Genetic alterations in Adult T cell leukemia

By

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Genetic alterations in Adult T cell leukemia

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Abstract

Human T-cell leukemia virus type 1 (HTLV-1), which infects more than 20 million people worldwide, is known to cause adult T-cell leukemia (ATL). Even those patients treated with intense chemotherapy have a poor prognosis [1]. Although the detailed mechanisms on how HTLV-1 transforms T cells are unclear, it is believed that the viral oncoprotein Tax and the accumulation of somatic alterations lead to ATL [2]. In 2015, Seishi Ogawa and colleague used whole-exome sequencing and whole-genome sequencing to comprehensively analyze ATL genetic alterations [2, 3]. They found that fifty genes are significantly mutated, with 13 genes (PLCG1, PRKCB, CCR4, CARD11, STAT3, TP53, VAV1, TBL1XR1, NOTCH1, GATA3, IRF4, FAS, CCR7) affecting more than 10% of ATL patients [2].

In our previous study, we found Notch1 mutations in 30% of ATL patients leading to reduced Fbw7-mediated degradation and stabilization of the intracellular cleaved form of Notch1 (ICN1). In addition, Notch inhibitors reduced ATL tumor formation in a xenograft model [4]. Since FBXW7 has been reported to be mutated in 6% of human tumors, we hypothesized that the deregulation of FBXW7 can accelerate ATL proliferation and transformation. In my first study, we found that FBXW7 is down-regulated and mutated in ATL patients. In contrast to the tumor suppressor role of FBXW7 wild-type, FBXW7 D510E increased cell proliferation and transformation both in vitro and in an ATL xenograft model [5].

Genome-wide H3K27 me3 accumulation has been observed in ATL patients, which can be explained by Polycomb repressive complex 2 hyperactivation [6]. In addition, EZH2 suppressed Fbxw7 expression via H3K27me3, resulting in Notch activation [7]. We hypothesized that the mutations of epigenetic regulators can reduce the FBXW7 expression in ATL. In my second study, we applied whole-genome next-generation sequencing (NGS) of
uncultured freshly isolated ATL samples and identified the presence of mutations in SUZ12, DNMT1, DNMT3A, DNMT3B, TET1, TET2, IDH1, IDH2, MLL, MLL2, MLL3 and MLL4. TET2 was the most frequently mutated gene, occurring in 32% (10/31) of ATL samples analyzed. Consistent with the previous report, Seishi Ogawa demonstrated hypermethylation in promoter-associated CpG islands in ATL [2]. Since the FBXW7 promoter hypermethylation has been reported [8] and a DNA methyltransferase inhibitor can restore the expression of FBXW7, the correlation of TET2 mutations and FBXW7 down-regulation needs to be further examined.

FBXW7α R465C/+ knockin mice increased T-ALL formation when in cooperation with a Notch1 mutation [9]. Mechanically, FBXW7α R465C/+ stabilized c-Myc protein half-life, therefore increasing leukemia-initiating cells (LICs) in FBXW7α R465C/+ knockin mice [9]. In my third study, we confirmed the existence of side populations having both self-renewal and leukemia-renewal capacity and representing cancer stem cells (CSC)/leukemia-initiating cells (LIC) in ATL cell lines and patient samples. We further show that PI3K and the NOTCH1 signaling pathway have opposite functions on the ATL side population. Constitutive activation of NOTCH1 signaling depletes the pool of side population cells in ATL-derived cell lines.

Since Notch1 signaling is deregulated and essential for ATL progression, our results indicate another mechanism to explain how Notch1 signaling is constitutively active in ATL patients, implying a unique therapeutic opportunity to target FBXW7 in the future.
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Chapter I: General Introduction
HTLV-1

Human T-lymphotropic virus type I (HTLV-1) infects 20 million people worldwide [1]. While most people remain asymptomatic, about 5% of HTLV-1-infected people eventually develop adult T-cell leukemia-lymphoma (ATL). The clinical and hematologic characterization of ATL was first reported in 1977 as a distinct T-cell leukemia [10]. The RNA retrovirus HTLV-1 was then isolated from a cutaneous T-cell lymphoma patient by Gallo in 1980 [11]. In addition to ATL, HTLV-1 is causative for HTLV-1-associated myelopathy / tropical spastic paraparesis (HAM-TSP) [12], a chronic inflammatory disease and opportunistic infection. It is believed that low immune response to HTLV-1-infected cells is related to ATL development and high immune response is correlated with HAM-TSP [13].

In a clinical setting, the features of ATL are diverse. According to the Shimoyama classification, ATL patients can be divided into 4 different subtypes: acute, lymphoma, chronic, and smoldering [1]. Among those subtypes, acute, lymphoma, and unfavorable chronic are thought to be aggressive forms of ATL and favorable chronic and smoldering are classified as indolent forms of ATL.
Virology

HTLV-1 is a positive, single-stranded RNA virus belonging to deltaretrovirus [14]. Deltaretrovirus consists of exogenous horizontally-transmitted viruses found in a few groups of mammals, including bovine leukemia virus, Human T-lymphotropic viruses (HTLV-1 and 2), and Simian T-lymphotropic viruses (STLV 1-4). HTLV-1 genome encodes 3 structure proteins (Gag, Pol, and Env) and many regulatory proteins such as Tax, p30 and HBZ (Figure 1).
Figure 1. HTLV-1 genome

The gene of HTLV-1 encodes 3 structural proteins (Gag, Pol, and Env) and many regulatory proteins such as Tax, p30 and HBZ, which can activate viral/host gene expression, escape host immune response and maintain viral infection (Adapted from [14]).
**Virus infection**

After infection, the virus uses reverse transcriptase to generate DNA from virus RNA and integrate into host genomic DNA, which is referred to as provirus [14]. HTLV-1 preferentially replicates in CD4+ T cells, but other cells like stem cells and CD8+ T cells can also be infected by HTLV-1. Viral *env* gene, which encodes gp21 and gp46, is important for HTLV-1 infection [15]. In addition, it is found that glucose transporter (GLUT-1), heparan sulfate proteoglycans (HSPG), neuropilin 1 (NRP1) are host receptors for HTLV-1 infection [16]. The initial virus attachment is mediated by the interaction between gp46 and HSPG which get virus closer to host cell [15]. Then gp46 binds to NRP1, which causes structural change of gp46 and exposes GLUT-1 binding site [15]. Finally, the interaction between gp46 and HSPG/NRP1/GLUT-1 forms multi-receptor complexes resulting HTLV-1 fusion and host cell infection [15]. Antibody targeting GLUT-1 reduced HTLV-1 fusion and infection, but not binding, indicating that interaction with GLUT-1 is essential for fusion [15].
HTLV-1 encoding proteins

The genome of HTLV-1 encodes the following proteins: 1. envelope proteins (encoded by env): surface glycoprotein (gp46) and transmembrane protein (gp21); 2. Structural proteins (encoded by gag): matrix layer, capsid and nucleocapsid; 3. Functional proteins (encoded by pol): protease (p14), reverse transcriptase (p95) and integrase; and 4. Regulatory proteins like Tax, Rex, p12, p13, p30 and HTLV-1 basic leucine zipper (HBZ), which is a minus-strand gene protein [14]. The functions of HTLV-1 protein are summarized in the Table 1 [14].
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**Table 1. HTLV-1 proteins**

Functions of HTLV-1 encoding protein (Adapted from [14])
The virus synthesizes a full-length messenger RNA (mRNA) encoding a large Gag-PR-Pol precursor polyprotein, which uses different translation starting sites for protein translation, a single spliced mRNA encoding Env, and many mRNAs encode different regulatory proteins [14].

**p12**

p12 mainly localizes to the endoplasmic reticulum (ER) that involves viral replication and T cell activation [17, 18]. p12 interacts with β and γ chains of the interleukin-2 receptor (IL-2R), which activates Janus kinase/signal transducer and activator of transcription 5 (Jak/Stat5) signal transduction pathway [18]. In addition, by interacting with calreticulin and calnexin, p12 increases Ca$^{2+}$ release from the ER and activates nuclear factor of activated T-cells (NFAT), which increase T cell proliferation [18]. p12 and its proteolytic cleavage product p8 has been reported to be critical for HTLV-1 viral persistence and spread *in vivo*[17]. In addition, p12 is known to reduced NK cell mediated cytotoxicity of HTLV-1 infected T cell by down-regulation of intercellular adhesion molecules ICAM-1 and ICAM-2. Consistently, p12 can bind and induce major histocompatibility complex (MHC) class I heavy chain degradation, which helps virus escape host immune response [17].

**p13**

p13 is an inner mitochondria membrane protein that reduces Tax activity through disrupting Tax-CREB binding protein (CBP)/p300 interaction. Therefore, p13 reduced cell proliferation by inhibiting Tax-mediated viral and cellular transcription [17]. In addition, p13 enhances mitochondrial permeability to K$^+$ and activates the electron transport chain, resulting in up-regulation of reactive oxygen species (ROS) [18].
p30

p30 is a nuclear protein that regulates viral replication. p30 reduces the expression of Tax and Rex by retaining doubly-spliced tax/rex mRNA in the nucleus therefore down-regulating viral gene expression [17]. Therefore, p30 plays a role in escaping host immune surveillance [18].

Rex

Rex promotes the nuclear exportation of unspliced and single spliced HTLV-1 mRNA to cytoplasm therefore increases the stability of HTLV-1 mRNA and HTLV-1 protein translation [18]. Initially, Rex interacts with HTLV-1 mRNA by binding to Rex responsive element (RxRE). Then, Rex interacts with nuclear exporter CRM1 and promotes nuclear exportation of HTLV-1 mRNA [18]. The cytoplasm exportation of unspliced and partially spliced HTLV-1 mRNA protects further mRNA splicing and increasing the translation of Gag and Env for viral replication [18].

The RxRE sequence is located within the retroviral 3’ LTR and is known to for highly stable stem-loop structure that is essential for Rex binding [19]. In addition to being the binding site for Rex, RxRE is indispensable for optimal positioning of the HTLV-1 mRNA ploy-A signal and poly-A binding site in the HTLV-1 mRNA, which brings two separate sequence together through RNA stem-loop structure [18].

Tax

Tax is a strong transcriptional activator for viral genes and host genes [20, 21].
**Tax and viral transcription**

HTLV-1 genome contains a long terminal repeat (LTR) at N- and C-terminals, which consists of unique 3’ (U3), the repeated (R) and the unique 5’ (U5) [22]. U3 contains Tax responsive element I (TRE-1), which consist of 3 discontinuous 21-base pair repeats. Each of the 3 discontinuous 21-base pair repeats is sufficient for Tax-mediated transcription [22]. Furthermore, discontinuous 21-base pair repeats are composed of 3 domains (Domain A, B and C) [22]. Among 3 domains, domain B contains viral cAMP response element (vCRE) that is important for Tax-mediated transcription [22]. Tax forms complex with cAMP response element binding protein (CREB), CREB-binding protein (CBP) and p300 at vCRE and initiate viral gene transcription [22].

**Tax and NF-κB**

Tax can interact with IKK regulatory component NEMO and recruits the IKK complex to perinuclear compartment where IKK is phosphorylated and activated [23]. Consistently, loss of Tax-mediated NF-κB activation was observed in cell depleted NEMO. There are two models explain how Tax activates IKK complex [23]. The first model is that Tax forms self-dimerization and brings different IKK complexes closely together. The adjacent IKK complexes cross-phosphorylate and activate each other. Therefore, Tax M22 mutation deficient in self-dimerization lost it NF-κB activation [23]. Another model is Tax acts as a scaffold protein that brings IKK complex and its upstream kinase together. IKK upstream kinases such as mitogen-activated protein kinase kinase kinases (MAP3Ks), MEKK1, NIK, Tpl2, and TAK1 have been reported to interact with Tax and increase Tax-mediated NF-κB activation [23]. The activation
of IKK complex results in IκB degradation and NF-κB nuclear translocation. In the nucleus, Tax forms Tax nuclear bodies with RelA and other cellular transcriptional components, which inducing high NF-κB transcriptional activity [23].

In addition to canonical NF-κB signaling pathway, Tax can activate non-canonical NF-κB signaling pathway by processing p100 to p52 [23]. NEMO is essential for Tax mediated non-canonical NF-κB activation by recruiting Tax/IKK complex, which contains only IKKα, but not IKKβ [23]. On the other hand, NIK has been shown to be dispensable for Tax mediated non-canonical NF-κB activation [23]. The formation of Tax/NEMO/IKKα activates IKKα. The activated IKKα phosphorylates p100 and leads to β-Trcp mediated ubiquitination of p100. Ubiquitylated p100 is then partial degraded by proteasome and form p52 to activate non-canonical NF-κB signaling pathway [23].

**Tax and host gene transcription**

The promoters of c-fos, Erg-1, Erg-2, Fra-1, c-Jun, and JunD contain serum response factor (SRF) binding sites are known to be activated by Tax [22]. Mechanically, Tax interacts with Elk-1 and SAP-1, which belong to transcription factor family (TCF) that binds to promoters with serum response element (SRE). The deregulation of growth regulator genes responsive to SRF signaling increased cell proliferation and cell transformation [22]. In addition, Tax is known to increase the binding of SRF proteins to more diverse group of DNA sequences compared to canonical serum response element [22].

**HBZ**
HTLV-1 basic leucine zipper (HBZ) is an antisense mRNA transcribed from 3’-LTR and is constantly expressed in fresh ATL cells and HTLV-1-infected cells. In addition, HBZ inhibits Tax activity through interacting with CREB and CBP/p300, resulting in disrupting their binding with Tax responsive element I and cAMP response element (CRE) [22]. Silencing HBZ gene expression inhibits ATL cell proliferation and expression of HBZ enhances ATL cell proliferation [24]. In addition, enhancing TGF-beta signaling and Foxp3 expression by HBZ is a feature observed in ATL cells and is important for HTLV-1 persistence [25].

HTLV-1 mainly exists in a host-integrated provirus form and HTLV-1 infected cells virtually do not produce cell-free infectious HTLV-1 particles. HTLV-1 uses virus-induced polarization of the cytoskeleton to spread between lymphocytes. When infected cells come into contact with uninfected cells, HTLV-1 genome transfer to the uninfected lymphocyte through the viral synapse [26], which is composed of microtubule-organizing center (MTOC) [21] (Figure 2).
Figure 2. HTLV-1 viral synapse

Viral synapse for HTLV-1 transmission (Adapted from [21])
The interaction between intercellular adhesion molecule 1 (ICAM1) and lymphocyte function-associated antigen 1 (LFA1) enhances the polarization of the MTOC at the cell-cell contact point and facilitates HTLV-1 transmission [21].

During early infection, HTLV-1 virus spreads through cell-cell contact and causes heterogeneous infected clones. When the virus production and host immune response reach balance, HTLV-1 duplication is mainly dependent on clonal expansion by host cell mitosis. The selective maintenance of certain clones and higher number of infected cells in late infection stage may play a role in the beginning of ATL [27]. Interestingly, HTLV-1 has a relatively low evolutionary rate compared to HIV because of the mitosis-dependent survival strategy of HTLV-1. The low genetic variability of HTLV-1 in vivo can be used to study the origin and evolution of HTLV-1 [28, 29].

Immune response is important for the control of provirus load and causes different clinical outcomes. 1. CD8+ T cells control the virus by lysing infected cells expressing viral antigen. 2. When the immune system is overactivated and produces too many inflammatory factors, it causes HAM-TSP and other inflammatory diseases. 3. The survival and proliferation of infected cells can accumulate genetic mutations and lead to ATL. The balance between immune response and provirus replication leads to a stable provirus load over time in any given individual [14].
**Epidemiology of HTLV-1**

About 20 million people worldwide are infected with HTLV-1. The distribution of HTLV-1 is endemic. There are some highly endemic regions in the world, wherein HTLV-1 infection can be as high as 5%, including: southwestern Japan, the Caribbean, intertropical Africa, the Middle East, South America, and Papua New Guinea [30] (Figure 3).
Figure 3. World Distribution of HTLV-1 Infection

Prevalence of HTLV-1 (Adapted from [31]).
Sex workers and people injecting drugs are at high risk. The major ways to transmit virus are breastfeeding, unprotected sex, blood transfusion, organ transplantation, and needle sharing.

In Japan, there are many public health measures to prevent HTLV-1 infection, including testing donated blood, screening pregnant women and promoting condom use to prevent sexual transmission of HTLV-1. However, the effect of these measurements is unknown due to the lack of data for incidence of new infections.

The origin of HTLV-1 can be traced back to primate T-cell lymphotropic virus (PTLV) in African non-human primates. PTLV spread to Asia with simian and evolved into simian T-cell leukaemia virus type-I (STLV-1). Then, STLV-1 migrated to Japan, India, and Indonesia where it evolved into the HTLV-1c (Australo-Melanesian) subtype. Later, STLV-1 came back to Africa from Asia and evolved into different subtypes of HTLV-1: HTLV-1a (the cosmopolitan subtype), Ib (the central African subtype), Id, Ie, and If. The slave trade and the increased mobility of humans helped spread HTLV-1a to the USA, Japan, the Middle East, and North Africa [1].
ATL related oncogenesis and pathogenesis

The detailed mechanisms on how HTLV-1 transforms T cells is unknown. However, viral proteins and host genetic and epigenetic modification have been shown to be involved in HTLV-1 mediated transformation (Figure 4).
Figure 4. Accumulation of somatic alterations in ATL

The involvement of viral protein and host genetic and epigenetic modification in HTLV-1 mediated transformation (Adapted from [21]).
Tax is known to activate various cellular signaling pathways, such as the NF-κB and Akt signaling pathway [20], which are important for tumor cell proliferation and survival. Except for the deregulation of protein coding genes, miRNA is also found to be aberrant in ATL patients [32].

Tax is a strong transcriptional activator for viral genes and host genes. It is known to activate various cellular signaling pathways, such as the NF-κB, cyclin dependent kinase and Akt signaling pathway (Table 2) [20, 21].

Tax and cell cycle

Tax binds with cyclin dependent kinase (CDK) 4 and Cdk6, and increases the formation of CDK4/cyclin D and CDk6/cyclin D [22]. CDK/cyclin D complex then phosphorylates Retinoblastoma protein (Rb) and release E2F to promote cell cycle progression through G1/S transition [22]. In addition, Tax has been shown to induce Rb degradation via proteasome [22]. In addition, Tax can interact with and inactivate Chk1 and Chk2, which increases the activation of Cdc25 and CDK1/cyclin B [22]. Increased CDK1/cyclin B activation move cells pass through M phase of cell cycle. Furthermore, Tax increased the expression of cyclin D2, CDK4 and CDK6, which are important for cell cycle progression [22].

Tax and aneuploidy

Tax has been reported to interact with MAD1, a mitotic spindle checkpoint (MSC) protein, which leads to cytoplasm translocation of MAD1 and MAD2 [22]. The translocation of MAD1 and MAD2 by Tax results in chromosomal segregation error and aneuploidy [22]. In addition to MAD1, Tax can interact with Tax1BP2 [22]. Increasing Tax1BP2 expression is
correlated with reduced aneuploidy. Tax mutations deficient in interacting with Tax1BP2
reduced aneuploidy compared with Tax wild-type, which implied Tax interacts and inactivates
Tax1BP2 [22].

In addition, Tax can also transform cells by inactivating p53 activity [1]. It is thought that
Tax is important for ATL oncogenesis in the early stages because Tax can immortalize T cells
[33] and transform cells [34]. Importantly, in transgenic mice, expressing Tax in T cells is
sufficient for the development of leukemia in mice [35]. Although Tax can promote tumor
progression, Tax is also an antigen recognized by host cytotoxic T cells. In order to escape the
immune system and survive in the host, most of the ATL cells lose Tax expression by nonsense
mutations, deletion/insertion or DNA methylation [1].

**Tax and DNA repair**

Tax has been reported to impair multiple DNA repair pathways such as base excision
repair (BER), nucleotide excision repair (NER), DNA homologous recombination repair and
non-homologous end joining (NHEJ) [22]. Tax inhibits base excision repair by down-regulation
of DNA polymerase β expression [22]. Inhibition of nucleotide excision repair by Tax is through
p53 inactivation and up-regulation of proliferating cell nuclear antigen (PCNA) expression. In
addition, activation of NF-κB signaling pathway by Tax skewed DNA repair from homologous
recombination to error-prone non-homologous end joining (NHEJ) pathway [36]. Finally, Tax
inhibits non-homologous end joining (NHEJ) pathway by targeting DNA-dependent kinase
(DNA-PK) and Ku80 [22]. Tax constitutively activates DNA-PK in cells, which de-sensitizes
cells to sense real DNA damage [22]. Furthermore, Tax reduced the expression of Ku80, resulting
in accumulation of DNA damage in nucleus [22].
In addition to DNA repair signaling pathway, Tax inhibits the DNA damage sensing system by targeting ataxia telangiectasia mutated (ATM) and CHK2, which impairs DNA damage sensing and correcting, and leads to cell cycle progression through G1/S checkpoint [22]. Specifically, Tax de-phosphorylates ATM, which inactivates the ATM and inhibits the accumulation of ATM on DNA damage site [22]. In addition, Tax expression reduced ATM-mediated phosphorylation of the pre-existing phosphorylated H2A.X (γH2A.X) and CHK2, resulting in reduced the recruitment of effector proteins such as MDC1 and CHK2 [22].
Table 2. Tax activated and inactivated genes

The effect of Tax on host gene expression (Adapted from [21])

<table>
<thead>
<tr>
<th>Activated by Tax</th>
<th>Consequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell-cycle related proteins (CDK2 and CDK4; cyclin D2; cyclin D3; WAF1; E2F1)</td>
<td>Accelerated cell cycle and DNA replication</td>
</tr>
<tr>
<td>Growth factors and cytokine receptor (IL2 and IL15; IL2Ra and IL15Ra)</td>
<td>Enhanced cell proliferation</td>
</tr>
<tr>
<td>Transcription factors (CREB; AP1; SRF)</td>
<td>Enhanced cell proliferation</td>
</tr>
<tr>
<td>Survival factors (Akt; NF-κB)</td>
<td>Inhibit cell apoptosis and senescence; drug resistance</td>
</tr>
<tr>
<td>Centrosome amplification (RANBP1; TAX1BP2)</td>
<td>Aneuploidy</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Inactivated by Tax</th>
<th>Consequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell-cycle checkpoint proteins (p15, p16 and p18; RB; DLG1)</td>
<td>Accelerated cell cycle and DNA replication</td>
</tr>
<tr>
<td>DNA repair factors (DNA polymerase β; MMR)</td>
<td>Increased DNA damage and gene instability</td>
</tr>
<tr>
<td>DNA damage response (p53; CHK1; CHK2; KU80)</td>
<td>S Increased DNA damage and gene instability</td>
</tr>
<tr>
<td>Chromosome instability checkpoint (MAD1; CHK1)</td>
<td>Aneuploidy</td>
</tr>
</tbody>
</table>
**HBZ and cell proliferation**

HTLV-1 basic leucine zipper factor (HBZ) is an antisense viral protein that interacts with CREB-2 and inhibits viral transcription at the 5’ LTR [22]. In addition, HBZ inhibits Tax activity through interacting with CREB and CBP/p300, resulting in disrupting their binding with Tax responsive element I and cAMP response element (CRE) [22]. Although HBZ protein suppresses viral transcription and Tax activity, HBZ mRNA plays an opposite role in cell proliferation [22]. Spliced HBZ mRNA, but not un-spliced HBZ mRNA, increased the proliferation of ATL [22]. Further studies are needed to figure out detailed mechanisms in HBZ-mediated cell proliferation.

The multi-step carcinogenesis model, wherein HTLV-1-immortalized T cells accumulate multiple changes during the latency period explains the age-specific occurrence of ATL [37, 38]. Therefore, the development of ATL is thought to be the combination result of Tax [21], HTLV-1 basic leucine zipper factor (HBZ) [24], miRNA [39], tumor suppressor [40-42] and oncogenes [4].

When using oligo-array comparative genomic hybridization (CGH) to analyze ATL patients’ peripheral blood (PB) and lymph node (LN) samples, Umino found that different subclones in the LNs come from a common clone and one of the selected subclones then appears in the PB. Their findings suggest the accumulation of multiple changes and the clonal evolution of ATL cells in LN.
HTLV-1 and ubiquitin-proteasome

Tax is known to be able to manipulate the host ubiquitin-proteasome system to activate NF-κB signaling [43] and stabilize Mcl-1 [44] to transform the cells and prevent apoptosis.

Tax can activate the canonical and non-canonical NF-κB signaling pathway [43]. In the canonical NF-κB signaling pathway, Tax directly interacts with NEMO and induces K63-polyubiquitination of NEMO [45]. In addition, Tax can sustain the IKK activation by inhibition of protein phosphatase 2A (PP2A). The activation of IKKβ phosphorylates IκBα and triggers its degradation. Then, the RelA/p50 dimer translocates into nuclear and activates downstream gene expression. Moreover, in the non-canonical NF-κB signaling pathway, Tax can activate the IKKα and trigger the process of p100 to p52. The Tax-mediated induction of p100 processing is dependent on beta-transducin repeat-containing protein (β-Trcp), a component of the SCF E3 ubiquitin ligase complex. IKK inhibitors or dominant-negative IκB mutants impeded the Tax-mediated transformation, which indicated the dependence of the NF-κB signaling pathway in Tax transformation.

Preventing apoptosis is important for tumor formation and resistance to chemotherapy. Mcl-1 belongs to the Bcl-2 family and is highly expressed in human tumors[46]. Mcl-1 has a very short half-life, which is known to be post-transcriptionally regulated by E3 ubiquitin ligases MULE, β-TRCP and FBW7 [44]. Using mass spectrometry, Choi found that Tax can stabilize Mcl-1 by adding lysine 63 (K63)-linked polyubiquitination [44] in a TRAF6-and IKK-dependent manner (Figure 5) [44]. K63- polyubiquitination of Mcl-1 prevents its binding with the core 20S proteasome, thus blocking the proteasome-dependent degradation of Mcl-1. The identification of Mcl-1 inhibitor may provide an opportunity to overcome the resistance caused by Mcl-1 overexpression in the future [47].
Figure 5. Tax and Mcl-1 stability

Tax stabilizes Mcl-1 through TRAF6-mediated K63 polyubiquitination (Adapted from [44])
Clinical features of ATL

The clinical features of ATL are diverse from patient to patient. The clinical features of ATL include general lymphadenopathy, skin lesions, hepatosplenomegaly, leukocytosis with abnormal flower-like lymphocytes [14] (Figure 6) or with an increased number of neutrophils, hypercalcemia, and opportunistic infection [1].
Figure 6. Flower-like ATL cells

Image of abnormal flower-like lymphocytes (Adapted from [14])
According to Shimoyama criteria (Figure 7) [1], ATL patients can be classified into 4 different subtypes (acute, lymphoma, chronic, and smoldering) according to their prognostic factors and clinical observation. The acute, lymphoma and unfavorable chronic subtypes largely define poor prognosis. On the other hand, favorable chronic and smoldering subtypes are associated with better clinical outcome.
**Figure 7. Shimoyama criteria**

ATL clinical classification (Adapted from [1])
Most ATL cells express cell surface markers: CD3, CD4, CD25, CCR4 [48] and FOXP3 [49], and monoclonal integration of HTLV-1 DNA. Although ATL cells functioning as regulatory T cells is controversial, ATL cells from a subset of patients are hypo-responsive to stimulation and repress the proliferation and IFN-gamma production of autologous CD4+ non-ATL cells [50]. That may at least explain part of the immunosuppression observed in ATL patients.
Clinical treatment for ATL

Most ATL treatments are based on other malignant lymphoma/leukemia treatments, such as diffuse large B-cell lymphoma and chronic lymphocytic leukemia (Table 3) [1].
<table>
<thead>
<tr>
<th>Therapeutic choices for ATL</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aggressive ATL (acute, lymphoma, and unfavourable chronic types)</td>
<td>- VCAM-AMP-VECP=sequential combination chemotherapy consisting of VCAP (vincristine [VCR], cyclophosphamide [CPM], doxorubicin [DOX], and prednisolone [PSL]), AMP (DOX, ranimustine [MCNU], and PSL), and VECP (vinodesine [VDS], etoposide [VP-16], carboplatin [CBDCA], and PSL)</td>
</tr>
<tr>
<td></td>
<td>- CHOP=combination chemotherapy consisting of CPM, DOX, VCR, and PSL</td>
</tr>
<tr>
<td></td>
<td>- ATL-GCSF=combination hemotherapy consisting of VCR, VDS, DOX, mitoxantrone (MIT), CPM, VP-16, MCNU and PSL with prophylactic support by granulocyte-colony stimulating factor</td>
</tr>
<tr>
<td></td>
<td>- mEPOCH=combination chemotherapy consisting of VP-16, PSL, VCR, CPM, and DOX with modification</td>
</tr>
<tr>
<td></td>
<td>- Hyper CVAD=combination chemotherapy consisting of hyperfractionated CPM, VCR, DOX, and dexamethasone</td>
</tr>
<tr>
<td></td>
<td>- IFN-AZT=combination of interferon α and zidovudine</td>
</tr>
<tr>
<td></td>
<td>- HSCT=haemaopoietic stem-cell transplantation</td>
</tr>
<tr>
<td>Indolent ATL (smoldering and favourable chronic types)</td>
<td>- Watchful waiting</td>
</tr>
<tr>
<td></td>
<td>- IFN-AZT</td>
</tr>
</tbody>
</table>

**Table 3. Therapeutic choices for ATL**

ATL treatments (adapted from [1])
Chemotherapy

The treatment of aggressive ATL and indolent ATL are usually based on the treatment used for other malignant lymphomas. The VCAP-AMP-VECP regimen is a series of combination chemotherapy composed of vincristine, cyclophosphamide, doxorubicin, and prednisolone (VCAP); doxorubicin, ranimustine, and prednisolone (AMP); and vindesine, etoposide, carboplatin, and prednisolone (VECP). The rate of complete response (CR) and overall survival are improved by the VCAP-AMP-VECP regimen compared with biweekly cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP). However, the 13-month median survival time is still shorter compared with other hematologic diseases and there is no treatment for relapsed or resistant ATL patients [1, 51].

Interferon \( \alpha \) and zidovudine

Interferon \( \alpha \) is secreted by many cells like lymphocytes and is known to stimulate immune response to eradicate virus infection. Interferon \( \alpha \) has been used in clinical settings for cancer (renal cell carcinoma and melanoma) and virus infection (hepatitis B and C virus).

Zidovudine is a nucleotide analog reverse-transcriptase inhibitor that is used for HIV treatment.

Treatment of ATL with a combination of interferon \( \alpha \) and zidovudine was first reported in 1995 [52, 53]. 58% of ATL patients receiving the combination of interferon \( \alpha \) and zidovudine have major response and 57% of ATL patients in whom prior cytotoxic therapy has failed also have major response. In a meta-analysis, five-year overall survival rates are 2 times higher in ATL patients receiving a combination of interferon \( \alpha \) and zidovudine compared with patients receiving chemotherapy. When analyzing different subtypes of ATL, acute, chronic, and smoldering ATL patients are more responsive to interferon \( \alpha \) and zidovudine treatment compared with chemotherapy, but the ATL lymphoma patients got a better prognosis with chemotherapy.
Importantly, the 5-year survival rate is 82% in acute ATL and 100% for chronic and smoldering ATL patients with complete remission by interferon α and zidovudine treatment [54]. In addition, combining chemotherapy with interferon α and zidovudine increased the overall response rate and median overall survival in acute and lymphoma ATL [55].

**Allogeneic hematopoietic stem cell transplantation (HSCT)**

All ATL patients receiving autologous stem cell transplantation had ATL relapse or died because of transplantation complications, which shows little benefit for ATL patients [56]. Allogeneic HSCT is an effective treatment for ATL patients with 3-year overall survival and relapse-free survival at 45.3% and 33.8%, respectively [57]. Mild-to-moderate acute graft-versus-host disease (GVHD) is associated with increased overall survival and lower disease-associated mortality. However, moderate-to-high acute GVHD is correlated with a higher risk for treatment-related mortality [58]. The results above indicate that allogeneic HSCT can benefit ATL patients, but the treatment-related mortality needs to be carefully managed. Another factor affects the successful rate of allogeneic HSCT is graft-versus-HTLV-1 response [59]. The restoration of immune response against the HTLV-1 antigen after HSCT leads to graft-versus-leukemia effects and may improve the outcome of ATL patients.

**Novel treatment**

CCR4 is a G-protein coupled receptor that is expressed on Th2 cells and regulatory T cells [1]. 88.3% of ATL cells from patients are CCR4+ and the positive CCR4 expression is correlated with unfavorable prognosis [60]. Mogamulizumab (KW-0761) is an anti-CCR4 monoclonal antibody with a defucosylated Fc region to increase binding affinity to the Fc receptor on the effector cells. Mogamulizumab can effectively treat against ATL *in vitro* and *in vivo* through activating antibody-dependent cellular cytotoxicity (ADCC). The efficiency of
Mogamulizumab is highly dependent on the amount of effector natural killer cells present [61]. In the phase I clinical trial of Mogamulizumab, 31% of ATL patients showed objective response and the toxicity was acceptable at all the doses tested [62]. In the phase II clinical trial of Mogamulizumab, the overall response rate is 50% and median progression-free and overall survival were 5.2 and 13.7 months, respectively. Collectively, Mogamulizumab showed effective anti-ATL activity in clinical ATL patients with tolerable toxicity [63].

Other clinical trials like Bortezomib (proteasome inhibitor), Lenalidomide (immunomodulatory drug), Panobinostat (histone deacetylase inhibitor), Alisertib (Aurora A kinase inhibitor) and Ruxolitinib (JAK1/2 inhibitor) are ongoing [1]. The research of novel treatments for ATL may one day improve the ATL survival and reduce the drug toxicity.
HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP)

In addition to ATL, HTLV-1 infection is associated with inflammatory disease HAM/TSP [14].

The clinical observation for HAM/TSP is a chronic inflammation of the white and grey matter of the spinal cord. Mononuclear cells, mainly T cells, are the source causing perivascular cuffing and infiltrating the parenchyma [14]. In clinic, HAM/TSP patients showed infected T cells in the spinal cord lesion and HTLV-1 Tax specific CD8+ T cells in the cerebrospinal fluid [14]. The spinal cord impair mainly happens at the white matter of the lower thoracic spinal cord, which explains the clinical observation of spastic paraparesis in the lower limbs [14]. Since there is no evidence showed that HTLV-1 infects neuronal cells, astrocytes, or microglia, the damage of spinal cord is caused by the bystander effect of T cell immune response [14].

When compare HAM/TSP patients with HTLV-1 infected asymptomatic carriers, HAM/TSP patients showed higher provirus load, inflammatory cytokines such as IFN-γ and TNFα and HTLV-1 specific CD8+ T cells [14]. This clinical observation implies that higher HTLV-1 burden and immune response skews the HTLV-1 infected patient toward developing HAM/TSP [14]. Consistently, HTLV-1 infected patients with polymorphism in the TNFα promoter and the chemokine gene SDF-1α is associated with HAM/TSP development [14].
Notch signaling pathway

The Notch signaling pathway is involved in many different cellular functions, such as cell cycle [64], apoptosis [65], and differentiation [66]. In Drosophila melanogaster, there is one Notch receptor and two ligands (Delta and Serrate (Ser)). Furthermore, there are 4 Notch receptors (Notch 1-4) and 5 ligands (Delta-like (DLL1, DLL3 and DLL4) and Jagged (JAG1 and JAG2)) in mammals (Figure 3) [67, 68]. Mature Notch receptor on the membrane is a non-covalently linked heterodimers. The extracellular and transmembrane (intracellular) portion connect with each other through heterodimerization domain (HD) (Figure 8). The mutations in the HD domain cause ligand-independent activation of Notch receptors [67]. The extracellular portion has many epidermal growth factor (EGF)-like repeats. The transmembrane and intracellular portion is comprised of RBP-J-associated molecule (RAM) domain, Ankyrin (ANK) domain, nuclear localization sequences (NLSs), transactivation domain (TAD) and PEST (proline-, glutamate-, serine- and threonine-rich) domain. PEST domain is known to regulate NICD degradation and is mutated in many tumors [4, 67].
Figure 8. Notch receptors

Notch receptors in mammals (Adapted from [67]).
Every Notch ligand has an EGF-like repeat region and Delta/Serrate/Lag (DSL) except for the domains Jagged1 and Jagged2, which have a uniquely conserved cysteine-rich (CR) domain [67].
Figure 9. Notch ligands

Notch ligands in mammals (Adapted from [67]).
Upon binding with a ligand, the Notch receptor starts a series of proteolytic cleavages (Figure 10) and releases Notch intracellular domain (NICD) from the membrane. Once released, the intracellular domain of Notch (ICN) releases from the cell surface and translocates into the nucleus. In the nucleus, ICN forms a transcriptional activation complex with DNA-binding protein CSL (CBF1/Suppressor of Hairless/LAG-1; also known as RBPJ) and MAML (Mastermind-like protein) to induce the expression of Notch downstream target genes [69-71]. At the end, CycC:CDK8 recruits to ICN and causes ICN hyperphosphorylation. The phosphorylation of ICN at the PEST domain causes ICN to be ubiquitylated by CDC4/FBXW7 ubiquitin ligase and finally degraded by proteasome to terminate the Notch signaling pathway [72].
Figure 10. Notch activation

Notch receptor and ligand interaction induced series cleavage (Adapted from [66]).
**Notch signaling pathway and T-cell development**

The first link between Notch signaling and the T cell is found in T-cell acute lymphoblastic leukemia (T-ALL) patients with chromosomal translocation t(7;9)(q34;q34.3). Later, gain-of-function and loss-of-function experiments indicated Notch signaling in αβTCR⁺, γδTCR⁺, CD4⁺ and CD8⁺ T-cell development [66]. Ectopic expression of NICD increase pre-T cells in the bone marrow and the ablation of Notch in hematopoietic stem cell (HSC) result in loss of T-cell differentiation, increased pool of granulocyte-monocyte progenitors and thymic atrophy. In addition to development, Notch1 signaling also affects pre-T cell metabolism through the PI3K/AKT signaling pathway [66, 69].
Notch Signaling Pathway in Cancer

The genetic alteration of Notch has been demonstrated in many hematopoietic and solid tumors (Table 4) [69, 73].
<table>
<thead>
<tr>
<th>Tumor Type</th>
<th>Oncogene or Tumor Suppressor</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult T cell leukemia (ATL)</td>
<td>Oncogene</td>
<td>Increase cell proliferation and tumor formation in xenograft model</td>
</tr>
<tr>
<td>T cell acute lymphoblastic leukemia (T-ALL)</td>
<td>Oncogene</td>
<td>Cancer initiation and maintenance</td>
</tr>
<tr>
<td>Chronic lymphocytic leukemia (CLL)</td>
<td>Oncogene</td>
<td>Cancer initiation and survival</td>
</tr>
<tr>
<td>Melanoma</td>
<td>Oncogene</td>
<td>Metastasis</td>
</tr>
<tr>
<td>Lung adenocarcinoma</td>
<td>Oncogene</td>
<td>Tumor initiation, maintenance and metastasis</td>
</tr>
<tr>
<td>Glioblastoma</td>
<td>Oncogene</td>
<td>Tumor propagation and radioresistance</td>
</tr>
<tr>
<td>Ovarian cancer</td>
<td>Oncogene</td>
<td>Cancer maintenance and therapy response</td>
</tr>
<tr>
<td>Pancreatic ductal adenocarcinoma (PDAC)</td>
<td>Oncogene and Tumor Suppressor</td>
<td>Tumor progression and maintenance</td>
</tr>
<tr>
<td>B cell acute lymphoblastic leukemia (B-ALL)</td>
<td>Tumor Suppressor</td>
<td>Growth arrest and death</td>
</tr>
<tr>
<td>Small cell lung carcinoma (SCLC)</td>
<td>Tumor Suppressor</td>
<td>Inhibits tumor maintenance</td>
</tr>
</tbody>
</table>

**Table 4. Notch and human cancers**

Notch signaling in human cancers (Adapted from [73])
The involvement of Notch in tumors was first reported by Ellisen et al, who observed the chromosome translocation in T-cell acute lymphoblastic leukemia (T-ALL) that causes the truncated form of Notch1 [74]. The direct evidence of truncated Notch involved in leukemia comes from in vitro and animal studies. First, when rat kidney cells express truncated Notch1, active Notch1 transforms the rat kidney cells [75]. Second, the mice developed T-cell leukemia after transplanting bone marrow expressing activated Notch1 [76]. However, only rare cases of T-ALL have chromosome translocation involving Notch1. Over 50% of the Notch1 mutations found in T-ALL are activating mutations in the extracellular heterodimerization (HD) domain and PEST domain [77]. The mutations in the HD domain cause ligand-independent activation or hypersensitivity of the ligands by reducing S2 cleavage site protection [73, 78]. In addition, the PEST domain is important for CDC4/FBXW7 mediated degradation of ICN1. Mutations in the PEST domain impair ICN1 degradation by FBXW7 and result in higher ICN1 expression [77]. Finally, studies from different groups also show the involvement of FBXW7 mutations in T-ALL. 8.6% of primary T-ALL samples show a mutation or homozygous deletion of FBXW7. The FBXW7 mutations impair FBXW7’s ability to bind ICN1 and result in ICN1 stabilization. Cell lines with a FBXW7 mutation also show Y-secretase inhibitor (GSI) resistance [79, 80].

In addition to activating canonical Notch downstream targets, the Notch signaling pathway can interact with numerous signaling pathways such as TGF-β, PI3K, NF-κB, and WNT signaling pathways [73]. For example, activated TGF-β signaling pathway is known to induce Jagged 1 expression and the phosphorylation of Notch intracellular domain by GSK3β is essential for FBXW7-mediated degradation of Notch intracellular domain.
Notch and ATL

The involvement of Notch in cancer is also observed in human T-cell leukemia virus type 1 (HTLV-1)–associated adult T-cell leukemia (ATL) [4, 81]. ICN1 expression is higher in ATL patients when compared with peripheral blood mononuclear cells (PBMCs) from a healthy donor. The increased level of ICN1 expression is correlated with higher HES1 expression, a Notch target gene, in 58 % of ATL patients when compared with normal PBMCs [4]. In addition, Notch ligand JAG1, JAG2 and DLL1 are found highly expressed in ATL-2 patients when compared with normal PBMCs. Increased cell surface and the intracellular level of JAG1 and JAG2 expression is correlated with high ICN1 expression in ATL patients [81].

When we sequenced Notch1 in thirty-two ATL samples, we found 30% of ATL samples harboring Notch1 mutations. Interestingly, all the Notch1 mutations found in ATL samples are located in the PEST domain, which is different from T-ALL where the Notch mutations are in the HD and PEST domains. Consistent with a previous study, the PEST domain mutations found in ATL samples increase Notch activity and half-life, and impair the CDC4/FBXW7-mediated proteasomal degradation [4].

The in vitro and in vivo studies further support the importance of Notch1 in ATL proliferation and survival [4, 81]. Inhibition of Notch1 leads to G0/G1 cell cycle arrest and decreases cell survival and proliferation in ATL cells. The effect of GSI in cell cycle arrest is through up-regulating p21 and p27 cyclin-dependent kinase inhibitor expression and reducing cyclin E expression, which promotes cell cycle progression from G1 to S [4]. In addition, silencing Notch1 in ATL cells results in increased cell apoptosis [81]. When treated mice engrafted ATL cells with GSI, GSI can reduce tumor size and increase the survival times compared with control mice [4, 81]. Importantly, using GSI to treat PBMCs from high ICN-1
ATL patients causes cell apoptosis. Additionally, combining GSI with bortezomib and romidepsin blocks the spontaneous proliferation of PBMCs from 8 ATL patients [81].

Until now, there is no effective treatment for ATL and the resistance to chemotherapy is the main reason for poor prognosis of ATL. The studies above show that Notch is highly active in ATL and targeting Notch can be a potent treatment for ATL patients. Knowing that the Notch signaling pathway interconnects with many other signaling pathways in ATL, like NF-κB and c-Myc, shows that combination therapy may be a promising direction for ATL therapy.
Ubiquitin consists of 76 amino acids and is a protein involved in protein post-translational modification. The ubiquitin-proteasome system (UPS) is involved in multiple aspects of cellular processes, such as cell cycle, differentiation and proliferation [82-84]. The most common protein ubiquitination is through the covalent conjugation between ε-NH2 group of Lys in the targeted proteins and the C-terminal carboxyl group of Gly76 in the ubiquitin [85]. There are three enzymes participating in protein ubiquitylation: E1 (ubiquitin-activating) enzyme, E2 (ubiquitin-conjugating) and E3 ubiquitin ligase (Figure 1) [83]. First, the C-terminal carboxyl group of ubiquitin is activated by E1 by ATP to AMP hydrolysis. Second, the activated ubiquitin is transferred to E2 and then added to the target protein by E3, which interacts with substrate and E2 and catalyzes ubiquitin transfer from E2 to substrate. Except monomer, repeating the process can form polyubiquitin chains on the substrate, which are linked by seven internal lysine (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48 and Lys63) or N-terminal Met1 (M1) [85]. Different polyubiquitin chains are involved in a wide range of cellular functions. For example, monoubiquitination and multi-monoubiquitinations are known to participate in membrane trafficking and endocytosis. In addition, the Lys48-linked polyubiquitylation chain is involved in protein degradation by 26S proteasome and the K63-linked polyubiquitin chain is related to signal transduction and DNA repair [83]. The K11-linked polyubiquitin chain serve as degradation during cell cycle progression and NF-kB signaling [86].
Figure 11. Ubiquitin-proteasome system (UPS)

Ubiquitin-proteasome system (UPS) (Adapted from [83])
In humans, there are two classes of E3 enzymes (RING and HECT E3 ligase). RING-domain E3 interacts with substrate and E2, but does not contact the ubiquitin directly. In contrast, HECT E3 transfers the ubiquitin onto itself and then transfers the ubiquitin to the substrate. There are many different RING E3 enzymes; some are single subunit enzymes and the others are multiple subunit enzymes. SKP1–CUL1–F-box protein (SCF) belongs to the multiple subunit RING E3 enzymes. It consists of core scaffold proteins and F-box protein to recognize specific substrates. In humans, there are sixty-nine F-box proteins and they can be divided into 3 classes according to their protein interaction domain. Among those F-box proteins, FBXW7 is known to target many key oncoproteins, like c-Myc and Notch1 (Figure 12) [82]. In humans, FBXW7 is located at chromosome 4q32, which has deletion in more than 30% of human cancers [87]. Importantly, overall, 6% of FBXW7 mutation is observed in human tumors and 31% of T-cell acute lymphocytic leukemia (T-ALL) harbors FBXW7 mutations [88].
**FBXW7 substrate recognition**

FBXW7 recognizes its substrate through conserved FBXW7 phosphodegron (CPD) motif, which requires the substrate to be phosphorylated (Figure 12) [89-91]. The CPDs and FBXW7 interaction sites are the phosphorylated threonine or serine in the “0” and “+4” position. Therefore, the signaling pathways that affect substrate CPDs phosphorylation can regulate substrate degradation. For example, Glycogen synthase kinase 3 beta (GSK3β) phosphorylates c-Myc [92, 93], cyclin E [91] and MCL1 [94] at “0” position, which is important for FBXW7-mediated degradation. The PI3K/AKT pathway can phosphorylate and inactivate GSK3β; therefore the PI3K/AKT pathway can affect the stability of FBXW7 substrates in cell proliferation and survival. Interestingly, some FBXW7 substrates, such as cyclin E and KLF2, have more than one phospho-degron (Figure 12B). In those cases, the dimerization of FBXW7 accelerates the substrate degradation.
Figure 12. FBXW7 and its substrate

FBXW7 recognizes its substrate through conserved FBXW7 phosphodegron (CPD) motif

(Adapted from [66])
In addition, R465, R479, and R505 are three arginine residues in FBXW7’s substrate binding domain (WD40 domain) that are important for interaction with CPDs. Importantly, these 3 arginine residues are FBXW7 hotspot mutations in many human cancers [82]. The dimerization of FBXW7 also controls the substrate degradation. FBXW7 dimerization increases binding strength between substrates and FBXW7, which is particularly important for substrates with weak CPDs, like cyclin E. The dominant-negative effect of FBXW7 mutants comes from the FBXW7\textsuperscript{WT}-FBXW7\textsuperscript{mutant} heterodimer that can no longer degrade substrates with weak CPDs [82].
Critical FBXW7 substrates

FBXW7 is a tumor suppressor known to target many different proteins (Table 5) [95]. Most of the substrates need to be further verified in different systems and in animal models. Below, we discuss the substrates that have been proved in many studies and animal models.
<table>
<thead>
<tr>
<th>Substrates</th>
<th>Phospho-degron (Phosphorylation sites)</th>
<th>Kinase(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclin E</td>
<td>LLTPQPSG, ITPPDKEED</td>
<td>Cdk2, GSK3</td>
</tr>
<tr>
<td>Aurora A</td>
<td>LGTVYREL</td>
<td>GSK3</td>
</tr>
<tr>
<td>c-Myc</td>
<td>LPTPPLSP</td>
<td>GSK3</td>
</tr>
<tr>
<td>c-Jun</td>
<td>GEPPTPLSP</td>
<td>GSK3</td>
</tr>
<tr>
<td>Notch1</td>
<td>FLTPSPES</td>
<td>Cdk8</td>
</tr>
<tr>
<td>SREBP1</td>
<td>TLTPPPSD</td>
<td>GSK3</td>
</tr>
<tr>
<td>p100</td>
<td>LPSPPTSD</td>
<td>GSK3</td>
</tr>
<tr>
<td>C/EBPδ</td>
<td>QPTPPTSP</td>
<td>GSK3</td>
</tr>
<tr>
<td>c-Myb</td>
<td>LMTVPSEED</td>
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<td>ATPPAS</td>
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<tr>
<td>Mcl-1</td>
<td>DGSLPSTP, IMSPEEEL</td>
<td>GSK3</td>
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</table>

**Table 5. FBXW7 substrates**

Potential substrates for FBXW7 (Adapted from [95])
c-Myc

c-Myc is a transcription factor that is deregulated in many tumors. Silencing c-Myc expression blocks cell growth in HTLV-1-transformed human T-cell lines, which implies the importance of c-Myc in ATL [96]. Tax is known to upregulate c-Myc expression through the NF-κB signaling pathway [20] and tumors from Tax transgenic mice also highly express c-Myc [97]. In addition, Tax alone is not sufficient to allow the hematopoietic cell line BAF-B03 to proliferate in the absence of cytokines, but it can do so when Tax cooperates with c-Myc [98]. The ubiquitination of c-Myc by FBXW7 reduces c-Myc’s ability to induce cell growth [93, 99, 100]. The phosphorylation of Threonine-58 and serine-62 in c-Myc is essential for FBXW7-mediated degradation. However, threonine-58 and serine-62 are often mutated in cancer, which implies impaired c-Myc degradation by FBXW7 [100, 101].
**Notch**

The Notch signaling pathway is involved in many different cellular processes, like cell cycle, apoptosis, and differentiation. Over 50% of the Notch1 mutations found in T-ALL are activating mutations in the extracellular heterodimerization (HD) domain and PEST domain [77]. The PEST domain is important for FBXW7/FBXW7 mediated degradation of ICN1. Mutations in the PEST domain impair ICN1 degradation by FBXW7 and result in higher ICN1 expression. FBXW7 is mutated in more than 30% of primary T-ALL samples [102]. The FBXW7 mutations impair FBXW7’s ability to bind ICN1 and result in ICN1 stabilization [79, 80].
**cyclin E**

Cyclin E- cyclin-dependent kinase Cdk2 (cyclin E/CDK2) is known to regulate cell cycle entry and progression by initiating DNA replication at the G1-S phase transition [103]. Abnormal cyclin E expression is observed in many cancers and is known to cause genomic instability [104-106]. Expression of Tax in human T cells induces the expression of cyclin E and CDK2, and reduces the expression of CDK inhibitors p19 and p27 [107]. The expression of p27 is lower in HTLV-1-transformed cells (IL-2-independent) compared with immortalized (IL-2-dependent) HTLV-1-infected T cells. The low expression of p27 is correlated with constitutive activation of cyclin E/CDK2 in HTLV-1-transformed T cells [108]. Cyclin E/CDK2 forms a complex with p300 and RNA Pol II in HTLV-1 infected cells. The complex has kinase activity and can phosphorylate the carboxyl terminal domain of RNA Pol II. Importantly, inhibition of cyclin E/CDK2 activity by specific CDK chemical inhibitor (Purvalanol A) can reduce the transcription of the HTLV-1 promoter and induce higher levels of growth inhibition and apoptosis in HTLV-1 infected cells than uninfected cells [109, 110]. The phosphorylation of cyclin E by CdK2 and GSK3 is essential for FBXW7-mediated degradation [89, 91]. In the FBXW7 knockout mice, the elevated levels of cyclin E are correlated with increased DNA synthesis in placental giant trophoblast cells [111]. The mutations of FBXW7 in cancers impaired its function to degrade cyclin E [80] and leads to high levels of cyclin E expression and chromosome instability [104, 105, 112, 113].
**Mcl-1**

Mcl-1 belongs to the Bcl-2 family and regulates apoptosis in normal and cancer cells [114]. Transgene mice expressing Mcl-1 ended up developing lymphoma [115]. In clinical studies, Mcl-1 is highly expressed in various leukemias [114] and the expression of Mcl-1 is correlated with chemotherapy response [116]. Mcl-1 expression is higher in primary human T cells immortalized by HTLV-1 than the parental uninfected primary T cells [44]. Induction of Tax in CD4+ T cells causes T-cell hyper-proliferation and immortalization with increased expression of Mcl-1 [117]. Collectively, Tax stabilizes Mcl-1 by promoting TRAD6-mediated K63-linked polyubiquitination to increase cell survival and transformation [44]. Mcl-1 can induce drug resistance to ABT-373, which blocks Bcl-2, Bcl-XL, and Bcl-w. Bortezomib is a proteasome inhibitor that can increase the expression of Noxa and the formation of the Mcl-1-Noxa complex, which neutralizes the function of Mcl-1, therefore synergistically inducing apoptosis in HTLV-1 infected T-cell lines and fresh ATL cells when combined with ABT-737 [118]. The regulation of Mcl-1 by GSK3 leads to the finding that FBXW7 is a ubiquitin ligase targeting Mcl-1 degradation [119]. Importantly, FBXW7 deletion or loss-of-function mutations from patient-derived cancer cells impair the Mcl-1 degradation and result in resistance to chemotherapy drugs [119, 120].
**FBXW7 and human cancers**

About 6% of primary tumors carry a FBXW7 inactivated mutations. Among those primary tumors, cholangiocarcinomas and T-ALL have the highest mutation rates, which is 35% and 31%, respectively [88]. Importantly, clinical studies found 3 FBXW7 arginine hotspot mutations (R465, R479, and R505) that are critical for FBXW7 interaction with its substrates [82]. FBXW7 hotspot mutations impair the ability of FBXW7 to bind and add ubiquitin to the substrates [80]. From the TCGA data, it shows that the majority of tumors with FBXW7 hotspot mutations have a normal second FBXW7 allele. On the other hand, some tumors harbor a nonsense mutation and homozygous null mutations. Importantly, the frequency and the FBXW7 mutation patterns are different from organ to organ, which imply the context-dependent outcome of FBXW7 mutations [82]. The related low frequency of single-FBXW7 substrate CPD mutations compared with FBXW7 mutations implies the requirement of dysregulation of many oncoproteins in FBXW7-related tumorigenesis [82].

Conditional knockout of FBXW7 in hematopoietic cells or T cells is sufficient to cause T-ALL or thymic lymphoma by increased expression of Notch1 and c-Myc in more than 50% of FBXW7-deficient mice [121, 122]. However, FBXW7 hotspot mutation knockin mice (FBXW7mut/+ ) did not develop leukemia spontaneously, indicating the difference between FBXW7 missense mutation and homologous deletion. When combining FBXW7 deletion/mutation with suppression of p53 [121] or loss of PTEN [123] or active Notch [9], FBXW7 deletion/mutation enhances the tumorigenesis.
**FBXW7 and prognosis**

The role of FBXW7 in the prognostic implication remains controversial. Consistent with the tumor suppressor role of FBXW7, FBXW7 expression was deficient in hepatocellular carcinoma (HCC) tissues and correlated with poor clinical pathology features including large tumor size, high pathological grading and advanced TNM stage. Importantly, patients with positive FBXW7 expression show a better 5-year survival and FBXW7 is an independent factor to predict the outcome of HCC patients [124]. In non-small cell lung cancer (NSCLC), loss of FBXW7 increases the sensitivity of a class I-specific histone deacetylase (HDAC) inhibitor, MS-275, and upregulates Mcl-1 expression. In addition, patients with low FBXW7 expression have more aggressive cancers and shorter survival time [125]. c-Myc amplification and FBXW7 deletion was found in gastric tumor samples at 51.5% and 45.5%, respectively. Abnormal c-Myc and FBXW7 expression is correlated with lymph node metastasis and tumor stage III-IV [126]. In high histological grade breast cancer patients, FBXW7 mRNA is significantly lower. Moreover, lower FBXW7 expression is correlated with high Ki-67 and cyclin E expression [127].

There is no difference between colorectal cancer (CRC) patients with and without FBXW7 mutation in 5-year overall survival [128], which is further confirmed by Mouradov [129]. In 822 patients from the VICTOR trial of stage II/III colorectal cancer, FBXW7 mutation is not associated with disease-free survival. In 47 patients with pediatric T-cell acute lymphoblastic leukemia (T-ALL), FBXW7 mutations were not associated with treatment outcome [130], which is the same as 88 adult patients with T-ALL treated on the
UKALLXII/ECOG E2993 protocol. Notch1 and FBXW7 mutations did not predict the clinical outcome [131].

However, in T-ALL patients treated in either the Lymphoblastic Acute Leukemia in Adults (LALA)-94 (n = 87) or the GRAALL-2003 (n = 54) trials, Notch1/FBXW7-mutated patients have better overall survival and event-free survival compared with other patients [132], although there is no correlation between Notch1/FBXW7 mutation and clinical biologic features. Glucocorticoid receptor alpha (GRα) may explain the better clinical outcome in patients with FBXW7 mutations. FBXW7 is known to ubiquitylate GRα and cause GRα to be degraded by proteasome. Inhibition of FBXW7 leads to increased glucocorticoid sensitivity, but not to other chemotherapy drugs for T-ALL [133].

The same was observed in 157 patients with T-ALL in the pediatric ALL-Berlin-Frankfurt-Munster (BFM) 2000 study [134]. Patients with Notch1 mutations show better response to treatment and a favorable long-term outcome, which is independent of sex, age, white blood cell count, and T-cell immune-phenotype at the time of diagnosis. Further study shows that the effect of Notch1 mutations in ALL-BMF is limited to patients with rapid early treatment response [135]. FBXW7 inactivated mutations are correlated with rapid early treatment response and act together with Notch1 mutations. However, FBXW7 inactivated mutations have no effect on long-term outcome by themselves or synergizing with Notch1 mutations [135]. In addition to Notch1, FBXW7 inactivated mutations can also affect c-Myc, cyclin E and Mcl-1, and this may explain why FBXW7 mutation loses synergistic effect with Notch1 mutations at late stage.

Although the studies above support the favorable outcome for Notch1 mutation patients, other studies show different results. Zhu found that in adult T-ALL patients, but not in pediatric
patients, Notch mutations are associated with high WBC count and poor survival time [136]. In 88 adult patients with T-ALL treated on the UKALLXII/ECOG E2993 protocol, Notch1 and FBXW7 mutations did not predict the clinical outcome [131]. The same results are observed from van Grotel [137] and Larson Gedman [130] that Notch1 mutations are not predictive for clinical outcome.

In conclusion, the patient cohort size, the sub-population of patients (adult or pediatric patients), the tumor type, other gene mutation, like p53 [138], and the drugs for treatment are factors that may affect Notch and FBXW7 clinical outcome prediction. Further large-scale studies are needed to further confirm the role of FBXW7 and Notch1 in clinical prognosis.
**Epigenome**

The Epigenome is defined by the changes in gene expression and cellular phenotype that are not caused by genomic sequence change. More than half of human cancers have mutations in enzymes involved in chromatin organization [139]. Epigenetic deregulation not only causes aberrant gene expression in cancer cells but also renders tumor cells with the ability to survive chemotherapy and immune surveillance. Therefore, targeting the epigenome, such as DNA methylation and histone modifications, represents a promising strategy for treating the disease.

Epigenetic modification can occur at the DNA, RNA and protein level. The system consists of a “Writer Enzyme” that adds the specific covalent modification to DNA, RNA and histone, a “Reader Enzyme” that recognizes the modification and activates the downstream effects, and an “Eraser Enzyme” that removes the epigenetic modification (Figure 13). Therefore, epigenetic modifications in cells are dynamic and cell-content dependent [140].
Figure 13. Epigenetic modulator

Three main components of epigenetic modulators. (Adapted from [141])
Importantly, a large number of mutations in writer, reader and eraser enzymes has been reported after genomic sequencing for large tumor sample size, which explains the epigenetic deregulation in cancer [139]. For example, the mutation of DNA methyltransferase 3A (DNMT3A) leads to DNA hypomethylation pattern in AML [139]. In addition, histone H3 lysine 36 (H3K36) and H3.3K27 mutations has been reported in sarcoma and gliomas, respectively, and have led to global histone modification reprogramming [139]. These findings may have implications for treatment of epigenetic-mutant cancers and help clinical patient classification.
DNA methylation

Methylation of DNA at the CpG island is associated with gene expression silencing. There are three DNA methyltransferase in mammalian cells: DNMT1, DNMT3A and DNMT3B. DNMT1 methylates newly synthesized DNA after DNA replication to maintain DNA methylation, whereas DNMT3A and DNMT3B de novo methylate the unmethylated DNA to silence gene expression [142] (Figure 1). To remove the DNA methylation, 5-methylcytosine (5-mC) is converted to 5-hydroxymethylcytosine (5-hmC) by ten-eleven translocation (TET) family (TET1, TET2 and TET3). Because DNMT1 cannot recognize 5-hmC, the methylation of DNA will be lost following DNA replication [142] (Figure 1).
Figure 14. DNAMT and TET in DNA methylation

DNA methylation and demethylation (Adapted from [142]). DNAM, DNA methyltransferases (DNMT1, DNMT3A and DNMT3B); 5mC, 5-methylcytosine; 5hmC, 5-hydroxymethylcytosine; TET, ten-eleven translocation family
Histone modification

Compared to DNA, histone has different types of modification including methylation, acetylation and ubiquitylation. In addition, different modifications on specific histone proteins and lysine residue will have a unique effect. For instance, histone 3 Lys9 dimethylation (H3K9me2), H3K9me3 and H3K27me3 modification are known to silence gene expression. On the other hand, H3K4me3 and histone acetylation are related to transcriptional activation [142].

Histone modifications are performed by various multi-subunit complexes. G9A and/or GLP are known to make H3K9me2, and H3K9me3 modification is catalyzed by SUV39H1 and/or SUV39H2. In addition, Polycomb repressive complex 2 (PRC2) is composed of EED, SUZ12 and a SET-domain containing EZH protein (EZH1 or EZH2), and can process H3K27me3 modification. Histone demethylases JMJD3 and UTX have been reported to reverse H3K27me3 modification and activate the transcription activity [142]. In contrast to reduced transcriptional activity, H3K4me1 and H3K4me3 are associated with enhancers and active gene expression, respectively. In human cells, there are six lysine-specific methyltransferases: MLL1, MLL2, MLL3, MLL4, SET1A and SET1B. MLL3 and MLL4 are responsible for H3K4me1 deposition at enhancer elements in mammalian cells, whereas MLL (KMT2A or MLL1) and MLL2 (KMT2B) are in charge of H3K4me3 modification (Figure 1) [143]. The acetylation of histone is catalyzed by histone acetyltransferases (HATs), which accumulates negative charge on the histone and leads to chromosome loosening. On the other hand, histone deacetylases (HDACs) are known to remove histone acetylation and result in gene silencing [142].
Epigenetic alteration in ATL

DNA hypermethylation at the CpG island has been reported in ATL samples compared with normal controls [2]. About 40% of ATL samples showed extensive hypermethylation at the CpG island, known as the CpG island methylator phenotype (CIMP). Importantly, CIMP in ATL samples is associated with poor clinical prognosis. Among those hypermethylated genes, MHC class I is known to be involved in immune surveillance to eliminate HTLV-1 infected cells, which can explain loss of MHC class I in 90% of ATL cases [2].

Combining microarray to comprehensively analyze gene expression and ChIP-on-chip to study the ATL epigenome, Fujikawa found that the Polycomb repressive complex 2 (PRC2) which is composed of EED, SUZ12 and a SET-domain containing EZH protein (EZH1 or EZH2) is up-regulated in acute ATL cells compared with normal CD4+ T cells [144]. Importantly, depletion of EZH2 and SUZ12 by shRNA reduced ATL proliferation and inhibition of EZH2 by GSK126 induced apoptosis in ATL cells. Consistent with the function of PRC2, ATL cells exhibit higher H3K27me3 modification at half of the genes. Among those genes with up-regulation of H3K27me3, NDRG2, CDKN1A, ZEB1, BCL2L11 (BIM) and CD7 are known to be involved in ATL progression.
Targeting the epigenome

Many drugs targeting the epigenome are under development and in clinical trials (Table 6) [139].
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<th>Inhibitor</th>
<th>Mechanism</th>
<th>Drug</th>
<th>Target</th>
<th>Cancer type</th>
<th>Approval and trial status</th>
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<td>Pan-DNMT</td>
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<td>AML and small-cell lung cancer</td>
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**Table 6. Epigenome targeting drugs**

Drugs targeting the epigenome (Adapted from [139])
DNMT inhibitors (DNMTi), histone deacetylase inhibitors (HDACi) and inhibitors of the bromodomain and extra-terminal motif proteins (iBETs) are pan-inhibitors that target more than one enzyme in the same family, which result in global changes in gene expression. In clinics, treatment of myelodysplastic syndrome (MDS) or AML patients with 5-azanucleoside drug azacitidine (also known as 5-azacytidine; Vidaza, Celgene) and its deoxy derivative decitabine (also known as 5-aza-2’-deoxycytidine; Dacogen, Otsuka) reduced tumor burden and improved patient prognosis [139]. HDACi such as Vorinostat (Zolinza; Merck & Co.), belinostat (Beleodaq; Spectrum Pharmaceuticals) and romidepsin (Istodax; Celgene) has been approved by the FDA for the treatment of cutaneous or peripheral T-cell lymphomas. Finally, iBET, which targets the bromodomain-containing protein (BRD), has been shown to reduce MYC expression and is in clinical trial [145-147]. In addition to pan-inhibitors, the H3K27 histone N-methyltransferase EZH2 inhibitor is showing promising results in cell lines bearing EZH2 mutations.

In addition to chemotherapy drugs, the advance of chromatin-modifying technology such as Transcription activator-like effector nucleases (TALEN) and bacterial Cas9 nuclease (dCas9) has improved our ability to modify the epigenome at specific sites [140]. Importantly, in addition to its nuclease activity, inactivated dCas9 can be fused with epigenetic modifying enzymes and enable us to pinpoint each particular epigenetic modification site in gene expression regulation (Figure 15).
Figure 15. dCas9 chimera

Site-specific modification of the epigenome (Adapted from [140])
Chapter II: Oncogenic mutations in the FBXW7 gene of adult T-cell leukemia patients

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Abstract

Human T-cell leukemia virus type 1 (HTLV-1) is associated with adult T-cell leukemia (ATL), an aggressive lymphoproliferative disease with a dismal prognosis. We have previously described the presence of Notch1 activating mutations and constitutive Notch1 signaling in patients with acute ATL. In this study, we report a high frequency of F-box and WD repeat domain containing 7 (FBXW7)/hCDC4 mutations within the WD40 substrate-binding domain in 8 of 32 acute ATL patients (25%). Functionally, ATL FBXW7 mutants lost their ability to interact with intracellular Notch (NICD), resulting in increased protein stability and constitutive Notch1 signaling. Consistent with the loss-of-function found in ATL patients, expression of WT FBXW7 in several patient-derived ATL lines demonstrated strong tumor-suppressor activity characterized by reduced proliferation of ATL cells. Remarkably, two FBXW7 mutants, D510E and D527G, demonstrated oncogenic activity when expressed in the presence of HTLV-1 Tax, mutated p53 R276H, or c-Myc F138C found in human cancers. Transforming activity was further demonstrated by the ability of the FBXW7 D510E mutant to provide IL-2–independent growth of Tax-immortalized human T cells and increase the tumor formation in a xenograft mouse model of ATL. This study suggests that FBXW7, normally a tumor suppressor, can act as an oncogene when mutated and may play an important role in the pathogenesis of ATL.
Introduction

Human T-cell leukemia virus type 1 (HTLV-1)–associated adult T-cell leukemia (ATL) carries a dismal prognosis and limited treatment options [51]. Although the molecular events leading to T-cell transformation are not fully elucidated, the low incidence and long latency of HTLV-1–associated ATL suggest that acquisition of genetic defects in virally infected cells are required for cellular transformation, and profound epigenetic changes have been reported in ATL cells [148]. Although the viral Tax protein has a low transforming efficiency in human T cells, Tax plays a major role by altering signaling pathways, such as NF-kB, p53, and telomerase [149, 150]. In addition, Tax expression impairs DNA replication forks, increases DNA breaks, and simultaneously inhibits faithful repair through homologous recombination [36, 151]. We have previously reported that HTLV-1–transformed ATL cells frequently display constitutive activation of the Notch signaling pathway and activating mutations within the proline, glutamic acid, serine, and threonine (PEST) domain of Notch1 [4]. Constitutive activation of Notch signaling is relevant to ATL and its inhibition reduced proliferation and survival of ATL cells in vitro and significantly reduced tumors in an engrafted ATL mouse model [4]. Because most mutations found in Notch disrupted F-box and WD repeat domain containing 7 (FBXW7)-mediated ubiquitination, degradation, and turnover of intracellular Notch (NICD), we hypothesized the presence of mutations in the FBXW7 gene in ATL patient samples. FBXW7 (also known as Sel-10, hCdc4, or hAgo) is a component of a S-phase kinase-associated protein 1 (Skp1)-Cul1-F-box protein ubiquitin ligase complex that regulates the degradation of Notch, cyclin E, c-Myc, mammalian target of rapamycin, myeloid cell leukemia 1 (Mcl-1), and c-Jun, most of which possess oncogenic functions [82]. Loss of FBXW7 by means of mutation or deletion has been reported in various human cancers and has been linked to severe chromosomal
instability. In addition, inactivation of FBXW7 by miR-223, a microRNA deregulated in ATL, has also been reported [152]. Importantly, several studies have demonstrated that mutations in FBXW7 have clinical significance and can provide resistance to γ-secretase inhibitors (GSI) [79].

In this study, we report a high frequency of FBXW7 mutations in primary acute ATL patients, 8 of 32 (25%). The biological significance of these mutations is suggested by the fact that these mutations were generally associated with an increase in Notch1 signaling. Although WT FBXW7 acted as a tumor suppressor in ATL cells, we identified two FBXW7 mutants, D510E and G527G, with transforming activities when expressed along with another oncogene, such as Tax, p53, or c-Myc. To our knowledge this is the first example of FBXW7 mutants with oncogenic properties.
Materials and Methods

Patients and Cell Lines.

Samples from 32 ATL patients were obtained after informed consent in a study approved by the Institutional Review Board to the National Institutes of Health and the Necker Hospital, and were used for FBXW7 sequencing as previously reported [4]. The 293T, U2OS, and Rat1 cells were cultured in DMEM with 10% (vol/vol) FBS. ATL cell lines MT1, ATL-T, ED40515(−), TL-Om1, and ATL-25 were cultured in RPMI-1640 with 10% (vol/vol) FBS. ATL cell line ATL-43T and Tax-immortalized WT4 T cells were grown in 20% (vol/vol) FBS and IL-2.

Animal Studies.

All animal studies were performed by Advanced Bioscience Laboratories. Protocols were reviewed and approved by Advanced Bioscience Laboratories’s Institutional Review Board. To study the oncogenic role of FBXW7 D510E in ATL tumor growth, 8-wk-old female NOG mice were used. Before injection of ATL cells, mice were given 2 mg/mL doxycycline (Vibramycin Monohydrate) in the drinking water with 2% sucrose for 7 d. The water was thereafter changed every day. Each animal received 5 × 10^7 ATL cell controls MT1-TripZ-WT and MT1-TripZ-D510E in the right and the left flank, respectively. Tumor growth was monitored three times a week for 21 d.

Plasmids and Transfections.

Polyfect (Qiagen) was used for 293T transfection. Calcium Phosphate Transfection Kit (Invitrogen) was used for Rat1 cell transfection and for lentivirus production. FBXW7 mutant plasmids were generated by QuikChange Site-Directed Mutagenesis Kit (Agilent). FBXW7 WT and mutants were cloned into the pTripZ inducible vector (Thermo).
**Western Blot and Coimmunoprecipitation.**

Cells were lysed in RIPA lysis buffer. Antibodies: anti-Myc (9E10; Roche), anti-actin (Santa Cruz), anti-Flag (Sigma), and anti-HA (3F10) (Roche) were used for the Western blots. For FBXW7 and NICD interaction by coimmunoprecipitation, 293T cells were cotransfected with the Myc-tagged NICD and Flag-tagged FBXW7 plasmids. After 48 h, cells were harvested in Nonidet P-40 lysis buffer, IP with anti-Myc antibody and Western blot. Nuclear protein extracts of MT1 cells expressing FBXW7 WT or mutants were used for endogenous NICD Western blot analyses.

**Cycloheximide Chase.**

293T cells were cotransfected with 100 ng Myc-tagged ICN1 and 1 μg Flag-tagged FBXW7 WT or mutants for 48 h. Before harvest, cells were treated with 100 μg/mL cycloheximide for 0, 2, 4, and 6 h. Anti-Myc and anti-Flag antibodies were used for the Western blots.

**Luciferase Assays.**

Luciferase assays were performed with the Luciferase Reporter Assay Kit (Promega) according to the manufacturer’s instructions. Luciferase assays were repeated at least twice from independent experiments and results were normalized to protein concentration.

**Ubiquitination Assays.**

293T cells were transfected with Myc-tagged NICD, Flag-tagged FBXW7, and HA-tagged Ub (K48 or K63) for 48 h. Cells were treated with 10 μM MG132 for 6 h. Cells were collected with RIPA lysis buffer containing N-ethylmaleimide, iodoacetamide, and EDTA, then immunoprecipitated with anti-Myc antibody and immunoblotted.
**Rat1 Cell Transformation Assays.**

After 72-h transfection, Rat1 cells transfected with indicated plasmid were subjected to puromycin selection. Cell culture medium was changed once a week. After a month, the colonies were fixed and stained with Crystal violet.

**ChIP Assays.**

FBXW7 WT or mutant MT1 cells (seeded at $1 \times 10^6$ cells per milliliter) were treated for 48 h with 2 μg/mL doxycycline or 72 h with ± Compound E (1 μM). Cells were cross-linked 10 min with 1% (CF) formaldehyde, stopped with 0.125 M glycine (CF), washed, and DNA was sheared by sonication. ChIP was performed with the ChIP Assay Kit (Millipore), anti-Notch (ab27526; Abcam), according to the manufacturer’s instructions. CHIP-quantitative PCR (qPCR) was performed with SYBR green qPCR using Hes1-CHIP primers (F:CTGTGGGAAAGAAAGTTTGGG and R:GACCAAGGAGAGGTTGAGG). Data were normalized using a percent input method ($= 100 \times 2^{[\text{Adjusted input-Ct (IP)]}}$). Amplified products were cloned into pGEM-T easy vector (Promega) to verify sequence.
Results

FBXW7 Inhibits the Proliferation of ATL Cells.

Disruption of FBXW7 has been reported in various human cancers, and because FBXW7 can target many proteins involved in proliferation and survival for degradation, it has been classified as a tumor suppressor. However, its role in HTLV-1–transformed ATL cells has never been investigated. Generally ATL-transformed cell lines demonstrated significantly reduced mRNA expression levels of FBXW7 compared with normal peripheral blood mononuclear cells (PBMCs) (Fig. 16A). In contrast, freshly isolated uncultured ATL samples display variable levels of FBXW7 mRNA and about one-third of samples had reduced FBXW7 mRNA expression (Fig. 16A). To study the function of FBXW7 in ATL cells, we used lentiviral delivery and puromycin selection to generate stable Tet-inducible FBXW7-expressing cells. Four distinct patient-derived ATL lines (MT1, ATL-T, ATL-25, and ATL-43T) were used to investigate the effects of FBXW7 expression in ATL cells. Induction with doxycycline resulted in re-expression of FBXW7 (Fig. 16B). FBXW7-transduced lines and matched-control cell lines were treated with the same dose of doxycycline and cellular proliferation was measured by cell counts every other day for 10 d. Our results demonstrate that restoring expression of FBXW7 resulted in inhibition of ATL cell proliferation (Fig. 16B). These data suggest that FBXW7 exerts a tumor-suppressive effect in HTLV-1–transformed ATL cells.

FBXW7 Mutations in Patients with Acute ATL.

With the exception of T-cell acute lymphocytic leukemia (T-ALL), the mutation of FBXW7 is usually rare in human leukemia. T-ALL mutations in FBXW7 are predominantly located at arginine residues R479Q, R505C, and R465H and result in FBXW7 mutants unable to bind to or degrade targets [79]. We sequenced FBXW7 in a cohort of 32 acute ATL samples of
Caribbean origin previously characterized for the presence of Notch1 mutations (Table 7). Our results revealed mutation of the FBXW7 gene in 8 of 32 acute ATL patients. The mutations were located in the propeller domain in proximity to the pocket for substrate binding (Fig. 16C). Remarkably, 4 of 32 (12.5%) ATL patients with mutations in FBXW7 also carried a mutation in the NICD PEST domain [4]. This observation is interesting because previous studies in T-ALL have shown that the combined effect of mutation in both NICD and FBXW7 improves a patient’s prognosis. We also sequenced the WD40 domain of FBXW7 from HTLV-1–transformed cells MT-2, MT-4, and C8166, and from ATL-derived cell lines but found no mutations. We next investigated the biological consequences of FBXW7 mutations. To this end, we used a transient assay in which FBXW7 WT or FBXW7 mutants are expressed along with NICD and a CSL [CBF-1, Su(H), LAG-1]-luciferase reporter construct (Fig. 16D). In this assay expression of NICD activates the CSL-Luc vector leading to higher luciferase values. However, when WT FBXW7 is coexpressed it targets NICD for degradation, lowering luciferase activation. The FBXW7 mutant R505C was used as a negative control. Our experiments demonstrated that six of eight FBXW7 mutations detected in ATL tumor cells resulted in a complete loss-of-function as demonstrated by luciferase activity similar to that of the R505C mutant (Fig. 16D). Two FBXW7 mutants, T416A and W406R, were partially inactive (Fig. 16D). Some FBXW7 mutants (H468R, S462P, and W425R) had higher activity, suggesting a dominant-negative effect (Fig. 16D). Because FBXW7 dimerization is essential for stable interaction with its substrates, we then tested if the above-mentioned mutants can dimerize with and antagonize WT FBXW7. In fact, dimer formation between FBXW7 and mutant W425R were readily observed upon coexpression in transient assays (Fig. 16E). Furthermore, a dose increase of W425R was able to
completely block WT FBXW7-mediated degradation of NICD, confirming its dominant-negative effect (Fig. 16E).
Figure 16. FBXW7 is a tumor suppressor in ATL cells.

The expression of FBXW7 in ATL-transformed cell lines and normal PBMCs (A) or healthy donors and freshly isolated ATL samples were evaluated by real-time RT-PCR. The arrow indicates the patients carrying the D510E mutation. (B) Cellular proliferation of FBXW7 transduced ATL lines were evaluated by cell count after reexpression of FBXW7. The data were from two independent experiments and shown as the average with SD. Western blot showed the expression of FBXW7. Actin served as a loading control. (C) The localizations of FBXW7 mutations found in primary ATL samples (Table 1) are highlighted in yellow on the FBXW7 protein 3D structure. (D) Transcriptional activities of NICD in cells expressing FBXW7 or FBXW7 mutants were measured by CSL-luciferase reporter assay. Luciferase activities were normalized to the CSL-luciferase reporter vector alone. The data are from two independent experiments and are shown as the average with SD. Western blot showed the expression of
tagged-FBXW7 and mutants. (E) Dimer formation between FBXW7 and mutant W425R was analyzed in 293T cells transfected with Myc-tagged FBXW7 and Flag-tagged W425R. IP FBXW7 and Western blot W425R showed the dimerization. NICD expression in 293T cells transfected with NICD, FBXW7 WT and a dose increase of W425R were analyzed by Western blot (Lower). Dox, doxycycline.
Table 7. FBXW7 mutations in ATL samples

Samples from 32 ATL patients were analyzed for mutations in the substrate binding domain (WD40 domains) of FBXW7 and compared with previously identified Notch1 mutations in the same acute ATL patient samples [4].

<table>
<thead>
<tr>
<th>Patients</th>
<th>NICD-PEST domain</th>
<th>FBXW7</th>
</tr>
</thead>
<tbody>
<tr>
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<td>No mutation</td>
</tr>
<tr>
<td>ATL 2</td>
<td>No mutation</td>
<td>No mutation</td>
</tr>
<tr>
<td>ATL 3</td>
<td>No mutation</td>
<td>S462P</td>
</tr>
<tr>
<td>ATL 4</td>
<td>No mutation</td>
<td>W425R</td>
</tr>
<tr>
<td>ATL 5</td>
<td>Q2546R</td>
<td>No mutation</td>
</tr>
<tr>
<td>ATL 6</td>
<td>No mutation</td>
<td>No mutation</td>
</tr>
<tr>
<td>ATL 7</td>
<td>G2427S</td>
<td>No mutation</td>
</tr>
<tr>
<td>ATL 8</td>
<td>aa 2440-TAG</td>
<td>L443F</td>
</tr>
<tr>
<td>ATL 9</td>
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<td>No mutation</td>
</tr>
<tr>
<td>ATL 10</td>
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</tr>
<tr>
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<td>H468R</td>
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<td>No mutation</td>
</tr>
<tr>
<td>ATL 13</td>
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<td>No mutation</td>
</tr>
<tr>
<td>ATL 14</td>
<td>No mutation</td>
<td>D510E</td>
</tr>
<tr>
<td>ATL 15</td>
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<td>No mutation</td>
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<tr>
<td>ATL 16</td>
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<tr>
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</tr>
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</tr>
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<tr>
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<tr>
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<tr>
<td>ATL 31</td>
<td>No mutation</td>
<td>W406R</td>
</tr>
<tr>
<td>ATL 32</td>
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<td>No mutation</td>
</tr>
</tbody>
</table>

The boldface text presents the mutations and where they lie.
ATL FBXW7 Mutants Activate the Notch Signaling Pathway.

To confirm that FBXW7 mutants stimulate Notch1 signaling in human T cells, an ATL-transformed MT1 cell line, carrying WT FBXW7, was used to generate stable Tet-inducible cell lines expressing WT or FBXW7 mutants W425R, S462P, and D510E. Induction and expression of WT and mutant FBXW7 proteins were confirmed upon the addition of doxycycline to the media (Fig. 17A). The ability of WT FBXW7 but not mutants W425R, S462P, and D510E to degrade endogenous NICD in the context of ATL cells was also demonstrated by Western blot (Fig. 17B). Activation of the Notch signaling pathway was confirmed by increased expression of the target gene, hes family bHLH transcription factor 1 (Hes1), in cells carrying W425R, S462P, and D510E compared with WT (Fig. 17B). To further demonstrate activation of Notch1 in cells with mutated FBXW7, we next investigated the presence of NICD onto the Hes1 promoter by chromatin immunoprecipitation (ChIP). To demonstrate specificity, we first treated MT1 cells with GSI compound E, resulting in loss of NICD expression as shown by Western blot (Fig. 17C). This result was accompanied by a 70% decrease in the amount of NICD bound to the Hes1 promoter as determined by ChIP (Fig. 17C). Finally, the PCR product amplified by ChIP was cloned and sequenced to confirm the identity of the Hes1 promoter. Consistent with the data described above and the reduced degradation of NICD by FBXW7 mutants (Fig. 16D), we confirmed an increase in NICD promoter occupancy of the Hes1 gene by ChIP in cells carrying mutated FBXW7 W425R, S462P, and D510E compared with WT (Fig. 17D). Together, these results suggest that FBXW7 mutations acquired in ATL cells increase Notch1 signaling.
Figure 17. FBXW7 mutants activate the Notch signaling pathway.

(A and B) RT-PCR for expression of FBXW7 and Hes1 after 48 h of doxycycline (Dox) induction in MT1 stable lines. Results were repeated at least twice and fold-change was calculated compared with GAPDH expression. Western blots confirm the induction of FBXW7 (A) and reduction of endogenous nuclear NICD in WT-expressing cells but not mutants of FBXW7 (B). Actin and cyclin A served as loading control. (C and D) ChIP assays on FBXW7 WT and mutant following induction in stably transduced MT1 lines using RT-PCR. ChIP assays were prepared using anti-Notch and amplified with Hes-1 specific primers. (C) MT1 cells cultured with or without GSI (1 µM for 72 h) served as an assay control for loss of NICD-Val1744 and Hes1 binding. Fold-change was calculated as a percent of the initial input material. Sequencing of the amplified product verified Hes1 amplification.
ATL FBXW7 Mutants Demonstrate Reduced Binding and Ubiquitin-Mediated Degradation of NICD.

We next investigated the ability of FBXW7 mutants to interact with NICD and promote ubiquitin-mediated turnover of NICD. FBXW7 and FBXW7 mutants were coexpressed along with NICD in 293T cells. Consistent with the reporter assay described in Fig.1D, our results showed that only FBXW7 mutants T416A and W406R retained their ability to degrade NICD (Fig. 18A). Accordingly, the half-life of NICD was extended in the presence of the FBXW7 ATL mutants (Fig. 18B). As expected from these data, most FBXW7 mutants found in ATL patients lost the ability to interact with NICD, and the FBXW7 mutants T416A and W406R retained binding (Fig. 18C). Surprisingly, despite the lack of NICD degradation, FBXW7 mutant D510E was still able to interact with NICD to a similar extent as the WT FBXW7 (Fig. 18C). The lack of NICD degradation was not a result of a defect in the ability of D510E to recruit SKP1 (Fig. 18D). We think that a conformational change of D510E may affect ubiquitination. FBXW7-mediated ubiquitination of NICD on Lysine K63 was not affected by the D510E mutation (Fig. 18E). However, FBXW7-mediated ubiquitination of NICD on Lysine K48, generally associated with proteasomal degradation, was significantly decreased for D510E compared with WT FBXW7 (Fig. 18E).
Figure 18. FBXW7 mutants reduced binding and ubiquitin-mediated degradation of NICD.

(A) FBXW7-mediated degradation of NICD was analyzed by Western blot (WB) and compared with control plasmid (pcDNA). FBXW7 WT and R505C were used as positive and negative controls, respectively. The NICD expression was quantified as relative to control normalized as 100% (WT 53%, R505C 115%, W406R 56%, T416A 61%, W425R 80%, L443F 100%, S462P 117%, H468R 111%, D510E 105%, and D527G 91%). (B) Expression of FBXW7 mutants increased the half-life of NICD. The effect of FBXW7 or mutants on the half-life of NICD was analyzed by Western blot after 100 μg/mL cycloheximide (CHX) treatment for 0, 2, 4, and 6 h. Western blot for transfected NICD, FBXW7, and actin are presented. (C) The interaction between NICD and FBXW7 was analyzed in 293T cells transfected with Myc-tagged NICD and Flag-tagged FBXW7 WT or mutants. Immunoprecipitated NICD and WB FBXW7
showed the interaction between NICD and FBXW7 WT. (D) Interaction between S-phase kinase-associated protein 1 (SKP1) and FBXW7 was analyzed in 293T cells transfected with HA-SKP1 and Flag-tagged FBXW7 WT or mutants. IP SKP1 and Western blot FBXW7 showed the interaction between SKP1 and FBXW7. (E) FBXW7-mediated NICD ubiquitination was analyzed in 293T cells transfected with FBXW7 WT (lane 2) or D510E (lane 3), NICD, and HA-Ub (K63 or K48). Cells were treated with MG132 for 6 h before harvest. Immunoprecipitated NICD and Western blot Ub showed the ubiquitination level of NICD.

HTLV-1 Tax’s transforming abilities have been characterized in vitro using a Rat1 fibroblast colony formation assay (16, 17). We next tested whether ATL FBXW7 mutants could increase Tax-transforming activity. Interestingly, two ATL FBXW7 mutants, D510E and D527G, were able to stimulate the transforming activity of Tax and increase colony formation by 50% and 35%, respectively (Fig. 19 A and B). These mutants had no transforming effects on their own (Fig. 19 A and B). Increased Tax transforming activity was not related to changes in Tax expression as demonstrated by Western blot assays (Fig. 19C). In addition, as reported above for the W425R mutant, the D510E mutation acted as a dominant-negative preventing WT FBXW7-mediated degradation of NICD (Fig. 19C). Previously characterized FBXW7 mutant R505C was not able to cooperate with Tax in transformation assays. Interestingly, the oncogenic activity of the D510E mutant was also observed when coexpressed with either p53 R276H or c-Myc F138C (Fig. 19D), two mutants found in human cancers (18, 19). Our results suggest that FBXW7 has a tumor-suppressor effect in ATL cells but acquisition of specific mutations may result in shifting to a transforming phenotype. We next validated our results in the context of human T cells. In the early stages of the disease, chronic or smoldering ATL cells rely on autocrine loops IL-2/IL-2Rα and IL-15/IL-15Rα for their proliferation. In vitro, transformation by HTLV-1 is characterized by IL-2–independent growth and acquisition of constitutive JAK/STAT activation (20). We cloned FBXW7 and the D510E mutant into a lentiviral vector for delivery into the IL-2–dependent Tax-immortalized WT4 T cells. Two days after infection, cells were grown in the absence of IL-2. After 8 wk in culture, all cells infected with FBXW7 had died but WT4 D510E
cells were still alive and proliferating in the absence of IL-2 (Fig. 19E), suggesting that D510E is able to facilitate transformation of IL-2–dependent Tax-expressing human T cells.
Figure 19. FBXW7 mutants promote cellular transformation.

(A) FBXW7 mutants increase Tax-transforming activity. Rat1 cell colony formation was evaluated after cells were transfected with the indicated plasmids and puromycin selection. The transformation efficiencies were normalized to Tax alone, which was set as 100%. The data were from two independent experiments and shown as the average. Increased number of transformed foci observed for D510E and D527G were statistically significant. (B) Photographs of transformed foci were shown (AMG EVOS XL core with 20× objective). (C) Western blot showed the expression of Tax and FBXW7. Actin served as a loading control. NICD expression in 293T cells transfected with NICD, FBXW7 WT, and a dose increase of D510E were analyzed by Western blot (Lower). (D) FBXW7 mutants increase p53 R276H and c-Myc F138C transforming activities analyzed as in A. The transformation efficiencies were normalized to p53 R276H and c-Myc F138C, respectively. The data were from two independent experiments and
shown as the average with SD. (E) WT4 cells infected with lentivirus encoding FBXW7 WT or D510E were cultured in medium with or without IL-2 for 8 wk. Photographs of cell colonies are shown (AMG EVOS XL core with 20× objective). (F) FBXW7 WT and mutant-mediated substrate degradation were analyzed by Western blot after transient transfection. (G) Cellular proliferation of MT1 cells expressing FBXW7 WT and D510E were evaluated by cell count. The data were from two independent experiments and shown as the average with SD. (H) Degradation of endogenous substrates NICD, c-Myc, and cyclin E by FBXW7, W425R, S462P, and D510E mutant were analyzed by Western blot in ATL cells (MT1).
**FBXW7 D510E Mutant Increases the Tumor Formation in an ATL Mouse Model.**

To gain some insight into the D510E mutant, we performed transient transfection assays and found that FBXW7 D510E retained its ability to target c-Myc and Cyclin E for degradation, but not NICD (Fig. 19F). WT FBXW7 and previously characterized R505C, which are unable to target proteins for proteasome degradation, were used as controls (Fig. 19F). To determine the physiological relevance of our studies, we next investigated whether FBXW7 D510E can promote ATL tumor cell growth in vivo. MT1 ATL-transformed cells were stably transduced with a TET-inducible vector expressing the FBXW7 D510E mutant or FBXW7 WT. In vitro proliferation assays demonstrated that MT1 cells expressing the D510E mutant expanded more rapidly (Fig. 19G). Importantly, the selective defect of D510E mutant for NICD degradation was also confirmed on endogenous proteins (NICD, c-Myc, and Cyclin E) in MT1 ATL cells (Fig. 19H).

To next investigate the ability of D510E to stimulate ATL tumor growth in vivo, we injected an equivalent number of MT1 FBXW7 or D510E cells in the right or left flanks of NOG (NOD/Shi-scid/IL-2Rγnull) mice. Mice received daily doxycycline in the drinking water and tumor volume was recorded every 3 d. After 21 d, the animals were killed and tumors excised (Fig. 20A). As expected from the data presented in Fig. 19, levels of NICD were higher in cells expressing D510E compared with WT FBXW7 (Fig. 20A). Examination of tumors revealed a significant increase in volume and weight for ATL cells expressing the D510E mutant compared with WT FBXW7 control (Fig. 20 B–C). Overall, these results confirm our in vitro transformation assays and suggest that the FBXW7 D510E mutation has tumor-forming potential in cooperation with other oncogenes.
Figure 20. FBXW7 D510E increased the tumor formation in vivo.

(A) MT1 FBXW7 (n = 5) or D510E (n = 5) TET-On cells were injected into the right or left flank of NOG mice. Pictures are representative of excised tumors from injected mice. Western blot for NICD (Val-1744) expression in tumor samples is shown. (B) In vivo tumor growth curves, plotted as the average tumor volume (mm$^3$) [calculated as the (width$^2 \times$ length)/2]. P values were calculated using a two-sided student’s t-test between the tumor volumes for FBXW7 vs. D510E. (C) Tumor weight (in grams) for FBXW7 and D510E tumors taken at the time of sacrifice. P values were calculated using a two-sided student’s t-test between the tumor weight for FBXW7 vs. D510E. The mean tumor weight was indicated with a bar and green square, with the SD indicated.
Discussion

In this study we report genetic mutations in the FBXW7 gene of acute ATL patients. In vitro established HTLV-1–transformed ATL cells demonstrate low levels of FBXW7 mRNA but similar mRNA half-life compared with PBMCs, suggesting transcriptional repression of the FBXW7 promoter in these cells. In contrast, analyses of freshly isolated and uncultured ATL samples demonstrated variable levels of FBXW7 mRNA expression, which was reduced in approximately one-third of ATL patients. Because ATL cells expressing or not expressing Tax had low levels of FBXW7, we think the mechanism is largely Tax-independent. Several reports suggest that a low level of FBXW7 expression is associated with increased malignancy potential, lymph node metastasis, and poorer prognosis in cancer patients [138]. The existence of additional posttranscriptional mechanisms to reduce FBXW7 expression, such as miR-223, miR-92a, and miR-27a regulation, has also been reported. Of note, we previously found that miR-223 is up-regulated in uncultured ATL samples, suggesting that miR-223 may contribute to suppression of FBXW7 in ATL cells in vivo. Our data confirmed that FBXW7 acts as a tumor suppressor in ATL cells and significantly reduces tumor cell proliferation, thereby justifying the requirement for its inactivation. In a recent study by Kataoka et al. [2], 81 ATL samples were analyzed by exome sequencing and the rate of mutation for FBXW7 was found to be much lower than in our study. However, using the same patient cohort we used in this study, we recently found a similar rate of mutation for STAT3 (25.5% in Caribbean vs. 21% for Japanese samples). Therefore, we believe the rate of mutation may be higher for FBXW7 in the Caribbean population. In support of this notion, using the 1000 Genomes Project Consortium database, we found the frequency of FBXW7 single nucleotide polymorphism (SNPs) to be higher in Caribbean samples compared with Japanese samples. In the total population the frequency of
SNPs at FBXW7 was 1.05% similar to Japanese 1.08%. However, the Caribbean frequency of FBXW7 SNPs was significantly higher, 1.42%. In addition, we observed that Caribbean individuals had a greater amount of unique SNPs (1,174) at the FBXW7 locus compared with Japanese samples (762). The data demonstrate that individuals of Caribbean descent have a greater frequency of FBXW7 SNPs. In addition, another difference between the two studies is that we analyzed only acute ATL, whereas Kataoka et al. [2] analyzed mixed acute, chronic, smoldering, and lymphoma ATL (acute 47%, lymphoma 16%, chronic 31%, and smoldering 6%). If the mutation occurs in a later stage of the disease, that may also explain the different rate of mutation between the studies.

Importantly, our study identified two mutants, FBXW7 D510E and D527G, which retained their ability to target endogenous Cyclin E, MCL-1, and c-Myc for proteasome degradation but were unable to degrade NICD in ATL cells. This observation contrasts with a previously reported phenotypes of FBXW7 mutants found in other cancers for which mutations abolish all substrate degradation. The reason why the D510E mutation selectively abolishes degradation of NICD but no other substrates is unclear. D510E retains its ability to interact with NICD but was much less efficient in promoting K48 ubiquitination of NICD. The mechanism underlying D510E and D527G oncogenic activity remains to be demonstrated. Other mutations may also play a different role. For example, several reports indicate that a mutation in FBXW7 can contribute to drug resistance [79] or promote metastasis. Deregulated MYC and FBXW7 has been associated with the presence of lymph node metastasis and poor prognosis in gastric cancers, and a distinctively poor prognosis in gastric cancer patients who had low FBXW7 expression levels and mutated p53 [138]. In addition, several studies showed that FBXW7 gene mutation and hyperphosphorylation of cyclin E, which usually correlates with FBXW7 mutation,
have a significant association with polyploidy and aneuploidy [112]. Because aneuploidy is also a characteristic of acute ATL, it will be important to determine if loss of FBXW7 affects chromosome segregation in ATL cells. In vivo FBXW7 mutation knock-in mice showed cooperation with specific signaling pathways, such as activated Notch or mutated adenomatous polyposis coli and accelerated tumor formation [9, 153]. However, to our knowledge, this study is the first example describing cooperative oncogenic activities of mutated FBXW7 with a viral oncogene (HTLV-1 Tax), p53, or c-Myc. Given the high rate of mutation of both p53 and c-Myc in human cancers, selective loss of FBXW7 functions may play a more active role than previously anticipated in the transformation process and warrants more study. Finally, an FBXW7 mutation may also provide novel therapeutic opportunities inasmuch as ablation of FBXW7 abrogates quiescence in leukemia-initiating cells, thereby increasing sensitivity to Imatinib. In fact, the combination of FBXW7 ablation with Imatinib treatment resulted in a greater depletion of leukemia-initiating cells than of normal hematopoietic stem cells in mice [154]. Glucocorticoid receptor-α is a substrate of FBXW7 and inactivation of FBXW7 has been linked to a higher level of glucocorticoid receptor-α expression in T-ALL. This result was associated with a better prognosis and an increased sensitivity of leukemia cells to steroid therapies [133], suggesting that loss of FBXW7 may create opportunities for specific anticancer therapies.
Chapter III: Mutation of epigenetic regulators TET2 and MLL3 in patients with HTLV-1-induced acute adult T-cell leukemia.

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Abstract

Epigenetic regulators play a critical role in the maintenance of specific chromatin domains in an active or repressed state. Disruption of epigenetic regulatory mechanisms is widespread in cancer cells and largely contributes to the transformation process through active repression of tumor suppressor genes. While mutations of epigenetic regulators have been reported in various lymphoid malignancies and solid cancers, mutation of these genes in HTLV-1-associated T-cell leukemia has not been investigated. Here we used whole genome next generation sequencing (NGS) of uncultured freshly isolated ATL samples and identified the presence of mutations in SUZ12, DNMT1, DNMT3A, DNMT3B, TET1, TET2, IDH1, IDH2, MLL, MLL2, MLL3 and MLL4. TET2 was the most frequently mutated gene, occurring in 32 % (10/31) of ATL samples analyzed. Interestingly, NGS revealed nonsense mutations accompanied by loss of heterozygosity (LOH) in TET2 and MLL3, which was further confirmed by cloning and direct sequencing of DNA from uncultured cells. Finally, direct sequencing of matched control and tumor samples revealed that TET2 mutation was present only in ATL tumor cells. Our results suggest that inactivation of MLL3 and TET2 may play an important role in the tumorigenesis process of HTLV-1-induced ATL.
Introduction

Human T-cell leukemia virus type I (HTLV-1) is associated with fatal lymphoproliferative disorders known as adult T-cell leukemia/lymphoma (ATL) [1, 155]. The disease is classified into distinct subtypes - smoldering, chronic, acute and lymphoma - that differ in their clinical presentation and in their response to treatment [51]. Since the clinical subtypes of ATL have distinct genomic alterations and different clinical progression, these diseases require a different approach for treatment. However, current therapies for ATL do not result in long-term remission and even the clinically less aggressive forms ultimately evolve to the acute. The 4 year survival rate for acute-, lymphoma-, chronic- and smoldering-type ATL is 11, 16, 36, and 52 %, respectively [156]. The viral oncoprotein Tax plays an important role in initiation of events leading to cellular transformation [36, 151]. However, the fact that the disease has a low penetrance and is observed after a long latency period of several decades has led to the hypothesis that the virus initiates oncogenic events but is not sufficient for cellular transformation [148, 157]. In support of this notion epigenetic alterations are required for the development of ATL. Promoter hyper-methylation associated with loss of SHP1 expression coincides with the IL-2-independent transformation of T cells by HTLV-1 in vitro. SHP1 is one of the most frequently altered genes in ATL patients, with an overall hyper-methylation rate of 90 %; other tumor suppressor genes inactivated by methylation in ATL include p53-related p73, CDKN2A and p21CIP1/WAF1 [158]. The fact that histone methyl-transferase EZH2 has been demonstrated to repress p57KIP2 expression through histone H3 lysine 27 trimethylation (H3K27me3), and that p57KIP2 is methylated in nearly 50 % of newly diagnosed ALL patients, prompted us to analyze the status of cellular genes involved in chromatin silencing. In this study we use next generation sequencing (NGS) to characterize the genetic mutations in EZH1, EZH2,
EED, SUZ12, DNMT1, DNMT3A, DNMT3B, TET1, TET2, TET3, IDH1/2, MLL, MLL2, MLL3, MLL4 and ASXL1. Our study revealed a high frequency of mutation in epigenetic regulators in ATL samples, suggesting that chromatin remodeling by some of these genes may play a role in the pathogenesis of ATL.
Materials and Methods

ATL patient samples

All patient samples were obtained after informed consent was provided and in agreement with regulations for the protection of human subjects according to the National Institutes of Health (NIH) guidelines. As for the samples from the Japanese material bank, they were provided from the biomaterial bank of the Japanese nationwide cohort study (Joint Study of Predisposing Factors for ATL Development, JSPFAD) that is approved by the ethical committee of the University of Tokyo (No. 14-15, No. 07-07 and No. 10-50). Genomic DNA was extracted using DNAZol (Invitrogen) from uncultured acute and lymphoma ATL samples. DNA samples 1–7, 10 and 11 were isolated from patients diagnosed with acute ATL. DNA samples 8, 9, 12 and 13 were isolated from patients with lymphoma ATL. HTLV-1 proviral load was calculated by TaqMan real time PCR and compared with a standard curve established using C91PL HTLV-1 transformed cell line harboring one proviral copy (Fig.21a). High tumor grade lymph node biopsy was used for ATL lymphoma patients as confirmed by real time PCR compared with B cells isolated from matched patient (Fig.21b).
Figure 21. ATL samples for sequencing

a A set of standard samples was prepared through dilution of HTLV-1 transformed cell line DNA (TL) containing a single copy of integrated HTLV-1 with HTLV-1 negative 293 T cell DNA. Real time PCR was performed with 100 ng of mixed DNA. Both GAPDH and gag were detected and ΔCt was calculated by Ct gag - Ct GAPDH. The standard curve was created with ΔCt (X) and the percentage of HTLV-1 viral load relative to TL cell (Y). b Proviral load was calculated in DNA samples isolated from high grade ATL lymphoma and matched-control B cells by real time PCR. Relative proviral loads were calculated using the standard curve established above.
Next generation sequencing (NGS)

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

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

Despite profound epigenetic alterations in the genome of ATL cells, the genetic status of chromatin modifiers has not been investigated. In this study we performed next generation exome sequencing (NGS) to identify novel mutations in epigenetic regulators in ATL samples. The polycomb repressive complex 2 (PRC2) has histone methyltransferase activity and primarily trimethylates histone H3 on lysine 27 (H3K27me3), a mark of transcriptionally silent chromatin. The PRC2 complex has four subunits: SUZ12, EED, EZH1 and EZH2. LOH mutations of EZH2 or SUZ12 have been reported in 25 % of T-ALL. In addition, the loss of PRC2 activity cooperates with mutated Notch1 by allowing recruitment of the intracellular domain of Notch onto the promoter of target genes. Along these lines, activated Notch is also required for ATL cell growth and tumor formation in an ATL mouse model [4]. While the EZH2 gene was not mutated in our study, 1/13 ATL sample had a mutation in SUZ12. It will be interesting in future studies to investigate if there is any cooperation of EZH2 and/or SUZ12 with activated Notch in a larger cohort of acute ATL patients. The coding sequence of the other two subunits, EED and EZH1, was not mutated in any of the ATL samples tested. The possibility that some members of PRC2 may be regulated post-transcriptionally by microRNA or LncRNA in ATL cells is under investigation. Similarly, miR101, miR-26 and miR208b have been shown to target EZH2, miR-323-3p to target EED, and miR-200b to target SUZ12. An earlier study demonstrated that decreased expression of miR-101, but not MiR-26b, in acute ATL is in part responsible for elevated expression of EZH2 in these cells [159]. Consequently, increased expression of the EZH2 protein induced the silencing of miR-31, resulting in NIK-mediated activation of NF-kB in ATL cells [39]. Additional sex combs like transcriptional regulator 1 (ASXL1) interact with PRC2 and are likely involved in a cross-talk between chromatin silencing systems, PRC1/PRC2,
the HP1α/CBX5 heterochromatin repressive complex and the polycomb repressive
deuibiquitinase (PR-DUB) complex. Mutation of ASXL1 has been reported in AML and chronic
myelomonocytic leukemia (CMML) patients. Our study revealed mutations of ASXL1 in 2/13
ATL samples. Interestingly, an ASXL1 somatic mutation, V1092M, detected in one ATL patient
has also been reported in myeloproliferative neoplasms (MPN) and myelodysplastic syndromes
(MDS).

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

The Mixed Lineage Leukemia (MLL) family of genes (also known as lysine (K)-specific
methyltransferases (KMT2)) plays an important role in histone methylation and transcriptional
activation and is involved as a regulator of growth of hematopoietic precursor cells. Mutation of
MLL and MLL2 was observed in 7.5 % (1/13) of ATL patients. The MLL3 gene, which encodes
a component of a histone H3 lysine 4 methyltransferase complex named the ASC-2- and Mll3-
containing complex (ASCOM), has been implicated as a tumor suppressor gene due to its
frequent mutations in multiple types of human tumors. Exome sequencing has recently been used
to identify an MLL3 germ line mutation in a pedigree of colorectal cancer and acute myeloid leukemia [160]. Mutations and LOH in MLL3 has been reported in various human cancers. Our initial NGS analyses identified a high rate of nonsense mutations in MLL3 at position R904* of ATL samples (Fig.22a). This was interesting because early termination of MLL3 is predicted to produce a dominant negative form with oncogenic activities [161]. The presence of R904* on a highly conserved sequence homologous to MLL3 present on chromosome 13 likely contributed to the wrong assignment of a snp (rs200662726) in position R904* of the MLL3 gene in the NCBI database. Nevertheless, direct sequencing for all ATL DNA samples confirmed LOH for MLL3 in one ATL patient (Fig.22 b and c).
Figure 22. MLL3 mutations in ATL

a Schematic representation of the MLL3 protein and distribution of mutations found in ATL patients. Nonsense and missense mutations found in ATL patients are shown in red and blue, respectively. b Chromatogram of the sequence of MLL3 from normal PBMC DNA (top) and MLL3 from ATL DNA with amino acid 904 nonsense mutation (bottom). c Alignment of ATL patient DNA KOE with MLL3 demonstrates the presence of a stop codon TGA in position 904. SNPs identifying the MLL3 sequence (different from Chr13) are labeled in red.
Ten-eleven translocation methylcytosine dioxygenase genes (TET1-3) are involved in DNA demethylation. Our investigations reveal mutation in the coding sequence of TET1 in 15% (2/13). The mutation I1229M has been reported in the cosmic database. We also noticed the presence of TET1 single nucleotide polymorphism (snp rs3998860) I1123M. This snp has a global minor allele frequency (MAF) of 0.3067/1536 but was detected in all ATL samples tested. A larger cohort study is needed to confirm these data. No mutations were detected for TET3. Interestingly, TET2 was mutated at a high frequency of 38% (5/13) in ATL patients. These results are in line with the high rate of somatic TET2 inactivation observed in MDS, MPN, chronic myelomonocytic leukemia (CMML) and AML [162], and they suggest that TET2 may play an important role in ATL pathogenesis. TET2 LOH was found in two ATL patients with nonsense mutations at positions Q876* and Q414* (Fig.23a), two mutations previously reported in CML patients. For these two ATL patients, we PCR amplified the TET2 region overlapping these mutations and cloned and sequenced five clones for high tumor grade and matched samples. ATL12 DNA was extracted from a high grade lymph node biopsy from megakaryocytes as a tumor negative control. For ATL11 DNA was extracted from high proviral load (high grade tumor sample) samples before therapy and control sample DNA obtained after complete remission. Proviral loads were confirmed by quantitative real time RT-PCR for all samples (Fig. 11). Both mutations, Q876* and Q414*, were somatic mutations found in TET2 of ATL cells and not detected in control samples (Fig.23 b and c). We then analyzed an additional 18 acute ATL patients by direct PCR, cloning and sequencing and found 6/18 (30%) with the mutation (Fig.24). Among missense ATL mutations only a mutation in position Q414R has previously been reported, although it was Q414L (COSM1618223). Of note, we found another unrelated
ATL patient with a Q876* mutation suggesting this may represent a frequently mutated region for ATL (Fig. 24).
Figure 23. TET2 mutations in ATL

a Schematic representation of TET2 protein and distribution of mutations found in ATL patients. (b and c) Somatic LOH Q414* and LOH Q876* were confirmed by direct sequencing and analyses of TA clones from high grade tumor and matched non-tumor of two ATL samples.
Figure 24. Independent patient cohort for TET2 sequencing

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

In this study we report a high frequency of TET2 missense mutations (8/31 (25%)) and LOH of TET2 (3/31 (10%)) in acute ATL patients. These data suggest that TET2 may be involved in HTLV-1 pathogenesis and warrant additional studies. Studies have shown that the TET2 mutation results in global low levels of 5hmC compared with normal controls, supporting a functional relevance of TET2 mutations in leukemogenesis. Activating mutations of IDH1/2 have been shown to be mutually exclusive with mutations of TET2 [163]. Although mutations of IDH1/IDH2 have the same final epigenetic effect as TET2 inactivation, mainly a global promoter hypermethylation, mutation in IDH1/2 was not observed in any ATL samples tested here.

Consistent with this notion, increased methylation of CDKN2A promoter has been associated with the progression of ATL disease. Wilms tumor (WT1) mutant AML patients have reduced 5hmC levels similar to the TET2/IDH1/IDH2 mutant in AML, suggesting that WT1 may also play an important role in control of the epigenome. WT1 and TET2 interact with one another. Although there are no reports regarding the genetic status of WT1 in ATL cells, we have previously shown PI3K-dependent cytoplasmic retention and inactivation of WT1 in HTLV-1 transformed cells. It will be interesting to evaluate the role of cytoplasmic WT1 in the regulation of TET2 functions in HTLV-1 transformed T cells. Although a number of genes have been shown to be hypo- or hypermethylated in ATL cells, a direct implication of these genes in cellular transformation and/or ATL pathogenesis is lacking. In this study, we also found LOH in 1/13 ATL patient for MLL3. ASCOM-MLL3 has a redundant but crucial role in transactivation of p53 and participates in DNA damage-induced expression of p53-targeted genes. Notably, p53 transcriptional functions are impaired in ATL patients in the absence of genetic mutations in p53
and the possibility that loss of MLL3 participates in this process for some ATL patients warrants future studies.
Chapter IV: NOTCH1 activation depletes the pool of side population cells in ATL.

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Abstract

**Background:** HTLV-I infection is associated with the development of adult T-cell leukemia (ATL), a malignancy characterized by a high rate of disease relapse and poor survival. Previous studies reported the existence of side population (SP) cells in HTLV-I Tax transgenic mouse models. These studies showed that these ATL-like derived SP cells have both self-renewal and leukemia renewal capacity and represent cancer stem cells (CSC)/leukemia-initiating cells (LIC). Since CSC/LIC are resistant to conventional therapies, a better characterization is needed.

**Methods:** We isolated, sorted and characterized SP cells from uncultured PBMCs from ATL patients and from ATL patient-derived cell lines. We then identified several specific signaling pathways activated or suppressed in these cells. Expression of viral gene HBZ and Tax transcriptional activity was also investigated. Using gamma-secretase inhibitor (GSI, Calbiochem) and stably transduced ATL cell lines expressing TET-inducible NOTCH 1 intracellular domain (NICD), we characterized the role of activated NOTCH 1 in the maintenance of the SP cells in ATL.

**Results:** Our studies confirm the existence of SP cells in ATL samples. These cells demonstrate lower activation of NOTCH1 and Tax, and reduced expression of STAT3, β-catenin/Wnt3 and viral HBZ. We further show that PI3K and the NOTCH1 signaling pathway have opposite functions, and constitutive activation of NOTCH1 signaling depletes the pool of SP cells in ATL-derived cell lines.

**Conclusions:** Our results suggest that in ATL, a balance between activation of the NOTCH1 and PI3K signaling pathway is the key in the control of SP cells maintenance and may offer therapeutic opportunities.
**Key words:** HTLV-I, ATL, side population (SP) cells, NOTCH, PI3K
Introduction

Limiting dilution transplantation demonstrated that only a small percentage of cells within a cell line population can give rise to tumors in vivo. These observations suggest that tumors and cell lines are composed of cells that are heterogeneous in terms of tumor-forming potential [164, 165]. Numerous studies demonstrate that side population (SP) cells identified by ABC pump-mediated exclusion of Hoechst can be referred to as Leukemia-initiating cells (LIC) or Cancer stem cells (CSC). These cells have the unique ability to regenerate full leukemia and self-renewal of the SP compartment in xenograft models [166]. SP analysis has also been used to identify CSC in a wide variety of human solid tumors, including breast, colon, ovarian and hepatic cancers [167-170]. These cells are relatively resistant to commonly used therapies. In addition, SP cells have also been reported in several hematologic malignancies, including but not limited to acute myeloid leukemia [171], chronic myeloid leukemia [172], and acute lymphoblastic leukemia (ALL) [173, 174].

Human T-cell Leukemia Virus type I (HTLV-I) infection is associated with an aggressive and fatal form of T-cell leukemia/lymphoma known as adult T-cell leukemia/lymphoma (ATL) [11, 175]. The mechanism by which HTLV-I engenders ATL is not fully elucidated, but numerous studies have demonstrated involvement of genetic and epigenetic events [5, 148, 176-178]. Overall, survival of ATL patients treated with various chemotherapy regimens is poor, with survival in several cohorts of patients presenting predominantly with acute leukemia or lymphoma ranging between 5.5 and 13 months [1]. Although most therapies initially result in a partial or complete remission, the vast majority of patients relapse and die, suggesting that current treatments do not completely eradicate ATL tumor cells. Consistent with these observations, published data suggest the existence of a slowly dividing cell subpopulation called
LIC, which is highly resistant to apoptosis following treatment with various chemotherapeutic regimens. Therefore, a major barrier impeding the cure of ATL patients may be the failure to effectively eliminate these LICs. In fact, studies demonstrated that combination therapy using arsenic trioxide and interferon-alpha (IFN) triggers proteasome-mediated Tax proteolysis and apoptosis and cures Tax-driven ATL in mice. This combination therapy of primary donor mice eliminated LIC engraftment and hampered ATL development in untreated secondary recipient mice [179]. Although this treatment showed promising results for long-term remission of ATL patients in the chronic phase of the disease, it did not benefit patients in the acute stage [180].

A study demonstrated that Hoechst-sorted SP cells correspond to CSC/LIC and investigated their role using a Tax-transgenic mouse model that causes T-cell lymphomas with characteristics similar to that of ATL [181]. The authors demonstrated that injection of non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice with as few as \( 10^2 \) CSC/LIC was sufficient to recapitulate the original lymphoma and reestablish CSC/LIC in recipient NOD/SCID mice, suggesting a role for CSC/LIC in this ATL malignancy. However, it is important to bear in mind the limitations of data derived from transgenic mouse models. Enforced overexpression of the Tax oncoprotein in mature T cells is not reflective of the interactions between a complete HTLV-I virus and targeted signaling pathways \textit{in vivo}. Tax expression is limited or absent in many ATL patients [182]. This approach also does not account for the role of the other viral accessory genes, such as p12, p30 and HBZ [183-185], in modulating viral leukemogenesis or playing a role in the CSC/LIC compartment.

In this study, we demonstrated the existence of SP cells in all ATL fresh samples and ATL patient-derived cell lines tested. We used FACS cell sorting to characterize signaling pathways modulated in SP cells and show that the activity of NOTCH1 and Tax, and the
expression of STAT3 and β-catenin/Wnt3, are predominantly decreased in ATL SP cells. Consistent with these results, ectopic expression of a constitutive active form of NICD significantly reduced the SP population while, on the other hand, inhibition of NOTCH1 signaling led to enrichment of the SP cells. These results suggest that targeted inhibition of NOTCH1 may reduce tumor burden but may not eliminate CSC/LIC. This is important because numerous studies suggest that leukemia relapse occurs because standard chemotherapy fails to eradicate CSC/LIC [186]. Therefore, elucidating the specific nature and properties of ATL CSC/LIC self-renewal and resistance to apoptosis represents an essential step towards curing ATL.
Materials and Methods

Cells and reagents

HTLV-I-transformed cell lines ED, MT1, ATL-T, and ATL-25 were cultured in RPMI-1640 with 10% fetal bovine serum, L-glutamine, 100U/ml penicillin and streptomycin and maintained in 5% CO₂ at 37°C. Vybrant® DyeCycle™ (DCV) was obtained from Invitrogen. Verapamil hydrochloride was purchased from Sigma-Aldrich. ED or MT1 cells were treated with either 10 μM LY294002 (Sigma-Aldrich, St Louis, MO) for 3 days or 1μM gamma-secretase inhibitor (GSI, Calbiochem) for 5 days as indicated in the figure legends.

Patient samples

ATL cryopreserved samples were obtained after informed consent and institutional IRB approval as described in the previous study [4].

Side Population (SP) Analyses

For DCV staining, cells were pelleted and resuspended in pre-warmed DMEM with 10% FBS and 10mM HEPES at a concentration of 1X10⁶ cells/ml. Before incubation with DCV, cells were pre-incubated for 30 minutes in 200 μM Verapamil at 37°C. DCV was added at a final staining concentration of 10μM. The cells were stained for 60 min at 37°C while gently vortexing every 15 min. Then the cells were washed 2 times with pre-warmed PBS and resuspended in pre-warmed DMEM with 10% FBS and 10mM HEPES at a concentration of 1X10⁶ cells/ml. After 1 hour, the cells were either analyzed on a BD™ LSR II cytometer or flow sorted on a BD FACS™.

RNA extraction and real-time quantitative RT-PCR

Acute ATL samples were previously published [4]. Total mRNA was isolated from HTLV-I cell lines and cells using TRIzol Reagent (Ambion) according to manufacturer's
instructions. After DNase I treatment, the RNA was reverse transcribed and the cDNA was used for real-time PCR. Real-time PCR was performed with the following sets of primers:

\[ \text{ABCG2F: } \text{CCTGAGATCCTGAGCCTTTGG} - 3', \text{ABCG2R: AGGTCATTGGAAGCTGTCGC; Hes1F: CTGTGGGAAAGAAAGTTTGGG; Hes1R: GACCAAGGAGAGGAGTGCAC; HBZF: CGGCCTAGGGCTGTTTTC; HBZR: CGCGGGTTCCCTTTCTAAGGA; GAPDHF: GAAGGTGAAGGTCGGAGTC; GAPDHR: GAAGATGGTGATGGGATTTTC} \]

The relative mRNA levels in each sample were normalized with GAPDH and were calculated using the 2−ΔCt method.

**Production and transduction of recombinant lentivirus**

Lentivirus vector SMPU-18x21-EGFP was kindly provided by Dr. C. Z. Giam [187]. The VSV-G pseudo-typed pSIH-H1-GFP and SMPU-18x21-EGFP viruses were produced and concentrated as previously reported [32]. ATL-25 and MT1 cells were infected in the presence of polybrene and spinoculated at 1200 relative centrifugal force (rcf) at room temperature for 1 hour. The cells were cultured for 2 days, followed by the SP analyses.

**Western blot**

MT1 cells were treated with 1μM GSI for 5 days. Whole cell extracts were prepared with radioimmunoprecipitation assay (RIPA) buffer (50 mm Tris-Cl, pH 7.5, 150 mm NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS) containing Complete Protease Inhibitor cocktail (Roche Diagnostics). Anti-NOTCH1 (#2421; Cell Signaling) and anti-actin (C-11; Santa Cruz Biotechnology) were used.
Results

Characterization of SP cells in ATL fresh samples and patient-derived ATL cell lines.

Numerous studies have shown that side population (SP) cells are enriched for cancer stem cells (CSC)/leukemia-initiating cells (LIC), which have both self-renewal and tumor-regenerating potential [166]. The SP phenotype is based on the ability of these cells to proficiently efflux fluorescent dyes such as Hoechst 33342 or DCV through the multidrug ABC transporter, such as ABCG2. This property allows the characterization and isolation of SP cells using fluorescence-activated cell sorting (FACS). To identify and characterize SP cells in ATL, we investigated the SP cells by efflux of DCV dye in several ATL-derived cell lines (ED, ATL-T, MT-1, MT-2 and C91PL) as well as freshly isolated uncultured PBMCs from acute ATL patients. Our results demonstrate the presence of a small percentage of SP cells, from 3% to 5.6%, in all ATL lines and in freshly isolated uncultured ATL primary samples (Fig.1A).

Verapamil, an irreversible inhibitor of ABCG2, confirmed loss of SP cells and was used for gating of the cell population in further experiments. We cell-sorted SP cells (SP+) and non-SP cells (SP-) cells and extracted RNA and genomic DNA (Fig. 1B-E), and ABCG2 expression was compared between SP- and SP+ cells in ED. In keeping with earlier reports, there was almost two times the ABCG2 expression in SP+ cells compared with SP- cells (Fig1C). Notably, PCR-based analyses of T-cell receptor (TCR) gamma gene rearrangement in DNA extracted from SP+ and SP- cell populations indicated that these two populations have the same clonal origin (Fig.1E).
Figure 25: The presence and expression profile of side population in ATL cell lines and primary ATL samples.

(A) The representative SP analysis of ATL cell lines and primary ATL samples. (B and C) The gates used to sort both SP+ and SP- cells were plotted as rectangles. The expression of ABCG2 in SP- and SP+ cells were analyzed by RT-PCR. The results were from two independent experiments and normalized to GAPDH expression. Data are mean ± SD. *P<0.05, two-tailed Student’s t-test. (C and D) SP+ and SP- cells were sorted from patient samples. The gates used to sort both SP+ and SP- cells were plotted as rectangles. DNA were extracted and analyzed by PCR. (E) Relative expressions of viral HBZ gene, cellular ABCG2, HOXB3, β-catenin, Hes1, BMI1, OCT-4, Nanog, Wnt3, Wnt5a, STAT3 and c-Myc were tested using RT-PCR. The results were from two independent experiments and normalized to GAPDH expression. Data are mean ± SD. *P<0.05, two-tailed Student’s t-test.
Signaling pathways involved in the development and maintenance of the malignant ATL cells.

Several signaling pathways such as Bmi-1, Notch, Wnt/β-catenin, Sonic hedgehog and NF-kB have been implicated in CSC/LIC self-renewal and survival in leukemia and other solid cancers [188-193]. Previous studies have reported an increased expression of c-Kit and decreased expression of Tax, NOTCH1, and Bmi1 in CSC/LIC isolated from a Tax transgenic mouse model [181]. FACS-sorted SP+ and SP- cells were used to extract RNA for RT-PCR analyses of selected genes previously shown to play a role in the development and maintenance of the malignant ATL cells. Among the targets tested, HOXB3, Hes1, a downstream target of Notch 1, and STAT3 were downregulated in SP+ cells (Fig.1F). Since we have reported somatic mutations of the NOTCH1 and STAT3 signaling pathway in ATL patients [4, 191], the effect of these signaling pathways in SP cells should be considered when applying targeted therapy.

Consistent with previous studies, we found no significant change in the expression of FLT3, N-cadherin, Oct-4, and Nanog (Fig.1F and data not shown). In our experiments, however, c-Kit (CD117) expression was not elevated in SP cells, suggesting differences between Tax-derived ATL-like transgenic models and patient-derived ATL cells, highlighting the need for further investigation.

In HTLV-I-transformed ATL cells, the most frequently expressed viral genes are HBZ and Tax. Interestingly, the HBZ mRNA was significantly downregulated in SP+ cells (Figure.1F). These data suggest that loss of HBZ may play a role in the maintenance of SP cells in ATL.

Next, we sought to analyze viral Tax activity in SP+ and SP- cells. Unlike HBZ mRNA generally expressed in most ATL cells, only about one-fourth of ATL samples have detectable
expression of tax viral mRNA [1]. Tax is a potent transcriptional trans-activator for a 21bp repeat motif found in the viral HTLV-I LTR promoter. To detect Tax activity in ATL cells, we used a previously characterized lentiviral vector known as SMPU-18x21-EGFP reporter construct [32, 187]. We first demonstrated that SP+ and SP- cells are equally susceptible to lentivirus infection using concentrated virus particles generated with pSI-H1-GFP. As demonstrated in Figure 2A, approximately 50% of cells in each population were transduced with pSI-H1-GFP. To validate our approach we used Tax- cells (MT1) and Tax+ cells (ATL-25), and measured GFP activity by FACS. Our results suggested that the GFP signal was comparable for both cell lines when using the pSI-H1-GFP particles, which indicated equal transduction efficiency (Fig.2B). However, SMPU-18x21-EGFP signaling was only detected in Tax+ ATL-25 cells, but not Tax- MT1 cells, which demonstrates the specificity of the reporter (Fig.2B). In order to demonstrate the correlation between Tax-transcription activity and SP, we gated the ATL cells according to Tax-transcription activity (SMPU-18x21-EGFP signaling) and then performed SP assay. A lower percentage of SP cells was found in the high Tax-transcription activity population compared with the low Tax-transcription activity population (3.2% vs. 3.9%) (Fig.2C).
Figure 26: Tax-transcription activity and SP cells.

(A) MT1 was first gated for SP+ and SP-, and then the SP+ and SP- cells were gated based on GFP expression level. (B) Both MT1 and ATL-25 were infected with SMPU-18x21-EGFP virus; SMPU-18x21-EGFP can only be detected in Tax+ ATL-25 cells (lower panel), but not Tax- MT1 cells (upper panel). (C) ATL-25 cells are first gated according to Tax-transcription activity and then SP assays were performed to analyze SP cells in each subgroup and total cells.
Activated Notch signaling depletes SP cells

Previous studies have shown that NOTCH1 signaling plays an important role in CSC/LIC homeostasis [192]. Since our studies revealed Hes-1 as one of most deregulated genes in ATL SP cells and NOTCH1 signaling has been implicated in ATL tumor growth \textit{in vitro} and \textit{in vivo} [4], we next investigated the role of NOTCH1 signaling in SP cells maintenance. Notch signaling, initiated by receptor-ligand interactions, requires subsequent proteolytic cleavage of the receptor, resulting in the intracellular cleaved form of NOTCH1 (hereafter referred to as NICD) which translocates to the nucleus and up-regulates the transcription of Notch-regulated genes (3-5). Treatment with gamma secretase inhibitor (GSI, Calbiochem) prevents cleavage of the receptor and interrupts NOTCH1 signaling. Effectiveness of the treatment was confirmed by decreased NICD expression in western blotting (Val1744 Ab, Fig.3B) following incubation with 1 μM GSI. Treatment of ATL cells with GSI resulted in a significant increase in SP cells from 5.4% to 16.3% (Fig.3A). This increase was not observed after a short incubation of 3 hours with GSI, suggesting that an increase in SP cells is specific to GSI-mediated loss of NICD rather than an effect on ABCG2 pump activity (Fig.3A). In contrast, treatment of cells with LY294002, a PI3K inhibitor, resulted in a drastic loss of SP cells (Figure.3C). The treatment was effective in ATL cells as demonstrated by reduction of pAKT by western blot (Fig.3D). Together these data suggest that the NOTCH1 and PI3K signaling pathways have antagonizing effects on SP cells maintenance.
Figure 27: The treatment of LY294002 decreases percentage of SP cells, while the treatment of GSI increases the SP percentage.

(A and B) ED cells were treated with 1μM GSI for 5 days or 3 hours, followed by SP analysis (A) and Western blot for cleaved Notch1 (B). Actin served as a loading control. (C and D) ED cells were treated with 10 μM LY294002 for 3 days, followed by SP analysis (C) and Western blot for phospho-AKT (D). Actin served as a loading control.
To eliminate the possibility of GSI off-target effects and further demonstrate the role of NOTCH1 activation in SP cells, we generated Tet-inducible ATL lines carrying a non-degradable constitutive active form of NICD (*2403 and *2466) [4]. Induction of NICD mutant expression (Fig.4A) with Doxycycline was, as expected, associated with increased Hes1 gene expression (Fig.4A). Consistent with results presented in Fig. 3, expression of constitutive NOTCH1 in MT-1 (MT-1 *2403 and MT-1 *2466) was associated with a significant loss of the SP cells from 11.6% to 1.6% and from 14.4% to 6.5% (Fig.4B). However, the SP cells was not affected in MT1 control cells (MT-1 pTripZ) (Fig.4B). These results were further confirmed in a different ATL patient-derived cell line (ED) using the *2466 NICD mutant (ED *2466) (Fig.4B). Finally, if the GSI effect described above occurs through NOTCH1 signaling, one would expect that expression of NICD mutants *2403 and *2466 would prevent an increase of the SP cells following GSI treatment. Western blot analyses confirmed that *2403 and *2466 expression is not affected by GSI, while endogenous levels of NICD are reduced after treatment (Fig.4C). FACS analyses further demonstrate that the SP cells significantly increases only in MT1 control ATL cells (2% to 11.8%) (Fig.4D) but not in MT1 cells carrying constitutive active NICD *2466 and *2403 mutants (3.7% to 4.8% and 6.5% to 6.4%, respectively) (Fig.4D). Altogether our studies suggest that activation of NOTCH1 is critical for SP cells maintenance.
Figure 28: The regulation of SP cells by Notch signaling.

(A) MT1 *2403 and *2466 stable cell lines were induced with doxycycline for 48 hours and western blot showed the expression of NICD. Actin acted as a loading control. The expression of Hes1 was analyzed by RT-PCR. The results were from two independent experiments and normalized to GAPDH expression. Data are mean ± SD. *P<0.05, two-tailed Student’s t-test. (B) SP assay was performed after MT1 and ED stable cell lines were induced with doxycycline for 48 hours. (C) Ectopic and endogenous expression of NICD were analyzed by Western blot after indicated treatment. (D) SP assay was performed after MT1 stable cell lines were treated with Dox alone or Dox + GSI.
Discussion

HTLV-I-associated ATL has limited therapeutic options, a very poor prognosis and a dismal survival rate. The 4-year survival rate for acute-, lymphoma-, chronic- and smoldering-type ATL is 5.0, 5.7, 26.9 and 62.8%, respectively [148]. The poor prognosis of ATL patients is associated with the resistance of tumor cells to the conventional combination of high-dose chemotherapy and radiotherapy, in addition to ATL being associated with a high rate of disease relapse. The failure of first line therapies to completely eliminate cancer cells likely contributes to acquisition of chemoresistance [194]. Hence, a better understanding of cell population and the genetic and epigenetic events providing chemoresistance is critical for the design of novel therapeutic strategies to successfully treat cancer. In recent years, the role of CSC/LIC in cancer resurgence and resistance to treatment has been the focus of many investigations. High levels of ABC-transporter-mediated efflux, such as ACBG2, facilitate but do not solely explain the acquisition of mechanisms of drug resistance. Defects in DNA repair pathways, control of apoptosis cell death and genetic mutations observed in ATL cells [151] may be contributing factors leading to CSC/LIC escape from chemotherapy. CSC/LIC has been poorly characterized in HTLV-I-associated ATL.

In this study, we demonstrate that HTLV-I-transformed ATL cells freshly isolated or cell lines derived from patient samples contain a small variable population of SP cells. As mentioned above, SP analysis has been used to identify CSC/LIC in a wide variety of leukemia and solid tumors. Our studies demonstrate that ATL SP cells display a lower expression of Hes1, STAT3, \(\beta\)-catenin and Wnt3. This is of interest because we have previously identified a high rate of somatic mutations in NOTCH1 and STAT3 in acute ATL patients [4, 191]. These mutations could trigger rapid proliferation and expansion of leukemia cells. Whether mutations occur in the
CSC/LIC or leukemic cellular compartment remains to be determined. In contrast, we have
previously shown that ATL patient-derived leukemia cells activate the non-canonical Wnt
pathway and overexpress Wnt5 but do not present activation of β-catenin or Wnt3 [188]. In this
study, we also investigated the functional expression of the two viral genes most frequently
expressed in ATL cells isolated from patients, Tax and HBZ. Our investigations revealed a
reduced expression of viral HBZ and lower Tax activity in SP+ cells. These data are consistent
with the fact that CSC/LIC are slowly dividing cells and viral proteins have an opposite effect.
Tax is a potent transcriptional activator of cellular genes involved in cell proliferation and it
favors genome instability by targeting DNA repair pathways [17, 36, 151]. Similar to Tax, HBZ
has been shown to stimulate the growth of ATL cells and activate the non-canonical NF-κB
pathway[21]. Our investigations suggest that the PI3K and NOTCH1 signaling pathways have
opposite functions in SP cells homeostasis. While constitutive activation of NOTCH1 signaling
depletes the pool of SP cells in ATL-derived cell lines, PI3K signaling seems to increase the pool
of SP cells. Additional experiments will be needed to further characterize the role of these
signaling pathways in CSC/LIC and identify effective therapeutic targets. While inhibition of the
NOTCH1 signaling pathway may be effective in eliminating ATL leukemia cells, this strategy
may increase the SP cells and lead to disease relapse, suggesting that combination therapies
targeting both cellular compartments may be more effective in curing ATL.
Chapter V: Clinical significance of microRNAs in chronic and acute human leukemia.

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Abstract

Small non-coding microRNAs (miRNAs) are epigenetic regulators that target specific cellular mRNA to modulate gene expression patterns and cellular signaling pathways. miRNAs are involved in a wide range of biological processes and are frequently deregulated in human cancers. Numerous miRNAs promote tumorigenesis and cancer progression by enhancing tumor growth, angiogenesis, invasion and immune evasion, while others have tumor suppressive effects [195, 196]. The expression profile of cancer miRNAs can be used to predict patient prognosis and clinical response to treatment [197]. The majority of miRNAs are intracellular localized, however circulating miRNAs have been detected in various body fluids and represent new biomarkers of solid and hematologic cancers [198, 199]. This review describes the clinical relevance of miRNAs, IncRNAs and snoRNAs in the diagnosis, prognosis and treatment response in patients with chronic lymphocytic leukemia (CLL), chronic myeloid leukemia (CML), acute lymphocytic leukemia (ALL), acute myeloid leukemia (AML) and acute adult T-cell leukemia (ATL).
Chronic lymphocytic leukemia (CLL)

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

miRNA signature in CLL

The miR-15/16 cluster, miR-34b/c, miR-29, miR-181b, miR-17/92, miR-150, and miR-155 represent the most frequently deregulated miRNAs reported in CLL and these microRNAs have been associated with disease progression, prognosis, and drug resistance [200]. Nearly two-thirds of CLL cases presented a down-regulation of miR-15a/16-1 expression. In fact, miR-15a and miR-16-1 are located in the locus 13q14.3, a genomic region frequently deleted in CLL patient samples [201]. However, additional mechanisms, such as overexpression of histone deacetylases (HDACs), also down-regulate expression of miR-15 and miR-16 [202]. Another point to highlight is that there is an inverse correlation between miR-15a/16-1 and BCL2 expression reported in CLL, as inhibition of this microRNA expression in leukemic cell lines leads to increased BCL2 expression and resistance to apoptotic signals. In addition, comparative microarray analysis in CLL patients with high or low levels of miR-15a/16-1 identified a gene signature that contains the anti-apoptotic BCL2 family member MCL-1, which is associated with B-CLL cell survival and chemotherapy resistance [203-205]. Down-regulated miR-15a and miR-16-1 in CLL patients is associated with a good prognosis, consistent with previous reports that
correlate 13q14.3 deletions with a favorable course of CLL [206]. On the other hand, the miR-29 family, which includes miR-29a, miR-29b and miR-29c, is significantly down-regulated in a subset of CLL patients and is associated with an unfavorable prognosis. miR-29b targets DNA methyltransferase (DNMT) isoforms and inhibition of miR-29b expression may lead to hypermethylation and epigenetic silencing of several tumor suppressors [206]. In addition, evidence shows that miR-29 targets the oncogene T-cell leukemia 1 gene, TCL1A. This TCL1 oncogene is involved in translocations and inversions characteristic of mature T-cell prolymphocytic leukemia (PLL) and is overexpressed in patients with unmutated immunoglobulin heavy chain variable regions (IgVH). Transgenic mice that overexpressed TCL1 in B cells display a similar phenotype to aggressive forms of human CLL [206]. Another genomic region frequently deleted in CLL patients is found in the 11q region where a miR-34 cluster is located. In fact, down-regulation of miR-34a in CLL is associated with p53 inactivation, impaired DNA damage response, and apoptosis resistance [207-209]. Since miR-34a also inhibits E2F1 and B-Myb [210], loss of miR-34a expression may increase tumor cell proliferation. Alternatively, the miR-17/92 polycistronic microRNA cluster is overexpressed in several lymphoid malignancies and studies have shown that miR-17/92 inhibited the expression of the pro-apoptotic factor Bim and the tumor suppressor PTEN [211]. In addition, STAT3-induced IL-6 stimulates the expression of miR-17 and miR-19a, resulting in lower expression of TLR7 and TNFα. Also of interest, CLL patient cells expressing zeta-chain-associated protein 70 kDa (ZAP-70) demonstrate significantly lower levels of miR-150 when compared with ZAP-70-negative CLL cells. In CLL cells miR-150 targets forkhead box P1 (FOXP1) and GRB2-associated binding protein 1 (GAB1), thereby reducing B-cell receptor signaling [212].
Another STAT3-activated microRNA, miR-155[213], is overexpressed in cells and in circulating microvesicles in CLL samples [213]. Induction of the onco-miR-155 in the plasma of CLL patients correlates with poor response to treatment and disease progression. Consistently, patients who achieved complete remission presented low levels of miR-155 in the plasma [200]. In addition, the expression of miR-155 is increased with disease progression from monoclonal B-cell lymphocytosis (MBL) to CLL and is higher in MBL and CLL than normal controls [214]. High expression of miR-155 is associated with a more aggressive disease and a poor clinical prognosis in CLL [215, 216]. Another miRNA, miR-181b, is frequently down-regulated in CLL patients with disease progression [217, 218]. Important targets of miR-181b include MCL-1 and BCL2 [217], which, as stated before, are important for cancer cell survival, and low expression of miR-181b is associated with poor prognosis as indicated by treatment-free survival [217, 218].

miRNA expression and drug response in CLL patients

Higher expression of miR-650 and miR-708 is associated with a favorable CLL prognosis [219] and affects B-cell proliferation [220]. On the other hand, overexpression of miR-21, miR-148a, miR-155 and miR-222 in CLL patients is associated with poor therapeutic response and prognosis [214-216, 221, 222]. For example, the expression of miR-155 is higher in CLL patients that failed to achieve a complete response to a chemo-immunotherapy combination of fludarabine [222, 223], cyclophosphamide, and rituximab (FCR) [214] and, as stated in the previous section, it is associated with poor clinical prognosis in CLL [215, 216]. The relapsed patients have higher miR-155 expression compared to baseline despite reduced expression at the beginning with response. Importantly, when the gene expression profile is analyzed, p53 downstream genes are only detected in fludarabine-responsive patients, but not resistant patients [224].
The mutation of TP53 in CLL is associated with unfavorable treatment response and clinical outcome [225], and in some CLL patients inactivation of TP53 correlates with reduced miR-34 expression, which is associated with an unfavorable prognosis [226]. In addition, miR-132 and miR-212 expression is lower in progressive CLL patients compared with stable CLL patients [227]. Gene expression profiling showed that the miRNAs miR-132 and miR-212 affect the Rb or TP53 signaling pathway, which may explain the clinical observation [227].

The expression of miRNAs can also be used as a biomarker to monitor CLL progression. For example, there is reduced expression of miR-181b, miR-29c and miR-223 with disease progression in CLL patients and this correlates with unfavorable prognosis, such as shorter progression-free survival and overall survival [221, 228-230]. miR-150 is highly expressed in both cellular and serum samples of CLL patients [231]. It is interesting to note, though, that low cellular but high serum expression of miR-150 is associated with poor prognosis as indicated by tumor burden, treatment-free survival (TFS) and overall survival (OS) [231]. This could be because lower expression of cellular miR-150 may enhance cell survival and proliferation in response to BCR signaling stimulation, which worsens the patient prognosis [231]. Furthermore, high serum miR-150 is correlated with high lymphocytosis, which contribute to high tumor burden and poor clinical outcome [231].
Chronic myeloid leukemia (CML)

Chronic myeloid leukemia (CML) is a hematopoietic malignancy characterized by abnormal expansion of immature hematopoietic progenitor cells in the bone marrow and increased levels of myeloid cells in the peripheral blood. The genetic hallmark of CML is a $t(9;22)(q34;q11)$ reciprocal translocation, also called 'Philadelphia chromosome'. This translocation results in a BCR-ABL fusion gene that leads to constitutive tyrosine kinase activation [232].

miRNA signature in CML

The most frequently down-regulated miRNAs in chronic myeloid leukemia include miR-10a, miR17–92, miR-150, miR-203, and miR-318 [233]. Several oncogenic miRNAs such as the miR17–92 cluster have been found to facilitate cellular transformation and to be up-regulated by the BCR-ABL fusion protein in CML [234]. Induction of this miRNA cluster is usually observed in the chronic phase of CML, but not in the blast crisis phase [235]. Similarly, loss of miR-328 is observed in blast crisis CML in a BCR/ABL dose- and kinase-dependent manner. Ectopic expression of miR-328 in cell lines restores differentiation of leukemic blasts by induction of the survival factor PIM1 and inhibition of the hnRNP E2 interaction with the hematopoietic transcription factor CEBPA. Down-regulation of miR-328 is essential in CML blast crisis [233]. The differentiation arrest observed during the blastic phase of CML requires the activity of hnRNP E2, a poly(rC)-binding protein, which behaves as a translational regulator. Finally, expression of miR-130a and miR-130b controlled by BCR-ABL down-regulates the expression of CCN3, a growth inhibitory protein [236], whereas some miRNAs inhibit BCR-ABL
expression. For instance, miR-203 and miR-451 act as tumor suppressors in CML and suppress ABL and BCR-ABL protein expression [237]. Consistent with this notion, miR-203 is frequently silenced by monoallelic loss and hypermethylation of the remaining allele [238]. Another consistency found in CML patients is the reduction of miR-150 and miR-10a expression [239, 240]. CML patients display inverse expression levels of miR-150 and the transcriptional activator MYB, which correlates with BCR-ABL (fusion gene) transcript levels [241], while miR-10a is associated with increased proliferation by targeting the upstream stimulatory factor 2 (USF2) [239]. Finally, recently miR-362-5p was found to act as an oncomiR by down-regulating GADD45α, which in turn activated the JNK1/2 and P38 signaling in CML patient samples [242].

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

As discussed with CLL, the expression of miRNAs can be used as a biomarker to monitor CML progression as well. For example, the expression of miR-15a and miR-145 is higher in CML patients at chronic phase than in normal controls, but lower at acute phase than chronic phase [243]. The expression profile of some miRNAs can predict the Imatinib therapy response in CML patients[244]. For example, expression of miR-26a, miR-29c, miR-130b and miR-146a is lower in patients with an Imatinib response than in patients with Imatinib-resistant treatment [243]. A loss of or reduced expression of miR-23a, miR-30a, miR-30e, miR-203, miR-320 and miR424 have been associated with resistance to Imatinib in CML, and re-expression of these miRNAs targets BCR-ABL and restores sensitivity to Imatinib treatment [245-249]. In contrast, loss of miR-217 and miR-199b correlates with resistance to ABL tyrosine kinase inhibitors [250, 251]. It should be noted that the tyrosine kinase inhibitor (TKI) Dasatinib affected miR-let-7d, miR-let-7e, miR-15a, miR-16, miR-21, miR-130a and miR-142-3p expressions while Imitanib
affected miR-15a and miR-130a levels [243]. Consistent with the notion that miR-130a can act as a tumor suppressor by targeting BCL2 and MCL-1 expression, lower expression of miR-130a is associated with poor prognosis as indicated by shorter overall survival and treatment-free survival in CML patients [252]. Importantly, a low expression of miR-148b is found in a subset of CML patients with stable complete molecular responses after stopping Imatinib treatment[253]. These studies suggest that expression or lack thereof of some miRNA may predict the ability of some CML patients to be safely removed from TKI treatments.
**Acute lymphoblastic leukemia (ALL)**

ALL is a lymphoid malignancy affecting the B or T lineages. Distinct microRNA signatures are reported in different ALL subtypes and can be used for the diagnosis and classification of ALL. ALL can be divided into T-cell, MLL-rearranged, TEL-AML1-positive, E2A-PBX1-positive, hyperdiploid ALL, BCR-ABL-positive, and “B-other” ALLs. Studies of the distinct microRNA signatures of ALL subtypes can be used for the diagnosis and classification of the disease [254].

**miRNA signature in ALL**

The B and T lineages of ALL can be distinguished by miR-148, miR-151, miR-424, miRNA-425-5p, miRNA-191, miRNA-146b, miRNA-128, miRNA-629, and miRNA-126. In addition, miRNA-708 was found highly expressed in TEL-AML1, BCR-ABL, E2A-PBX1, hyperdiploid, and B-other cases [254, 255]. The miRNA signature in hyperdiploid and TEL-AML1-positive patients partly overlap, suggesting a common underlying biology. Mavrakis et al. identified five miRNAs – miRNA-19b, miRNA-20a, miRNA-26a, miRNA-92, and miRNA-223 involved in T-ALL development in a mouse model. These five miRNAs have been shown to target T-ALL tumor suppressors such as IKAROS, PTEN, BIM, PHF6, NF1 and FBXW7 [256]. The expression pattern of these miRNAs can be used as a biomarker to distinguish the B and T lineages of ALL. Higher expression of miR-128b and lower expression of miR-223 has independently been reported for human ALL cell lines and ALL cells isolated from pediatric patients [257]. In a different study, single nucleotide polymorphisms (SNPs) analyses of precursor miRNAs (pre-miRNA) and miRNA-processing genes revealed eleven SNPs associated
with ALL susceptibility [258]. Among them, eight are located at miRNA biogenesis pathway genes (**TNRC6B, DROSHA, DGCR8, EIF2C1, CNOT1**, and **CNOT6**) and three at miRNA genes (**miR-612, miR-499**, and **miR-449b**). Interestingly, miRNA-612 and miRNA-499 have significant correlations with ALL susceptibility [254]. In addition, miRNA profiles can be useful to distinguish myeloid or lymphoid lineages of leukemia. De Leeuw et al. identified miRNA-23a, miRNA-27a, miRNA-199b, miRNA-221, and miRNA-223 as the most lineage-discriminative miRNAs between AML and ALL [259]. AML patients present down-regulation of let-7b and miRNA-223 and overexpression of miRNA-128a and -128b compared to ALL. Consistently, Wang et al. [260] identified miR-23a, miR-27a, miR-27b, miR-150, miR-199a, miR-199b, miR-221 and miR-340 as miRNAs differentially expressed in patients with ALL compared to AML.

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

Epigenetic deregulation is one of the mechanisms that accelerates ALL progression. miR-124a is methylated in 59% of ALL patients and down-regulation of miR-124a increased the expression of its target CDK6 resulting in phosphorylation of retinoblastoma (Rb), which is involved in cell proliferation. As a result, hypermethylation of miR-124a in ALL patients is correlated with a higher relapse and mortality rate and can be used as an independent prognostic factor for disease-free survival (DFS) and overall survival (OS) in the multivariate analysis [261]. microRNA analysis shows that expression of miR-10a, miR-134, miR-214, miR-221, miR-128b, miR-484, miR-572, miR-580, miR-624 and miR-627 are significantly correlated with a favorable clinical outcome [257, 260, 262]. In contrast, deregulation of the expression of miR-9, miR-33, miR-92a, miR-142-3p, miR-146a, miR-181a/c, miR-210, miR-215, miR-369-5p, miR-335, miR-454, miR-496, miR-518d, and miR-599 are associated with an unfavorable long-
term clinical outcome in ALL patients [260, 262-268]. Low expression of miR-151-5p and miR-451, and high expression of miR-1290 or a combination of all three, predicted inferior relapse-free survival (RFS) in pediatric B-ALL [269]. Importantly, activation of NOTCH intracellular domain (NCID) signaling leads to transcriptional repression of miR-451 and miR-709, two tumor suppressor microRNAs in T-ALL [270]. Furthermore, different independent analyses identified relapse-related miRNAs. When globally analyzed the relapse-related miRNAs – miR-7, miR-100, miR-216 and let-7i – were up-regulated, and miR-486, miR-191, miR-150, miR-487 and miR-342 were down-regulated in early relapse ALL patients[271]. In addition, overexpression of miR-708, miR-223 and miR-27a is associated with lower relapse-free survival in patients [272], possibly through regulation of FOXO3, BMI1 and E2F1. Expression of miR-126, miR-345, miR-222, and miR-551a are reduced in ALL patients with central nervous system (CNS) relapse compared to non-CNS-relapsed ALL patients [271]. Furthermore, higher expression of miR-7, miR-198 and miR-633 was found in patients with CNS relapse compared with non-CNS-relapsed ALL [271].

Glucocorticoids can be used to treat ALL because they induce apoptosis in lymphoid lineage cells [273]. In ALL patients, sensitivity to prednisone treatment is an important indicator for treatment outcome [271]. While miR-16 is lower in ALL patients with low leukocytes and good cytogenetic characteristics [274], higher expression of miR-16 is found in patients with corticosteroid resistance [274] and is correlated with shorter disease-free survival and overall survival. The expression of miR-223 and the miR-15/16 family is increased in ALL patients treated with systemic glucocorticoid monotherapy [257, 273]. In contrast, the expression of miR-548d-1 and miR-106b~93 is reduced after ALL patients are treated with glucocorticoids [273]. Differential expression of miR-18a, miR-532, miR-218, miR-625, miR-193a, miR-638, miR-550
and miR-633 can be used as a marker to predict prednisone response in pediatric ALL patients[271]. For example, high miR-18a but low miR-193a expression is associated with good prednisone response. Although up-regulation of miR-128a [275, 276] and miR-128b [257] is frequently found in childhood ALL patients, patients with poor prednisolone response are often associated with lower miR-128b expression, with higher expression of miR-128b correlated with a longer disease-free period [257]. miR-128b expression is higher in the bone marrow of relapsed ALL patients compared with the values detected at diagnosis [257]. Increased miR-708 expression is detected in childhood ALL with a good response to prednisone and with better remission status after 15-day and 33-day chemotherapy protocol [272]. The expression of let-7e is generally reduced in pediatric ALL patients [275, 276], but higher expression of let-7e is associated with positive minimal residual disease (MRD) at day 14 after treatment [276].
Acute myeloid leukemia

Adult acute myeloid leukemia (AML) presents abnormal miRNA expression diversely expressed in the different subtypes. Both the t(8;21) and inv(16) chromosomal aberration is associated with the formation of novel chimeric fusion genes that involve the core-binding factor (CBF) complex, an important regulator of hematopoiesis, providing the designation CBF-AML[277].

miRNA signature in AML

A distinct miRNA signature characterized by an alteration of miR-29, miR-125, miR-142, miR-146 and miR-155 expression has been reported to play a role in AML progression and pathogenesis [278]. miR-29 family members miR-29a, miR-29b, and miR-29c act as oncogenes and tumor suppressors in myeloid malignancies [279]. miR-29b targets DNA methyltransferase DNMT3A, DNMT3B, and Sp1 (a transcriptional regulator of DNMT1) [280]. Inhibition of miR-29b promotes DNA hyper-methylation in AML and contributes to methylation status in AML cells, suggesting its potential role as a therapeutic target in AML. In addition, miR-29a and miR-29b affect the expression of genes involved in apoptosis, cell cycle progression, and cellular proliferation. Consistently, altered expression of MCL-1 and CDK6 was reported in primary AML blasts following ectopic expression of miR-29b [279]. Interestingly, injection of precursor miR-29b oligonucleotides in mice engrafted with K562 cells reduce their tumor sizes [278]. The miR-125 family includes miR-125a/miR-99b/let-7e, miR-125b-2/miR-99a/let-7c-1, and miR-125b-1/miR-100/let-7a-2 located on human chromosomes 19, 21, and 11, respectively. The mir-125 family is involved in self-renewal, both in hematopoietic stem cells (HSC) and
Megakaryocyte-Erythroid Progenitor Cells (MEC) [281]. Overexpression of miR-125 enhances the development of an MPN-like phenotype, which progresses to AML. Based on the cellular context, miR-125b can act as a tumor suppressor or an oncogene[282]. In acute myeloid leukemia, miR-125b is significantly overexpressed in patient blasts and can promote the transformation of normal hematopoietic cells into malignant cells in an *in vitro* and *in vivo* model. miR-125b is located on chromosome 21 and involved in the development of a rare subtype of AML, acute megakaryocytic leukemia (AMKL), especially in patients with Down’s syndrome (DS). The trisomy chromosome 21, typical of DS, is associated with overexpression of miR-125b in both DS- and non-DS-related AMKL patients [283]. Down-regulation of miR-146a promotes AML disease progression by TRAF6-mediated induction of NF-kB [284]. miR-142 promotes the development of lymphoid and myeloid leukemia and is found recurrently mutated in AML [285]. miR-155 is located on human chromosome 21 in the B-cell integration cluster (BIC) gene[286]. BIC cooperates with c-Myc to induce lymphomas[286]. In addition, miR-155 inhibits the cell-cycle regulators WEE1 and the mismatch repair genes hMLH1, hMSH2, and hMSH6, resulting in an increase in spontaneous mutation rates in hematopoietic stem and progenitor cells (HSPC) and solid tumor cell lines [278, 287, 288]. In contrast, other studies suggest that in FLT3-wildtype AML cells, miR-155 induces myelomonocytic differentiation and apoptosis[289].

**MicroRNAs in the diagnosis of AML**

Up-regulated let-7a-2-3p is associated with a favorable prognosis, longer overall survival and event-free survival in cytogenetically normal AML [290]. The effects of let-7a-2-3p are possibly through regulating miRNAs (miR-135a, miR-335 and miR-125b and all members of the
miR-181 family) and genes (FOSB, IGJ, SNORD50A and ZNF502, and FOSB) that are favorable signatures in AML. The role of miR-181 in AML is controversial. High miR-181 expression is associated with a better clinical outcome in cytogenetically normal acute myeloid leukemia patients [291, 292] through reverse regulation of toll-like receptors and interleukin-1β. In addition, miR-181 contributes to a better clinical outcome in cytogenetically abnormal AML patients [293] by regulation of HOXA7, HOXA9, HOXA11, and PBX3. Drug resistance is the main reason for AML relapse and poor prognosis. miR-181b can increase AML drug sensitivity through down-regulation of HMGB1 and MCL-1. Therefore, miR-181b is found to be down-regulated in relapsed and refractory AML patients [294].

**MicroRNA expression associated with favorable prognosis in AML**

In analysis of the expression of the meningioma 1 (MN1) gene and MN1-associated microRNA in Chinese adult de novo acute myeloid leukemia (AML) patients, Xiang found that increased expression of MN1 is associated with reduced miR-20a expression and increased miR-181b expression. In further analyzing the clinical outcome, miR-20a up-regulation is associated with a higher complete remission rate and longer overall survival [295]. In contrast, high miR-181b expression is found in patients with a lower complete remission rate, shorter relapse-free survival and shorter overall survival [295].

Cytogenetic risk factors and molecular markers are important factors for AML prognosis [296]. Expression signatures of a minimum of two (miR-126/126*), three (miR-224, miR-368, and miR-382), and seven (miR-17-5p and miR-20a, along with the aforementioned five) miRNAs could correctly distinguish CBF, t(15;17), and MLL-rearrangement AMLs, suggesting that these microRNAs may cooperate with specific translocation in leukemogenesis [297]. In
fact, KIT-mediated upregulation of miR-17, which targets RUNX1-3’UTR, mimic the effects of CBF-AML fusion protein[298]. The expression of miR-29a is lower in the bone marrow of pediatric AML patients compared with normal controls [299], and reduced miR-29a expression is associated with unfavorable karyotypes and shorter relapse-free and overall survival in pediatric AML patients [299]. Importantly, the association of miR-29a and prognosis is more apparent in intermediate-risk cytogenetic AML patients [299]. The same is true for miR-29b in that AML patients with low miR-29b expression have an unfavorable overall survival [300].

Analyses of 238 intermediate-risk cytogenetic AML patients, reduced expression of miR-135a and miR-409-3p is associated with a higher risk of relapse [296]. Higher miR-142 expression was associated with a better overall survival in AML patients with intermediate cytogenetic risk [301]. In AML patients with complex karyotype, p53 status plays a role in determining miR-34a’s role in clinical prognosis. Up-regulation of miR-34a expression is correlated with unfavorable overall survival in TP53 (unaltered)-AML with complex karyotype, but is correlated with favorable overall survival and chemotherapy sensitivity in TP53 (biallelic altered)-AML with complex karyotype [302]. miR-96 is down-regulated in newly diagnosed AML patients and is associated with a higher white blood cell (WBC) count, bone marrow blast count, and lower hemoglobin and platelet count. Importantly, the expression of miR-96 increased after patients were treated with standard cytarabine plus daunorubicin induction chemotherapy [303]. When analyzing the relapse-free survival (RFS) and overall survival (OS), low expression of miR-96 is associated with shorter RFS and OS [303]. miR-204 expression is reduced in AML patients and low miR-204 expression is correlated with short patient survival [304]. After patients received induction chemotherapy (daunorubicin plus cytarabine), high expression of miR-204 is associated with complete remission [304]. In addition, increased expression of miR-212, miR-25
and/or miR-203 has been associated with a favorable overall survival, event-free and relapse-free survival in AML patients independent of cytogenetic subtypes [260, 305-307].

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

miR-378 is increased in 31% of AML patients and is associated with lower hemoglobin levels and shorter relapse-free survival [308]. There is a positive correlation between miR-155 expression and white blood cell (WBC) count, serum lactate dehydrogenase (LDH), C-reaction protein (CRP) value in peripheral blood (PB), and miR25 and miR-196b expression in AML [309]. miR-126 is highly expressed in hematopoietic stem cells and leukemic stem-like cells. In AML patients high miR-126 expression is correlated with poor survival, higher chance of relapse and expression of stem cell related genes [310, 311]. *In vitro*, overexpression of miR-126-5p increased the phosphorylation of Akt and caused cytarabin resistance. Increased miR-124, miR-128–1, miR-194, miR-219–5p, miR-220a and miR-320 expression are associated with increased risk in AML [291]. The expression of miR-320d is increased in AML patients [312] and higher expression of miR-124-1 is associated with shorter overall survival and relapse-free survival [313]. AML patients with worse overall and event-free survival are known to have higher expression of miR-191 and miR-199a [314]. In de novo AML patients, miR-9-5p and miR-155-5p are independent unfavorable prognostic factors [306]. miR-155 is up-regulated in AML patients compared to normal controls [309, 312]. Consistent with this finding, high miR-155 expression is associated with an unfavorable prognosis, including lower complete remission rate and shorter disease-free survival and overall survival in AML [306, 309, 315]. The deregulation of miR-155 is associated with a gene expression profile enriched for genes involved in apoptosis, nuclear factor-kappaB activation, and inflammation [315]. Analyzing 53 AML patients,
increased expression of miR-26a, miR-29b, miR-146a, and miR-196b is associated with an unfavorable overall survival [260]. The role of miR-196b was further supported by analyzing 238 intermediate-risk cytogenetic AML patients, whereby high miR-196b and miR-644 expression is associated with shorter overall survival [296]. In 40 non-M3 AML patients, high expression of miR-26a, miR-29b and miR-146a is associated with short overall survival [260]. It is worth noting that miR-146a expression is reversely correlated with prognosis in both ALL and AML patients [260]. The opposite role of miR-29b in AML prognosis has been reported. miR-29b expression is inversely associated with MLLT11 expression, which is a poor prognostic biomarker for AML patients. Low miR-29b and elevated MLLT11 expression are found in patients with poor overall survival [300]. Whether the cooperation between miR-29b and MLLT11 caused poor prognosis needs to be further confirmed. Reduced miR-188-5p expression is associated with favorable prognosis as indicated by longer overall survival (OS) and event-free survival (EFS) in cytogenetically normal AML patients [290]. The effects of miR-188-5p are possibly through regulating miRNAs (miR-135a, miR-335 and miR-125b and all members of miR-181 family) and genes (FOSB, IGJ, SNORD50A and ZNF502, and FOSB) that are a favorable signature in AML. Up-regulated miR-3151 is found in AML patients with an unfavorable prognosis, such as short overall survival and leukemia-free survival, and higher relapse risk [316, 317]. High expression of miR-3151 is associated with low expression of genes involved in transcriptional regulation, posttranslational modification, and cancer pathways, such as FBXL20 and USP40 [317]. High miR-3151 expression is associated with high miR-501-5p and low miR-590, miR-135a, miR-100*, miR-186* and let-7a* expression [316]. The expression of let-7a-3 is increased in 25% of de novo AML patients and is associated with shorter overall
survival and relapse-free survival [318] in AML patients with complete remission. Further studies are needed to confirm the opposite role of let-7a-3 and let-7a-2-3p.

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

Higher expression of miR-29b is found in older AML patients with single-agent decitabine response compared with non-response patients [319]. The ability of miR-29b to target DNA methyltransferases may explain the role of miR-26b in decitabine response. miR-29c expression is higher in AML patients compared with healthy controls and is associated with poor survival [320]. Reduced miR-29c expression is associated with complete remission after initial treatment (intensive chemotherapy: daunorubicin plus cytarabine or low dose chemotherapy (low dose cytarabine or azacitidine)). Higher miR-29c expression was associated with relapse after patients achieved complete remission. Importantly, low miR-29c expression is associated with better response to azacitidine treatment and remission achievement in elder AML patients who are not suitable for intensive chemotherapy [320]. The increased expression of miR-181a is associated with a higher complete remission rate, longer overall survival and disease-free survival [292, 293] in AML patients treated similarly with intensive induction chemotherapy and consolidation with autologous peripheral blood stem-cell transplantation on Cancer and Leukemia Group B (CALGB) protocols 9621 and 19808. In addition, after AML patients received double induction and one consolidation therapy, increased miR-181b expression was associated with worse complete remission rates, relapse-free survival and overall survival in adult patients with de novo AML [295]. In contrast, up-regulation of miR-20 is associated with better complete remission rates and overall survival [295]. The following drugs are included in the induction therapy and consolidation therapy: daunorubicin, cytarabine, idarubicin, and
fludarabin [295]. miR-204 expression is reduced in AML patients and low miR-204 expression is correlated with short patient survival [304]. After patients received induction chemotherapy (daunorubicin plus cytarabine), high expression of miR-204 is associated with complete remission [304]. miR-331 is up-regulated in AML patients. AML patients with longer complete remission after induction chemotherapy have reduced miR-331 expression [321]. miR-335 is up-regulated in pediatric AML patients both in bone marrow and serum [322]. High serum miR-335 is associated with poor relapse-free and overall survival after patients received 10 days of induction chemotherapy [322]. However, a separate study reported no association between serum miR-335 expression and Ara-C-based chemotherapy response. However, high expression of miR-335 in the bone marrow was indicative of poor Ara-C-based chemotherapy response, lower relapse-free survival and overall survival in AML patients [323]. High expression of the miR-10 family is associated with complete remission after AML patients received induction chemotherapy [324].
Adult T-cell Leukemia

Adult T-cell Leukemia: signature and prognosis

Adult T-cell Leukemia is a fatal malignancy of mature CD4+, CD25+ T lymphocytes induced by the retrovirus Human T-cell leukemia virus (HTLV)-1[158, 325]. Several studies have reported deregulated microRNAs in ATL patient samples and HTLV-1-transformed cells, among them miR-155, miR-146a, miR-150, miR-223 were found up-regulated and miR-31 and miR124a down-regulated [39, 152, 326, 327]. Interestingly, a recent study demonstrated that virus-encoded protein HBZ targets the expression of DICER thereby modulating the expression of a subset of microRNAs [328]. Deregulation of miR-146a, miR-155, miR-150 and miR-223 is reported to affect cellular proliferation [329-331] and alteration of miR-31, miR-130b and miR-93 are involved in apoptosis resistance [332], suggesting a possible role of miRNA expression in ATL progression and pathogenesis. Differential analyses of microRNA expression in non-infected healthy individuals, chronic ATL patients and acute ATL patients revealed an increased number of up-regulated miRNAs in acute ATL patients when compared with chronic ATL patients [333]. Among these, increased miR-155 expression correlates with disease progression from HTLV-1 carrier to chronic ATL and then to acute ATL [333]. Both STAT3 and Myb, which transcriptionally up-regulate miR-155, are activated in HTLV-1-transformed cells and ATL samples [327, 334, 335]. On the other hand, let-7g is highest in healthy donors and its expression is significantly reduced in an HTLV-1 carrier, chronic and acute ATL patient samples [333]. For clinical outcomes, high miR-155 and low miR-126 is associated with a poor prognosis [333]. High miR-130b and low miR-145 and miR-223 expression in aggressive-type ATL are associated with shorter overall survival. Among miR-130b, miR-145 and miR-223, only miR-145 can act as an independent risk factor for ATL prognosis by a multivariate prognostic
analysis. An *in vitro* study showed that overexpression of miR-145 in ATL cells reduced cell proliferation [336]. A recent study demonstrated that epigenetic silencing of miR-124-1 resulted in STAT3-mediated Pim1 kinase activation and increased tumorigenic potential [327].
Role of circulating RNA

The majority of miRNAs are cellular miRNAs, however an emerging class of circulating miRNAs has been described. Circulating miRNAs have been observed in various body fluids[337]. They are involved in proliferation and differentiation. Recent evidence shows elevated expression of the miR-29 family (miR-29a, miR-29b and miR-29c), miR-150 and miR-155 in CLL-derived exosomes compared to healthy donors [338]. The plasma expression of miR-29a and miR-150 is associated with absolute lymphocyte count in the blood [339]. The miR-29 family is significantly down-regulated in a subset of CLL patients and is associated with an unfavorable prognosis [206]. miR-150 is highly expressed in cellular and serum samples of CLL patients [231]. Interestingly, low cellular expression of miR-150 but high serum expression of the same is associated with poor prognosis as indicated by tumor burden, treatment-free survival (TFS) and overall survival (OS) [231]. The expression of miR-155 is increased with disease progression from monoclonal B-cell lymphocytosis (MBL) to CLL and is higher in MBL and CLL than normal controls [214]. In addition, high plasma miR-155 expression is associated with CLL patients poorly responding to fludarabine, cyclophosphamide, and rituximab (FCR) chemotherapy [214]. Therefore, high expression of miR-155 is associated with more aggressive disease and poorer clinical prognosis in CLL [215, 216].

Consistent with the finding in CLL patients, the expression of miR-150 and miR-155 is higher in AML-derived exosomes and can act as a biomarker to distinguish AML patients from normal controls [324]. There is a positive correlation between miR-155 expression and white blood cell count, serum lactate dehydrogenase (LDH) and C-reaction protein (CRP) value in peripheral blood in AML patients [309]. High miR-155 expression is associated with an
unfavorable prognosis, such as lower complete remission rate and shorter disease-free survival and overall survival in AML patients [306, 309, 315]. However, the role of miR-150 in AML is controversial. The expression of miR-150 has been reported to be lower in AML-derived plasma compared to healthy donors [340]. Up-regulation of miR-150 after treatment is associated with AML patients with complete remission [340]. In addition, circulating miR-155-5p and miR-181b-5p are up-regulated in AML patients when compared with normal controls [312]. Up-regulated circulating miR-181b-5p is associated with shorter overall survival [312] and is found in patients with a lower complete remission rate, shorter relapse-free survival and shorter overall survival [295].

Other circulating miRNAs can also act as biomarkers for AML prognosis. For instance, miR-210 is up-regulated in the bone marrow and serum of AML patients compared with normal controls. Reduced serum miR-210 expression is found in patients with complete remission, while high miR-210 expression is correlated with poor relapse-free survival and overall survival in AML patients [341]. Similarly, the expression of miR-375 is higher in the serum and bone marrow of pediatric AML patients and is associated with unfavorable karyotypes and poor prognosis as indicated by shorter relapse-free survival and overall survival [342]. Like miR-29a [299], the association of miR-375 and prognosis is more apparent in intermediate-risk cytogenetic AML patients [342]. Plasma miR-511, miR-222, and miR-34a are up-regulated in B-ALL patients compared with normal controls, whereas plasma miR-199a-3p, miR-223, miR-221, and miR-26a are lower in B-ALL patients [343]. Moreover, the low expression of miRNA-100 and miRNA-146a is associated with poor clinical outcome in ALL patients [257, 260, 262, 320]. Together these studies clearly demonstrate that detection of circulating miRNAs has significant
value for detection of disease progression and can also serve as indicators of therapeutic response.
Clinical significance of other non-coding RNAs

In addition to microRNA, other non-coding RNAs have been reported to play a role in human leukemias. Long non-coding RNAs (lncRNAs) are RNA molecules longer than 200 nucleotides with undefined open reading frames involved in gene expression regulation. A small subset of lncRNAs have been reported in leukemia and an lncRNA expression profile correlated with treatment response and survival in AML patients [344]. The X-inactive specific transcript Xist lncRNA, involved in epigenetic regulation of transcriptionally inactive chromatin, is overexpressed in some leukemias [345]. NOTCH-regulated lncRNA LUNAR1 (leukemia-induced non-coding activator RNA) has been shown to have oncogenic effects in T-ALL [346]. LUNAR1 has been demonstrated to increase IGF1R mRNA expression and IGF1 signaling [346]. Another NOTCH-related lncRNA, RP11-611D20.2 (NOTCH-associated lncRNA in T-ALL (NALT)), has been found to be overexpressed in pediatric ALL and may play a role in the leukemia stem cell compartment [347]. In CML patients with BCR-ABL translocation, deregulation of two lncRNAs has been described: the Beta Globin Locus 3 (BGL3) lncRNA [348] and the imprinted H19 lncRNA [349]. Little is known about the function of these lncRNAs in CML. BGL3 lncRNA has been shown to increase the expression levels of the tumor suppressor PTEN by acting as a competing endogenous RNA (ceRNA) [350]. In contrast, lncRNA H19, which is transcriptionally activated by the oncogene c-Myc [349], has been shown to inhibit the expression of the onco-suppressor let-7 microRNA family [351]. In AML patients, lncRNA IRAIN [352], which is transcribed from the IGF1R imprinted locus, is down-regulated in patients with high-risk AML, while urothelial carcinoma-associated 1 (UCA1) lncRNA is specifically up-regulated in AML [353], although its role in the pathogenesis is unclear. Finally,
the IncRNA B-ALL-associated long RNAs-2 (BARL-2) was found to affect B-ALL patient response to corticosteroid treatment [354]. Another class of non-coding ncRNAs, the small nucleolar snoRNAs, is also affected in cancers and leukemia. Elevated levels of SNORD112–114 snoRNAs has been found in acute promyelocytic leukemia (APL) [355]. In a different study, Affymetrix GeneArray screening identified snoRNA SNORA70F as significantly down-regulated in poor prognostic subgroups of CLL patients. In addition, high expression of SNORA74A and SNORD116-18, and low expression of SNORD56 were associated with shorter progression-free survival (PFS) in these patients [356]. Although IncRNA and snoRNA are not as greatly studied as miRNA, they are likely to play an increasing role in the future and eventually become a part of patients’ genetic signatures for individualized targeted medicine.
Chapter VI: Perspectives and Conclusions
**CRISPR screen to prevent HTLV-1 infection**

HTLV-1 preferentially replicates in CD4+ T cells, but other cells like stem cells and CD8+ T cells can also be infected by HTLV-1. It is found that the glucose transporter glucose transporter 1 protein (GLUT1) and surface heparin proteoglycan are receptors for HTLV-1 infection [16, 21]. In addition, when infected cells come into contact with uninfected cells, HTLV-1 genome transfer to the uninfected lymphocyte through the viral synapse [26], which is composed of microtubule-organizing center (MTOC) [21]. The interaction between intercellular adhesion molecule 1 (ICAM1) and lymphocyte function-associated antigen 1 (LFA1) enhances the polarization of the MTOC at the cell-cell contact point and facilitates HTLV-1 transmission [21].

In 2014, the team led by June used zinc-finger nuclease (ZFN) to target CCR5 in T cells and infused ZFN-modified autologous CD4 T cells back into patients [357]. The results demonstrate that it is safe to genetically modify CCR5 in autologous CD4+ T cells. Consistently, using CRISPR-base screening, Ryan identified host genes such as CCR5, tyrosylprotein sulfotransferase 2 (TPST2) and solute carrier family 35 member B2 (SLC35B2) that are essential for HIV infection but dispensable for host cell proliferation and survival [358]. In the future, the same method can be used to identify HTLV-1 infection essential genes. By using small molecule and/or antibody blockage, it is possible to prevent the infection or viral spreading at the beginning of HTLV-1 infection. Determining the indispensable proteins for HTLV-1 infection has the potential to inform the development of new targeting strategies that may have significant therapeutic implications.
**HTLV-1 patient classification**

The clinical features of ATL are diverse from patient to patient. The clinical features of ATL include general lymphadenopathy, skin lesions, hepatosplenomegaly, leukocytosis with abnormal flower-like lymphocytes or with an increased number of neutrophils, hypercalcemia, and opportunistic infection [1]. According to Shimoyama criteria (Figure 1) [1], ATL patients can be classified into 4 different subtypes (acute, lymphoma, chronic, and smoldering) according to their prognostic factors and clinical observation. Acute, lymphoma and unfavorable chronic subtype largely defines poor prognosis. On the other hand, favorable chronic and smoldering subtypes are associated with a better clinical outcome. However, the molecular basis for patient classification is still missing. Therefore, comprehensive studies such as RNA-sequencing, whole-genome sequencing [2] and mass-based proteomic study will provide the molecular mechanisms by which HTLV-1 transforms T cells. A recent comprehensive genomic study of 426 ATL cases revealed activating mutations and gene fusions. These genetic changes result in deregulation of signaling pathways: TCR–NF-κB, JAK-STAT, Notch, and phosphoinositide 3-kinase (PI3K)-AKT signaling [2]. These results identify potential biomarkers and druggable targets for ATL patients and provide the opportunity to identify ATL patients who might best respond to precise therapy such as a PI3K/AKT inhibitor and JAK/STAT inhibitor.

**Combinational therapy**

Although the combination chemotherapies, antiviral therapies, and allogeneic HSCT have improved ATL patient prognosis, the overall ATL prognosis is still dismaying. Most of the ATL patients receiving general chemotherapy (CHOP) eventually develop drug resistance and relapses in a few months. The detailed mechanism underlying drug resistance is still poorly understood [359]. Most of the drug resistance comes from the mutation in the drug targets, such
as the mutation of epidermal growth factor receptor (EGFR) in lung cancer causing resistance to EGFR inhibitor. In addition, reactivating the downstream signaling pathway of the chemotherapy target can bypass the dependence on the specific target. For example, the activation of the ERK signaling pathway in melanoma results in RAF inhibitor resistance [360]. Understanding the molecular mechanisms underlying drug resistance can help us develop new inhibitors or combine specific target chemotherapies to circumvent or postpone resistance.

In our study, we found that the mutation of FBXW7 in ATL cell lines increased downstream oncoproteins, such as Notch, c-Myc and cyclin E [5]. Although it is still impossible to target FBXW7 mutants, the deregulation of diverse downstream targets caused by FBXW7 inactivation may reveal unique cancer dependencies and provide multiple downstream opportunities for therapeutic intervention. It will enable identification of patient populations that may benefit from specific chemotherapy interventions. Therefore, targeting the FBXW7 substrate can be a therapeutic strategy in a clinical setting (Figure 25). For example, JQ1 is a bromodomain and extra terminal protein (BET) inhibitor that can reduce the expression of c-Myc. JQ1 inhibitor has anti-tumor effects both in vitro and in vivo for AML-T-ALL and ATL [147, 361].
Figure 29. Targeting FBXW7 substrate to reverse the effect of FBXW7 mutant
In addition, it is possible to target FBXW7 mutant cells through synthetic-lethal methods (Figure 26) or oncogene dependence, such as targeting genes that are selectively essential in cell lines carrying particular driver mutations [82] that prevent the damage to normal cells with wild-type FBXW7. For example, FBXW7 has been demonstrated to target glucocorticoid receptor alpha (GRalpha) in T-ALL. Reduced FBXW7 expression or function increased the glucocorticoid sensitivity in T-ALL, but not sensitivity to other chemotherapy drugs [133]. Thus, glucocorticoid treatment can provide a therapeutic window to block FBXW7 mutant cells and spare normal FBXW7 wild-type cells.
Figure 30. Schematic of synthetic lethality.

Synthetic lethality means when 2 or more genes have loss-of-function at the same time leading to cell death, but not causing cell death when only one gene has loss-of-function.
FBXW7 substrates

The mutations of FBXW7 can affect the interaction between FBXW7 and SKP1 (Figure 27A), inactivating FBXW7 without affecting interaction (Figure 27B), impairing the interaction between FBXW7 and substrates and creating a novel interaction between FBXW7 and new substrates (Figure 27C).
Figure 31. The effect of FBXW7 mutation.

The star indicates the mutation site of FBXW7
Until now, approximately 20 different FBXW7 targets have been identified [82]. As each of the substrates may be differentially affected by specific FBXW7 mutations and the cross-talk between signaling pathways, it is difficult to identify the contribution of each pathway to the overall survival and proliferation of cancer cells in FBXW7 mutant ATL. To comprehensively determine cellular proteins affected by FBXW7 mutation in ATL, ATL cells stably expressing FBXW7 mutant can be used to globally analyze protein expression levels by mass spectrum [362]. In addition, UbiScan ubiquitination proteomics platform can be used to unbiasedly determine protein ubiquitylation changed by FBXW7 mutation [44]. Furthermore, systematic screening such as CRISPR-Cas9 library or shRNA pool will identify the signaling pathway that is essential for specific FBXW7 mutant effect and reveal the synthetic lethal partners of FBXW7 inactivation.

In addition to the natural FBXW7 substrates, it is possible to use small molecular that links both a novel substrate and FBXW7. The chimeric adaptor binds to novel substrate and FBXW7. The recruitment of FBXW7 to the novel substrate causes novel substrate ubiquitination and eventually is degraded by proteasome (Figure 28). This strategy can be used to target oncoproteins that are unable to be targeted by small molecular inhibitor.
Figure 32. Novel substrate for FBXW7
**Immunotherapy for ATL**

Although ATL cells functioning as regulatory T cells is controversial, ATL cells from a subset of patients are hypo-responsive to stimulation and repress the proliferation and IFN-gamma production of autologous CD4+ non-ATL cells [50]. That may at least explain part of the immunosuppression observed in ATL patients. In a clinical setting, HTLV-1 infection can cause opportunistic infection, which is also associated with immunosuppression and could be the reason for poor ATL prognosis [1]. Recently, the success of an antibody against programmed cell death 1 (PD-1; also known as PDCD1) and its ligand (PD-L1; also known as CD274) has represented a promising strategy for ATL treatment. However, Keisuke reported that 27% of ATL patients have structural variations (SVs) at the 3′ region of the PD-L1 gene. The structural variations can increase PD-L1 transcription and cause immunosuppression in animal models [363]. Therefore, it is important to exclude ATL patients with PD-L1 aberrant expression before immunotherapy. In the future, it is possible to use CRISPR-Cas9 to permanently eliminate PD-L1 in T cells to overcome this problem. Indeed, CRISPR-Cas9 mediated knockout of PD-1 has been shown to increase cellular immune response and does not affect the viability of primary human T cells [364].
**Oncogenic activity of FBXW7**

About 6% of primary tumors carry an FBXW7 inactivated mutation [88]. Conditional knockout of FBXW7 in hematopoietic cells or T cells is sufficient to cause T-ALL or thymic lymphoma by increased expression of Notch1 and c-Myc in more than 50% of FBXW7-deficient mice [121, 122]. In previous studies, however, FBXW7 hotspot mutation knock-in mice (FBXW7\textsuperscript{mut/+}) did not develop leukemia spontaneously, indicating the difference between FBXW7 missense mutation and homologous deletion. When combining FBXW7 deletion/mutation with suppression of p53 [121] or loss of PTEN [123] or active Notch [9], FBXW7 deletion/mutation enhances the tumorigenesis.

In our study, we found two FBXW7 mutants, D510E and D527G, that demonstrated oncogenic activity when co-expressed with HTLV-1 Tax, mutated p53 R276H, or c-Myc F138C found in human cancers. Transforming activity was further proved by the ability of the FBXW7 D510E mutant to provide IL-2-independent growth of Tax-immortalized human T cells and increase the tumor formation in a xenograft mouse model of ATL. This study suggests that FBXW7, normally a tumor suppressor, can act as an oncogene when mutated and may play an important role in the pathogenesis of ATL.

Although most of the studies indicated FBXW7 as a tumor suppressor, there are few reports that demonstrated the oncogenic activity of FBXW7 [365]. In epidermal cells, loss-of-presenilins (PS), which is a catalytic component of gamma-secretase led to up-regulation of FBXW7 and subsequent stabilization of EGFR [365]. Furthermore, ablation of FBXW7 in embryo fibroblast reduced EGFR expression. In chronic myelogenous leukemia (CML), loss of FBXW7 resulted in c-Myc stabilization and subsequent CML leukemia-initiating cell (LIC) exhaustion. Mechanically, the up-regulation of c-Myc after FBXW7 deletion caused p53-
dependent apoptosis in CML leukemia-initiating cells [366] and disrupted the quiescence of CML leukemia-initiating cell [154]. Importantly, the depletion of CML leukemia-initiating cells by FBXW7 deletion increased the sensitivity of CML to chemotherapy drugs such as imatinib [154]. Further studies are needed to know if FBXW7 functions as an oncogene in other tumors.
Reduced FBXW7 expression in ATL

Consistent with the tumor suppressor role of FBXW7, FBXW7 expression was deficient in many cancers and correlated with clinical outcomes. For example, loss of FBXW7 expression in hepatocellular carcinoma (HCC) tissues is correlated with poor clinical pathology, with features including large tumor size, high pathological grading and advanced TNM stage [124]. In addition, in high histological grade breast cancer patients, FBXW7 mRNA is significantly lower [127]. Moreover, lower FBXW7 expression is correlated with high Ki-67 and cyclin E expression. In ATL, down-regulation of FBXW7 expression was found in all the ATL cells lines, including MT-1, MT-2, MT-4, C8166, TL, ATL-25, ATL-T, ED, SP, ATL-43T and ATL-55T, compared to normal PBMC.

Shahab showed that FBXW7β expression was reduced in primary breast cancer by promoter hypermethylation [367]. To determine the relationship between promoter methylation and low FBXW7 expression in ATL, we treated MT-2 and MT-4 with 5-Aza-2'-deoxycytidine, which causes DNA demethylation. After 5-Aza-2'-deoxycytidine treatment, FBXW7 expression is increased in both MT-2 and MT-4, which implied the promoter methylation of FBXW7.

In addition to mutations and promoter methylation, FBXW7 is targeted by miRNAs such as miR-27, miR-92 and miR-223 in various tumors [82]. Among those miRNAs, miR-223 is up-regulated in many cancers, such as gastric cancer and esophageal squamous cell carcinoma [368, 369]. Overexpression of miR-223 promotes cell proliferation in vitro and in vivo, and inhibits apoptosis [368]. On the other hand, inhibition of miR-223 inhibits cell proliferation and induces apoptosis in T-ALL cells [370]. Importantly, in our previous study, we found miR-223 was highly expressed in all ATL patients tested [152]. Whether miR-223 regulates FBXW7 in ATL is still unclear.
Although the idea is attractive, increasing FBXW7 expression remains a challenge. Thus, a better understanding of the mechanisms regulating FBXW7 abundance is required.
**Restore the function of FBXW7**

FBXW7 is a tumor suppressor known to target many oncoproteins, such as cyclin E, c-Myc and Notch 1. Overall, 6% of human cancers harbor FBXW7 mutations. Among those mutation points, R465, R479, and R505 are three arginine residues in FBXW7’S substrate binding domain (WD40 domain) that are important for interaction with substrates (Figure 29A). Importantly, in the COSMIC database, more than half of the FBXW7 mutations happened on these 3 arginine residues [82]. Furthermore, we found 25% of ATL patients have mutations in the WD40 domain. The FBXW7 mutants lost their ability to interact with and degrade intracellular domain of Notch (NICD). In addition to the FBXW7 mutations, the mutations on the substrate’s phosphodegron also have been reported. For example, the mutation of c-Myc Thr58 depletes its Thr58 phosphorylation, which is the phosphodegron for FBXW7, and leads to c-Myc stabilization in Burkitt's lymphoma [101]. In addition, the mutations in T-ALL are shown to ablate Notch interaction with FBXW7 [134].

Proteolysis targeting chimaeras (PROTACs) and small molecules can be used as therapeutic applications to restore interaction between FBXW7 and its substrates [371, 372]. PROTACs are heterobivalent, chimeric molecules that bring together the E3 ligase and substrate (Figure 29 B). Besides, it has been reported that conjugation of JQ1 with phthalimide led the Cereblon E3 ubiquitin ligase complex to degrade the BET protein [371]. In addition, small molecules can act as molecular glue to bring together FBXW7 and its substrates (Figure 29C), although it may have off-target effects [83].
Figure 33. Restore interaction between FBXW7 and substrates

a. Interaction between FBXW7 and its substrates in physical condition. b. In case the mutations disrupt the interaction between FBXW7 and its substrates, PROTAC can be used to recruit substrates to FBXW7. c. Small molecules act as molecular glue to connect FBXW7 and its substrates. (Adapted from [83])
The advance of CRISPR-Cas9 RNA-guided DNA endonuclease (CRISPR-Cas9 system) has improved our ability to edit genomes in living cells [373]. The CRISPR-Cas9 system consists of guide RNA, which can guide Cas9 DNA endonuclease specifically to target DNA by Watson-Crick base-pairing and cause DNA double-strand break. When the repair template is available, homology-directed repair (HDR) will be used to repair DNA double-strand breaks and lead to desired genomic editing. Although the CRISPR-Cas9 system is an easier method to do genomic editing compared to zinc-finger nucleases and transcription-activator-like effector nucleases, the target DNA sequence (the protospacer) must contain a unique DNA sequence called protospacer-adjacent motif (PAM), which is a short DNA sequence that is CRISPR nuclease-specific [373]. In case FBXW7 is inactivated by mutations or the viral oncoprotein Tax [5], CRISPR-Cas9 system can be used to correct mutations in FBXW7 genes and reverse the oncogenic activity of an FBXW7 mutation or specifically ablate Tax expression in ATL cells.

In addition to editing genomic DNA, modified CRISPR-Cas9 system has also been used to alter the epigenome in living cells. The combination of DNA methyltransferase enzyme DNMT3A to inactivated Cas9 can specifically edit epigenetic modification at the target CpG islands [373]. Furthermore, the fusion of DNA demethylase Tet1 to inactive Cas9 leads to target DNA demethylation [373]. When FBXW7 is inactivated by promoter methylation [367], the modified CRISPR-Cas9 system can be applied to specifically de-methylate FBXW7 promoter methylation.
Patient-derived xenografts model for ATL

The heterogeneous clinical phenotype and recurrence of ATL highlight the difficulty in developing ATL therapy. Thus, there is an eager need for preclinical assessments that can efficiently and specifically predict treatment response. Cancer cell lines cannot represent clinical observation well because they have been cultured and adapted in the dish for decades. In addition, the microenvironment, which is important for tumor progression and drug resistance, is missing in a cell line culture system. Patient-derived xenografts (PDXs) that implant tumor samples from clinical patients directly into immune-deficient mice or humanized mice are believed to better predict clinical prognosis compared to \textit{in vitro} dish culture. PDXs maintain the tumor heterogeneity and each PDX represents an individual patient that can be used to study inter-tumor heterogeneity [374]. Recently, PDX-base drug screen led by EurOPDX Consortium included more than one thousand PDX models from different solid cancers. Importantly, the results obtained from PDXs are highly correlated with clinical observation in patients [374]. For example, the response to EGFR antibody in colorectal PDXs is consistent with the clinical observations, which are indicated by tumor regression, disease stabilization and progression. The first ATL PDX model has been reported by Public Repository of Xenografts (PRoXe) [375]. In this study, they found PDXs can not only be used for randomized phase II-like preclinical drug trials, but also for identifying transcriptional, functional, and proteomic biomarkers. In the future, more and more ATL PDX models will be developed and used for the drug screen. Furthermore, the humanized PDX model can provide the opportunity to uncover immune escape in ATL patients and provide a therapy window to use immune therapy in ATL patients.
Epigenetic modification in ATL

Most human cancers harbor both genetic and epigenetic alterations. The cross-talk between genetic and epigenetic aberrance has been reported [139]. In acute myeloid leukaemia (AML), the mutations in isocitrate dehydrogenase-encoding genes IDH1 and IDH2 inactivate histone demethylases and DNA demethylases, leading to changes in DNA and histone methylation that drive the disease phenotype. In our study, we found TET2 was the most frequently mutated gene, occurring in 32% (10/31) of ATL samples analyzed. Next-Generation Sequencing revealed nonsense mutations accompanied by loss of heterozygosity (LOH) in TET2, which was further confirmed by cloning and direct sequencing of DNA from uncultured cells. Importantly, TET2 mutation is known to cause hypermethylation of haematopoietic-specific enhancers in myeloid malignancies. Whether TET2 mutations in ATL caused hypermethylation of ATL driver genes remains to be defined. However, targeting the epigenome can be a promising therapeutic strategy for a considerable cohort of patients with ATL when coupled the genomics-guided patient selection and observation.


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