure 6A and 6C).

DISCUSSION:

Recent studies have found that short telomeres and high telomerase activity are associated with tumorigenesis and poor prognosis in cancer patients. In myelodysplastic syndromes, short telomeres correlated with an increased risk for transformation to acute leukemia and in chronic myeloid leukemia with a worse prognosis (41). In

myeloma, cases with high telomerase activity and short telomeres had an increased mortality rate (42), and in chronic lymphocytic leukemia (CLL) shorter survival was associated with short telomeres and high telomerase activity (43).

Additional reports have shown telomere shortening to be associated with the up-regulation of telomere-binding proteins, TRF1, TRF2, and TIN2. Gastric carcinomas with short telomere lengths expressed significantly stronger telomerase activities along with higher TRF1 expression (44). TRF2 and TIN2 also tended to be expressed at higher levels than those with longer telomere lengths. In addition, human multistep hepatocarcinogenesis has been shown to express higher levels of TRF1, TRF2, and TIN2 (45).

Our study is the first to examine the expression of telomerase and telomere binding factors in HTLV-I infected cells *in vitro* and in *in vivo* patient's samples. We found that *in vivo* patient's samples from ATL, but not asymptomatic carriers, have high telomerase activity in absence of an alteration in *PinX1* mRNA expression. The telomeres of many human tumor cell lines, in which a high telomerase activity is present, do not grow but remain stable at a setting characteristic for each individual cell line. Results from our quantitative FISH analysis indicated a telomeric length of 10-20 kb in normal human T-cells not infected with HTLV-I. However, in all

ATL patients analyzed, telomere lengths were significantly shorter suggesting that despite an increased expression of telomerase, the latter may not gain access to the telomeric ends in ATL cells. The telomere size was shown to be dependent upon telomerase activity, as treatment of HTLV-I transformed cell lines with a pharmacological inhibitor, AZT, caused drastic reduction in telomerase activity, followed by a significant decrease in telomere lengths. To account for the shorter telomere lengths seen in ATL patients with an increase in telomerase activity, we investigated whether telomerase is being limited in its access to telomeric DNA. We investigate the expression of telomere-binding proteins TRF1, TRF2 and POT1, known to prevent recruitment of telomerase to the telomeric ends. While POT1 expression was not specifically affected, both TRF1 and TRF2 were over-expressed in ATL samples when compared to asymptomatic carriers. It has been reported that over-expression of TRF1 and TRF2 leads to a progressive shortening of telomere length in telomerase-positive cells, possibly facilitating genetic instability and tumor progression. However, after an initial period of telomere shortening, the telomeres stabilized at a reduced length even though TRF1 levels remained high. Thus, increased expression of TRF1 and TRF2 may explain the shorter telomeres found in ATL cells.

Our results are also consistent with a recent report showing that tumor cells with short telomere lengths exhibited higher TRF1 expression and tended to express TRF2. Current research demonstrates TRF2's likely role in regulating the replicative state of the cell. Studies have shown that TRF2 inhibition results in the induction of apoptosis in a p53- and ATM-dependent manner (46). Furthermore, overexpression of TRF2 likely protects short telomeres by reducing the senescence set point in p53-deficient cells, thereby extending the proliferative capacity of pre-cancerous cells by preventing replicative senescence(47). We have found TIN2 expression to be increased in all ATL patients when compared to asymptomatic carriers, consistent with similar observations demonstrating an increase in TIN2 expression during carcinogenesis (48). TIN2 has been shown to bind TRF1 and TRF2, thereby stabilizing TRF2 onto telomeric DNA. The up-regulation of TIN2 expression, along with TRF1, in patients infected with HTLV-I, may therefore, aid in stabilizing TRF2 onto telomeres, consequently preventing apoptosis and the activation of the DNA damage response pathway in tumor cells with short telomeres (49). We postulate that by protecting such cells, TRF2 and related telomere accessory proteins may extend the proliferative capacity and decrease the senescence set point in ATL cells.

Our findings suggest that increased expression of TRF1, TRF2, and TIN2 may be associated with the establishment and the maintenance of short telomeres in ATL patients, which may increase chromosomal instability and the risk of progressing to the acute stage of ATL. The maintenance of high TRF1 and TIN2 expression may prevent apoptotic signals resulting from short telomeres. As telomere length is significantly higher in non-tumoral cells from ATL patients, interfering with TRF1 or TIN2 expression could lead to specific death or senescence of ATL cells, suggesting that these genes constitute potential targets for future ATL therapy.

MATERIALS AND METHODS:

Cell Culture: Human leukemia cell lines Jurkat and HL60 and HTLV-I-transformed cell lines MT-2, HUT102, C91PL, C10MJ, MT-4 and C8166 were cultivated in RPMI 1640 (GIBCO) with 10% fetal bovine serum (FBS, GIBCO), supplemented with 2mM glutamine, 1% penicillin-streptomycin and 0.4% gentamicin. HTLV-I-immortalized cell lines 1185, SP, and LAF were cultivated in the presence of IL-2 (50U/ml, Roche Molecular). Cells from exponentially growing lines were used in the different assays described in the study.

Patient Samples:

All patients' samples were obtained after informed consent was received. Clinical data is provided in Table 1. HTLV-I infection was confirmed by ELISA and detection of proviral DNA. Acute ATLL was defined according to the Lymphoma Study Group of the Japan Clinical Oncology Group. Lymphocytes from the peripheral blood of healthy volunteers, asymptomatic carriers, or ATL patients were purified by Ficoll-Hypaque gradient. Cells were lysed in TriZol reagent (Invitrogen) for RNA and DNA extractions. HTLV-I infected (CD25+) and uninfected (CD25-) cells, of the same donor, were used for telomere length analysis. Sorting was performed using anti-CD25 magnetic beads (Dynall) according to manufacturer's instructions. Purity was confirmed by staining with anti-CD25 and FACS analysis.

RNA Extraction and RT-PCR:

Total RNAs were extracted from exponentienally growing cells. Potential DNA contaminants were removed by treatment of RNA samples with 10U of Deoxyribonuclease I (Invitrogen) in100 µl of DEPC treated water containing 4 µl of ribonucleoside vanadyl complexes (Sigma) and 160U of RNaseOUT (Invitrogen). Absence of DNA contamination in RNA preparations was confirmed by RT-PCR reactions without a reverse-transcription step (data not shown).

RT-PCR was carried out using the OneStep RT-PCR kit (Qiagen) according to manufacturer's instructions. Appropriate primers, indicated in Table 2, were added at a final concentration of 1 μM. A reverse transcription step temperature was set at 50˚C for all samples and GAPDH amplification in non-saturating conditions was used as a control to monitor RNA quantity and quality. cDNA products were visualized by electrophoresis using 1.8% agarose gels stained with Ethidium Bromide.

Telomeric repeat amplification protocol (TRAP) assays

TRAP assays were performed using the TRAPeze telomerase detection kit according to manufacturer's instructions (Intergen, NY). Patient samples were lysed in CHAPS buffer and 400 ng (5μl) was used per reaction. Extension was performed for 30 minutes at 30°C followed by polymerase chain reaction (PCR) as previously described. Telomeric products were separated on 8% Tris boric acid EDTA (TBE) gel and stained with SYBR Green.

Telomere length assay by quantitative fluorescence in situ hybridization (Q-FISH) and Southern blot.

The telomere length of CD25 positive and negative cells from ATL patients were quantitatively determined by flow cytometry using

Telomere PNA /FITC kit (Dako Cytomation, Carpinteria, CA) according to manufacturer's instructions. Briefly, the sample DNA was denatured by incubating cell suspensions at 82˚C for 10 minutes and hybridized at room temperature overnight in the dark with or without FITC-conjugated PNA telomere probe in the hybridization solution. The cells were washed twice with the provided wash solution and analyzed by flow cytometry (FACSCalibur, Becton Dikinson, San Jose, CA). Southern blots were performed as previously reported (50).

Western Blots:

Antibodies were as follow: α-actin (C-11, Santa Cruz Biotechnology, Santa Cruz, CA), at a dilution of 1:1200. α-TRF1 (C-19, Santa Cruz Biotechnology), α-TRF2 (IMG-124, Imgenex, San Diege, CA), and α-TIN2 (AM77, Oncogene, San Diego, CA) antibodies were used at dilutions of 1:1500, 1:1500, and 1:4000, respectively. α-POT-1 (#978, a gift from T. de Lange) was used for cell line immunoblots, using the Guanidine-HCl western protocol. α-POT-1 (a gift from C. Harris), used at 1:2000, was used for patient immunoblots. After blocking in 5% nonfat dry milk, all blots were incubated overnight at 4^{o}C with primary antibody. Appropriate secondary antibodies were applied for 2 hours at room temperature.

Proteins were detected using chemiluminescent substrate solution (Pierce, Rockford, IL).

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CHAPTER II

Central Role of PI3K in Transcriptional Activation of hTERT in HTLV-I Infected Cells

This work has been submitted for publication to *Blood* 2008.

Central Role of PI3K in Transcriptional Activation of hTERT in HTLV-I Infected Cells.

ABSTRACT:

The persistence of human T-cell leukemia/lymphoma virus-I (HTLV-I) infected cells is dependent upon clonal expansion and up-regulation of telomerase (hTERT). We previously found that in IL-2-independent transformed HTLV-I cells, Tax strongly activates the hTERT promoter through NF-κB-mediated Sp1 and c-Myc activation. However, in IL-2-dependent cells and ATLL patient's samples, Tax expression is very low, yet these cells retain strong telomerase activity suggesting the possible existence of compensatory mechanisms. In this study, we demonstrate that telomerase activity is significantly decreased upon IL-2 withdrawal from immortalized HTLV-I cell lines. Inhibition of PI3K or AKT signaling pathways drastically reduced telomerase activity in HTLV-I cells. We found that IL-2/IL-2R signaling was associated with a PI3K-dependent/AKT-independent transcriptional up-regulation of the endogenous hTERT promoter. We also found that the Wilm's Tumor (WT1) protein strongly suppressed hTERT promoter expression but not an hTERT promoter mutated for the WT1 binding site. These data demonstrate that activation of the PI3K pathway

promotes cytoplasmic retention of WT1, thereby inhibiting WT1 binding to the hTERT promoter. The importance of this regulatory pathway for telomerase expression is underscored by the findings that the PI3K pathway is commonly found activated in cancer cells.

INTRODUCTION:

IL-2 (interleukin-2) is required for the differentiation and long-term proliferation of T-cells. IL-2 induces activation of janus kinases and signal transducers and activators of transcription (JAK/STATs), leading to the induction of Shc/Ras/Raf/mitogen-activated protein kinase (MAPK) and PI3K/AKT pathways (1). These pathways promote cellular proliferation, survival, and differentiation (2). Consequently, they are frequently deregulated in hematological malignancies, including B-cell acute and chronic lymphoblastic leukemias, T-cell childhood and adult acute lymphoblastic leukemias, and various myeloid/lymphoid leukemias.

HTLV-I is the causative agent of adult T-cell leukemia/lymphoma (ATLL) (3). During initial infection, HTLV-I infected cells are dependent upon IL-2, possibly contributing to the early clonal expansion of infected T-cells through an IL-2/IL-2R autocrine/paracrine loop. Disease progression, however, occurs in the

absence of IL-2 secretion or expression, and when HTLV-I infected cells become transformed the cells no longer require IL-2. While the steps leading to IL-2 independence remain to be elucidated, HTLV-I transformed cells constitutively express the IL-2R and acquire constitutive activation of PI3K and JAK/STAT pathways required for the growth of HTLV-I infected cells (4-7).

In order to sustain long term proliferation, leukemic cells must acquire several oncogenic events – two of which bypass apoptosis and replicative senescence. In *in vivo* HTLV-I infected ATLL cells apoptosis is in part inhibited by increased expression of the anti-apoptotic protein, Bcl-xL (8). The avoidance of replicative senescence in ATLL cells is associated with an increase in telomerase activity (9), a cellular reverse transcriptase that prolongs the lifespan of cells by extending the ends of chromosomes, or telomeres. During successive replication cycles telomerase (hTERT) lies down repetitive TTAGGG repeats provided by the template RNA (hTR). While *hTR* is constitutively expressed in all cells, the catalytic subunit of telomerase, *hTERT*, is transiently expressed, and its expression is the rate-limiting step for telomerase activity (10,11).

We have shown that *hTERT* mRNA is overexpressed in HTLV-I infected cells and in ATLL patients (12). In these cells, Tax stimulation of NF-κB induced activation of c-Myc and Sp1, thereby

upregulating hTERT promoter expression. We also demonstrated that IL-2 dependent and independent HTLV-I cell lines and ATLL cells possess strong levels of telomerase activity, independent of their transformation status (13). More importantly, treatment with interferon and azidothymidine (AZT), which inhibits telomerase activity, results in cellular senescence of HTLV-I infected cells, and disease remission in ATLL patients carrying a functional p53 (9). In the present study, we demonstrate that upon IL-2 withdrawal telomerase activity is rapidly and substantially reduced in IL-2-dependent HTLV-I infected cells. Using inhibitors of downstream IL-2R targets, we identified a novel PI3K-dependent/AKT-independent pathway that potently regulates transcription from the hTERT promoter.

RESULTS:

In agreement with previous studies (14), immunoblot analysis revealed high levels of Tax in HTLV-I transformed cells lines (MT-2 and MT-4) compared to HTLV-I immortalized cell lines (LAF, 1185, SP) (Figure 1A).

Figure 1. Removal of IL-2 from HTLV-I immortalized cells decreases telomerase activity. (A) Total cell extracts from IL-2 independent (MT2 and MT4) and IL-2 dependent (LAF, 1185, and SP) cell lines were immunoblotted with anti-Tax. Actin served as a loading control. (B) LAF, 1185, and SP cells were cultured in the presence (+) or absence (-) of IL-2 for 24 hours followed by TRAP analysis for telomerase activity. TPGs are calculated as the percentage of activity compared to cells grown with IL-2 (100%), as previously reported[9]. (C) LAF cells were analyzed at either 4 hours or 24 hours following growth in either IL-2-containing or IL-2-deprived media. For each time point, cells were collected and analyzed by FACS for cell cycle and TRAP for telomerase activity. TPGs are calculated as the percentage of activity compared to cells grown with IL-2 (100%).

Figure 1A:

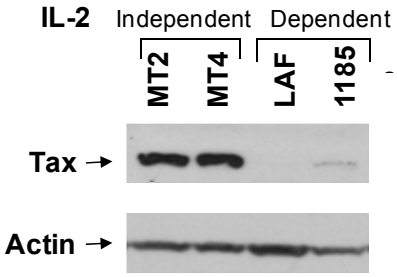


Figure 1B:

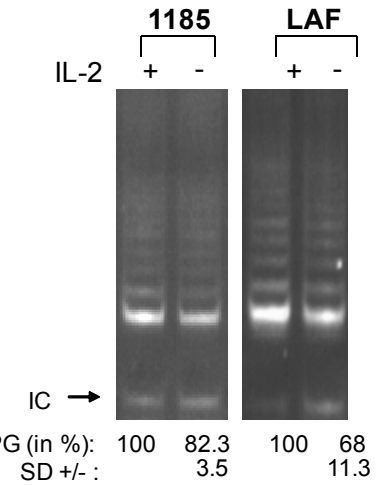
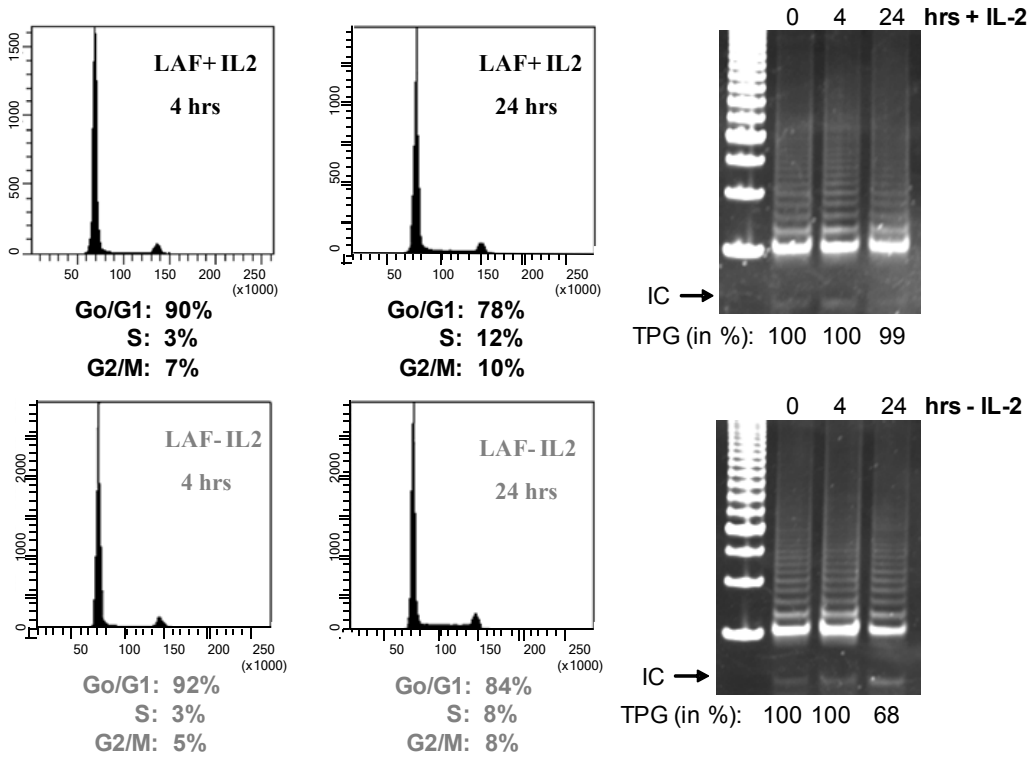


Figure 1C:



However, HTLV-I immortalized cell lines have levels of telomerase activity similar to those of transformed cell lines (13), suggesting that in HTLV-I immortalized cell lines, activation of telomerase occurs in part through a Tax-independent pathway. Since HTLV-I immortalized cells are strictly IL-2-dependent for long term but not short term growth, we hypothesize that IL-2/IL-2R may transduce a signal that compensates for the low levels of Tax expression to stimulate telomerase activity in pre-leukemic stages. Consistent with this hypothesis, when IL-2 was removed from HTLV cell lines (LAF, 1185, SP), telomerase activity was significantly decreased (Figure 1B). IL-2 withdrawal was less sensitive to 1185 cells compared to other HTLV-I immortalized cell lines, probably due to higher levels of Tax expression.

To determine if IL-2 removal affected cell cycle distribution, LAF and 1185 cells were grown in the presence or absence of IL-2 for 4 and 24 hours. FACS analysis revealed marginal differences in cell cycle distribution between cells cultivated with or without IL-2 (Figure 1C and data not shown). Our results demonstrate that IL-2 withdrawal had no significant effect on cell cycle distribution. More importantly, when cells were grown in the same conditions, telomerase activity was decreased but there was no significant effect on cell cycle (Figure 1C). Thus, in agreement with published studies

(15,16), hTERT enzymatic activity is constitutive and not significantly affected throughout the cell cycle, independent of IL-2. In contrast, accessibility of the telomeric ends by hTERT is restricted to the S phase (17). In addition, the TRAP assay used in this study is an *in vitro* measurement of hTERT activity on a template provided by the manufacturer. Therefore, the TRAP assays are independent of cell cycle distribution, as shown by our data.

PI3K is a downstream target of IL-2R signaling and is constitutively activated in HTLV-I infected cells, where it may be required for T-cell transformation (18). To determine if PI3K has a role in telomerase activity, two HTLV-I, IL-2-dependent cell lines and two HTLV-I, IL-2-independent cell lines, with constitutive PI3K activation, were assayed for telomerase activity following LY294002 (LY29) treatment, a pharmacological inhibitor of PI3K (19). Our results show a significant decrease in telomerase activity in all cell lines tested, confirming that PI3K activation is essential for sustaining strong telomerase activity in HTLV-I infected cell lines (Figure 2A).

Figure 2. The PI3K and AKT pathways are associated with increased telomerase activity in HTLV-I immortalized and transformed cells. (A and B) IL-2 independent (MT4 and C8166)

and dependent (LAF and 1185) cells were treated with LY29 (LY294002- 10 μM) or control, LY30 (LY303511- 10 μM), for 48 hours, followed by TRAP analysis for telomerase activity. In (B), cells were treated with AKT Inhibitor II (20 μM) or DMSO control (solvent control). TPGs are calculated as the percent activity compared to cells grown without inhibitor (100%). To verify inhibitor efficacy, total cell extracts were immunoblotted with anti-phosphorylated-AKT (P-AKT). Anti-AKT served as a loading control. The percentage of decreased expression of P-AKT was calculated by spot densitometry as indicated, with cells grown in either LY30 or DMSO considered to be 100%. (C) HTLV-I cell lines, MT4 and LAF, were treated for 48 hours with either LY29 (10μM), AG490 (50 μM), or control DMSO. Cells were subsequently stained for Annexin V and propidium iodine (PI), and analyzed for apoptosis by FACS analysis. The percentage of dead cells for each treatment is indicated. (D) 1185 and LAF cells were cultivated without IL-2 and in the presence of LY29 (10 μM) for 48 hrs. Following the 48 hr incubation period, the cultures were split and IL-2 was added in the absence or presence of LY29 (10 μM) for an additional 24 hrs. TRAP analysis was performed and TPGs are calculated as the percentage of activity compared to cells grown without treatment (100%). For a positive control, cells were grown

for 48 hrs without IL-2, then treated an additional 24 hrs without IL-2, and with or without LY29 (10 μM). Cells grown continuously in IL-2 served as a negative control.

FIGURE 2A

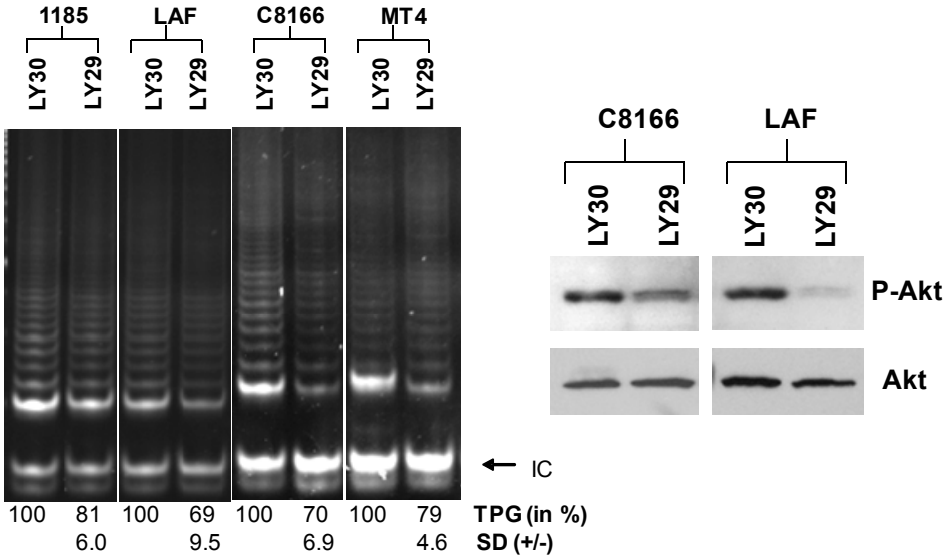


FIGURE 2B

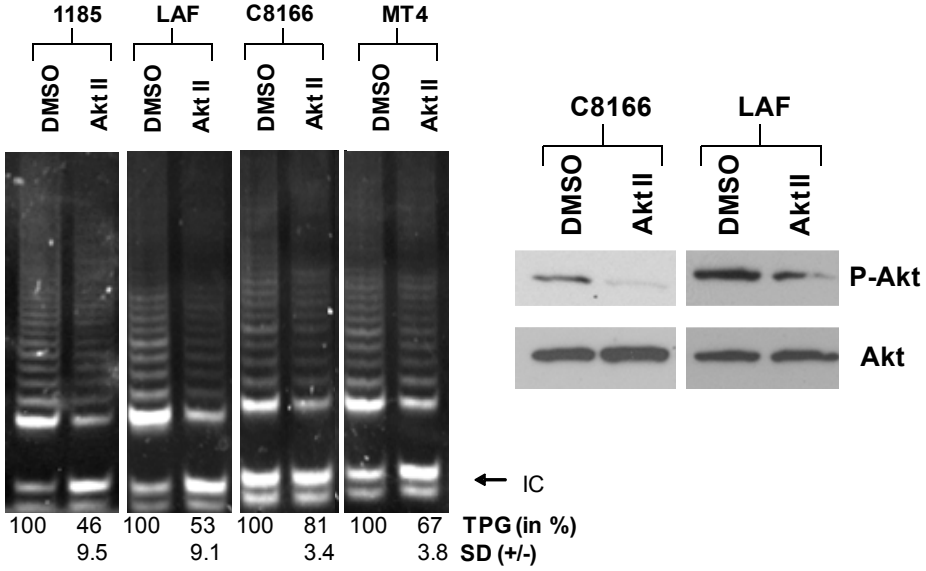
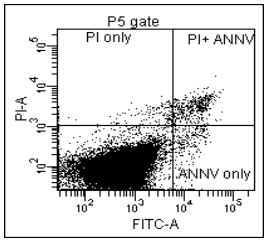


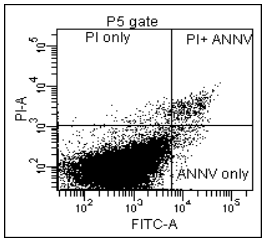
FIGURE 2C

MT4: DMSO



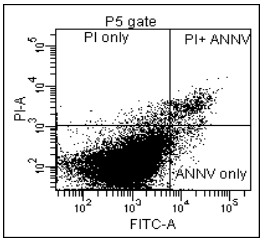
PI: 0.6%
PI +ANNV: 1.7%
NEG: 96.8%
ANNV: 1.0%

MT4: LY29 (10μM)



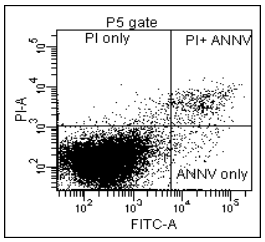
PI: 0.6%
PI +ANNV: 1.7%
NEG: 96.7%
ANNV: 1.0%

MT4: AG490 (50μM)



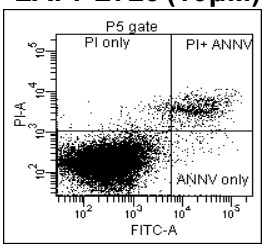
PI: 1.3%
PI +ANNV: 2.6%
NEG: 94.5%
ANNV: 1.5%

LAF: DMSO



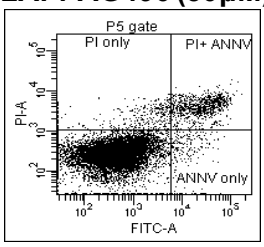
PI: 1.5%
PI +ANNV: 2.6%
NEG: 94.5%
ANNV: 1.5%

LAF: LY29 (10μM)



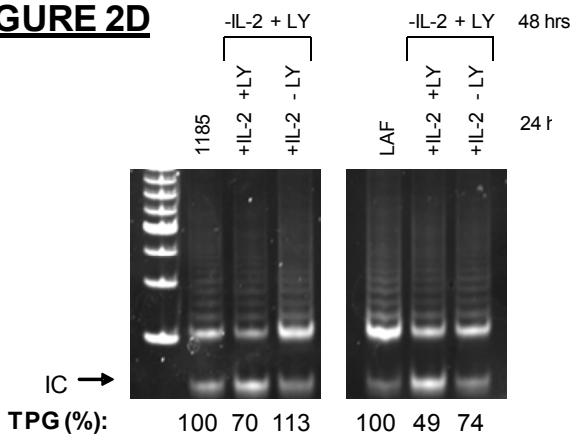
PI: 1.4%
PI +ANNV: 4.5%
NEG: 93.7%
ANNV: 0.7%

LAF: AG490 (50μM)



PI: 2.6%
PI +ANNV: 6.5%
NEG: 89.2%
ANNV: 1.6%

FIGURE 2D



To verify the efficacy of LY29 treatment, cell extracts were assayed for AKT phosphorylation, a downstream target of PI3K. Western blot analysis showed decreased AKT phosphorylation at Ser473, while levels of AKT remained unchanged.

The Tax protein of HTLV-I has been shown to stimulate AKT activity (20), a target of PI3K signaling. Inhibition of AKT, following treatment with the specific inhibitor, AKT Inhibitor II (AKT II), resulted in decreased telomerase activity in both immortalized and transformed HTLV-I cell lines (Figure 2B). This is in agreement with published reports, in which AKT increased telomerase activity by phosphorylating hTERT, thereby increasing hTERT activity at the posttranscriptional level (21).

Previous studies have reported growth inhibition and induction of apoptosis upon treatment with LY29 and AG490, a Jak inhibitor (22,23). To demonstrate that loss of PI3K signaling, and not an increase in cellular death, caused the decrease in telomerase activity, we treated MT4 and LAF cells with LY29 or AG490 for 48 hours and analyzed cells for apoptosis by annexin V/propidium iodine (PI) staining. AG490 was used as control, because our data showed no effect of AG490 on telomerase activity (data not shown). FACS analysis demonstrated marginal increases of 0.1% and 0.8% in cell death for MT4 and LAF cells, respectively, upon treatment with

LY29 (Figure 2C). Treatment with AG490, caused a slightly greater increase in MT4 and LAF cell death, (0.8% and 4.5%, respectively). These results are in agreement with a previously published report, in which treatment with AG490 did not inhibit proliferation in HTLV-I cell lines (24). Since AG490 had no effect on telomerase activity, these data demonstrate that inhibition of telomerase activity by LY29, is due to inhibition of the PI3K-pathway and independent from cellular death.

To confirm the role of the IL-2/IL-2R/PI3K pathway in stimulation of telomerase activity, we cultured 1185 and LAF cells in media without IL-2 and with LY29 for 48 hours. These cells were then divided into cultures with IL-2 in the presence or absence of LY29 for an additional 24 hours. IL-2 pulse increased hTERT activity only when LY29 was absent (Figure 2D). Unlike 1185 cells, LAF cells pulsed with IL-2 did not completely rescue telomerase activity in 24 hours. This could be do to the lower amount of Tax in LAF cells, or a longer period of recovery is needed. These data suggest that most of the IL-2R signaling involved in hTERT promoter regulation occurs through the IL-2/IL-2R/PI3K pathway. As a control, cells were grown in the absence of IL-2 for 48 hours, followed by treatment with or without LY29. As was expected,

telomerase activity decreased following IL-2 removal, which was further decreased upon LY29 treatment (Figure 2D).

The PI3K pathway transcriptionally regulates *hTERT* expression in HTLV-I infected cells.

To determine whether PI3K and AKT signaling acted at the transcriptional and/or post-transcriptional levels, we treated 1185 cells with PI3K and AKT inhibitors for 4 hours. Quantitative real-time PCR revealed a rapid and considerable loss of *hTERT* expression following treatment with LY29, which was not seen to the same extent with treatment with AKT II (Figure 3A).

Figure 3. Telomerase activity is regulated at the level of transcription by a PI3K-dependent pathway in HTLV-I cell lines. (A) 1185 cells were treated with LY29 (10 μM), AKT II (20 μM), LY29 (10 μM)/AKT II (20 μM) or DMSO (solvent control) for 4 hours, followed by analysis of *hTERT* RNA expression by quantitative real-time PCR. *GAPDH* expression was used as a control. Time 0 hrs indicates cells prior to inhibitor treatment. (B-D) 1185 cells were treated with LY29 (10 μM), AKT II (20 μM), LY29/AKT II (10 μM/20 μM), or DMSO (solvent control) for 48

hours, followed by TRAP analysis for telomerase activity (B), FACS analysis for cell cycle (C), or FACS analysis for apoptosis (D). TPGs are calculated as the percentage of activity compared to DMSO (100%). (E) 1185 cells were grown with or without IL-2 for 4 hours followed by western blot analysis. Nuclear extracts were probed with anti-NF-κB RelA/p65, and cytoplasmic extracts with anti-IκB-α.

FIGURE 3A

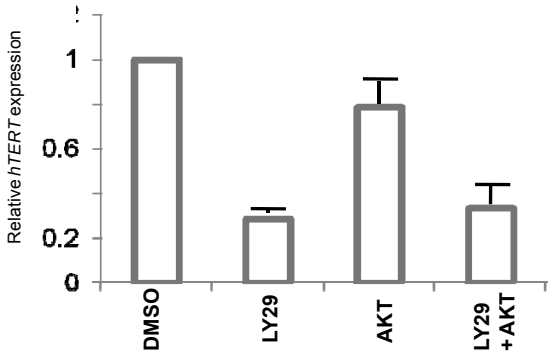


FIGURE 3B

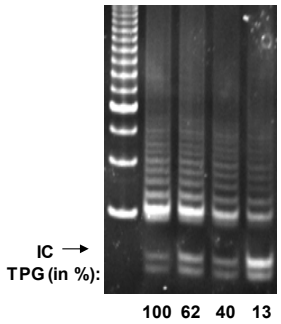


FIGURE 3C

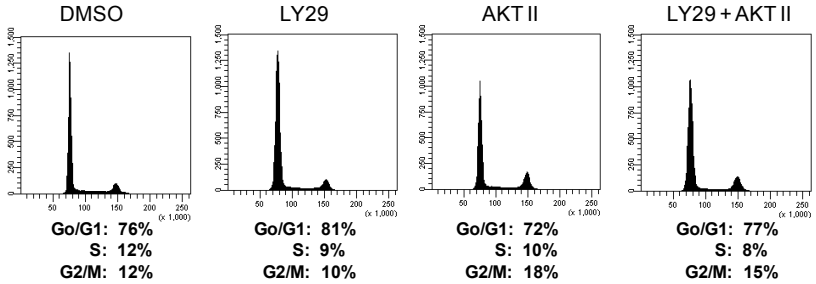


FIGURE 3D

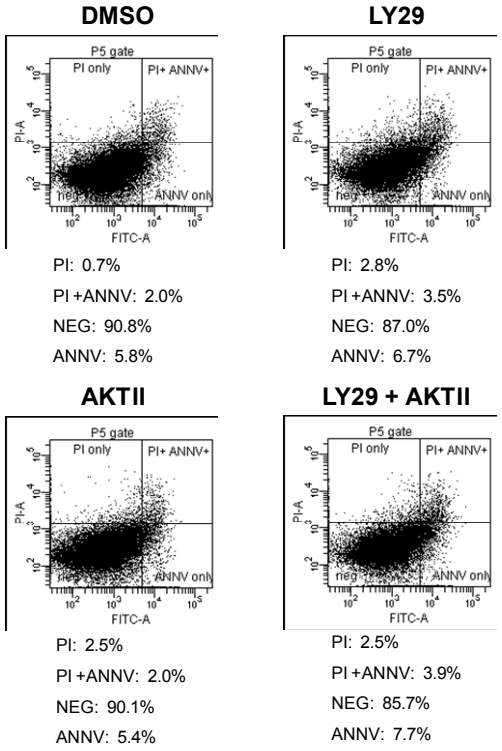
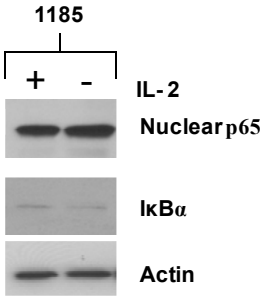


FIGURE 3E



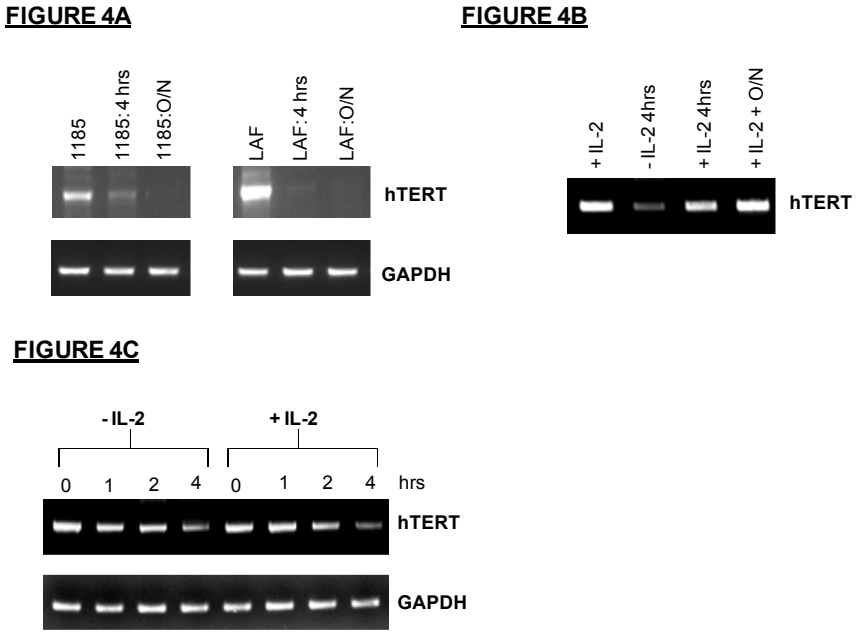
Treatment with a combination of LY29 and AKT II lead to a decrease in *hTERT* expression comparable to treatment with LY29 alone, suggesting that PI3K is the primary regulator of *hTERT* expression at the transcriptional level. Because PI3K, and not AKT inhibition, led to significant transcriptional repression of *hTERT* (approximately 80%), our results suggest that PI3K regulates telomerase activity through an AKT-independent pathway (transcriptional). To verify this hypothesis, 1185 cells were treated with a combination of PI3K and AKT inhibitors. Results indicated that treatment with both inhibitors affected telomerase activity to a

much larger extent than treatment with either drug alone (Figure 3B), thereby demonstrating that PI3K and AKT have an additive, independent effect on telomerase activity. These effects were independent of cell cycle distribution (Figure 3C) or cell death (Figure 3D), as demonstrated by FACS analysis of PI staining and AnnexinV/PI staining, respectively.

A downstream target of PI3K/AKT signaling is NF-κB. NF-κB increases hTERT activity transcriptionally and post-transcriptionally: the former, by nuclear translocation of hTERT through p65-mediated interactions (25), and the latter, by increases in c-Myc and Sp1 expression (26). Since Tax can directly stimulate NF-κB through activation of PI3K, AKT, and IKK, we did not expect to see a difference in NF-κB-mediated control of hTERT upon IL-2 withdrawal. As expected, 1185 showed no significant differences in nuclear RelA/p65 or IκB-α expression following removal of IL-2 from the culture media (Figure 3C).

We next assayed the effect of IL-2 on hTERT promoter expression in HTLV-I infected cells. We found a strong decrease in *hTERT* expression only 4 hours after IL-2 removal in both 1185 and LAF cells, and *hTERT* expression was completely suppressed after 24 hours (Figure 4A).

Figure 4. IL-2R/IL-2 signaling regulates hTERT promoter expression. (A) IL-2 was withdrawn from 1185 and LAF cells for 4 or 24 (overnight – O/N) hours, followed by *hTERT* RNA expression by RT-PCR. (B) LAF cells were cultured in the absence of IL-2 for 4 hours, and subsequently pulsed with IL-2 for 4 and 24 hours prior to hTERT RT-PCR analysis. Cells grown continuously in IL-2 media served as a control (first lane) (C) 1185 cells were treated with actinomycin D (5 μg/ml) in the presence or absence of IL-2 for 0, 1, 2, and 4 hours. RNA was extracted and analyzed for *hTERT* expression by RT-PCR. For (A-C), *GAPDH* was used as an amplification control.



In order to demonstrate that IL-2 removal was responsible for transcriptional control of hTERT expression, LAF cells were

cultivated in the absence of IL-2 then pulsed with IL-2. Results indicated that *hTERT* expression levels were increased in less than 4 hours and restored to the original levels after overnight culture with IL-2 media (Figure 4B). This confirms a potent and rapid effect of IL-2R signaling on hTERT expression. Because our data showed a sudden loss of hTERT RNA levels in only 4 hours, we next looked at the stability of the RNA transcript by measuring the half life of the *hTERT* mRNA in1185 cells cultured in the presence or absence of IL-2. Our results indicated no difference in *hTERT* half-life (around 2 hours) in 1185 cells cultivated in the presence or absence of IL-2 (Figure 4C). These results are consistent with the previously reported half-life of *hTERT* mRNA (about 2 hours) (27) and suggest that the effect of IL-2 removal mainly occurs at the transcriptional level in absence of *hTERT* RNA destabilization.

The activation of PI3K signaling pathways in HTLV-I infected cells, results in cytoplasmic retention of WT1, a negative regulator of *hTERT* expression.

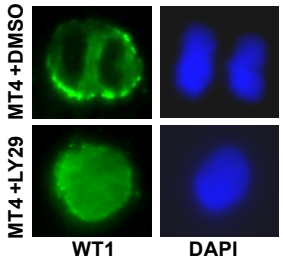
Protein phosphatase 2A (PP2A) lies directly downstream of PI3K and targets AKT and protein kinases A and C (PKA and PKC). PKC acts as a post-transcriptional regulator of telomerase, by

phosphorylating hTERT and allowing for assembly of the holoenzyme complex (28). In contrast, PKA increases phosphorylation of the Wilm's tumor suppressor (WT1) protein, a potent transcriptional repressor that inhibits *hTERT* expression by direct binding to the hTERT promoter (29). PKA-mediated phosphorylation of WT1, sequesters WT1 in the cytoplasm (30,31). Consistent with this model, and the constitutive activation of PI3K found in HTLV-I infected cells, we observed a prominent cytoplasmic distribution of WT1 in MT4 and 1185 cells (Figure 5A).

Figure 5. Cellular localization of WT1 dictates repression of *hTERT* expression. (A) MT4 and 1185 cells were treated with LY29 (10 μM) or DMSO (solvent control) for 48 hours followed by immunostaining with anti-WT1 (Green). Nuclear staining was visualized by DAPI (Blue). (B) 1185 cells were cultured overnight with or without IL-2 and stained with anti-WT1, as described above. (C) The HTLV-I transformed cells, MT2 and MT4, or the HTLV-I immortalized cells, 1185, were electroporated with the hTERT-Luc-Promoter using the Amaxa transfection kit. 12 hours post-transfection, MT2 and MT4 cells were treated with either LY29 (10 μM) or DMSO (solvent control) for 8 hours. 1185 cells were electroporated and cultured in media with and without IL-2 for 24

hours. Cell extracts were normalized and assayed for luciferase activity.

FIGURE 5A



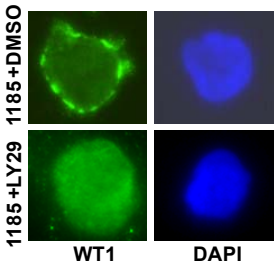


FIGURE 5B

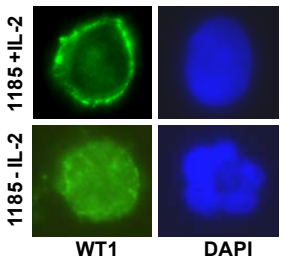
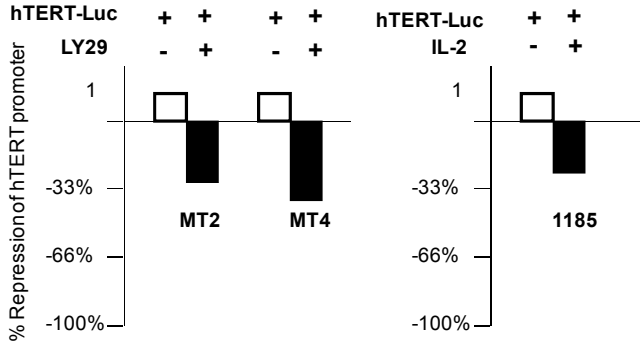


FIGURE 5C



However, inhibition of PI3K, by treatment with LY29 in HTLV-I infected cell lines, caused a redistribution of WT1 to the nucleus. Importantly, similar results were obtained upon IL-2 removal, which was not seen upon AKT inhibition, in 1185 cells (Figure 5B). These results demonstrate that IL-2R signaling, via PI3K, leads to cytoplasmic retention of WT1, effectively preventing WT1 from reaching and repressing the hTERT promoter.

To confirm the role of IL-2/IL-2R signaling through PI3K on *hTERT* expression in HTLV-I infected cells, we electroporated MT2

and MT4 cells with the hTERT luciferase vector. Consistent with the results described above, inhibition of PI3K by LY29, reduced hTERT luciferase activity by 32% and 34% for MT2 and MT4 cells, respectively (Figure 5C). In the HTLV-I immortalized cell line 1185, we found a 28% reduction in hTERT luciferase promoter transactivation when cells were grown for 24 hours in media without IL-2 compared to cells grown with IL-2. The differences in hTERT transactivation in the 1185 luciferase assays (28% reduction) compared to the endogenous levels of *hTERT* expression (approximately 80% reduction) seen in Figure 3A, are probably due to the presence of multiple copies of the hTERT luciferase plasmid upon Amaxa transfection. In such conditions, the limiting amounts of WT1 in HTLV-I infected cells would explain that the suppression of the hTERT luciferase promoter upon transfection is not as pronounced as compared to the endogenous hTERT promoter.

To further characterize the role of IL-2R signaling on WT1 activity, we performed a biotin-pull-down assay using the hTERT promoter as bait. We found that cells cultured in the absence of IL-2 had a significant increase (45%) in WT1 binding to the hTERT promoter, thereby explaining the repression of hTERT expression (Figure 6A).

Figure 6. IL-2-dependent WT1 binding to the hTERT promoter. (A) The hTERT-3915 promoter was biotin-labeled and bound to streptavidin-agarose beads, followed by incubation with 1185 cellular extracts from cells cultivated overnight in the presence or absence of IL-2. Bound proteins were visualized using anti-WT1 or anti-c-Myc. The percentage of increased binding of WT1 to the hTERT promoter was calculated by spot densitometry as indicated, with 1185 cells grown in the presence of IL-2 considered to be 100%. (B) 293T cells were transfected with wild-type WT1 (SS – normal serines) or mutant WT1 (FF – mutated serines to phenylalanines), along with the hTERT-Luc promoter, or wild-type WT1 (SS) or mutant WT1 (FF), along with the hTERT-Mut-Luc promoter (defective for WT1-binding). Extracts were assayed 48 hrs post-transfection for luciferase activity (experiments performed in duplicate). Cell extracts were probed with anti-WT1 and anti-actin to verify expression. Average luciferase values were as follows: hTERT-Luc promoter (150,518), +WT1(SS) (79,578), +WT1(FF) (241,727) and for the hTERT-Mut Luc promoter (890,681), +WT1(SS) (686,143), +WT1(FF) (2,184,084).

FIGURE 6A

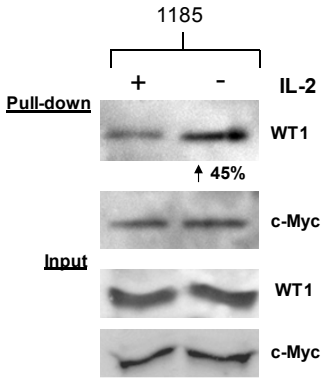
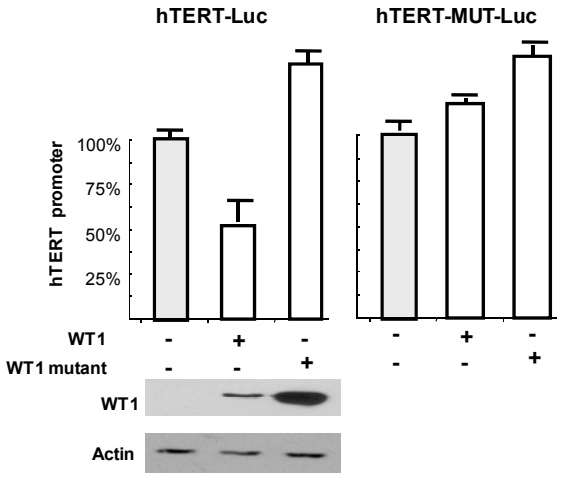


FIGURE 6B



Increased binding of WT1 was due to an increase in DNA binding affinity, rather than overall levels, as shown by equal input of WT1 and c-Myc. In fact, it has been shown that PKA-mediated phosphorylation of WT1 impairs both its nuclear localization and its DNA binding activity (30). As an internal control for this assay, the levels of c-Myc bound to the hTERT promoter did not change in response to IL-2 withdrawal. To confirm that WT1 is a transcriptional repressor of the hTERT promoter, we transfected wild-type WT1 into 293T cells along with an hTERT luciferase construct. hTERT promoter activity decreased by approximately 50% (Figure 7B). In contrast, a mutant WT1 (WT1-KK), which cannot be phosphorylated by PKA, was unable to repress the hTERT promoter. To further demonstrate that the WT1 effect was mediated by direct binding, we mutated the WT1 binding site

(GCG**CG**GGCG) (29) within the hTERT luciferase vector. This hTERT promoter luciferase construct (hTERT-MUT-Luc) was no longer repressed to the same extent as the wild-type hTERT promoter upon co-expression of WT1 (Figure 6B). Our data demonstrate that through activation of the PI3K pathway, WT1 is sequestered in the cytoplasm of HTLV-I infected cells contributing to the overall elevated telomerase activity seen during HTLV-I infection.

DISCUSSION:

Tax plays an important role in the early steps of transformation by HTLV-I through deregulating host cell signaling pathways, including those involved with cell cycle, apoptosis, and proliferation. We have previously shown Tax stimulates high telomerase activity in HTLV-I infected cells (12). However, IL-2 dependent cell lines express very little Tax, yet retain the high levels of telomerase activity seen in transformed cell lines. Here, we demonstrate that HTLV-I cell lines rely upon IL-2R signaling pathways to sustain high telomerase activity, in absence of high Tax expression.

In order to gain a better insight into IL-2- and Tax-mediated regulation of telomerase activity, we looked at the level of *hTERT*

expression. In absence of exogenous IL-2, Tax can lead to PI3K and AKT stimulation. In accordance, inhibition of downstream IL-2R targets, PI3K and AKT, leads to a significant loss of telomerase activity in both immortalized and transformed cell lines, from 25 to 75%. Importantly, such levels of repression were previously shown to be sufficient for telomere attrition and induction of senescence in HTLV-I infected cells (9). AKT inhibition had marginal effects on hTERT promoter expression, consistent with its role as a post-transcriptional regulator of telomerase activity (21). In contrast, we found that PI3K inhibition substantially decreased *hTERT* expression, suggesting that a PI3K-dependent/AKT-independent pathway controls *hTERT* transcription. PI3K stimulates PKA, which phosphorylates WT1, leading to its cytoplasmic retention (30,31). By sequestering WT1 in the cytoplasm, HTLV-I infected cells remove a negative regulator of *hTERT* expression. We previously demonstrated c-Myc and Sp1 have increased binding to the hTERT promoter upon transfection of Tax. We now report a new mechanism by which HTLV-I increases hTERT activity, by relieving WT1-mediated hTERT promoter repression. The biological significance of our data is underscored by the fact that similar levels of telomerase inhibition, as those reported here, by AZT have been shown to induce p53-dependent senescence and death of HTLV-I cells lines

and ATL patients (9). Therefore, IL-2 may be required in the early stages of transformation to ensure high telomerase activity and extend the life span of infected cells, therefore; increasing the risk of cumulative genetic defects and transformation.

WT1 is expressed in Wilms' tumors, colon cancer, breast cancer, and leukemia (32). In hematopoietic cells, WT1 behaves like a tumor suppressor, promoting differentiation by decreasing cellular proliferation, contributing to growth arrest, and reducing colony formation (33,34). However, it has been suggested that WT1 acts as an oncogene in leukemic cells as it is highly expressed in the peripheral blood of many leukemias (32). Our study supports a role for WT1 as a tumor suppressor gene in HTLV-I infected cell lines, by decreasing the level of telomerase expression. Decreased telomerase activity in cancer cells has been linked to cellular senescence and death. WT1 expression is elevated in leukemias beyond the levels found in normal hematopoietic cells. The use of RNA interference and antisense oligonucleotides against endogenous WT1 in acute myeloid leukemia (AML) and chronic myelogenous leukemia (CML) cell lines, including leukemia patient samples, leads to decreased growth and apoptosis (35,36). HTLV-I infection may allow for WT1 expression to enhance the proto-oncogenic effects required for tumor proliferation, while at the same time inhibiting

WT1's transcriptional suppressive functions by sequestering the protein in the cytoplasm to allow for elevated telomerase activity.

Our study was performed in HTLV-I infected cell lines, which express high levels of Tax. However, Tax is barely detectable in ATLL leukemic cells (37,38), yet these cells maintain high levels of telomerase expression and activity (12,13). We propose that ATLL patients may rely upon an IL-2R signaling pathway similar to those in HTLV-I immortalized cell lines to maintain high telomerase activity, in absence of Tax expression. This is supported by the fact that ATLL cells have constitutive activation of the high affinity IL-2R (39). Future studies, in which IL-2R components, especially the PI3K pathway, are inhibited through siRNA-mediated inhibition, could verify this. It remains to be clarified whether PI3K is responsible for retaining WT1 in the cytoplasm, and whether additional IL-2R signaling pathways are activated and/or repressed in order to enhance telomerase expression in ATLL patients.

In conclusion, our data suggests an important role for the PI3K pathway in transcriptional regulation of *hTERT* and underscores the importance of this regulatory pathway for telomerase expression in cancer cells, especially given the fact that the PI3K pathway is commonly found activated in tumor cells.

MATERIALS AND METHODS:

Cell Culture

The HTLV-I transformed cell lines, MT4, MT2, and C8166, and the HTLV-I immortalized cell lines, LAF, SP, and 1185, were cultivated in RPMI 1640 with 10% fetal bovine serum and, when indicated in the text, IL-2 (50 U/ml). 293T cells were grown in DMEM. HTLV-I cell lines were treated with either 20 μM AKT Inhibitor II (Calbiochem), 10 μM LY294002 (Sigma), or 50 μM AG490 (Biomol), as indicated in the figure legends. Treatment with either Dimethyl sulfoxide (DMSO) or LY303511 (Sigma) served as negative controls.

Telomeric Repeat Amplification Protocal Assays (TRAP)

TRAP assays were performed using the TRAPeze telomerase detection kit according to the manufacturer's instructions (Intergen, NY). Quantification of the total product generated (TPG) was performed using the following equation: $[(x-x_0)/c]/[(r-r_0)/c_R] \times 100$, where (x) is the TRAP product generated in the sample, (x_0) is the TRAP product generated in the heat-treated sample, (c) is the internal control band for the sample, (r) is the TSR8 quantitation control, (r_0) is the 1x CHAPS lysis buffer control, and (C_R) is the TSR8

quantitation internal control band. Telomeric products were separated on 8% tris boric acid EDTA gels and stained with SYBR green for visualization. Standardization of the assay and appropriate controls in our laboratory have been previously reported (9).

RNA extraction, RT-PCR, and Real-Time PCR.

Total RNA was extracted using Trizol (Invitrogen). Prior to analysis, DNA contamination was removed from RNA samples as previously described (13). RT-PCR was performed in non-saturating conditions using the OneStep RT-PCR kit (Qiagen), according to the manufacturer's instructions. RT-PCR was performed with the following sets of primers: hTERT, F: 5'-CTG GGT GGC ACG GCT TTT GTT C-3' and R: 5'-CCC CGG GAG CTT CCG ACT-3' and GAPDH, F: 5'-GAA GGT GAA GGT CGG AGT C-3' and R: 5'-GAA GAT GGT GAT GGG ATT TC-3'. RT-PCR was performed without the reverse transcriptase step to verify absence of DNA. cDNA for Real-time PCR was generated using the Transcriptor first strand cDNA synthesis kit (Roche). The cDNA was diluted (1:2) and Real-time PCR was performed using the RT^2 SYBR Green/ROX qPCR Master Mix (SuperArray), with the following hTERT primers, F: 5'-GCG GAA GAC AGT GGT GAA CT-3' and R: 5'-AGC TGG AGT AGT CGC TCT GC-3'

Western Blots

Cells were lysed in RIPA and subject to western blotting using the following sets of antibodies: monoclonal anti-Tax (NIH AIDS Reagent Repository), c-Myc (A-14, Santa Cruz), WT1 (C-19, Santa Cruz), α-actin (C-11, Santa Cruz), AKT (9272, Cell Signaling) and Phospho-Akt (Ser 473) (9271, Cell Signaling). For western blots following IL-2 withdrawal, the cell pellets were lysed in hypotonic buffer (10mM HEPES, pH 7.9, 1mM $MgCl_2$, 0.5mM NaCl, 0.5% NP-40), then centrifuged. The supernatant was collected for cytoplasmic proteins and the cell pellet was lysed in hypertonic buffer (5mM HEPES, pH 7.9, 5mM $MgCl_2$, 0.1mM EDTA, 0.4M NaCl, 1mM DTT). Following centrifugation, the supernatant was collected for nuclear proteins. Western blots were then performed using NF-κB p65 (A, Santa Cruz), c-Myc (A-14, Santa Cruz), and IκB-α (H-4, Santa Cruz). All secondary antibodies were from Santa Cruz.

Cell Cycle Analysis

Cells were cultured with and without IL-2 or treated with LY29 (10 μM) and/or AKT II (20μM) as indicated. Cells were fixed with 80% EtOH for 30 minutes on ice, followed by treatment with

RNase for 15 minutes at 37^{o}C. Cells were then stained with 50 μg/ml propidium iodine for 15 minutes at room temperature. Cell cycle was analyzed by flow cytometry.

Annexin V/PI Staining

HTLV-I infected cells were treated for 48 hours with either LY29 (10μM), AG490 (50 μM), AKT II (20μM), or DMSO control. Following treatment, cells were collected and stained with annexin V (FITC)/propidium iodine using the Vybrant Apoptosis Assay Kit no.2 (Molecular Probes), according to the manufacturer's instructions. Apoptosis was analyzed by flow cytometry.

Immunofluorescence

HTLV-I infected cells were treated with or without LY29 (10μM) for 48 hours and/or cultivated with or without IL-2 overnight. Following treatment, cells were fixed with 4% paraformaldehyde and permeabilized using 0.5% Triton X-100. Cells were then incubated in blocking buffer (10% BSA and 0.1% Tween 20), followed by incubation with WT1 antibody (C-19, Santa Cruz). Secondary body was Alexa Fluor 488 goat α-rabbit IgG (Molecular Probes). Slides were stained with DAPI and images were captured

using a Nikon EFD3 microscope (Boyce Scientific) and Nikon camera (100X Eplan (160/0.17) objective).

Biotin Pull-Down Assay

The hTERT promoter was isolated from the hTERT-Luc-3915 plasmid (a generous gift from I. Horikawa) and labeled with C14-biotin (Invitrogen). Labeled DNA was bound to streptavidin-agarose beads and incubated with protein extracts from cells cultured with or without IL-2 for 24 hours. The pull-down was subsequently washed in binding buffer (10 mM Tris [pH 7.05], 50 mM NaCl, 50 mM NaF, 0.2 mM Na_3VO_4, 30 mM $Na_2P_2O_7$, 5 μM $ZnCl_2$, 0.5% Triton X-100) and bound proteins were analyzed by western blot.

Luciferase

Luciferase assays were performed with the Luciferase Reporter Assay Kit (Promega). 293T cells were co-transfected with the hTERT-Luc-3915 vector, along with either wild-type WT1 (SS) or mutant WT1 (FF) expression vectors (generous gifts from T.Hunter from the Salk Institute). All transfections were performed using Polyfect (Qiagen). The binding site for WT1 in the hTERT-Luc-3915 promoter (GCG**CG**GGCG) was mutated to (GCG**AA**GGCG) using the QuickChange II XL site-directed

mutagenesis kit (Stratagene), according to the manufacturer's instructions. DNA was sequenced to verify the correct mutations. HTLV-I infected cell lines were transfected using Nucleofector Kit V (Amaxa Biosystems), according to manufacturer's instructions. 12 hours post-transfection, MT4 and MT2 cells were treated with either LY29 (10 μM) or DMSO control for 8 hours. For IL-2 withdrawal, 1185 cells were cultivated in media with and without IL-2 for 24 hours.

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CHAPTER III

Persistent Inhibition of Telomerase Reprograms Adult T-cell Leukemia to p53-dependent Senescence

This work was published in *Blood*: 2006; 108, 1021-1029.

Persistent inhibition of telomerase reprograms adult T-cell leukemia to p53-dependent senescence.

ABSTRACT:

The antiviral thymidine analog azidothymidine (AZT) is used to treat several virus associated human cancers. However, to date the mechanism of AZT action remains unclear and thus, reasons for treatment failure are unknown. Adult T-cell leukemia/lymphoma (ATL) is an aggressive malignancy of poor prognosis. Here, we report that enduring AZT treatment of HTLV-I infected cells, *in vitro*, and *in vivo* ATL patients, results in inhibition of telomerase activity, progressive telomere shortening and increased $p14^{ARF}$ expression. In turn, this elicits stabilization and reactivation of the tumor suppressor p53-dependent transcription, increased expression of the cyclin-dependent kinase inhibitor $p21^{Waf1}$ and accumulation of $p27^{kip1}$, thereby inducing cellular senescence and tumor cell death. While ATL patients carrying a wild type p53 enter remission following treatment with AZT, those with a mutated p53 did not respond and patient's disease relapse was associated with the selection of a tumor clone carrying mutated inactive p53.

INTRODUCTION:

Adult T-cell leukemia is etiologically linked to the human T-cell leukemia virus type I (HTLV-I) (1,2). HTLV-I-mediated T-cell transformation presumably arises from a multi-step oncogenic process in which the virus induces chronic T-cell proliferation resulting in an accumulation of genetic defects and the deregulated growth of infected cells [3]. Since its discovery many aspects of HTLV-I biology have been uncovered however treatments of the diseases remain disappointing with minimal improvement in the overall survival of patients. The poor prognosis of ATL patients is associated with the resistance of neoplastic cells to the conventional combination of high-dose chemotherapy and radiotherapy [4]. Recently, a higher response rate following AZT/ IFN alpha treatment of ATL patients has been reported in several human trials [5-7]. However, the mechanism of action of AZT/ IFN remains unknown and therefore predictive markers for prognostic therapy are not available. Several human cancers have been treated using anti-retroviral AZT, including, AIDS-related Kaposi sarcoma [8]; Kaposi sarcoma associated primary effusion lymphoma (PEL), EBV-associated lymphoma [9] and primary central nervous system

lymphoma (PCNL) [10]. In vitro treatment of PEL cell lines with AZT resulted in TRAIL-dependent apoptosis [11;12] along with NF-kB inhibition and apoptosis in EBV-associated Burkitt lymphoma [13]. In contrast, remission of ATL patients treated with AZT follows a slow kinetic over several months, suggesting that a non apoptotic pathway may be involved. These observations prompted us to investigate the long term potential effects of AZT on telomerase and telomere functions. Since DNA polymerase is unable to replicate the very end of linear DNA, every replication cycle leads to a progressive shortening of the telomeric ends and to the limited proliferative capacity of normal cells termed "replicative senescence." Activation of human telomerase, an RNA-dependent DNA polymerase that elongates telomeres, has been proposed as a mechanism for avoiding telomere shortening. Consistent with this model, most cancer cells, including HTLV-I infected cells, have detectable telomerase activity, as opposed to normal somatic cells [14]. In some cases, immortalized cells do not have any detectable telomerase activity in spite of long telomere length, suggesting the existence of an alternative mechanism referred to as ALT and characterized by specific makers [15].

Here we report that enduring treatment of HTLV-I infected cells with AZT result in telomere attrition and reactivation of p53

transcriptional activities leading to senescence of tumor cells. Importantly, in vivo treated ATL patients responded to therapy only when p53 was wild type in sequence and inversely disease relapse or absence of response to treatment was associated with mutation and inactive p53. Our results indicate that p53 is a predictive marker and a response to AZT therapy requires a functional p53 gene.

RESULTS:

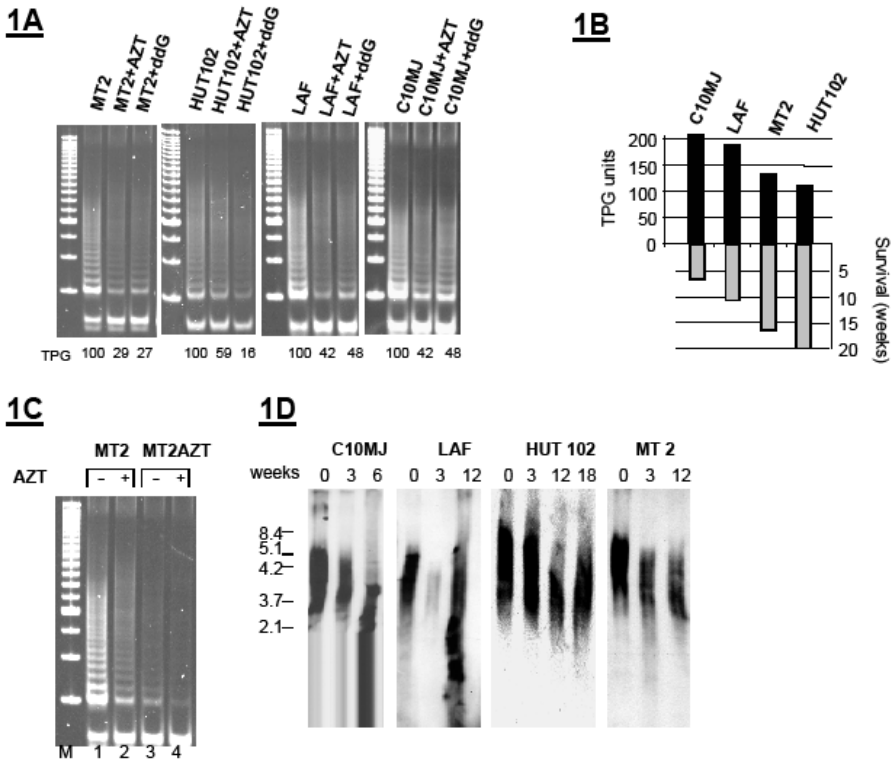
Inhibition of telomerase induces telomere attrition and cell death in HTLV-I transformed cell lines.

We and others have reported that HTLV-I infection is associated with increased telomerase activity in transformed cells *in vitro* and *in vivo* [16-19]. As expected, treatment of HTLV-I transformed cell lines with telomerase inhibitors, AZT or ddG, resulted in a marked reduction of telomerase activity in HTLV-I infected cells (Fig.1A).

Figure 1: Telomerase inhibition induces senescence in HTLV-I infected cells.

(A) Telomerase activity (expressed as telomeric product generated, TPG), measured by TRAP assay in HTLV-I cell lines in absence of

or presence of telomerase inhibitors AZT (50μM) and ddG (10μM) for 72 hours. **(B)** Correlation between telomerase activity (TPG) and survival of AZT treated HTLV-I cell lines (in weeks). **(C)** Inhibition of telomerase activity by AZT in the HTLV-I transformed MT-2 cell line untreated or treated with AZT for 18 weeks. **(D)** Southern blot analysis of telomere shortening following AZT treatment.



As telomerase activity is required for continual proliferation and avoidance of replicative senescence of tumor cells, we evaluated the long term effects of telomerase inhibition by maintaining HTLV-I cell lines in medium supplemented with AZT or ddG. While short

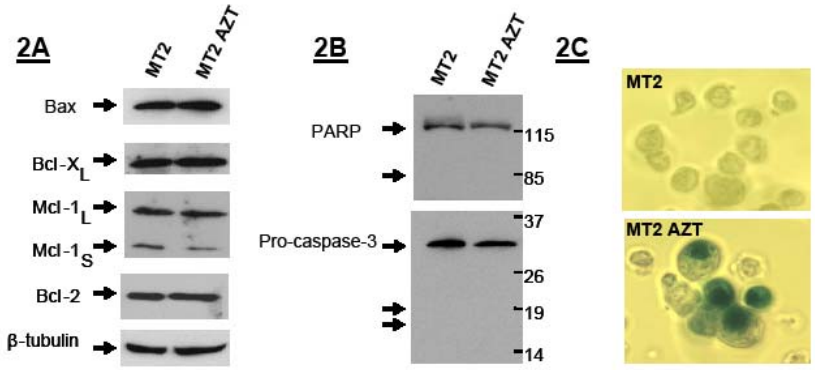
term treatment had no effect on proliferation or survival (data not shown and [20]), all HTLV-I cell lines ceased to grow, entered senescence (Fig.1B) and died after several weeks of culture in the presence of AZT or ddG, as observed by cell counts and trypan blue exclusion (data not shown). Longer survival, as observed in MT-2 and HUT-102, appeared to be associated with a lower telomerase activity compared to C10MJ and LAF (Fig.1B and data not shown). In contrast, the Jurkat T-cell line, treated under the same conditions, did not enter growth arrest (data not shown), in spite of significant telomere shortening as previously reported [21]. HTLV-I transformed cells do not acquire resistance to AZT-mediated inhibition of telomerase activity even after a prolonged period of treatment because residual activity present in long term treated cells (Fig1C, lane 3) can be further inhibited in MT-2 as well as in other HTLV-I cell lines by addition of AZT for 48 hours (Fig.1C lane4, and data not shown). Analysis of telomere length by Southern blot revealed a progressive shortening in all HTLV-I cell lines cultured in continuous presence of AZT (Fig.1D).

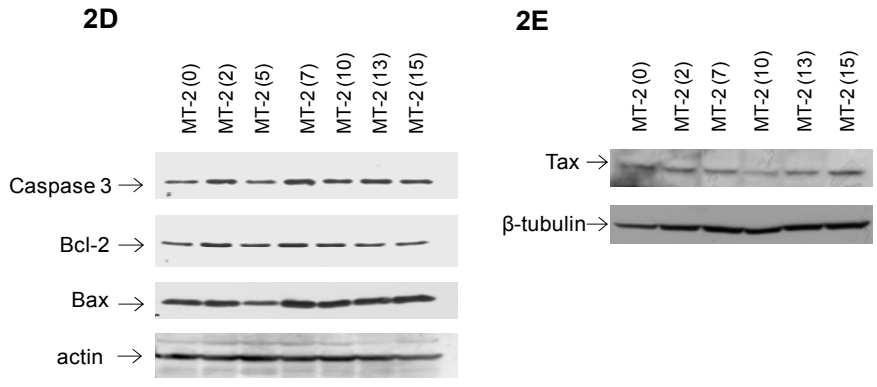
AZT induces senescence of HTLV-I infected cells

As telomere shortening has been associated with chromosomal instability causing either apoptosis or senescence [22;23] , we investigated the mechanism of tumor cell death in long term AZT-treated cells. Previous studies reported that EBV-positive Burkitt lymphoma cells from AIDS patients respond *in vitro* to AZT by undergoing caspase-dependent apoptosis. HTLV-I infected cells are protected from apoptosis in part through high levels of Bcl-2 and Bcl-xL expression [24]. However the expression of pro-apoptotic and anti-apoptotic proteins remained unchanged in long term AZT-treated MT-2 cells (Fig.2A).

Figure 2: Persistent telomerase inhibition is not associated with apoptosis but induces senescence in HTLV-I infected cells. (A) Western blots analysis for expression of apoptosis regulators in untreated MT-2 or treated with AZT (18 weeks). Equal amounts (50μg) of each extract was used and confirmed by β-tubulin. **(B)** Absence of cleveage for procaspase 3 or PARP in AZT treated MT-2 cells. **(C)** Senescence β-gal (SA-β gal) assay in MT-2 cells untreated and treated with AZT (18 weeks). A significant amount of senescence was detected only in end of cultures. **(D)** Western blots analysis of MT-2 cells treated with AZT. Samples were collected at different times after treatment from 0 to 15 weeks. Expression of

caspase 3, Bcl-2 and Bax was tested. Actin was used as loading control. **(E)** Western blots analysis of MT-2 cells treated with AZT. Samples were collected at different times after treatment from 0 to 15 weeks. Expression of Tax was analyzed as described under materials and methods. Beta-tubulin was used to confirm equal loading.





Absence of procaspase-3 and PARP cleavage (Fig.2B) along with negative AnnexinV staining (data not shown), further confirmed the absence of apoptosis in AZT-treated HTLV-I cells. Instead, we found that HTLV-I cells undergo senescence as shown by the senescence β-galactosidase assay, which was strongly positive in long term AZT treated MT-2 cells, but not in MT-2 control cells (Fig.2C). This was also confirmed in other treated HTLV-I cell lines (C10MJ, HUT102 and LAF) (data not shown).

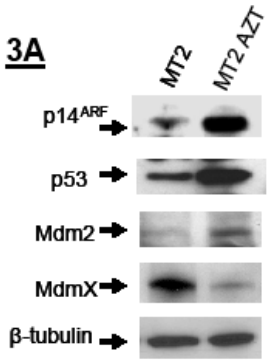
Analysis of samples collected at different intervals in MT-2 cells cultured in the presence of AZT showed no significant differences in the levels of expression of apoptotic markers (Fig.2D). In contrast to previous observations made in HTLV-I infected cells treated with arsenic trioxide and IFN [25;26], the levels of the viral oncoprotein Tax remain unchanged during AZT treatment (Fig.2E).

Continuing inhibition of telomerase by AZT in HTLV-I infected cells induces post transcriptional stabilization of p53.

To confirm these findings and gain further insights into the mechanisms involved, the expression level of $p14^{ARF}$, a well known senescence marker [27], was analyzed. We found significant

increased expression of $p14^{ARF}$ in long term AZT-treated MT-2 cells (Fig.3A). Consistent with its ability to prevent Mdm2-mediated degradation of p53 [28], levels of p53 expression were considerably increased in AZT-treated cells when compared to control MT-2 cells (Fig.3A).

Figure 3: Continuing inhibition of telomerase by AZT in HTLV-I infected cells induces post transcriptional stabilization of p53. (A) Western blot analysis for expression of p53 pathway regulators in untreated MT-2 and AZT-treated (18 weeks) MT-2 cells. Equal amounts (50μg) of each extract was used and confirmed by β-tubulin. **(B)** Analysis of *p53* mRNA expression by RT-PCR in MT-2 and after culture with AZT for 18 weeks. *GAPDH* was used as internal control for amplification. **(C)** Western blot analysis of p53 after 24 hours of AZT treatment. Equal amounts (50μg) of each extract was used and confirmed by β-tubulin. **(D)** Increased expression of p53 detected by western blot in several HTLV-I infected cell lines (1185, C10MJ, LAF) treated for 4 weeks with AZT.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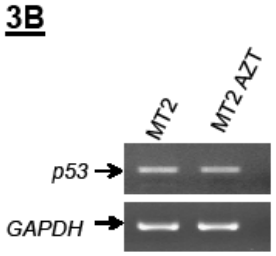
MT2 AZT
p14ARF
p53
Mdm2
MdmX
β-tubulin


3B
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MT2 AZT
p53
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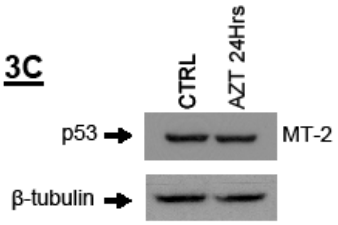
3C
CTRL
AZT 24Hrs
p53
MT-2
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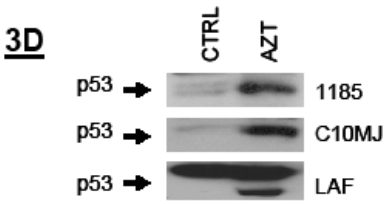
3D
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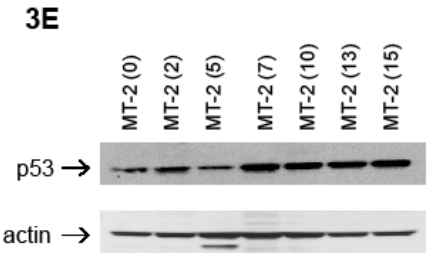
3E
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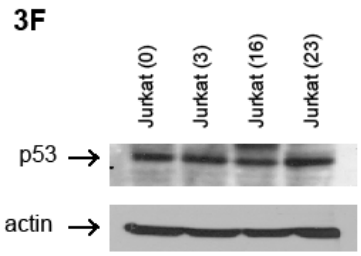
MT-2 (15)
p53
actin


3F
Jurkat (0)
Jurkat (3)
Jurkat (16)
Jurkat (23)
p53
actin

The underlying mechanism of increased p53 expression was post transcriptional, because the levels of p53 mRNA in MT-2 and AZT-treated MT-2 cells remained similar (Fig.3B). In addition, the effect on p53 protein stabilization was only detected after long term treatment with AZT, and treatment with AZT for 24 hours did not result in any significant increase in the levels of p53 expression (Fig.3C). These results exclude a potential direct effect of AZT on p53 and suggest that telomere attrition plays an essential role in increasing the levels of $p14^{ARF}$ and p53 expression. Importantly, long term effects of AZT on cell cycle arrest and increased expression of p53 were reproduced in two independent experiments using MT-2 and C10MJ cell lines, and were further confirmed in three other HTLV-I cell lines C10MJ, 1185 and LAF (Fig.3D), suggesting a general mechanism rather than an observation limited to MT-2 cells. Increased expression of $p14^{ARF}$ and p53 following long term AZT treatment was accompanied by an increase in Mdm2 expression (Fig.3A), and consistent with the previous findings that Mdm2 promotes ubiquitination and proteasome degradation of MdmX [29], our results also revealed significant decrease in the levels of MdmX expression in long term AZT-treated MT-2 cells (Fig.3A). Thus, by blocking the ability of Mdm2 to target p53 for degradation, $p14^{ARF}$ causes p53 stabilization, and, by stimulating MdmX degradation by

Mdm2, $p14^{ARF}$ concurrently eliminates another inhibitor of p53 function.

Analysis of samples collected at different intervals in MT-2 cells cultured in the presence of AZT showed an increase in p53 expression after 7 weeks of treatment and thereafter (data not shown). In contrast, in Jurkat cells treated in the same conditions, p53 levels of expression remained constant (data not shown).

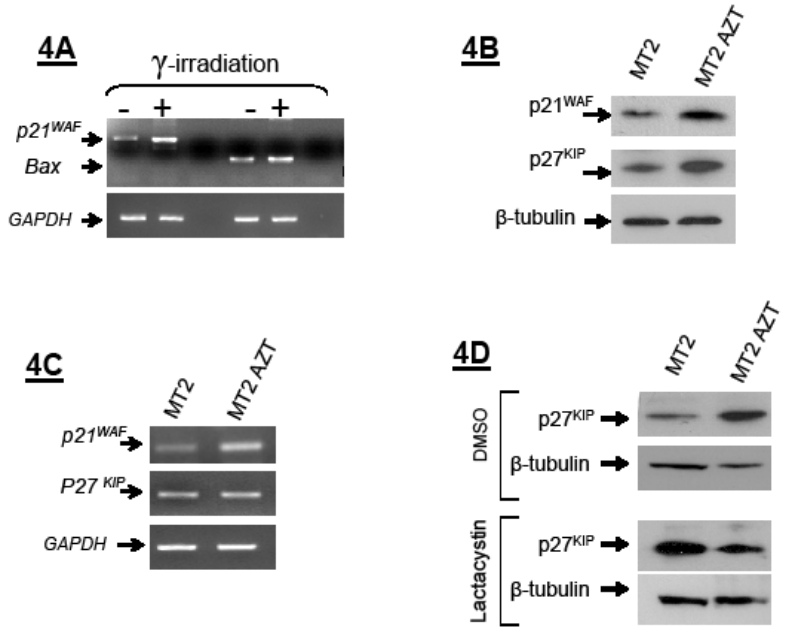
AZT triggers reactivation of p53-dependent transcription and accumulation of cyclin dependent kinase inhibitors p21waf and p27kip.

We hypothesized that the concerted actions of $p14^{ARF}$, Mdm2 and MdmX may lead to reactivation of p53 transcriptional activities. Previous studies have found that p53 is transcriptionally inactive in HTLV-I infected cells *in vitro* and in uncultured ATL cells, and that p53-responsive genes are not activated following ionization-radiation in these cells [30;31]. To test for reactivation of p53 transcriptional functions, long term AZT-treated MT-2 cells were subjected to mock or ionization-radiation, which produces DNA damage and induces transcription of p53 responsive genes. Our results confirmed the reactivation of p53 transcriptional activities, demonstrated by the

induction of p53-responsive genes *p21*^{WAF} and *Bax* following ionization-radiation (Fig.4A). Spot densitometry indicated a 3.2 and 1.8 fold increase for *p21*^{WAF} and *Bax* mRNA expression following irradiation. Proliferation assays along with propidium iodide staining revealed that long term AZT-treated MT-2 cells arrest in all phases of the cell cycle (data not shown), a signature of cyclin-dependent kinase inhibitors (CDKI). Consistently, protein expression levels of both p21^{WAF} and p27^{KIP} were significantly increased following prolonged AZT treatment (Fig.4B).

Figure 4: AZT-mediated reactivation of p53 functions and stabilization of CDKI p21^{WAF} and p27^{KIP}. **(A)** Expression of *p21waf* and *Bax* mRNA before and after ionizing radiation in AZT treated MT-2 cells (18 weeks). *GAPDH* was used as internal control for amplification. **(B)** Western blot analysis for expression of p21^{WAF} and p27^{KIP} in untreated MT-2 or after culture with AZT for 18 weeks. Equal amounts (50μg) of each extract was used and confirmed by β-tubulin. **(C)** Analysis of *p21*^{WAF} and *p27*^{KIP} mRNA expression by RT-PCR in MT-2 and after culture with AZT for 18 weeks. *GAPDH* was used as internal control for amplification. **(D)** Western blot analysis for expression of p27^{KIP} in untreated MT-2 or after culture with AZT for 18 weeks, in absence or presence of

proteosome inhibitor Lactacystin. Equal amounts of each extract (50μg) were used and confirmed by β-tubulin. **(E)** MT-2 derived p53 cDNA nucleotide sequence. **(F)** p53 amino acid sequence from MT-2 cells compared to wild type p53. **(G)** RT-PCR for p53 responsive genes before and after gamma irradiation as described in the materials and methods.



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4F

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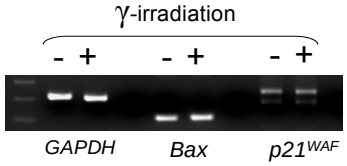


However, while increased expression of *p21*^{WAF} occurred at the transcriptional level (Fig.4C), presumably through p53, *p27*^{KIP} mRNA expression remained comparable in MT-2 control and long term AZT-treated MT-2 cells suggesting a post transcriptional mechanism (Fig.4C). In fact, when MT-2 and AZT treated MT-2 cells were incubated with lactacystin, a proteasome inhibitor; levels of p27^{KIP} expression significantly increased in MT-2 but not in MT-2 AZT treated cells (Fig.4D). These findings suggest that the p27^{KIP} degradation pathway is hampered in long term AZT treated cells which allows for its stabilization. Our results are consistent with previous observations that limiting amounts of p27^{KIP} correlate with

constitutive activation of the cyclin E-CDK2 complex and increased expression of $p27^{KIP}$ triggers cell cycle arrest [32]. We next confirmed that in the original MT-2 cells, p53 was wild type but transcriptionally inactive. To this end p53 cDNA was amplified, cloned and sequenced. As shown below we found three nucleotides changed in the p53 sequence from MT-2 cells (Fig.4E). However, these mutations were silent and did not affect the amino acid sequence which was wild type in MT-2 (Fig.4F). To confirm that p53 transcription was impaired we gamma-irradiated MT-2 cells and analyzed expression of p53-responsive genes *bax* and *p21waf* as reported in figure 4A. In clear contrast to AZT treated MT-2 cells, our results demonstrate that p53 is transcriptionally inactivated in untreated MT-2 cells (Fig.4G).

Overall our results suggest that prolonged AZT treatment and inhibition of telomerase activity leads to telomere attrition, restores p53's functions, and increases expression of senescence markers $p14^{ARF}$ and $p21^{WAF}$ in long term AZT treated MT-2 cells.

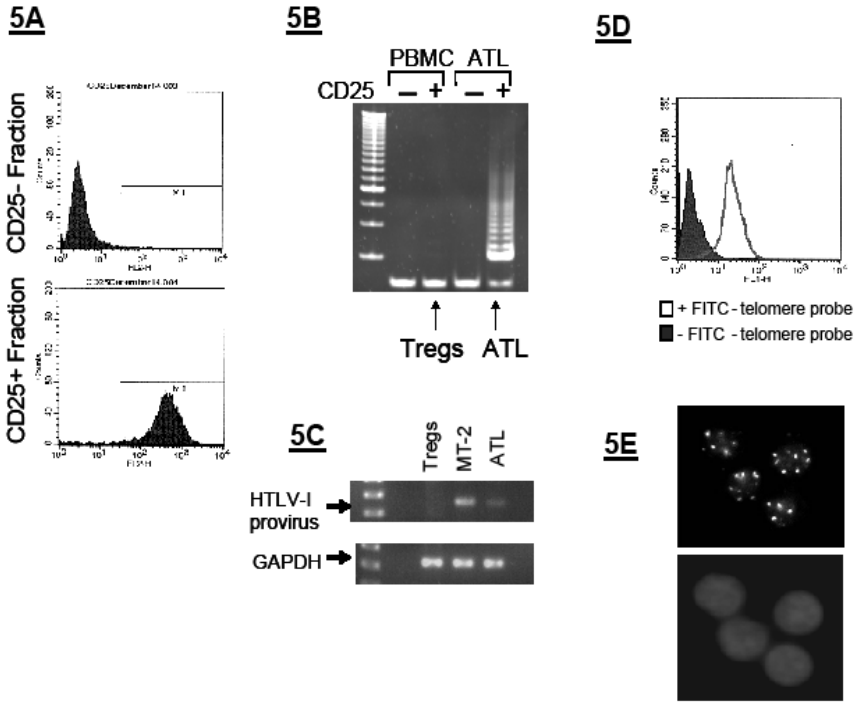
AZT based treatment induces telomerase inhibition and telomere shortening *in vivo* in ATL patients.

The effect of AZT *in vitro* requires several weeks of treatment [33], which parallels the slow kinetics observed in ATL patients treated with AZT (3). Strikingly, the estimated 30% failure rate of AZT treatment in HTLV-I infected ATL patients [34] coincides with the approximate percentage of ATL patients carrying a mutated inactive p53 gene [35]. These observations prompted us to investigate whether the effect of AZT in treatment of ATL patients also relied on telomerase inhibition, telomere attrition and p53 functions.

Samples were obtained from 18 ATL patients at different clinical stages, lymphomatous, smoldering, chronic and acute from two different origins (Necker hospital, France and NIH, USA). All samples were blind-tested and in each case the response to therapy was provided only after analysis. In the case of 3 ATL patients (ATL3, ATL4, ATL5) a paired sample, diagnostic and remission/relapse, could be obtained. Due to the intrinsic limitation in the amounts of each of the patient samples available, telomere sizes were measured by quantitative flow-FISH (Q-FISH) [36], before and after *in vivo* AZT treatment. To avoid potential variations between patient's sample origins, we cell-sorted HTLV-I-infected from non-infected cells of three uncultured ATL patient samples collected prior to and after several weeks of AZT treatment and compared the relative telomere size in each cellular fraction. As

HTLV-I infected ATL cells over express the CD25 activation marker on their surface [37], we used anti-CD25-coupled magnetic beads to sort HTLV-I infected from their non-infected counterparts. Successful sorting was confirmed by staining each sorted cell population with an anti-CD25 fluorescein-isothiocyanate (FITC)-conjugated antibody, and fluorescence-activated cell sorter scanner (FACS) analysis showed at least 95% purity (Fig.5A).

Figure 5: Analysis of CD25+ HTLV-I infected cells. (A) FACS analysis of the presence of CD25 marker expression from CD25+ and CD25- sorted cells. **(B)** Telomerase activity detected by TRAP assay in CD25+ and CD25- fractions isolated from an HTLV-I-donor and ATL PBMCs. **(C)** Integrated HTLV-I proviral DNA was detected by PCR using primers in the *tax* coding region. GAPDH was used as amplification control to ensure proper quality and quantity of extracted DNAs. **(D)** Staining control of PBMCs in presence or absence of FITC-telomere probe. **(E)** In situ hybrizidation of FITC-conjugated telomere probe (top) and DAPI (bottom).



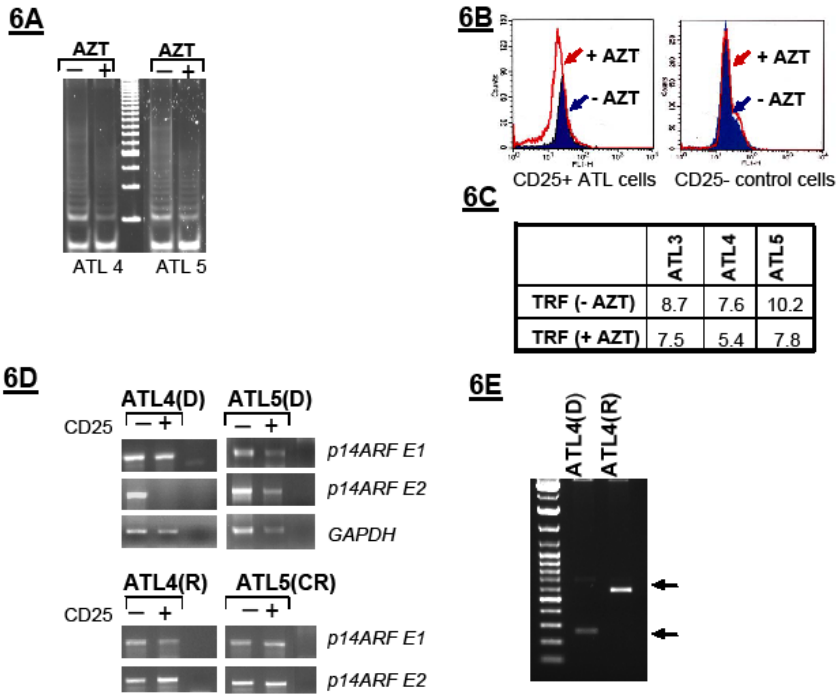
To ensure that CD25+ sorted cells correspond to HTLV-I infected cells and not uninfected CD25+ regulatory T-cells (Tregs), CD25+ and CD25- cells isolated from PBMCs of a healthy individual and those of an ATL patient were tested by TRAP assay. Significant telomerase activity was only detected in the CD25+ population isolated from the ATL patient and no or very low telomerase activity was detectable in either population isolated from the non-HTLV-I infected donor (Fig.5B), suggesting that Tregs are mostly telomerase negative and that the CD25+ cell fraction isolated from the ATL patients indeed corresponds to HTLV-I infected cells. In fact,

integrated HTLV-I provirus was detected by PCR in CD25+ cells isolated from the ATL patient only (Fig.5C). Specific hybridization of the FITC- labeled probe to the telomeric ends was confirmed by microscopic observation of a punctuated pattern consistent with telomere labeling (Fig.5D and 5E).

Telomerase activity was readily detected by TRAP assay in all tested ATL patients (data not shown). Treatment with AZT reduced telomerase activity *in vivo* (Fig.6A).

Figure 6: AZT-induced telomerase inhibition and telomere shortening in ATL patients. (A) Inhibition of telomerase activity measured by TRAP *in vivo* ATL patients before and during treatment with AZT. **(B)** FACS analysis of telomere size by Flow-FISH in CD25+ and CD25- cells isolated from uncultured ATL patient samples. **(C)** Average telomere size in CD25+ cells before and after AZT treatment calculated from two independent measurements. **(D)** Detection of exon 1 and 2 of the $p14^{ARF}$ gene by PCR in CD25+ cells from two ATL patients before AZT treatment or after AZT treatment, *GAPDH* amplification was used as control. (D) diagnosis, (R) relapse/death, (CR) complete remission. **(E)** Detection of

provirus integration site by inverse PCR before and after AZT treatment, (D) diagnosis, (R) relapse.



While average telomere size from CD25- cells was not affected by treatment with AZT, a 14% to 30% reduction in telomere length occurred in HTLV-I infected CD25+ cells after treatment with AZT (Fig.6B and 6C). Since $p14^{ARF}$ is an important player in p53 functions and overlaps the *p16ink* locus, a gene frequently methylated, deleted or mutated in ATL patients [38-41], we analyzed the integrity of the $p14^{ARF}$ gene. At the time of diagnosis $p14^{ARF}$ exons1 and 2 were present in ATL5 patient, but $p14^{ARF}$ exon 2 was deleted in HTLV-I infected cells of ATL4 patient (Fig.6D).

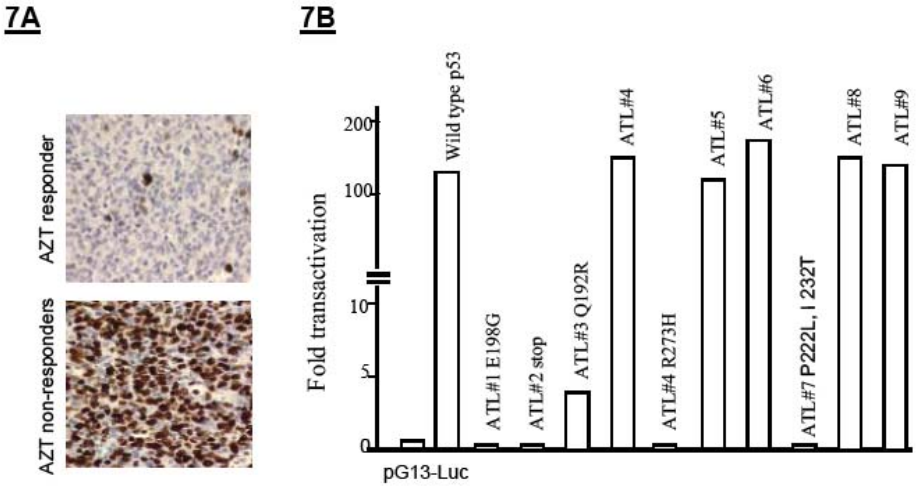
However, a recent study showed that exon 2 is dispensable for $p14^{ARF}$ function and the amino-terminal 29 residues of $p14^{ARF}$ are sufficient for stabilization of p53 [42]. Interestingly, analysis of the ATL4 patient's DNA revealed the presence of both $p14^{ARF}$ exons after the course of AZT treatment and patient disease relapse (Fig.6D). These findings suggest that in patient ATL4, treatment with AZT eradicated the initial tumor clone but resulted in the outgrowth of a different tumor clone at relapse. In fact, inverse PCR experiments and sequencing demonstrated a different provirus integration site in ATL4 patient's DNA samples collected at diagnostic and after disease relapse (Fig.6E).

Response of ATL patients to AZT based therapy correlates with their p53 transcriptional status.

As most mutations that inactivate p53-dependent transcription lead to protein stabilization, we investigated p53 stabilization by immunochemistry in four ATL patients. Many studies have reported stabilization and increased expression of transcriptionally inactive mutated p53. In three patients that did not respond to AZT treatment p53 expression was readily detectable by immuno histochemistry

(Fig.7A). In contrast, in patient responding to AZT, p53 expression was very weak, consistent with a wild type p53 sequence (Fig.7A).

Figure 7: Response of ATL patients to AZT based therapy correlates with their p53 transcriptional status. (A) Immuno-histochemistry detection of p53 expression in ATL samples collected from AZT responder (top) and non responders (bottom) performed as previously described [3]. **(B)** p53 from ATL patients was cloned into pCDNA3.1 and tested in a functional assay. Jurkat T-cells were transfected with a p53-responsive vector and p53 expression vectors and luciferase activity detected 24 hours later. Results are representative of two independent experiments.



In order to establish a correlation between the p53 status and the outcome of ATL patients that received AZT therapy the p53 hot spot region, exon 4 through 9, was cloned by RT-PCR. In each case, we sequenced two p53 hot spot clones from 14 ATL patients. For ATL3, 4 and 5; two p53 hot spot clones were sequenced both from before treatment and after remission/disease relapse. Whenever the p53 hot spot region was found to be wild type in sequence, the whole p53 cDNA was then cloned and two additional clones were sequenced to confirm the phenotype.

Our results demonstrate that all ATL patients carrying a wild type p53 gene responded to AZT treatment and went into partial or complete remission (Table 1). In contrast, all patients carrying a mutated p53 at diagnosis did not respond to AZT treatment and died within a short period of time (Table1). Thus, a total correlation between p53 and AZT response was found and makes the results highly significant.

The biological relevance of our finding is further supported in the case of ATL4, in which the initial response to treatment was associated with the presence of a wild type p53, but as indicated above, AZT treatment led to the selection of another tumor clone and disease relapse. Strikingly, the clone responsible for the relapse carried a p53 mutated at R273H, a well characterized mutation that

abrogates p53 transcriptional activities. All p53 mutations of unknown function were further tested in a luciferase functional reporter assay after transfection in Jurkat T-cells (Fig.7B). Results confirmed an absolute correlation between the p53 transcriptional status and the response of ATL patients to AZT treatment (Table1 and Fig.7B).

Table 1: Correlation between AZT treatment response and p53 status in ATL patients. Status of patients before AZT treatment, response to treatment, status of p53 gene and its transcriptional activity. The criteria for clinical therapeutic response were as follows: complete remission (CR) was defined as the disappearance of all measurable and assessable disease lasting more than 3 month; partial remission (PR) was defined as reduction of leukemic cell count lasting more than 1 month but less than 3 months; not responding (NR) was defined as increase in leukemic cell count.

Table 1

	Diagnostic	P53 status before AZT	Patient response to AZT	P53 status After AZT	P53 Transcriptional activity
ATL1	acute	Mut E198G	NR	-	No
ATL2	acute	Frameshift, stop aa 148	NR	-	No
ATL3	acute	Mut Q192R	PR	Q192R	Partial
ATL4	chronic	wt	R	R273H	No
ATL5	chronic	wt	CR	wt	Yes
ATL6	smoldering	wt	CR	-	Yes
ATL7	lymphoma	Mut P228L; I 232T	NR	-	No
ATL8	acute	wt	PR	-	Yes
ATL9	acute	wt	PR	-	Yes
ATL10	lymphoma	mut	NR	-	nd
ATL11	lymphoma	mut	NR	-	nd
ATL12	acute	mut	NR	-	nd
ATL13	acute	Mut R72P;S166P;R280G	NR	-	No
ATL14	chronic	Mut R72P;H178Y;I255V	NR	-	No

CR: complete remission; PR: partial remission; NR: not responding; R: relapse; nd: not determined

DISCUSSION:

Results described here provide a novel link between telomerase targeting, telomere attrition and reactivation of p53-dependent senescence pathways in HTLV-I infected cells. While it is

established that p53 is inactive in HTLV-I infected cells, the molecular mechanism of p53 transcriptional inactivation remains a matter of debate [43-49]. Our results clearly indicate that the p53 inactivation mechanism is reversible and could therefore be used to develop novel therapeutic interventions. Previous studies have shown that localization and DNA-binding activity of p53 is not altered in HTLV-I cells, and it is more likely that post transcriptional modification and/or association with specific cellular partners dictates the inhibition. Whether prolonged action of AZT modulates the phosphorylation or the acetylation status of p53 or its interaction with cellular partners warrants further studies.

Results obtained from patient ATL 4 further underscore the importance of a functional p53 (Fig.6 and Table 1). Initial responses to AZT treatment coincides with the presence of a major tumor clone that is wild type p53 in sequence. Eradication of that tumor clone however led to the emergence of a minor clone carrying a mutated and inactive p53. This selection also coincides with disease relapse and death of the patient. Analyses of the $p14^{ARF}$ locus and the provirus integration sites, at the initiation of treatment and at disease relapse, clearly indicate that the two tumor clones present in this patient are distinct.

Several studies have found that TGF-beta signaling is impaired in HTLV-I infected cells *in vitro* and *in ex vivo* ATL patient samples [50-52]. Since prolonged treatment of HTLV-I cells with AZT reduces MdmX protein expression which has been implicated in inhibition of Smad transactivation and TGF-beta signaling [53;54] we tested whether this pathway may be reactivated. However, transfection of MT-2 and AZT treated MT-2 cells with a TGF-beta reporter vector in the absence or presence of exogenous TGF beta did not show any difference in activity (data not shown), suggesting that either MdmX is not involved in TGF-beta inhibition or at least that down-regulation of MdmX is not sufficient to restore TGF-beta signaling in HTLV-I transformed cells.

Interestingly, a recent study reported complete response and suppression of HTLV-1 viral load following Alemtuzumab therapy in AZT/IFN refractory adult T-cell leukemia[55]. Since Alemtuzumab is active in a mouse model of ATL [56] and in patients with refractory T-cell malignancies or CLL with mutated or absent *p53* gene [57;58] it could be used in combination or in maintenance treatment to prevent emergence of p53 mutated clones in patients responding to AZT therapy. It should be noted that the majority of ATL are wild type p53 and die of their disease despite of AZT/IFN or other treatments. In this study we did not use IFN-alpha because

AZT has been shown to have some effect on its own in several virus-associated hematological disorders and to limit the number of variable parameters and facilitate analysis. However, synergies previously observed *in vivo* between AZT and IFN-alpha may in part result from the transcriptional inhibition of the hTERT promoter by IFN-alpha as significant down regulation of hTERT mRNA expression and telomerase activity has been reported in leukemic cell lines as well as in primary leukemic cells [35]. It is also possible that additional functions of IFN- alpha may be involved.

Our study identifies p53 status as an essential predictive marker for the response of HTLV-I infected ATL patients to AZT/IFN treatment and suggests that such therapy may not have any clinical benefits in patients carrying a mutated, inactive p53 gene for which alternative therapies such as allogenic bone marrow transplant, Alemtuzumab or radio-immunotherapy for IL-2Rα [59;60] should be considered. Patients treated with chemotherapy or radiotherapy usually select for tumor clones with mutated p53 and become non responders to AZT when used in the second line of treatment [34]. We propose that for patients carrying wild type p53, AZT/IFN should be considered as the first line of treatment. Whether the effect of AZT/IFN on AIDS-related Kaposi sarcoma, Kaposi sarcoma associated primary effusion lymphoma and AIDS-related primary

central nervous system lymphoma *in vivo* is also p53-dependent, is of significant importance and warrants further investigations. In addition, it is possible that other human cancers, such as HPV-associated cervical cancer or breast carcinomas, for which p53 remains wild type may benefit from AZT/IFN therapy deserve to be considered.

MATERIALS AND METHODS:

Cell culture:

HTLV-I cell lines were maintained in RPMI 1640-10% FBS with and without IL-2. Cells were treated with 50 μM AZT or 10 μM 2', 3'-Dideoxyguanosine, ddG (CalBiochem, La Jolla, CA). Medium containing AZT or ddG was replaced every 3 days until growth arrest.

Patients:

Samples were obtained after informed consent and in agreement with regulations for the protection of human subjects according to the NIH guidelines. Treatment regimens AZT and IFN were previously reported [3,4]. Clinical status of the patients and response to AZT treatment is reported in table 1. Patients 2 and 7 were initially treated with chemotherapy (CHOP) while patients 13 and 14 received

yttrium-90 monoclonal antibody therapy. All other patients received AZT therapy combined with IFN- α as a first line of treatment. PR refers to when ATL cells were still detectable by FACS analysis (>5%) while CR means no detectable ATL cells by FACS in the peripheral blood.

TRAP assay:

Telomerase activity was measured by TRAP assay using Trapeze Telomerase detection kit (Chemicon, Temecula, CA) followed by SYBR-green staining (Molecular Probes, Eugene, OR) and quantified as previously reported [18]. An equal amount of protein lysates in CHAPS buffer was used. Results are representative of three experiments.

Telomere length analysis:

Genomic DNA was isolated and telomere length was determined by Southern blot analysis using TeloTAGGG telomere length assay kit (Roche, Indianapolis, IN) according to manufacturers' instructions.

Flow Q-FISH:

Peripheral CD25+ T-cells were isolated from HTLV-I infected ATL patients using anti-CD25 magnetic Dynabeads (Dynal Biotech, Lake Success, NY). The telomere length of CD25+ cells from different patients were quantitatively determined by hybridization of Telomere PNA /FITC probe by flow cytometry (FACSCalibur, Becton

Dikinson, San Jose, CA) using Telomere PNA /FITC kit (DakoCytomation, Carpinteria, CA) as reported [61] and according to manufacturer's instructions.

Western blots:

Equal amounts of proteins from untreated and long-term AZT-treated MT2 cells were resolved by SDS-PAGE. All primary and secondary-HRP conjugated antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Bax (N-20), Bcl-xL(H-62), Mcl-1(S-19), Bcl-2 (N-19), PARP (H-250), caspase-3 (H-277), $p14^{ARF}$ (FL-132), p53 (FL-393), MDM2 (SMP-14), MDMX (D-19), $p21^{WAF}$ (C-19), $p27^{KIP}$ (C-19G), and β-tubulin (D-10) was used as loading control.

Senescence β-gal (SA-β gal) assay:

Untreated and long term AZT-treated MT2 cells were fixed in 2% formaldehyde-0.2% glutaraldehyde for 3 min, washed with PBS and incubated at 37°C with SA-β-gal staining solution, pH 6.0. Images were captured using a Nikon EFD3 microscope (Boyce Scientific, St Louis, MO) and Nikon camera with an Eplan 100X (160/0.17) objective. Acquisition software, Image-ProExpress version IV was from Media Cybernetics (Silver Spring, MD).

PCR and Semi-quantitative RT-PCR:

RT-PCR primers were as the following: p53 – forward primer F: (5′-GTCCCCGGACGATATTG-3′), reverse primer R:(5′-CCAGAATGCAAGAAGCCCAG-3′); $p21^{WAF}$ F:(5′-GAAGAAGGGTAGCTGGGGCT -3′), R:(5′-CTCTAAGGTTGGGCAGGGTG -3′); $p27^{KIP}$ F:(5′ TGCCCGAGTTCTACTACAGACC-3′), R:(5′CTTATTCCTGCGCATT GCTCCGC -3′); Bax F: (5′-GGGGACGAACTGGACAGTAA – 3′), R:(5′CAGTTGAA GTTGCCGTCAGA-3′); $p14^{ARF}$ exon1 F: (5′-CTGGAGGCGGCGAGAACATGG -3′), R: (5′-GGGCCTTTCCTACCTGGTCTT- 3′); $p14^{ARF}$ exon2 F: (5′-GCTCTACACAAGCTTCCTTTCCG-3′), R: (5′-CGGGCTGAACTTTCTGTGCTGG -3′**)** and GADPH F: (5′-GAAGGTGAAGGTCGGAGTC -3′), R: (5′-GAAGATGGTGATGGGATTTC-3′). Total RNA was isolated by Trizol reagent (Invitrogen, Carlsbad, CA) and RT-PCR was performed using the One-Step RT PCR kit (Qiagen, Valencia, CA).

Inverse LTR-PCR:

Proviral integration site was determined by digesting genomic DNA by SmaI, ligation and inverse PCR with HTLV-I LTR primers F: (5′-CCCCAAATATCCCCCGGG-3′) and R: (5′-

GGGGCTTATGGTCATTGTC- 3′). The amplified products were cloned into pCR2.1 TA vector (Invitrogen, Carlsbad, CA) and sequenced.

p53 status and functional assay:

Total RNA was isolated from ATL patients and the p53 hotspot region was amplified by RT-PCR using the following primers F: 5′-CCAGAAAACCTACCAGGGCAG -3′, R: 5′-GCTCGCTTAGTGCTCCCTGG-3′, cloned and sequenced. For p53 full length RT-PCR and cloning we used F: 5'-CGGAATTCATGGAGGAGCCGCAGTCAGATCC-3' and R: 5'-CCGCTCGAGTCAGTCTGAGTCAGGCCCTTCTG-3'. For the p53 functional assay, the p53 from MT-2 (wild type) was cloned into pcDNA3.1Zeo vector (Invitrogen, Carlsbad, CA) and the hotspot region from pcDNAp53MT2 was substituted by respective ATL p53 hotspot regions using DrdI-BsaI restriction enzymes. Luciferase assays were performed with Luciferase Reporter Assay kit (Promega, Madison, WI) by transfecting Jurkat E-6 cells with p53-responsive pGL13Luc and respective ATL pcDNA3.1Zeo-p53.

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CHAPTER IV

Regulation of Telomerase and Telomeres:

Human Tumor Viruses Take Control

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Regulation of Telomerase and Telomeres: Human TumorViruses Take Control.

ABSTRACT:

Human tumor viruses are responsible for one-fifth of all cancers worldwide. These viruses have evolved multiple strategies to evade immune defenses and to persist in the host by establishing a latent infection. Proliferation is necessary for pre-tumor cells to accumulate genetic alterations and to acquire a transformed phenotype. However, each cell division is associated with a progressive shortening of the telomeres, which can suppress tumor development by initiating senescence and irreversible cell cycle arrest. Therefore, the ability of virus-infected cells to circumvent the senescence program is imperative for the long-term survival/ proliferation of infected cells and the likelihood of transformation. We review the multiple strategies used by human DNA and RNA tumor viruses to subvert telomerase functions during cellular transformation and carcinogenesis. Epstein-Barr Virus, Kaposi Sarcoma-associated Herpes Virus, Human Papillomavirus, Hepatitis B Virus, and Human T-cell Leukemia Virus-I each can increase transcription of the telomerase reverse transcriptase. Several viruses appear to mediate

cis-activation or enhance epigenetic activation of telomerase transcription. Epstein-Barr Virus and Human Papillomavirus have each developed post-transcriptional mechanisms to regulate the telomerase protein. Finally, some tumor virus proteins can also negatively regulate telomerase transcription or activity. It is likely that, as future studies further expose the strategies used by viruses to deregulate telomerase activity and control of telomere length, novel mechanisms will emerge and underscore the importance of increased telomerase activity in sustaining virus-infected cells and its potential in therapeutic targeting.

INTRODUCTION:

Telomeres, which form the ends of eukaryotic chromosomes, are composed of tandem arrays of telomeric repeats (5'-TTAGGG-3' in humans) and help to preserve genome integrity and to prevent senescence (1,2). Somatic cells have a finite proliferative capacity, due largely to the inability of DNA polymerase to replicate the distal ends of chromosomes, leading to a progressive shortening of the telomeres after each cell division. Immortal tumor cells overcome this barrier mainly by increasing transcription of telomerase, a cellular reverse transcriptase that can extend telomeric ends (3-5).

Increased telomerase activity may contribute indirectly to the transformation process by sustaining the proliferation of pre-tumoral cells indefinitely, thereby increasing the potential risk of cumulative genetic defects. Only 10% of human tumor cells do not express telomerase; these cells use an alternative lengthening of telomeres pathway, referred to as ALT, to maintain telomere length. (6) Recent studies show that the ALT pathway involves the DNA repair and homologous recombination machinery (7,8). Telomerase is a holoenzyme that consists of several subunits, including hTR (human telomerase RNA), TEP1 (telomerase associated protein1), hTERT (human telomerase reverse transcriptase), hsp90 (heat shock protein 90), p23, and dyskerin (1). The ability of telomerase to extend telomere length is complex. Not only is the hTERT gene subject to transcriptional, post-transcriptional, and epigenetic control, but access of telomerase to the telomere ends is also regulated by the shelterin complex. Shelterin consists of the double-stranded DNA binding proteins, TRF1 and TRF2 (TTAGGG repeat binding factors -1 and -2), the single-stranded DNA binding protein, POT1 (protection of telomere), and their binding partners, TIN2 (TRF1-interacting nuclear protein 2) and PTOP (POT1- and TIN2-interacting protein) (9). Shelterin provides telomerase with stochastic signals regarding the length of telomeres, which then

allows or prevents telomerase from gaining access to the telomere ends. In addition, the shelterin components can form various subcomplexes that determine the structure of the telomere ends and preserve the integrity of telomeres by preventing DNA damage pathways from becoming activated (10).

Although telomere attrition can lead to chromosomal abnormalities, a direct role for telomeres or telomerase in the transformation process is not likely. Somatic cells expressing telomerase can extend their replicative lifespan but are unable to form tumors in nude mice and do not form colonies in soft agar (11). By contrast, normal epithelial and fibroblast human cells can be converted to tumorigenic cells with expression of transfected hTERT and Ras oncoproteins along with the large T-antigen of simian virus 40 (SV40) (12). Removal of SV40 proteins from normal human somatic cells could still lead to transformation as long as Rb and p53 were inactivated and Ras, c-Myc, and hTERT were overexpressed (13). Taken together, these findings suggest that overexpression of telomerase by itself is not sufficient to induce a tumorigenic state in normal cells, but it can contribute to tumorigenesis in the presence of additional oncogenes and/ or the disruption of tumor suppressors.

OVERVIEW OF TELOMERASE ACTIVITY AND TELOMERE LENGTH IN HUMAN ONCOGENIC VIRUS INFECTIONS:

Epstein-Barr Virus (EBV)

EBV infection is associated with Burkitt lymphoma, Hodgkin disease, nasopharyngeal carcinoma, and infectious mononucleosis, along with approximately 10% of gastric cancers and a subset of T-cell lymphomas (14). EBV mainly infects and immortalizes B-lymphocytes, but it can also produce latent infections in T-lymphocytes or natural killer cells. Analysis of samples from patients with nasopharyngeal carcinoma revealed that 94.9% were positive for telomerase activity, and analysis of EBV-immortalized B-lympboblastoid cell lines revealed increased telomerase activity, both supporting an important role for telomerase reactivation in EBV-infected individuals (15,16).

The EBV-encoded latent membrane protein-1 (LMP1) displays properties of a constitutively active member of the TNF receptor family. LMP1 is expressed during viral latency leading to the activation of NF-kappaB and Jak/STAT signaling pathways and oncogenic transformation (17). LMP1 is at least partially responsible for the increase in telomerase activity in nasopharyngeal epithelial

cells (18). LMP-1 also appears to enhance telomerase activity in B-cell lymphomas, in which expression of small interfering RNAs (siRNAs) directed against LMP1 substantially reduced hTERT protein levels and telomerase activity (19). Moreover, expression of LMP1 in an EBV-negative nasopharyngeal carcinoma cell line increased hTERT protein expression (19). Although hTERT has not been implicated in regulation of gene expression, a recent study suggests that hTERT expression decreases EBV lytic gene expression and promotes proliferation of latently infected B-lymphocytes (20). Other work in B-lymphoblasts suggests that immortalization may be associated with the reactivation of telomerase activity, along with aneuploidy and changes in p16, Rb, and p53 status; however, this finding has yet to be confirmed by independent laboratories (21,22).

EBV latent membrane protein 2A (LMP2A) induces constitutive activation of downstream effectors of the B-cell receptor, preventing infected cells from entry into the lytic replication cycle (23). LMP2A is also involved in transformation of epithelial cells. Transient transfection assays indicate that LMP2A may act as a negative regulator of the hTERT promoter (24).

Analysis of telomere lengths in EBV-positive Burkitt's lymphoma cell lines showed increases in telomere length compared

with that in EBV-negative cells (25,26). Although infections by many tumor viruses have been associated with increased telomerase activity, EBV is, to date, the only human oncogenic virus for which infection has been linked to an actual increase in telomere length.

Human Herpes Virus 8/ Kaposi Sarcoma–associated Herpes Virus (HHV-8/ KSHV)

HHV-8/ KSHV is the etiologic agent for Kaposi's sarcoma, multifocal Castleman's disease, and primary effusion lymphoma (27). KSHV-transformed endothelial cells have elevated telomerase activity compared with uninfected cells (28). The latency-associated nuclear antigen (LANA) protein of HHV-8/ KSHV plays an important role in latent episomal persistence of the viral genome in infected cells (29). LANA targets p53 and Rb and plays a role in B-cell lymphoma development (30). LANA has been found to increase hTERT promoter expression through modulation of Sp1 in fibroblasts 293, and B-cell lines BJAB and BCBL1 cells (31,32).

To date, the influence of infection on telomere length in HHV-8/ KSHV-infected cells has not been reported. However, it has been shown that an angiogenic lytic gene, the G protein–coupled receptor oncogene (vGPCR), of KSHV can immortalize human umbilical vein endothelial cells (HUVEC) in which telomere length

was maintained by the ALT pathway (33). A direct oncogenic role for vGPCR has not been established as it is an early lytic gene not expressed in HHV-8/ KSHV transformed cells.

Human Papillomavirus (HPV)

Over 100 types of HPV are responsible for a wide array of human diseases, ranging from malignant cervical cancer to benign warts (34). High-risk HPV16 and 18 are the genotypes most commonly associated with cancer (35,36).

Early studies with HPV16 found elevated telomerase activity in pre-crisis human cervical keratinocytes (HCKs) that express the viral protein E6 (37). HPV E6 cooperates with E7 to transform infected cells by targeting p53 and Retinoblastoma (Rb) for proteasomal degradation, respectively (38).

Further studies indicated that E6-mediated elevation of telomerase activity requires an additional protein, the E6-associated protein (E6AP) (39). E6AP is an ubiquitin-ligase that is involved in p53 degradation. Because E6AP is also involved in ubiquitination, it has been suggested that E6 targets a cellular inhibitor of telomerase activity for degradation using the ubiquitin machinery that is also used to degrade p53**.** A recent study demonstrated that E6 mutants, defective in E6AP binding retained their ability to stimulate hTERT

expression, (40) suggesting the existence of both E6AP-dependent and -independent mechanisms for E6-mediated increase in hTERT expression.

HPV16 E6-expressing fibroblasts had an increased lifespan and did not enter cellular senescence, unlike control cells that also had a marked decrease in telomere length (41). In these E6-expressing cells**,** the shortened length of the telomeres was eventually stabilized through an ill-defined mechanism.

A similar pattern—i.e., increased telomerase activity following viral infection and telomere shortening followed by stabilization—is found in HPV-infected cells, as in nearly all human infections by oncogenic viruses. For example, analysis of patient samples from early stages of cervical intraepithelial neoplasia to later, malignant stages of this disease demonstrated the persistence of shortened telomeres and strong telomerase activity in all stages of disease (42).

HPV early gene E2 is important for viral transcription through E2 responsive elements and replication (43). E2 can also suppress growth of HPV-positive cancer cells through transcriptional repression of E6 and E7 (44). Since E6 is a positive regulator of the hTERT promoter, it is expected that E2 would act as a negative

regulator in the context of HPV infected cells by preventing the action of E6.

Hepatitis B and Hepatitis C Virus (HBV/HCV)

HBV and HCV infect hepatocytes, leading to chronic liver disease and malignant transformation. Combined, they account for 70% of hepatocellular carcinomas worldwide (45). Tissue biopsies from HBV-positive patients exhibited strong telomerase activity compared with those from normal liver tissue, irrespective of disease stage (46). Short telomeres were present in HBV- and HCV-positive tissues despite elevated telomerase activity (47). Similarly, telomeres have been found to be shorter in hepatocellular carcinoma than in non-cancerous liver tissues isolated from the same patient (48,49).

HBV encodes two proteins with transcriptional activator functions – the HBV-X and preS2 activators (large surface proteins and truncated middle surface proteins) (50). The HBV-X transactivator oncoprotein (HBx) stimulates viral gene expression and cellular transformation by altering p53 functions and disrupting multiple cell signaling pathways (51). HBx has been shown to increase telomerase expression and telomerase activity in hepatoma cells (52). Transfection of the preS2 gene into a hepatocellular

carcinoma cell line was also associated with an increase in telomerase expression and activity (53).

Human T-cell leukemia virus type 1 (HTLV-I)

HTLV-I is the etiological agent of adult T-cell leukemia/lymphoma, a lymphoproliferative disorder of infected CD4+ T-cells (54). Similar to the pattern seen in HPV, HBV, and HCV infections, telomeres are short in HTLV-I infected cells despite the presence of strong telomerase activity (55). Telomerase activity was high in both HTLV-I-immortalized and HTLV-I-transformed cell lines as well as lymphocytes from patients with adult T-cell leukemia/lymphoma, including those with acute, smoldering and chronic disease, compared with peripheral blood mononuclear cells from uninfected individuals or samples from asymptomatic carriers (55-57). Disease progression, from the asymptomatic stages to acute and chronic disease, was correlated with increased telomerase activity, such that patients with acute and chronic disease exhibit a higher level of telomerase activity than asymptomatic carriers of the virus (55,57). Multiple other studies have confirmed that HTLV-I infected cells have elevated telomerase activation (58,59). Additional studies found that increased telomerase activity was not correlated with transformation status as measured by IL-2 dependence (55).

A direct role for HTLV-I in the elevation of hTERT expression was demonstrated by *in vitro* transmission of the virus to human primary T-cells (60), which led to increased hTERT promoter expression in infected cells only. Similar results were obtained by transduction of lymphocytes with a Tax-expressing vector. Although a study proposed that the HTLV-I-encoded Tax protein had a negative effect on an hTERT promoter reporter vector in transient assays (61), current data indicate that Tax is a strong positive regulator of the endogenous hTERT promoter (60,62).

The initial report that Tax acts as a repressor of the hTERT promoter turned out to be misleading because these experiments were performed in the presence of phytohaemagglutinin (PHA), a potent inducer of hTERT expression. Results showed that PHA-mediated induction of hTERT was indirectly lessened by Tax, however such effects were not seen when Tax was expressed in the absence of PHA stimulation. Thus, Tax should be considered as a positive regulator of the hTERT promoter. It remains to be demonstrated whether, in the context of HTLV-I infected cells, Tax prevents full induction of hTERT expression in physiological conditions which trigger cell activation (PHA or antigen stimulation),

Model for Telomere Maintenance in Virus-Infected Cells.

A list of the tumor virus-encoded regulators of telomerase activity that we have described is presented in Table 1.

Table 1: Human tumor virus-encoded regulators of telomerase activity.

	Viral-encoded Regulator	Impact on Telomerase
EBV	A) LMP1 B) LMP2A	A) Positive B) Negative
HHV-8	A) LANA	A) Positive
HPV	A) E6 B) E6AP C) E2	A) Positive B) Positive (through NFX1-123) Negative (through NFX1-91) C) Negative
HBV	A) HBX	A) Positive
HCV	A) HCV-C	A) Positive
HTLV-1	A) Tax	A) Positive

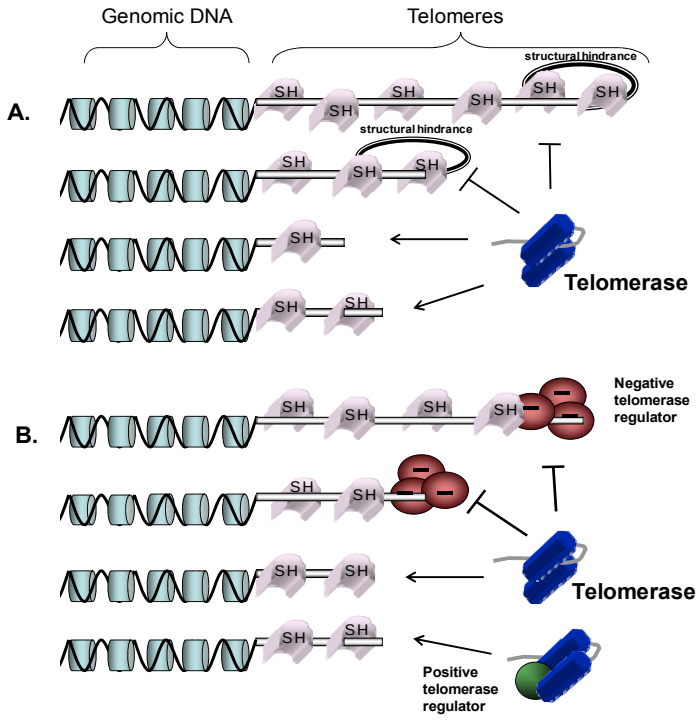
Current data support a model in which the initial stages of viral infection are associated with progressive telomere shortening despite an increase in hTERT expression (Fig. 1).

Figure 1: During most oncogenic viral infections, enhanced replication leads to a state of progressive telomere shortening, which if unchecked, promotes senescence/apoptosis of the infected cells. Oncogenic viruses protect infected cells with critically short telomeres by overexpressing telomerase, which can maintain telomere length. In this model, chromatin bound genomic DNA

(light blue) is protected by telomeres (white bar) bound by shelterin sub-complexes (light purple). Telomerase (dark blue) is either A. unable to elongate telomeres due to structural hindrance (black loop), or B. unable to elongate telomeres due to the presence of a negative telomerase regulator(s) (green), and/or the absence of a positive telomerase recruiter(s) (red). Critically short telomeres can lead to disruption of the structural interference or loss of the negative regulator(s), thereby allowing telomerase access to the telomere ends. Alternatively, critically short telomeres may signal the recruitment of a positive regulator(s), which delivers telomerase to telomeres. In this model, telomeres remain short in viral-infected cells, which are protected from senescence/apoptosis by the overexpression of telomerase. Telomerase can then maintain the shortened telomere length during each successive replication cycle and aid in establishing an oncogenic state.

Light blue: chromatin, white bar: telomeres, light purple: shelterin, dark blue: telomerase, red: negative telomerase regulator, green: positive telomerase regulator.

Figure 1:



Shortened telomere lengths are eventually stabilized, and maintenance of telomere size becomes dependent on elevated telomerase activity (55). The reasons for the initial telomere shortening and stabilization are unclear. Variations in the composition of the shelterin complex or accessibility of telomerase to the telomeres may explain this phenomenon and warrant further studies. The presence of shortened telomere and aneuploidy in cancer cells are circumstantial, and to date, there is no experimental evidence that telomere attrition induces chromosome instability.

In some instances, short telomeres may be poor prognosis makers for disease progression, as is the case in hepatocellular

carcinoma and several leukemias, including chronic myeloid leukemia, chronic lymphocytic leukemia, and HTLV-I associated adult T-cell leukemia/lymphoma (63-66). However, in other types of cancer, such as HPV-associated cervical cancer, there is no obvious relationship between telomere length and the clinical outcome (67). Although telomeres were substantially shortened in all cervical intraepithelial neoplasia samples, no further telomere shortening occurred in the majority of samples from patients during transition to a malignant phenotype (42).

MECHANISMS OF VIRAL ACTIVATION OF TELOMERASE:

hTERT expression is regulated primarily at the level of transcription (68). The hTERT promoter contains several E-boxes and five GC-rich elements, which can bind c-Myc and Sp1, respectively (69). It is well established that hTERT gene expression is increased following c-Myc binding to E-box elements present in the hTERT promoter (70,71). In fact, in most cells, c-Myc and Sp1 act cooperatively as the dominant determinants of hTERT expression (69,72). A more complete listing of various factors known to regulate hTERT transcription can be found in Table 2. The most

important tumor virus proteins, known to directly regulate hTERT transcription, are shown in boldface type in Table 2, and are discussed below.

Table 2: Tumor Virus Interactions with Transcriptional Activators and Repressors of the hTERT promoter.

* A selection of known regulators of hTERT promoter activity. Those viral proteins that have an established role in direct hTERT regulation are discussed in the text and shown in bold. Bmi-1: Polycomb-group gene, c-Myc: myelomonocytic leukemia gene, E2F-1: E2F transcription factor-1, IRF-1: IFN-regulatory factor-1, Sp1: Specificity protein-1, WT1: Wilm's tumor rrotein-1, USF-1/2: Upstream stimulatory factor-1/2. BZLF1: (bZIP transactivator of EBV), initiates the switch between latent and productive infection in B cells (124). vFLIP (viral FLICE inhibitory protein), activates NF-κB and protects from apoptosis (125). vIRF (viral homolog of the interferon regulatory factors), represses interferon antiviral response (126). NS4B (nonstructural protein of HCV), part of the replication complex of HCV (127). NS5A (nonstructural protein of HCV), aids in viral invasion of the immune system and NS3 (nonstructural protein of HCV), signals the immune response (128).

	Impact on hTERT	Mode of Action	EBV	HHV-8	HPV	HBV	HCV	HTLV-I
Bmi-1	Positive	-	(+) LMP1	-	(+) E6	-	-	-
c-Fos/c-Jun c-Fos/JunD	Negative	Direct	(+) LMP1	(+) LANA (+) vFLIP	(+) E6 (+) E7	(+) X	(+) NS4B (-) NS5A	(+) Tax
c-Myc	Positive	Direct	**(+) LMP1**	(+) LANA	**(+) E6/E6AP**	(+) X	(+) Core	**(+) Tax**
E2F-1	Negative Positive	Sp1 Direct	(+)BZLF1	(+) LANA	(+) E7	(+) X	(+) Core	(+) Tax
IRF-1	Negative	-	(-) BZLF1	(-) vIRF	(-) E7	-	(-)	-
p16^{INK4A}	Negative	Sp1	(-) LMP1	(-)	(+)	(-)	(-)	(-) Tax
p27^{KIP1}	Negative	c-Myc/Sp1	(-)	(-)	(-) E7	(-)	(-)	(-)
p53	Negative	p21 and E2F	(-)	(-) vIRF (-) LANA	(-) E6	(-) X	(-) NS5A (-) NS3 (+)/(-) Core	(-) Tax
p73	Negative	c-Myc	(-)	-	(-) E6	-	(-) p73α/Core	(-) Tax
Smad3	Negative	c-Myc	(-) LMP1	(-) VIRF1	(-) E7	-	(-) Core	(-)
Sp1	Positive	Direct	(+) EBNA2	**(+) LANA**	**(+) E6**	(+) X	(+) Core	**(+) Tax**
Survivin	Positive	c-Myc/Sp1	(+) LMP1	(+)	(+) E6	(+) X	(+) NS5A	(+) Tax
USF-1/2	Negative Positive	Direct	-	-	**(-) E6**	-	-	-
WT1	Negative	Direct	(+)/(-)	-	-	(-) X	-	**(-)**

Trans-Activation of the hTERT Promoter by viral oncoproteins

Most studies of viral transactivation of hTERT gene expression have been done with the papillomavirus system, in which the HPV E6 oncoprotein has been shown to directly transactivate the hTERT promoter. Mutations in either the GC or the E-box elements of the hTERT promoter modestly inhibited telomerase expression, demonstrating that independent binding of c-Myc or Sp1 to the hTERT promoter is not sufficient for E6-induced telomerase activation (73). However, telomerase expression is strongly induced by E6 when c-Myc and Sp1 are cooperatively bound to the hTERT promoter (73). Although E6 had no effect on levels of c-Myc gene expression, immunoprecipitation studies demonstrated that E6 forms a complex with c-Myc on the hTERT promoter (74). The use of

E6AP knock-out mice and E6AP siRNA has demonstrated that E6AP is important for hTERT promoter activation. Both E6 and E6AP are required for binding to the E-box elements and for the consequential activation of hTERT expression (75). Indeed, a yeast two-hybrid screen identified NFX1 as a binding partner of the E6/E6AP complex. NFX1 exists in two isoforms, NFX1-123 and NFX1-91. NFX1-123 has been shown to activate the hTERT promoter along with c-Myc (39).

In the case of papillomavirus infection, one possible model for the mechanism of hTERT transactivation is that E6 forms a tertiary complex with E6AP and c-Myc. Once the complex is bound to E-box elements, E6AP could target the degradation of possible negative regulators of the hTERT gene, such as NFX1-91 (39) or BRCA1 a tumor suppressor that has been found to suppress hTERT gene expression by binding to E-box elements (76). In addition, c-Myc, once activated, may itself be targeted for degradation to limit hTERT gene expression. In fact, the E6/E6AP complex has been shown to ubiquitinate c-Myc *in vitro* and *in vivo* (77). c-Myc has also been shown to interact with a transcriptional coactivator, TRRAP, that can activate a silent hTERT gene in fibroblasts by inducing acetylation of histones H3 and H4 at the hTERT promoter (78).

Upstream stimulatory factors -1 and -2 (USFs) have also been found to bind to the same E-boxes as c-Myc. USFs can suppress the hTERT promoter in nontransformed cells where no hTERT gene expression is detected (79). HPV E6 can relieve USF-1 or -2-mediated repression of telomerase activity by preventing USF-1/ -2 binding to the hTERT promoter. This interaction allows for an increase in c-Myc binding to the E-box (80). The exact role of c-Myc in E6-mediated transactivation of the hTERT gene remains to be seen, and additional transcriptional regulators required for HPV-mediated telomerase activation may remain to be discovered.

Much less is known about the mechanisms used by other viral oncoproteins to transactivate hTERT gene expresssion. The Tax protein of HTLV-I is able to stimulate the hTERT promoter through activation of the NF-κB pathway. Chromatin immunoprecipitation (ChIP) assays demonstrated increased binding of c-Myc and Sp1 at the hTERT promoter in response to NF-κB activation in both HTLV-I- infected and Tax- expressing cells (60). In fact, a Tax mutant unable to activate the NF-κB pathway could not stimulate hTERT expression (60).

The LMP1 protein of EBV has been found to induce c-Myc-mediated transactivation of the hTERT promoter in primary human nasopharyngeal epithelial cells and in a nasopharyngeal carcinoma

cell line that stably expresses LMP1 (18). In this case, the C-terminal portion of LMP1, which includes CTAR1 and 2 domains, was found to stimulate hTERT gene expression through NF-κB activation.

Lastly, the LANA protein of KSHV has been shown to transactivate the hTERT promoter in various cell lines (31). LANA activates hTERT gene expression through interactions with Sp1 (32).

Most studies on viral-induction/ repression of hTERT expression have focused on the core hTERT promoter. This site includes the GC and E-box elements that bind Sp1 and c-Myc, respectively. However, the distal promoter remains to be explored. The region between -2000 and -378 is responsible for JunD and c-Jun binding to the hTERT promoter. The expression of AP-1, a transcription factor that includes Jun and Fos, can lead to hTERT promoter repression in some cancer cells (81). In contrast, activation of JNK (c-Jun NH_2-terminal kinase) has been shown to increase hTERT gene expression (82). This suggests that hTERT regulation could occur by AP1-mediated repression or hTERT activation through JNK transactivation. In HTLV-I and EBV infected cells in which both AP1 and JNK pathways are activated (83-86), it seems that JNK activation has a dominant effect.

Epigenetic Control of hTERT in virally-infected cells

Activation of hTERT transcription may involve histone acetylation, and conversely, repression may involve histone deacetylation. Trichostatin A, which inhibits the family of mammalian histone deacetylates, activates hTERT gene expression in different cell types (87). However, trichostatin A may also activate additional genes that directly or indirectly derepress the hTERT gene, including c-Myc itself (88). HPV E6 and E6AP expression promote acetylation of histone H3, providing epigenic control of the hTERT gene (89). Acetylation of histone H3 at the hTERT promoter was increased in late passage E6- and E7-immortalized keratinocytes and p300 expression was decreased (89). E6 has been shown to target the coactivator/acetyl transferase p300 and cells that expressed E6 and p300 antisense RNA showed increased acetylation of histone H3 and activation of the hTERT gene (89). Therefore, p300 may act as a repressor of telomerase activation in the context of E6 expression. Other examples of this regulatory mechanism include adenovirus E1A binding to p300/CBP and the subsequent recruitment of its histone acetyltransferase activity to the hTERT promoter (90).

Further control of hTERT gene expression involves histone H3 phosphorylation mediated by the mitogen-activated protein kinase (MAPK) cascade. In normal human T lymphocytes and fibroblasts, growth or stress stimuli that are known to drive H3

phosphorylation through MAPK signaling induced hTERT gene expression, whereas inhibition of MAPK-triggered H3 phosphorylation substantially abrogated hTERT induction (91). MAPK-mediated control of H3 phosphorylation may have important consequences for hTERT gene expression during viral infection because c-Jun kinase (JNK) and extracellular signal-regulated kinases 1 and 2 (ERK1/2), members of the MAPK family, are constitutively activated in HTLV-I-, EBV-, and KSHV- infected cells (84,92,93).

Finally, the hTERT promoter was more frequently methylated in cervical cancer specimens than in normal cervical tissue isolated from different patients; however, there was no correlation between methylation status and hTERT gene expression (94). A similar phenomenon is seen in hTERT-expressing cells, in which a demethylated hTERT promoter does not necessarily correlate with an increase in hTERT gene expression and both methylation-dependent and -independent mechanisms are in place to modulate hTERT gene expression (95-97).

Cis-Activation of the hTERT Promoter through viral integration

In addition to encoding telomerase regulators that act in *trans* on the hTERT promoter, the HPV and HBV genomes have been

found in sporadic cases to integrate in proximity to the hTERT gene (98-100). In a subset of cervical and hepatocellular carcinoma tumors, the integration resulted in the placement of viral enhancers near the hTERT promoter without perturbing the hTERT coding region. Telomerase expression increased in all cases, though it was not clear whether the increase was due to viral integration or to the action of viral oncoproteins in transformed cells. Analysis of the hepatocellular carcinoma cell line huH-4 produced similar results, with HBV integration acting as an enhancer for cis-activation of the hTERT gene (101). Many different patterns of karyotypic abnormality have been reported in cervical carcinomas, but the most recurrent structural chromosomal aberration in cervical cancer is a gain of chromosome 3q copy number (102). A recent study demonstrated that the gene encoding the RNA component of telomerase (hTR) at 3q26 was progressively amplified with the development of HPV-associated cervical intraepithelial neoplasias to advanced invasive carcinomas (103). This gain of *hTR* was predominantly associated with integration of oncogenic HPV.

Therefore, it appears that expression of the genes for hTERT and/or hTR may be specifically activated by virus integration in a population of infected cells. In concert with other viral proteins, enhanced telomerase expression and the resultant increase in

telomerase activity could provide a selective advantage by promoting transformation.

Post-Transcriptional Regulation of Telomerase Activity

Telomerase activity can also be regulated at the post-transcriptional level. For example, protein kinase C (PKC) mediated phosphorylation of telomerase results in the interaction of telomerase and protein hsp90, the latter of which is necessary to maintain the integrity of the telomerase holoenzyme (104). Inhibitors of PKC substantially reduced telomerase activity present in human nasopharyngeal and head and neck cancer cells (104) and PKC has been shown to modulate telomerase activity in human cervical cancer cells (105), suggesting that EBV and HPV activate PKC to increase telomerase enzymatic activity

Some viruses have evolved mechanisms to exploit the post-transcriptional regulation of telomerase. These mechanisms include blocking access of the enzyme to its substrate by regulating the phosphorylation and nuclear translocation of the hTERT catalytic subunit and promoting increased translation of hTERT by regulating the poly-A binding proteins that stabilize hTERT messenger RNA transcripts.

In uninfected cells, AKT has been shown to elevate telomerase activity by phosphorylating the hTERT protein, leading to its accumulation in the nucleus (106). Similarly, the RelA/p65 subunit of NF-κB has been shown to directly bind to hTERT and to facilitate its translocation to the nucleus (107). On the other hand, protein phosphatase 2A (PP2A) has been shown to dephosphorylate and inactivate telomerase (108). The LMP1 protein of EBV has evolved to exploit this pathway. In nasopharyngeal carcinoma cells, LMP1 can increase telomerase activity post-transcriptionally, by promoting NF-κB RelA/p65-mediated binding to and nuclear localization of hTERT (109). To date, EBV is the only oncogenic virus that has been shown to regulate hTERT nuclear translocation.

In HPV E6-expressing cells, the E6AP-binding protein NFX1-123—in addition to its role as a transcriptional activator of hTERT—can also act as a post-transcriptional regulator of hTERT (110). NFX1-123 has been shown to interact with cytoplasmic poly (A) binding proteins (PABPCs) affecting RNA stability in the nucleus and cytoplasm, and thereby elevating telomerase activity.

Protection of Short Telomeres in Virus-induced Tumor Cells

As discussed above, in most tumor cells telomere lengths are shortened despite strong telomerase activity. This situation may seem

paradoxical; however, several positive and negative regulators of telomere length can bind and form a complex, shelterin, that can protect or sustain telomere length (10). Telomerase extension is often negated because shelterin blocks access of the telomerase reverse transcriptase to the now "closed" ends of telomeric DNA (111).

Three components of the shelterin complex, TRF1 and TRF2 and, TIN2—are overexpressed in tumor cells of HTLV-I infected adult T-cell leukemia/ lymphoma patients (55). Studies have shown that overexpression of either TRF1 or TRF2 results in rapid telomere shortening (112). In addition, TRF1, TRF2, and TIN2 can inhibit apoptosis and cell-cycle arrest due to DNA damage (113,114). It is possible that the telomeres in adult T-cell leukemia/ lymphoma cells reach a critically short length but that the cells are prevented from initiating apoptosis and senescence due to the overexpression of key members of the shelterin complex. Samples from multiple stages of human hepatocarcinogenesis and from patients with non-Hodgkins B-cell lymphoma also showed differential expression of shelterin components, but whether this was specific to viral infection was not elucidated (115).

A cellular protein, PINX1, has been shown to negatively regulate telomerase activity by binding to and inhibiting hTERT

directly (116). However, samples from HTLV-I- and HBV-infected patients showed no substantial differences in *PINX1* mRNA levels compared with uninfected control subjects (55,117).

MECHANISMS OF VIRAL NEGATIVE REGULATION OF TELOMERASE

Telomerase activity is inhibited by cellular differentiation and by down-regulation of hTERT transcription; and high levels of hTERT are a characteristic of immortalized cells (3). Several tumor suppressor pathways, such as Mad-1/c-Myc or TGF-β, repress hTERT in somatic cells, thereby preventing a critical component of tumorigenesis from becoming activated (118).

Thus it may be surprising, then that several oncogenic viruses encode negative regulators of telomerase activity. In fact, in addition to the positive regulators, there are two known negative regulators of hTERT gene expression. The EBV-encoded transmembrane protein, LMP2A, like LMP1, is expressed in the latent stages of disease and during cellular transformation. Surprisingly, LMP2A has been shown to reduce telomerase expression and activity in epithelial cells by repressing hTERT promoter activity through its tyrosine-based activation motif (ITAM) (24). Repression of the hTERT gene was not associated with changes in cell-cycle progression. It has been

suggested that LMP2A reduces hTERT gene expression to prevent B-cell activation and promote the viral latent state (24).

Reduced activity of E2, an HPV regulatory protein that represses E6/E7 expression, is obligatory for HPV-mediated carcinogenesis (119,120). In fact, expression of E2 induces senescence via pRb- and p21-associated pathways (121) and E2 represses hTERT gene expression through Sp1 (122). E2 may disrupt the interaction of positive-regulators at the hTERT promoter, such as the recruitment of histone-deacetylases (HDAC), thereby leading to a decrease in telomerase activity. The ability of E2 to repress E6/E7 and hTERT gene expression may have an important role in the induction of senescence.

NFX1 was identified as a novel telomerase target and as a possible candidate for E6/E6AP-mediated telomerase regulation (39). The NFX1-91 isoform represses promoter activity in HPV16 E6- or c-Myc-expressing keratinocytes by binding to an X-box motif adjacent to the E-box (123). NFX1-91 is also ubiquitinated by the E6/E6AP complex. Why NFX1-123, and not NFX1-91, stimulates telomerase expression is still unknown. Possible explanations include the rapid turnover of NFX1-91, the fact that NFX1-91 lacks a C-terminal domain similar to that of NFX1-123, or the fact that NFX1-91 is expressed predominantly in the nucleus, whereas NFX1-

123 is mostly cytoplasmic (110). All of these factors may affect the binding of NFX1-91 to stimulatory elements at the hTERT promoter. Whether NFX1-91 is necessary for the overall regulation of telomerase expression during HPV infection and whether additional proteins that are required for telomerase transactivation are ubiquitinated by E6AP still remains to be demonstrated.

Although it may seem counter-intuitive that viruses encode negative regulators of telomerase expression, two oncogenic viruses, EBV and HPV, have been shown to encode such viral negative regulators. In addition, the Tax protein of HTLV-I is a potent positive regulator of hTERT expression yet was able to limit hTERT gene activation following antigen stimulation. Thus, a balance between transcriptional activation and repression of telomerase may be important in viruses that require a latent infection. Elevation of telomerase expression guarantees that the infected cells can proliferate indefinitely in the absence of senescence and apoptosis, whereas repression of telomerase may play an important role in preventing an immune response against activated infected cells or create a state of transient genetic instability. It would be useful to know whether these viral inhibitors are expressed constitutively during latency or whether they repress telomerase gene expression during a particular stage of disease progression.

Conclusions

The human tumor viruses have evolved numerous strategies to constrain tumor suppressor pathways and to promote cellular transformation. Among these, elevation of telomerase transcription and/or activity can be used as mechanisms to bypass replicative senescence and to increase proliferative capacity, and these mechanisms, in turn, increase the cumulative risk of genetic alterations. A summary of the data concerning the relationships of six human tumor viruses to telomere length and telomerase activity is shown in Table 3.

Table 3:

	Telomerase Activity	Telomere Length	Epigenetic Control	Cis-Activation of the hTERT Promoter	Trans-Activation of the hTERT Promoter	Post-Transcriptional Modification of hTERT
EBV	Elevated	Long	-	-	Yes	Yes
HHV-8	Elevated	-	-	-	Yes	-
HPV	Elevated	Short	Yes	Yes	Yes	Yes
HBV	Elevated	Short	-	Yes	-	-
HCV	Elevated	Short	-	-	-	-
HTLV-1	Elevated	Short	-	-	Yes	Yes

Malignant cells infected by all six human tumor viruses studied exhibited elevated telomerase activity, yet in all but EBV- infected cells, telomere length was found to be shorter than that in uninfected control cells. In the case of EBV, HHV-8/ KSHV, HPV and HTLV-

I, elevated telomerase activity can be clearly ascribed, at least in part, to direct transactivation of the hTERT promoter by viral proteins. As discussed above, the LMP1 protein of EBV, the LANA protein of HHV-8/ KSHV, the E6 protein of HPV, the X transactivator of HBV, and the Tax transactivator of HTLV-I each can increase transcription of the telomerase reverse transcriptase. Whereas HPV and HBV may mediate cis-activation of telomerase transcription, HPV appears to enhance its epigenetic activation. EBV and HPV may regulate telomerase post-transcriptionally, by activation of its phosphorylation through PKC, promotion of its nuclear translocation through NF-κB, or stabilization of hTERT mRNA. However, viral regulation of telomerase activity is likely to be complex, and there are indications that at some stages of viral infection, some tumor virus proteins can also negatively regulate telomerase transcription or activity,

The diversity of strategies used by tumor viruses underscores the complexity of hTERT promoter and telomerase regulation. Further investigation in this area is likely to yield many new advances and to shed light on potential new therapeutic approaches for the treatment of human cancers.

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CHAPTER V

Telomerase: A Crucial Player in HTLV-I-induced Human T-cell Leukemia

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Telomerase: A Crucial Player in HTLV-I-induced human T-cell leukemia.

Abstract:

One in seven human cancers is associated with an oncogenic virus infection. Most human tumors have high telomerase activity but very short telomeres, yet the maintenance of these short telomeres is critical to avoid telomere end fusion or senescence and to support active proliferation. Oncogenic viruses have evolved a wide repertoire of strategies to stimulate telomerase functions at the transcriptional and post-transcriptional levels. Since telomerase activity is absent in somatic cells, the inhibition of telomerase is an attractive target for cancer therapeutics.

Telomerase and Cancer.

The "end replication" problem model states that the ends of chromosomes cannot be replicated due to the lack of a downstream template for DNA polymerase. This eventually leads to a loss of the very distal ends of DNA with successive replication cycles, leading to telomere attrition, cell cycle arrest, and cellular senescence or

apoptosis (1). This normal physiological aging process assures that proliferating cells that accumulate genetic defects over time are replaced by new undamaged cells and protects against potential pre-cancerous cells. However, this process needs to be tightly regulated because shortened telomeres can also lead to chromosomal instability characterized by end-to-end fusions, chromosomal translocations and DNA damage responses increasing the risk of cellular transformation (2).

In tumor cells, the existence of a mechanism for the maintenance of telomere length is critical and the upregulation of telomerase expression and activity has a well established role in carcinogenesis. In fact, nearly 90% of all cancers demonstrate an increase in telomerase expression (3,4). The remaining 10% of tumors use a telomerase-independent alternative lengthening of telomeres (ALT) pathway (5).

Telomerase is composed of *hTR*, a 451 nucleotide stretch of RNA, which serves as a template for the RNA-dependent DNA polymerase, telomerase (*hTERT)*. Through repetitive binding to *hTR*, hTERT is able to lie down tracts of TTAGGG, 10-15 kbp in length, which serve to protect and sustain chromosomal ends. Telomerase expression alone, however, is not sufficient to sustain telomeres, as the shelterin complex, composed of the DNA binding

proteins, TRF1, TRF2, and POT1, and their partners, TIN2, hRAP1, and PTOP, have been shown to have a definitive role in regulating and protecting telomere lengths (6,7). These telomere binding proteins prevent recognition of telomere structures by DNA damage response proteins, repress recombination mechanisms by altering the shape of telomeric DNA, and protect telomeres against the action of exonucleases.

A proven role for telomerase in the transformation process is still ongoing. The ability of telomerase to avoid cellular senescence is evident in early *in vitro* studies, in which telomerase-negative cells and human endothelial cells reconstituted with telomerase, and telomerase transduced CD8+ T-cells, were able to prolong growth, sustain longer telomeres, and in the case of CD8+ T-cells, maintain a normal karyotype, through successive cycles of growth (8-10). However, telomerase expression alone may not be enough to induce a tumorgenic state in normal cells, as somatic cells expressing telomerase are unable to form tumors in nude mice, nor colonies in soft agar (11). Recent studies have shown that forced overexpression of *hTERT* and Ras^{G12V} proteins, along with the small and large T-angtigens of simian virus 40 (SV40), are fully capable of transforming human cells. Transformation could also occur in the absence of the viral SV40 proteins, if RB, p53, Ras and c-Myc, are

overexpressed, along with hTERT (12). These findings suggest that the overexpression of telomerase can prevent cellular senescence, thereby contributing to the transformation process, and once fully transformed, can then extend the proliferative capacity of these cells, ensuring successive generations of growth. As the transformation process depends upon the deregulation of host cellular proteins and pathways, the ability to circumvent these processes is vital to the survival of the evading pathogen. Deregulating host mechanisms is a hallmark of all viruses, as they have proven exceptional at perturbing host cell homeostasis, and among them, deregulation of telomerase activity.

Transformation of ATLL cells by HTLV-I: the case for achieving clonal expansion.

Human T-cell leukemia virus type 1 (HTLV-I) is the etiological agent of adult T-cell leukemia/lymphoma (ATLL), a lymphoproliferative disorder of infected CD4+ T-cells (13). ATLL develops in less than 5% of infected individuals after a long latency of 20 to 30 years. The lack of proofreading and subsequent high error rates of viral reverse transcriptase (RT) enzymes have been associated with the diversity observed in retroviruses. HTLV-I is

unusual in that the observed genetic variability among isolates is unusually low. Thus, it has been hypothesized that HTLV-I provirus replication occurs mainly with the high-fidelity process of cellular DNA replication during division of infected cells (14). This concept is supported by the fact that treatment with AZT, a reverse transcriptase inhibitor, does not reduce proviral loads in infected patients over a short period of time.

ATLL disease progression is linked to the proviral load, which in turn depends on long term survival and proliferation of the infected cells. To efficiently achieve clonal expansion, HTLV-I-infected cells must gain an extended life span while avoiding elimination by the host immune responses. Increased longevity may in part be explained by an increased expression of anti-apoptotic Bcl-2 and Bcl-xL in HTLV-I-transformed T-cells *in vitro* and of Bcl-xL in uncultured PBMCs isolated from ATLL patients (15,16). Escape from immune defenses may result from a combinatorial effect of the regulatory proteins p30 and p12 that reduces viral expression and down-regulate MHC expression, respectively (17,18). Yet to achieve clonal expansion, HTLV-I-infected cells must also acquire an increased capacity to replicate their DNA and telomeric ends.

HTLV-I infection subverts transcriptional control of the *hTERT* promoter.

Regulation of the *hTERT* promoter is complex and subject to the positive and negative regulators, c-myc, Sp1, USF1/2, 14-3-3 and Mad1, phosphorylated-Rb, PTEN, respectively (19-20). Regulation of the *hTERT* promoter can also occur by hypermethylation or acetlyation (21). Many oncoviruses have evolved proteins that target the *hTERT* promoter to stimulate telomerase expression and promote transformation. The E6 protein of human papillomavirus (HVP) and the LMP1 protein of Epstein-Barr virus (EBV) have been shown to transactivate the *hTERT* promoter (22-24). HPV E6 was also found to promote acetylation of histone H3 and HPV can integrate near the *hTERT* gene (25,26). Hepatitis B virus (HBV) has also been shown to integrate viral DNA into the hTERT promoter, allowing for cis-activation of telomerase (27).

A recent report suggests that the HTLV-I oncoprotein Tax may repress transcription from the *hTERT* promoter and lower telomerase activity under specific cell culture conditions (28). Yet, such a function of Tax is difficult to reconcile with its capacity to immortalize T-cells and other studies showing increased transcriptional regulation of the *hTERT* promoter in HTLV-I and Tax

expressing human T-cells. These discrepancies may simply depend on the activation status of infected cells. We found that Tax suppresses *hTERT* mRNA expression only when Tax-expressing cells are subject to PHA mitogenic stimulation and that in the absence of exogenous stimulation, Tax always stimulates expression from the *hTERT* promoter (29). We propose that in response to antigen stimulation, interference of Tax with the full induction of hTERT expression may result in transient genetic instability during mitosis. Once the mitogenic effect has vanished Tax-mediated activation of *hTERT* gene expression offers a long term proliferative advantage to these cells that have acquired chromosomal abnormalities increasing their chances to repeat this cycle. Successive repetition of transient proliferation and stabilization phase may be required for the development of ATLL. This model is supported by *in vitro* HTLV-I infection of activated peripheral blood mononuclear cells (PBMCs), as PBMCs stimulated by coculture with MT2 (HTLV-I producer T-cell line), show a significant decline in telomerase activity by week 4, with undetectable levels reached during the 5^{th} and 6^{th} weeks of culture. By week 7, however, telomerase activity begins to increase and is significantly elevated by week 13. Telomerase detection by week 7 occurred before the cells escaped from crisis, and therefore, is not attributed to a general loss

of PBMC cells as they died, but rather as a possible mechanism for selection and maintenance of transformed cells (29,30). In addition, PBMCs cocultured with MT2 cells produce strong telomerase activity that correlates with viral load (29). Further analysis into this model of Tax-mediated *hTERT* regulation is required in order to more fully appreciate the contribution of telomerase in mediating transformation of HTLV-I infected T-cells. Real-time PCR analysis detected *hTERT* mRNA expression in all HTLV-I established cell lines, IL-2 dependent or independent. Increased expression of *hTERT* mRNA can be detected in PBMCs following coculture with lethally-irradiated MT2 cells, suggesting that HTLV-I activates endogenous *hTERT* expression (29). Surprisingly, our laboratory found that Tax-mediated NF-κB activation was responsible for the increased *hTERT* expression. Chromatin immunoprecipitation (ChIP) assays demonstrated an increased binding of the transcriptional activators, c-Myc and Sp1 onto the *hTERT* promoter, thereby acting as downstream effectors of the NF-κB responses (29). *In vivo* studies also support the idea of HTLV-I-induced telomerase expression, as telomerase activity is consistently detected in acute, smoldering and chronic ATLL patients (31,32). In fact, disease progression, as exemplified from asymptomatic to acute stages of disease, correlates with increased telomerase activity, as acute/chronic patients exhibit a

higher percentage of telomerase activity than asymptomatic carriers (33).

Post-transcriptional regulation of telomerase by HTLV-I Tax oncoprotein.

There is abundant evidence that telomerase expression and activity is constrained at multiple levels. Several reports have identified a lack of correlation between *hTERT* mRNA levels and telomerase activity measured by telomeric repeat amplification protocol (TRAP) assays. Although the half life of *hTERT* mRNA and the telomerase complex differ significantly, one and eighteen hours respectively, these observations suggest additional post-transcriptional regulation of the enzymatic activity. The region surrounding Ser-824 in hTERT contains a consensus sequence for phosphorylation by Akt, and Akt kinase enhances human telomerase activity through phosphorylation of hTERT (34). Telomerase must be translocated from the cytoplasm to the nucleus in order to act upon telomeric DNA. TNFα modulates telomerase activity by inducing nuclear translocation of hTERT protein bound to NF-κB p65/RelA phosphorylated on Ser-536. A specific IKK inhibitor, PS-1145 that prevent p65/RelA phosphorylation or a specific NF-κB

nuclear translocation inhibitor, SN-50, both block TNF$_\alpha$-induced hTERT nuclear translocation and stimulation of telomerase activity (35). In addition, EBV's LMP1 increases telomerase activity in nasopharyngeal carcinoma cells post-transcriptionally through NF-κB mediated phosphorylation and nuclear localization of hTERT (36). Finally, 14-3-3 protein can also enhance the nuclear localization and activity of hTERT (37).

ATLL cells have short telomeres despite strong telomerase activity.

Both HTLV-I immortalized and transformed cell lines and *in vivo* ATLL patient samples have high telomerase activity when compared to non-infected PBMCs and asymptomatic carriers. However, measurements of telomere lengths show a decrease in size in both *in vitro* and *in vivo* HTLV-I infected samples, relative to normal human T-cells (31,32). This paradox is not entirely surprising, as telomere length does not solely depend upon the expression of telomerase and most cancer cells have short telomeres.

Telomere length is also regulated by several key positive and negative regulators that have been discovered of late, which act either directly on telomerase or indirectly, by binding to the

telomeric DNA itself. These proteins can then prevent telomerase extension by blocking access of the reverse transcriptase to the now "closed" ends of telomeric DNA. While the functions of most of these telomeric regulators are still being elucidated, it is clear that the complex they form (shelterin), can negatively affect the extent of telomere elongation by inhibiting the access of telomerase to the ends of DNA (38). In fact, three components of the shelterin complex, TTAGGG repeat binding factors, TRF1 and TRF2, and the TRF1-interacting nuclear protein 2, TIN2, are over-expressed in ATLL patients. Studies have shown that over-expression of either TRF1 or TRF2, results in rapid telomere shortening. In addition, TRF1, TRF2, and TIN2 can inhibit apoptosis and cell cycle arrest due to DNA damage (39,40). It is possible that the telomeres of transformed ATLL cells reach a critically short length, but the cells are prevented from initiating apoptosis and senescence, due to the over-expression of key members of the shelterin complex. Further investigations into the roles of these proteins in allowing the continuous proliferation of ATLL cells is warranted as it could explain how ATLL cells continue to survive, despite short telomeres.

Treatment of ATLLpatients with Telomerase Inhibitors.

The clinical significance of telomerase activity in ATLL disease progression can readily be demonstrated upon treatment with telomerase inhibitors. Azidothymidine (3'-azido-3'-deoxythymidine, AZT), is a thymidine analog, that has been shown to inhibit cancer growth and telomerase activity. AZT is used to treat several virus associated human cancers including, AIDS-related Kaposi sarcoma, Kaposi sarcoma associated primary effusion lymphoma (PEL), EBV-associated lymphoma and primary central nervous system lymphoma (PCNL). However, until recently the mechanism of AZT action was unclear and thus, reasons for treatment failure unknown. The poor prognosis of ATLL patients is associated with the resistance of neoplastic cells to the conventional combination of high-dose chemotherapy and radiotherapy. Recently, a higher response rate following AZT/ IFN alpha treatment of ATLL patients has been reported in several human trials (41). Long-term treatment of HTLV-I infected cells with AZT (or ddG, an additional telomerase inhibitor) inhibits telomerase activity, induces telomere attrition, and promotes cellular senescence, in absence of apoptosis (42). HTLV-I infected cells undergo senescence during long-term AZT treatment, due to the reactivation of tumor suppressor p53 transcriptional activities. This effect is dependent upon telomere shortening. *In vivo* patient samples of AZT treated ATLL patients show decreases in telomerase

activity and telomere lengths. Further analysis of ATLL patients at varying disease stages demonstrates that those patients with a transcriptionally functional p53 gene are able to undergo partial or complete remission, following AZT/ IFN alpha treatment (43). However, those patients with a mutated p53 do not respond to AZT treatment and die (44). These results demonstrate that AZT treatment causes telomere attrition that leads to the reactivation of a functional p53. Reactivated p53 is then capable of driving senescence in ATLL cells, allowing for patient remission, underscoring the importance of telomerase and telomeres in ATLL disease status.

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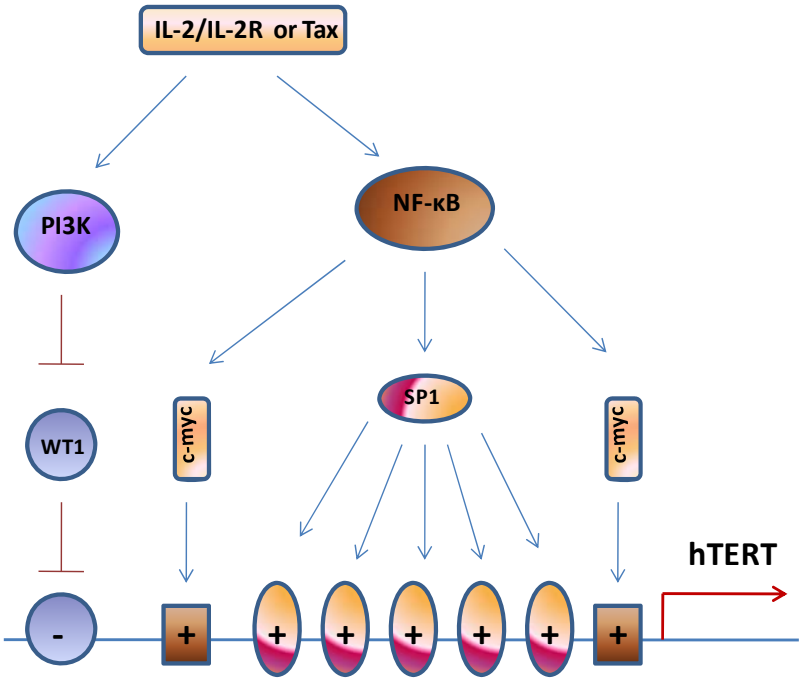
Conclusion and Future Directions

CONCLUSION*:*

The aforementioned collection of works establishes telomerase as a key component of HTLV-I-mediated cellular transformation. HTLV-I infected cell lines and ATLL patient samples display similar telomere dynamics: an increase in telomerase expression and activity, with a shortening of telomere lengths. This pattern is demonstrated in all oncogenic virus infections, including those induced by Human papillomavirus (HPV), Hepatitis B and C virus (HBV and HCV), and Human Herpesvirus 8/Kaposi Sarcoma-associated Herpesvirus (HHV-8/KSHV) (Bellon M, 2008). The only exception to this general rule is Epstein-Barr virus (EBV), where infection leads to an elongation of telomeres. Initial HTLV-I viral infection is associated with an increase in telomerase expression, either through transcriptional or post-transcriptional controls (Figure 9).

Figure 9: Transcriptional Regulation of hTERT during HTLV-I infection.



In HTLV-I infected cells, an increase in telomerase expression occurs by increased binding of positive telomerase regulators, c-Myc and Sp1, to the hTERT promoter through NF-κB (132). Activation of IL-2R signaling pathways, promote PI3K-mediated retention of the hTERT negative regulator, WT1, from the nucleus (Bellon M and Nicot C, Submitted). In transformed cell lines, that are IL-2 independent, Tax may compensate for IL-2R signaling, by increasing PI3K activity. Given the strong activation of AP-1 and STAT transcription factors during HTLV-I infection, it is likely that additional methods of transcriptional control of hTERT during HTLV-I infection still remain to be explored. Though post-

transcriptional regulation of telomerase was not tested in these studies, HTLV-I infected cells display constitutive activation of NF-κB and elevated AKT activity. This suggests that post-transcriptional control of hTERT may play a vital role in elevating telomerase activity during disease progression.

Telomere lengths are progressively shortened in HTLV-I infected cells, yet these cells do not undergo cellular senescence or apoptosis. Short telomeres are maintained through increased telomerase expression and elevated levels of TRF1, TRF2, and TIN2. These shelterin components have been shown to inhibit apoptosis and cell cycle arrest due to DNA damage, thereby protecting shortened telomeres. It is still unclear why telomeres initially shorten during cancer progression. It has been suggested that shortened telomeres lead to chromosome instability and aneuploidy, but this still remains to be proven. Shortened telomeres are prognostic indicators of disease progression in some cancers, including hepatocellular carcinoma, chronic myeloid leukemia, and chronic lymphocytic leukemia (120).

Treatment of HTLV-I infected cells with AZT leads to telomere shortening and reactivation of p53 transcriptional activities, which mediate cellular senescence. We have shown a direct correlation between patient response to AZT therapy and p53 status.

We have further demonstrated that p53 reactivation occurs in response to DNA damage following telomere attrition, leading to ataxia telangiectasia mutated (ATM) kinase activation (135). ATM causes phosphorylation and acetylation of p53, leading to p53 transcriptional activation of senescence genes.

Our study with AZT is the first of several reports indicating that telomere shortening leads to cellular senescence and reactivation of p53 transcriptional activities. Since our initial description, similar mechanisms demonstrating tumor suppression after telomere loss due to p53-mediated senescence have also been found (136-138).

In our study, treatment of one ATLL patient (ATL4) carrying wild-type p53, led to an initial response to AZT treatment, but the patient eventually underwent disease relapse. Proviral integration, before and after AZT treatment, demonstrated that during AZT treatment, the patient selected for a new clone, which carried a transcriptionally inactive p53. This clearly demonstrates the importance of p53 in AZT-mediated therapy and also reveals a limit to the use of AZT in treating ATLL: patients must remain wild-type for p53. Our work demonstrates that AZT/IFN treatment may not have success in patients with a mutated p53, and that these patients should be treated with conventional ATLL treatments, including allogenic bone marrow transplantation, radio immunotherapy for IL-

2Rα, or chemo- or radio-therapy (139, 140). We have also demonstrated *in vitro* success with Arsenic trioxide/interferon-α (ATO) in combination with emodin and docosahexaenoic acid (DHA) (141). The combination of these drugs leads to cell-cycle arrest and cell death of HTLV-I infected cells. The importance of ASO treatment is shown by the fact that AZT treatment, or any anti-hTERT therapy, is p53-dependent, whereas ASO treatment is p53-independent. Clinical trials of ASO in combination with DHA and emodin remain, in order to determine the efficacy of this treatment in ATLL patients.

Analysis of p53 status at the on start of treatment could be highly beneficial to disease outcome, as most patients that undergo chemotherapy or radiotherapy select for tumor clones that carry mutant p53 (142). Instead, patients with wild-type p53 should be placed on AZT/IFN as a first line of therapy. ATLL patients have demonstrated a complete response and suppression of HTLV-I while undergoing alemtuzumab therapy, a monoclonal antibody targeted to CD52 that is present on mature lymphocytes. It is possible that alemtuzumab could be used in conjunction with AZT, to prevent emergence of mutated p53 clones (143).

Future Directions:

We have extensively studied the core promoter of hTERT. We have found that in HTLV-I infected cells, there is increased binding of c-Myc and Sp1, two positive regulators, and decreased binding of WT1, a negative regulator of hTERT expression. However, the distal promoter of hTERT still remains to be studied in the context of HTLV-I infection. Studies have shown that the AP-1 family of transcription factors, which include c-fos/c-Jun and c-fos/JunD can bind to the distal hTERT promoter between -2000 and -378 of the transcriptional start site. The c-Jun NH –terminal kinase (JNK) increases hTERT expression, whereas AP-1 can repress the hTERT promoter. AP1 and JNK pathways are both activated in HTLV-I infected cells. In vitro biotin pull down assays or chromatin-histone immunoprecipitation assays (CHIP), using AP-1 transcription factors, would demonstrate whether there was an increase or decrease in these factors bound to the hTERT promoter in HTLV-I infected cells. Transient transfection assays in HTLV-I infected cells, with an hTERT luciferase construct and varying concentrations of AP-1 transcription factors, would aid in establishing whether these factors are positive or negative regulators of hTERT transcription in HTLV-I infected cells. In addition, inhibition of JNK or MAPK pathways with pharmacological

inhibitors, would give a view of the pathways involved in hTERT regulation in HTLV-I infected cells.

We have found that the distal hTERT promoter also contains a putative binding site for signal transducer and activator of transcription (STATs) transcription factors. HTLV-I infected cells *in vitro* and *in vivo* have constitutive activation of JAK/STAT pathways. Biotin pull-down assays with the hTERT promoter or CHIP analysis using STAT antibodies, and treatment with JAK inhibitors, would demonstrate whether there is an increase or decrease in these transcription factors in HTLV-I infected cells compared to normal PBMCs. In addition, mutation of the putative STAT binding site, within the hTERT luciferase promoter, would demonstrate the importance of the JAK/STAT pathway in promoting hTERT expression.

Using AKT inhibitors, we have shown that AKT acts as a potent post-transcriptional regulator of hTERT activity. However, our data has primarily focused on transcriptional regulation of hTERT expression. Further analysis into hTERT post-transcriptional regulation in HTLV-I infected cells would aid in understanding the role of telomerase in HTLV-I infected cells. Protein kinase C (PKC) phosphorylates hTERT and aids in holoenzyme integrity. PKC has been shown to modulate hTERT activity in cervical cancer cells, as

well as human nasopharyngeal and head and neck cancer cells. Given that PI3K is a strong regulator of hTERT activity in HTLV-I infected cells, and PKC lies downstream of PI3K, it is possible that PKC positively impacts hTERT during HTLV-I infection. Long-term inhibition of PKC, with pharmacological inhibitors, would demonstrate a role for PKC in post-trancriptionally regulating hTERT in HTLV-I infected cells. The RelA/p65 subunit of NF-κB can bind hTERT and aid in the nuclear localization of hTERT. While removal of IL-2, in HTLV-I infected cells, showed no short-term (4 hours) effects on NF-κB localization, demonstrated that NF-κB was not primarily involved in IL-2 regulation of hTERT transcriptional activation; it does have a role in regulating c-Myc and Sp1 activity on the hTERT promoter in HTLV-I infected cells. Treatment with NF-κB inhibitors, or inhibition of NF-κB nuclear localization, would demonstrate whether NF-κB plays a role in post-transcriptionally regulating hTERT in HTLV-I infected cells. Tax, like the LMP1 protein of EBV, may promote NF-κB RelA/p65-mediated binding to and nuclear localization of hTERT.

An area of hTERT regulation that has not been studied in-depth is epigenetic control of hTERT activity. Hyper-methylation of the promoter of genes often leads to a reduction in transcription of the gene. In contrast, hypo-acetylation of the promoter, leads to gene

activation. HPV proteins, E6 and E6AP, have been shown to promote acetylation of histone H3 of the hTERT promoter and adenoovirus E1A protein binds to p300/CBP to recruit histone acetyltransferases to the hTERT promoter. In addition, histone H3 phosphorylation by MAPK, leads to hTERT expression. JNK and ERK1/2, transcriptional factors activated by MAPK, are constitutively activated in HTLV-I infected cells. Treatment with trichostatin A, which inhibits histone deacetlyases, and subsequent analysis of hTERT activity, would demonstrate a role for epigenetic regulation of hTERT in HTLV-I infected cells. Epigenetic regulation of hTERT expression through methylation of the hTERT promoter is complicated, as, in contrast to the general pattern of gene regulation, methylation status does not correlate with hTERT gene expression. Treatment of HTLV-I infected cells with bisulfate (which leads to conversion of unmethylated cytosines to uracils, whereas methylated cytosines are unchanged), along with methylation-specfic PCR, would demonstrate whether the hTERT promoter is methylated or unmethylated. Further treatment with 5-azacytodine, which inhibits methylation, in HTLV-I infected cells, followed by TRAP analysis of telomerase activity, would aid in understanding whether methylation plays a role in hTERT regulation. However, it does not demonstrate a direct effect of promoter methylation on hTERT expression, as

treatment with 5-azacytodine, could effect the expression of known positive and/or negative transcriptional or post-transcriptional regulator of hTERT expression, therefore, having an indirect effect.

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