

β -catenin signaling inhibited effector differentiation and promoted the generation of self-renewing CD8⁺ memory stem cells and central memory T cells. In CD4⁺ T cells, Wnt/ β -catenin signaling facilitated Th2 polarization and enhanced the survival of naturally occurring regulatory T cells (nTreg). Canonical Wnt signaling has also been suggested as important for the self-renewal of hematopoietic stem cells (HSCs) and progenitor compartments. β -catenin deletion perturbs HSC survival, self-renewal, and their subsequent development into T cells [58,59].

Because the Wnt pathway is important for normal T cell development and signaling pathways, constitutively active Wnt signals can lead to T cell acute malignancies. An experimental model of Wnt activation demonstrated that the constitutively active form of β -catenin led to development of an aggressive T cell lymphoma that could invade the bone marrow and was transplantable into irradiated recipient mice [60]. These data suggest that Wnt-dependent c-Myc expression is required for lymphomagenesis. Interestingly, this appears to be independent of Notch signaling that has been thought to be an analog of the Wnt pathway. Crosstalk between these pathways will be discussed in a later chapter.

Interestingly, modest transgenic expression of β -catenin in thymocytes enhances the generation of CD8⁺ thymocytes and accelerates thymic involution [61]. Some evidence indicated that oncogenic β -catenin induced p53-independent oncogene-induced senescence and p53-dependent apoptosis, which protected developing thymocytes from transformation [62]. The molecular mechanisms induced by the Wnt pathway in T cells remain to be elucidated.

Hedgehog (Hh) pathway

The Hh family of secreted intercellular molecules, including Sonic, Indian, and Desert Hh, regulates target gene transcription through GLI transcription factors. This family is of pivotal importance in embryogenesis and the homeostasis of adult tissues, as well as cell differentiation, cell cycle progression, and cell survival [63].

Hh signaling is important in T cell development in both humans and mice [64]. The generation of mature functional T cells in the thymus requires sonic Hh (Shh) signals from the thymic epithelium. Shh-induced signaling is important for the differentiation and proliferation of early thymocyte progenitors. Shh also modulates TCR signaling during repertoire selection.

Hh signaling is involved in the conversion of immature DP cells to mature SP thymocytes and can affect TCR repertoire selection and CD4/8 lineage choices. Depletion of Hh signaling promotes CD4 lineage commitment and results in an increased CD4:CD8 ratio. In contrast, increased Hh signaling results in a reduced CD4:CD8 ratio, suggesting that Hh signaling is an inducer of CD8⁺ T cell development [64,65].

Hh is also involved in peripheral T cell activation. Additional Shh supports TCR/CD28 signaling and enhances T cell activation and subsequent proliferation [66-68]. In contrast, Hh activation by the expression of the constitutive active form of Gli2 results in inhibition of TCR/CD28-mediated T cell activation. In this case, Hh appears to inhibit TCR-dependent Erk phosphorylation [65]. Modulation of TCR signal strength by Hh has profound implications for immunity. Thus, the precise molecular involvement of Hh in T cell signaling needs to be examined.

Hh signaling is implicated in several hematological malignancies,

including chronic lymphocytic leukemia (CLL), plasma cell myeloma, mantle cell lymphoma, diffuse large B-cell lymphoma (DLBCL), ALK-positive anaplastic large cell lymphoma (ALCL), chronic myelogenous leukemia (CML), and acute leukemias (AML). ALK+ ALCL is an aggressive type of non-Hodgkin's lymphoma of the T cell/null lineage. One study demonstrated that Hh ligands and GLI1 were highly expressed in ALK+ ALCL [69]. NPM-ALK activates the PI3K-Akt pathway, which contributes to GSK-3 β inactivation. Because GLI1 and GLI2 are the targets of GSK-3-mediated proteasomal degradation, NPM-ALK activates Hh signaling through the PI3K-Akt-GSK-3 cascade. Of note, we have demonstrated that the NPM-ALK abrogates CD30 signaling and constitutive NF- κ B activation in ALCL cells [70]. The reciprocal relationship between Hh and NF- κ B signaling in T cells remains unclear.

TGF- β -mediated signaling

The TGF- β superfamily regulates many cellular functions, including cell growth, differentiation, migration, and apoptosis. TGF- β -mediated signaling is essential for embryonic development, embryogenesis, and cell fate decisions [71].

TGF- β is synthesized in an inactive form consisting of a TGF- β dimer in association with the latency-associated protein (LAP). Signaling is initiated by a conformational change in a tetrameric complex consisting of TGF- β receptor II (TGF- β RII), TGF- β receptor I (TGF- β RI), and activated TGF- β dimer. Activated TGF- β RI then phosphorylates the transcription factors Smad2 and Smad3 and triggers their translocation into the nucleus. Smads then bind to the specific regulatory sequences of target genes and regulate gene expression by recruiting transcription cofactors.

TGF- β regulates T cell development, homeostasis, tolerance, and immunity [72]. TGF- β signaling promotes the differentiation of thymic T cells into natural killer T cells, nTreg cells, and CD8⁺ T cells. TGF- β signaling is likely to be pivotal for the survival of peripheral CD4⁺ and CD8⁺ T cells with low affinity. In contrast, it inhibits the proliferation and differentiation of CD4⁺ and CD8⁺ T cells with high affinity.

In particular, TGF- β plays central roles in Treg cell regulation. It inhibits proliferation of Treg cells, although it supports their maintenance in peripheral lymphoid organs, which can control Treg cell survival. TGF- β promotes the differentiation of induced Treg (iTreg) cells, which is potentiated by IL-2 and retinoic acid (RA). In the presence of IL-6, TGF- β drives the differentiation of Th17 cells and supports their maintenance.

The interplay between TCR and TGF- β signaling has been implicated in the regulation of several genes, including FoxP3, which is important for Treg functions [73]. However, because most studies have mainly performed phenotypic analyses, the exact molecular signaling pathway induced by TGF- β in T cells is not completely understood.

Signalings from receptors of innate immune cells

Innate and adaptive immune responses have many interactions that are regulated by the balance of signals initiated by several activatory and inhibitory receptors. Toll like receptor (TLR) expression has been generally assigned to professional APCs, such as dendritic cells. However, several recent studies have demonstrated that the low expression levels of TLRs could also be detected on naïve and memory T cells [74,75]. In addition, TLR agonists can directly target TLRs expressed on T cells and function as signal inducers.

Engagement of the TLR1/2 complex was associated with T cell

proliferation, survival, and functions [76]. Ligation of TLR3 on effector CD8⁺ T cells increased IFN- γ secretion by these cells. Interestingly, the effects of CpG (TLR9 ligand) on CD4⁺ T cells were mediated by MyD88 and PI3K-dependent mechanisms [77]. Another study showed that ligands for TLR3 and TLR9 directly enhanced T cell survival, which was sustained by NF- κ B signaling [78]. Collectively, these findings suggest that TLR agonists can directly target TLR signaling in effector CD4⁺ and CD8⁺ T cells. We do not understand the molecular mechanisms by which TLR signaling can affect the other sustained T cell signaling.

NKG2 receptors are expressed by natural killer (NK) cells and a subset of CD8⁺ T cells. They regulate the development and function of NK cells by inhibiting and activating cytotoxicity and promoting cell survival. The CD94/NKG2 heterodimers consist of one CD94 molecule and one NKG2 family member, including NKG2A and its splice variant NKG2B, which form inhibitory receptors, as well as NKG2C, NKG2E, and NKG2H, which form activating receptors [79]. NKG2D is unique in that it does not dimerize with CD94; instead, it associates with the adaptor molecules DAP10 and DAP12 for its activating function [80]. Among these, the acquisition of CD94/NKG2A expression by CD8⁺ T cells could play a role during infection by pathogens. This has been observed in mice infected with a variety of pathogens, including lymphocytic choriomeningitis virus, herpes simplex virus (HSV)-1, influenza virus, polyomavirus, and *Listeria monocytogenes* [81]. Furthermore, HIV-1 infection has also been implicated in the upregulation of CD94/NKG2A [82,83]. The expression of CD94/NKG2A, which is acquired early during the infection and persists after clearance of the pathogen, does not interfere with the activity of CD8⁺ T cells. However, the CD94/NKG2A expression inhibits antiviral CD8⁺ T cell responses [84]. In addition, high level of CD94/NKG2A expression on CD8⁺ T cells protects them from apoptosis, thereby perhaps inducing in the generation and maintenance of memory cells.

Emerging Regulatory Factors: Learning from Malignancies

Recent experimental evidence indicates the presence of additional molecular hallmarks of T cells. miRNAs regulate gene expression patterns in all cell types. Epigenetic gene regulation is based on chromatin regulation. Both of these are pivotal for development, differentiation, proliferation, and other cellular processes in hematopoietic lineage. Their deregulation is frequently observed in T cell disorders, which strengthens the fact that miRNA-mediated gene regulation and epigenetic mechanisms may be important for maintaining T cell homeostasis.

miRNA in T cells

One of the most significant recent advances in biomedical research has been the discovery of the 22-nt-long class of non-coding RNA designated miRNA that post-transcriptionally regulates gene expression by binding to the target mRNAs. miRNA is expressed by all metazoans and plants, as well as by several DNA viruses; it regulates cellular processes such as development, differentiation, growth, homeostasis, stress responses, apoptosis, and immune activation [85].

Some recent extreme studies have collectively suggested several concepts. First, miRNAs have unique roles as time-sensitive gene regulators. Second, miRNAs have distinct functions in distinct cell types. Third, miRNAs regulate gene expression by incompletely repressing their targets. These specific functions of miRNAs as “fine-tuners” are apparently involved in immune systems as well as

hematological cancers. It is not surprising that miRNA deregulation appears to play important roles in human cancers.

miRNAs provide modest changes in gene expression [86]. However, it is also true that gene expression patterns can occasionally be changed drastically by a change in a single miRNA that regulates a key transcription factor. For example, a decrease in a miRNA, miR-150, leads to drastic deregulation of the B cell transcriptome by regulating c-MYB [87]. Furthermore, given that miRNAs are upstream of gene regulation systems, miRNAs confer robustness to several biological processes by controlling several feed-forward and feed-back loops [86]. Several recent reports including our own have demonstrated crosstalk between miRNA-mediated gene regulation and several signal transduction pathways in T cells.

Specific knockout of *Dicer*, which is essential for processing miRNAs and siRNAs in the T cell lineage, leads to impaired T cell development and aberrant Th cell differentiation and cytokine production [88,89]. In addition, peripheral CD8⁺ T cell development was severely blocked after *Dicer1* deletion in the thymus. These cells were defective in miRNA processing, and after stimulation, they proliferated poorly, which resulted in their apoptosis. *Dicer*-deficient Th cells preferentially expressed INF γ . The levels of secreted IL-2 in *Dicer*-deficient and control cultures were comparable after primary stimulation, and addition of exogenous IL-2 to the growth media did not rescue the proliferative defect of *Dicer*-deficient T cells, which suggested that *Dicer* might be involved in IL-2 receptor (CD25) signaling in T cells [89].

An early study revealed that miR-181a acted as an intrinsic antigen sensitivity “rheostat” during T cell development [90]. miR-181a expression enhanced the basal activation of TCR signaling and IL-2 production because miR-181a repressed multiple negative regulators of TCR signaling (Figure 7). Thus, several miRNAs probably participate in each step of T cell regulation. This in turn suggests that miRNA deregulation may cause catastrophic gene regulation in T cells.

Individual miRNAs have been implicated in T-ALL. miR-19b is

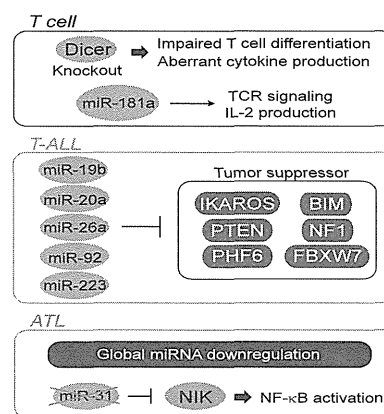


Figure 7: miRNA in T cell biology. Specific knockout of *Dicer* in T cell lineage leads to impaired T cell development and aberrant T helper cell differentiation and cytokine production. miR-181a expression enhances the basal activation of TCR signaling and IL-2 production (top box). In T-ALL cells, upregulation of five miRNA leads to repression of tumor suppressor genes implicated in T-ALL (middle box). Global downregulation of miRNA is the one of molecular hallmarks of ATL. In particular, miR-31 expression is genetically and epigenetically silenced, which is prerequisite condition of ATL cells. The loss of miR-31 consequently contributes to NIK over expression.

a member of the oncogenic miR-17-92 cluster, which is targeted by the t(13;14)(q32;q11) translocation in T-ALL [91]. Comprehensive methods have been used to address the miRNA candidates associated with abnormal gene expression and leukemogenesis. Recently, one study reported on miRNA signature in T-ALL [92]. A total of 430 types of miRNAs were analyzed in 50 clinical T-ALL and 18 T-ALL cell lines by quantitative RT-PCR, revealing that a small set of miRNAs was responsible for the cooperative suppression of several tumor suppressor genes. Cross comparisons of miRNA expression profiles in human T-ALL with the results of an unbiased miRNA library screen identified five miRNAs (miR-19b, miR-20a, miR-26a, miR-92, and miR-223), which were capable of promoting T-ALL development in a mouse model. Furthermore, these miRNAs produced overlapping and cooperative effects on tumor suppressor genes implicated in T-ALL, including *IKAROS*, *PTEN*, *BIM*, *PHF6*, *NF1*, and *FBXW7*. Thus, a comprehensive and unbiased analysis of miRNA actions in T-ALL reveals a striking pattern of miRNA–tumor suppressor gene interactions (Figure 7).

For ATL, some studies have reported several miRNA aberrations identified in HTLV-1-infected cells and ATL samples [93-95]. Very recently, we established global gene expression analysis in a large cohort ATL study that included analyses of mRNA expression, miRNA levels, and genomic copy numbers [34]. A strict threshold ($p < 1 \times 10^{-5}$) and two-dimensional hierarchical clustering analysis revealed 61 miRNAs with significantly altered expression levels in ATL cells ($n = 40$) compared with control CD4⁺ T cells ($n = 22$). It is most important that primary ATL samples show global miRNA downregulation, similar to observations in other cancer researches [96,97]. Of the 61 miRNAs, 59 (96.7%) exhibited decreased expression in ATL (Figure 7).

Among these, miR-31 was the most profoundly repressed miRNA in all ATL individuals (fold change of 0.00403). This is a known tumor suppressor that may also be associated with metastatic breast cancer [98]. Other downregulated miRNAs found in ATL patients may also be involved in the hallmark capabilities of ATL because they were uniformly decreased in tested ATL samples and each miRNA may regulate a large number of genes.

Several predictions and experimental approaches have defined a novel miR-31 target gene, *NIK*, which acts as a persistent NF- κ B activator in various malignancies (described above). Manipulation of the miR-31 level clearly indicated that the miR-31 level was negatively correlated with cellular NF- κ B activity. Importantly, enforced miR-31 expression in B cells attenuated both BAFF- and CD40L-mediated *NIK* accumulation and subsequent canonical and noncanonical NF- κ B signaling. Induced miR-31 expression or *NIK* knockdown reduces apoptotic resistant proteins such as *BCL-XL* and *XIAP*, which results in significant apoptosis among ATL cell lines as well as among primary leukemic cells from ATL patients [34]. Several lines of evidence definitively support two notions: (1) miR-31 acts as a tumor suppressor in T cells and (2) *NIK*-regulated NF- κ B is of pivotal importance to cancer cell survival (Figure 7). Taken together, based on the results of genetic studies and those on T-ALL and ATL, abnormal miRNA expression has a critical impact on the development of hematological malignancies.

Epigenetic regulation

Technological advances in genomics and epigenomics have provided new methods to distinguish one cell type from another. The epigenetic code consists of the combined on–off states of hundreds of genes, which coordinately dictate cellular identity and function.

Increasing attention is being paid to global regulatory factors and molecular mechanisms by which gene transcription control is regulated. Within these environments, genome programming operates fundamentally through DNA methylation, histone chemical modification, and protein complex binding.

The appropriate control of gene expression must be maintained in normal T cells by utilizing transcriptional networks as well as epigenetic controls. Indeed, several specific, comprehensive studies have demonstrated the epigenetic regulation of genes encoding for cytokines that are required for T cell development, differentiation, and activation [99]. Collectively, several global analyses have proposed that naïve T cell activation, differentiation, and lineage commitment result in epigenetic changes and that a fine balance between different histone modifications is required. In contrast, memory T cells are already poised and do not require epigenetic changes for short-term activation. It has been suggested that transcriptional memory, particularly epigenetic marks on chromatin, forms the underpinning of immunological memory [100-102].

Integrated histone modifications consequently decide the degrees of chromatin condensation and subsequent transcriptional sensitivity. Trimethylation of the histone H3 Lys27 (H3K27) mark plays a central role in repressing transcription, primarily in the euchromatin region. The polycomb family is a master regulator of the H3K27me3 level by inducing and maintaining the histone mark. Polycomb repressive complex 1 (PRC1) and PRC2 are recognized as essential molecular machines involved in polycomb-mediated gene silencing [103].

The polycomb family participates in the control of T cell activation. Enhancer of zeste homolog 2 (*EZH2*) serves as the catalytic subunit in PRC2 and mediates gene silencing by catalyzing H3K27 trimethylation in the promoters of target genes. One study showed that triggering of TCR and costimulation signaling in *Ezh2*-deficient T cells resulted in an impaired proliferative response [104]. The mechanism by which *Ezh2* can affect T cell signaling is not completely understood.

Deregulation by the polycomb family confers a specific gene expression pattern that is responsible for chronic proliferation, survival, peculiar development, and cancer-associated stemness in various cancer types, including ATL [105]. The involvement of the polycomb family in ATL was first revealed by global gene expression analysis. Significantly higher levels of *EZH2* as well as RING1- and YY1-binding protein (*RYBP*) transcripts with enhanced H3K27me3 levels were found in ATL cells compared with normal CD4⁺ T cells [106]. *EZH2* is highly expressed in many cancer types, including breast and prostate cancers and lymphomas, and its expression is often correlated with advanced stages of tumor progression and a poor prognosis. We recently identified a notable gene silenced by polycomb. A human gene that encodes for miR-31, *hsa-miR-31*, is located at 9p21.3, which is adjacent to clusters of the *CDKN2* and *IFNA* families. In addition to loss of this gene (12.5% of ATL cases), transcription of the miR-31 precursor was completely lost in ATL cells. Computational predictions and experimental evidence clearly demonstrated that an assembly of YY1-binding motifs upstream of the miR-31 region was responsible for the occupancy of the polycomb family at the target region, which resulted in H3K27me3-dependent transcriptional repression. Of note, given that miR-31 is a master regulator of the ATL-specific gene expression pattern described above, polycomb-mediated loss can influence gene expression downstream of miR-31 in T cells and other cell types (Figure 8) [34].

Intriguingly, several recent lines of evidence have shown two faces

of polycomb. It was surprising that these were some somatic mutations in the gene encoding EZH2 in some sets of mature B cell malignancies, including germinal center-type DLBCL (GCB-DLBCL) and follicular lymphoma [107]. However, subsequent studies have demonstrated that the mutations observed in B cell lymphomas sustained strong activity of histone methyltransferases.

Very recently, however, additional experimental evidence suggested that polycomb acted as a tumor suppressor in a cell type-specific manner. In disorders of HSCs or progenitors, such as myelodysplastic syndromes (MDS), myeloproliferative disorder (MPD), and AML, homozygous and heterozygous *EZH2* deletions or inactivating mutations are commonly observed [108], thus suggesting that loss of PRC2 contributes to oncogenesis in malignant myeloid diseases. Interestingly, inactivating mutations in H3K27 demethylase *UTX* have also been identified in myeloid malignancies. In immature T-ALL, *EZH2* is suggested to be a tumor suppressor. Several experimental approaches and clinical observations have indicated that a loss of *EZH2* contributes to the development of aggressive T-ALL in mice and humans [109-111].

Collectively, several studies of hematological disorders, including ATL and T-ALL, have indicated that the polycomb family acts as an oncogene in differentiated cells, such as mature T and B cells, and as a tumor suppressor in undifferentiated cells, such as hematopoietic stem/progenitors, which is an origin of acute leukemia (Figure 8). Importantly, the differentiation of these immune cells is directly regulated by polycomb-dependent epigenetic mechanisms [99,105]. The epigenetic background may be involved in distinct outputs. It must also be noted that the polycomb family regulates large numbers of genes in an epigenetic manner and by other routes (discussed below).

Novel Concept: Signal Cross Talking in T cell Regulation

Each of the signaling pathways described above contributes to T cell biology. However, physical and functional interactions between these pathways may be involved in complex T cell regulation. Based on this concept, we discuss the possible molecular interconnections that affect T cell functions and fates (Figure 9).

Notch and PI3K-Akt-mTOR

Several genetic lines of evidence and studies on T-ALL have demonstrated that the Notch pathway has central roles in T cells. Complex gene regulation through Notch may be sustained by signal crosstalk. As described above, the PI3K-Akt and mTOR pathways are connected to the Notch pathway through PTEN regulation. Notch-mediated Hes1 expression results in the transcriptional repression of PTEN [112]. PTEN negatively regulates the PI3K-Akt-mTOR pathway. In addition, a linkage between the Notch and mTOR pathways has been implicated. Treatment with a γ -secretase inhibitor

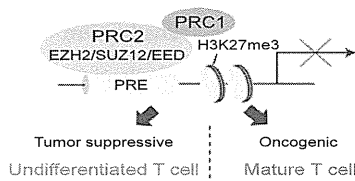


Figure 8: Mode of polycomb-dependent epigenetic gene regulation. Polycomb family is likely to act as an oncogene in differentiated cells such as mature T and B cells and as a tumor suppressor in undifferentiated cells such as hematopoietic stem/progenitors, which is an origin of acute leukemia.

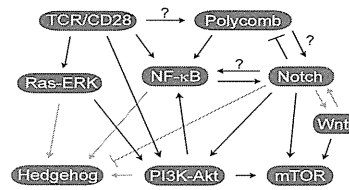


Figure 9: Crosstalk within signaling pathways. Each element contributes to regulation of gene required for T cell development, differentiation, and proliferation, respectively. However, substantially, they can interplay with each other, enabling T cell to form a more complicated regulatory network. The black lines represent relationship demonstrated in T cell. The gray ones show possible relationships suggested from non-T cell studies.

to inhibit the Notch pathway in T-ALL cell lines suggested a model in which Notch stimulated mTOR activity through a pathway that was independent of PI3K and Akt but dependent on c-Myc [113].

Notch and NF-κB

Crosstalk between Notch and NF-κB signalings has also been suggested. First, NF-κB2 (p100/p52) and IκBα have been identified as NOTCH1-targeted genes [114,115]. Second, the NF-κB component of the Rel family promoted the upregulation of Jagged1 expression and its functional interaction with Notch receptors on cocultured T cells [116]. Third, NF-κB subunits functioned as possible nuclear binding partners of intracellular Notch proteins. A complex containing Notch1, NF-κB1 (p50), and RelA could bind to DNA from the promoter region of the gene encoding IFN γ [117]. This study suggested that although an initial TCR-dependent NF-κB activation did not require the Notch signaling, intracellular Notch protein appeared to be necessary for sustained NF-κB activation in T cells. Finally, results of a recent study suggested that Hes1, which is positively regulated by Notch, appeared to sustain the NF-κB activation by repressing the expression of deubiquitinase CYLD, a negative regulator of the IKK complex, in T-ALL cells [118]. In contrast, some reports have indicated that Notch signaling could inhibit NF-κB activation by physical blocking mechanisms [119,120]. Delineation of the exact molecular mechanism involved in the crosstalk between Notch and NF-κB is urgently needed.

Notch and Wnt

The Notch and Wnt pathways, which were first discovered in *Drosophila*, are evolutionary conserved signaling pathways and control the earliest steps of T cell development. It would seem that the Wnt and Notch pathways have opposing functions in the thymus. Wnt signaling serves cell proliferation, whereas Notch signaling regulates cell differentiation. These processes are mutually exclusive. However, gain-of-function mutations in the positive effectors of the Notch (NOTCH1) and Wnt (β -catenin) pathways lead to cancer development, which indicates that both may participate in cell proliferation.

One report has suggested the molecular cooperation between the Notch and Wnt pathways in HSC self-renewal [121]. Notch signaling has a dominant function in inhibiting HSCs differentiation, in which Wnt signaling mediates cell proliferation. It is also suggested that Notch signaling is required for Wnt-mediated maintenance of undifferentiated HSCs but not for their survival or entry into the cell cycle. With regard to lymphoma development, β -catenin-dependent development of lymphoma appears to be independent of Notch signaling [60].

Molecular crosstalk between the Wnt and Notch pathways has

been described, including genetic interactions in *Drosophila*, physical interaction between Notch and β -catenin, and their associations with common cofactors. In mammalian cells, GSK-3 β directly phosphorylates Notch protein and modulates its transcriptional activity [122]. Moreover, β -catenin activates Jagged1 transcription thus leading to Notch activation in colorectal cancer [123]. Inhibition of Wnt and Notch results in downregulation of a common gene program. Conversely, in different types of tumor cells, Notch activates the Wnt pathway and stabilizes β -catenin by unknown mechanisms in melanoma [124] or by the transcriptional activation of Slug [125] in breast cancer. More intensive physiological and molecular studies regarding the associations between Notch and Wnt signaling should be conducted.

Notch and the polycomb family

A recent study suggested that NOTCH1 activation specifically induced the loss of the repressive H3K27me3 by antagonizing the PRC2 activity [111]. This suggests a tumor suppressor role for PRC2 in human acute leukemia and a novel concept regarding the dynamic interplay between oncogenic NOTCH1 and PRC2 function for the regulation of gene expression and cell transformation. In immature lymphoid and myeloid cells, the polycomb family might act as tumor suppressors [109-111]. Conversely, in mature T lymphocytes, the polycomb family clearly functions as oncogenes. In ATL, both the polycomb and Notch pathways are strongly activated, which contributes to the expression of several genes and signaling pathways [30,34,48]. The two faces of the polycomb family need to be addressed in the near future.

Wnt and mTOR

In response to Wnt activation, GSK-3 inhibition appears to result in the inactivation of the tumor suppressor complex TSC1/2, followed by activation of the mTOR signaling pathway [126]. GSK-3 can phosphorylate and stimulate the TSC2 in a coordinated manner, which results in TORC1 inactivation and reduced protein synthesis. Therefore, Wnt activation can inhibit the TSC1/2 complex and activate mTOR signaling through GSK-3. In T cells, both the Wnt and mTOR pathways are involved in T cell development, as described above. Thus, the crosstalk between these pathways might shed some light on the molecular basis of T cell development. In addition, from a pathological viewpoint, this provides a novel therapeutic concept for Wnt signaling pathway-dependent cancers. Rapamycin may prove to be efficacious for treating tumors with activated Wnt.

Hedgehog and other signaling pathways

Hh signaling is indeed physiologically interconnected with Ras-Raf-MEK, PI3K-Akt, TGF- β , Notch, and NF- κ B signaling in several types of cells [127]. In a complicated interaction, the Ras-Raf-MEK and PI3K-Akt pathways modulate the amount and activity of GLI, which results in the transactivation of Hh signaling-dependent gene expression. TGF- β also activates Hh signaling through GLI2 induction and binding with GLI family. In contrast, in mammalian skin, Notch deficiency increases GLI2 levels, which causes Hh activation and development of basal cell carcinoma-like tumors. This suggests opposing roles of Notch and Hh [128].

NF- κ B activation is one of the mechanisms underlying Shh overexpression in pancreatic cancer. It has been suggested that proliferation of pancreatic cancer cells is accelerated by NF- κ B activation, in part, through Shh overexpression [129]. These interconnections between numerous oncogenic pathways have important implications for T cell regulation. These connections

associated with Hh signaling have not yet been reported in studies of T cells. Complex, yet hierarchical, gene regulation may be achieved during appropriate T cell development and homeostasis.

Polycomb and NF- κ B

As described above, the polycomb family regulates the expression of a large number of genes by epigenetic molecular mechanisms. In turn, this suggests that gene expression fluctuations induced by the polycomb family may be involved in other signaling pathways that participate in T cell regulation.

Using ATL as a model of a mature T cell disorder, we have demonstrated that the cellular amounts of PRC2 components, EZH2 and SUZ12, indirectly affected the NF- κ B signaling pathway [34]. Expression levels of EZH2 and SUZ12 directly strengthened the cellular miR-31 depletion, which in turn activated the NF- κ B pathway through NIK induction and conferred anti-apoptotic features to T cells. The dynamic regulation of miR-31 by polycomb-dependent epigenetic machinery is critical for mature T cell-derived leukemic cell survival and other hallmark capabilities, such as promoting cell cycle, invasiveness, chronic proliferation, replicating immortality, and drug resistance, because both miR-31 and NF- κ B signaling can predominantly control the expression of genes responsible for these processes [30]. It is also noteworthy that the molecular and biological interconnections between polycomb-miR-31-NF- κ B are conserved in breast cancer cells and B lymphocytes. By organizing this new principle, various cell types may realize the more complex gene regulatory networks required for maintaining and executing various cellular functions. An imbalance in this network probably switches the cell fate from one to another.

Our findings on ATL study also provided new ideas: YY1 is one of the recruiters of PRC2 in T cells. In humans, polycomb response elements (PREs) have not been definitively identified. A good candidate for a mammalian recruiter of PRC2 is YY1, which is the homolog of *D. melanogaster* PHO and is abundantly expressed in almost all cell types [130]. The detailed mechanism by which YY1 mediates the recruitment of the polycomb family may be important with regard to epigenetic regulation of orchestrated gene expression and T cell functions. Identification of targets of polycomb, such as miR-31, will facilitate our understanding of the molecular mechanisms of T cell regulation.

Interconnections between the polycomb and NF- κ B pathways have been reported in other cell types. Epigenetic silencing of the RasGAP gene *DAB2IP* is a key mechanism by which EZH2 activates Ras and NF- κ B and triggers metastasis in prostate cancer [131]. In addition, physical interactions between EZH2 and RelA/RelB were suggested in basal-like breast cancer cells [132]. Thus, our findings and molecular mechanisms introduced from other cell types suggest that polycomb-mediated epigenetic changes modulate NF- κ B signaling activity in mature T cells.

miRNA control of signaling transduction

After the identification of hundreds of functional miRNAs, the challenge now is to understand their specific biological functions. Signaling pathways are ideal candidates for miRNA-mediated regulation owing to their robustness, which is the capacity of biological systems to generate invariable phenotypes even when encountering genetic or environmental perturbations. miRNAs have been proposed to contribute to robustness by regulating signaling pathways, including TGF- β , Wnt, Notch, Hh, and pathways driven by receptor tyrosine kinases [133]. A clear indication from current studies is that miRNAs

participate in signaling networks, both as backups for transcriptional control and as feed-forward or feedback devices that confer robustness to the output of cell signaling (Figure 10). Thus, the effects of a miRNA can be the result of the net effects of opposing regulations or of the activities against mutually inhibiting factors. Therefore, new miRNA functions may be revealed as we refine our understanding of the networks in which they operate.

Some recent T cell studies have provided support for this proposition. In T-ALL, miR-19 upregulation results in the enhancement of the activities of PI3K-Akt and mTOR pathways by regulating Bim (Bcl2L11), AMP-activated kinase (Prkaa1), and the phosphatases Pten and PP2A [91].

NF- κ B signaling is also linked to miRNAs. One study focused on the functional roles of miR-146a in T cell-mediated immune responses [134]. miR-146a was induced by TCR stimulation in naïve T cells. Moreover, NF- κ B- and c-ETS-binding sites were required for inducing miR-146a transcription after TCR engagement. Interestingly, miR-146a could impede both AP-1 activity and IL-2 production induced by TCR engagement. In contrast, miR-146a targeted TRAF6 and IRAK1, two key protein adapters that activate a spectrum of signaling pathways from NF- κ B to ERK [135]. It was also noted that HTLV-1 Tax could induce miR-146a expression through NF- κ B activation [93].

As described above, our comprehensive study revealed novel interconnections between miR-31 and noncanonical NF- κ B pathways in T cells [34]. The dynamic regulation of miR-31 by polycomb is critical for sustaining anti-apoptosis and proliferation capabilities in ATL cells. This polycomb-miR-31-NF- κ B linkage provides speculation that, in addition to controlling transcription, the polycomb group can modulate translation via miRNA regulation (Figure 10). This signaling circuit permits the regulation of multiple genes. Another comprehensive study revealed that lymphocyte-specific miRNAs were either tightly controlled by polycomb group-mediated H3K27me3 or maintained in a semi-activated epigenetic state prior to their full expression [136].

Concluding Remark

Gene expression control ultimately regulates T cell fate and activation. In reality, very complex signaling pathways appear to be involved in the biology of T cell commitment. Using mouse models, in vitro studies, as well as patient's samples, a large number of studies have revealed novel conceptual advances that explain the complex regulation of genes in T cells. However, some discrepancies have also been shown. It should be noted that although each of the signaling

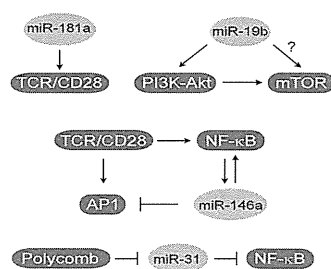


Figure 10: miRNA control of signal transduction. miRNAs participate in signaling networks, both as backups of transcriptional control and as feed-forward or feedback devices that confer robustness to the output of cell signaling.

pathways described in this review must contribute to gene expression, crosstalk between these pathways is required for realizing the complex gene regulatory network required for the complete functional activity in T cells. It also should be noted that the tumorigenic precursor T cells (i.e. T-ALL) are not terminally differentiated and their molecular setting does not appear to be directly associated with the mature TCR/CD28 signaling. It is required to further investigate physiological signaling pathways in terminally differentiated T cells to those observed in malignant T-ALL and ATL. To further our understanding of the precise molecular mechanisms involved in T cell activation and regulation in the immune system and disorders, the biological significance and interference with each other should be considered in future signaling research.

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Molecular hallmarks of adult T cell leukemia

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The molecular hallmarks of adult T cell leukemia (ATL) comprise outstanding deregulations of signaling pathways that control the cell cycle, resistance to apoptosis, and proliferation of leukemic cells, all of which have been identified by early excellent studies. Nevertheless, we are now confronted the therapeutic difficulties of ATL that is a most aggressive T cell leukemia/lymphoma. Using next-generation strategies, emerging molecular characteristics such as specific surface markers and an additional catalog of signals affecting the fate of leukemic cells have been added to the molecular hallmarks that constitute an organizing principle for rationalizing the complexities of ATL. Although human T cell leukemia virus type 1 is undoubtedly involved in ATL leukemogenesis, most leukemic cells do not express the viral protein Tax. Instead, cellular gene expression changes dominate homeostasis disorders of infected cells and characteristics of ATL. In this review, we summarize the state of the art of ATL molecular pathology, which supports the biological properties of leukemic cells. In addition, we discuss the recent discovery of two molecular hallmarks of potential generality; an abnormal microRNA pattern and epigenetic reprogramming, which strongly involve the imbalance of the molecular network of lymphocytes. Global analyses of ATL have revealed the functional impact of crosstalk between multifunctional pathways. Clinical and biological studies on signaling inhibitory agents have also revealed novel oncogenic drivers that can be targeted in future. ATL cells, by deregulation of such pathways and their interconnections, may become masters of their own destinies. Recognizing and understanding of the widespread molecular applicability of these concepts will increasingly affect the development of novel strategies for treating ATL.

Keywords: HTLV-1, ATL, genome, epigenetics, miRNA, signal transduction

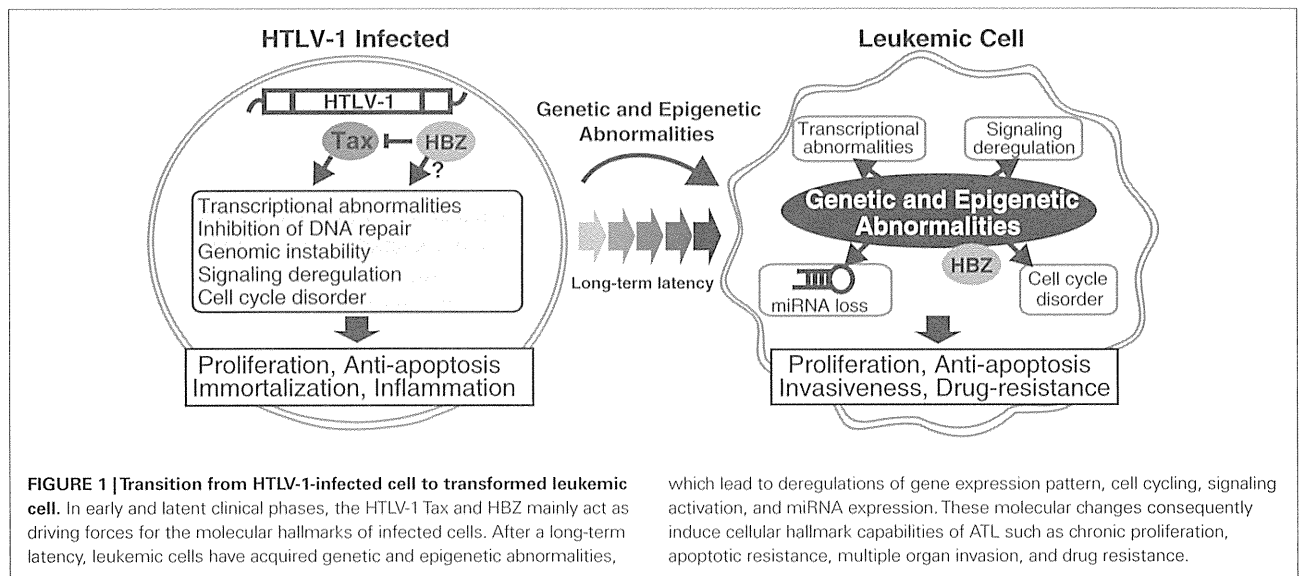
INTRODUCTION: CURRENT STATUS OF ADULT T CELL LEUKEMIA RESEARCH

Adult T cell leukemia (ATL), which is derived from human T cell leukemia virus type 1 (HTLV-1)-infected CD4⁺ T cells, is an aggressive T cell leukemia/lymphoma with the worst poor prognosis (Yamaguchi and Watanabe, 2002). Endemic expansion of HTLV-1 infection and related ATL onset have been observed in Japan (Iwanaga et al., 2010). The diverse clinical features of ATL have led to its sub-classification into acute, lymphoma, chronic, and smoldering subtypes. ATL has a poor prognosis with a mean survival time of 13 months and is refractory to currently available combination chemotherapy (Yamada et al., 2001). It is therefore essential to develop a novel treatment strategy, in particular, a molecular targeting therapy. ATL is incurable because we do not have complete understanding of its molecular basis, leading to the lack of molecular targeting. Although HTLV-1 is an apparent causative agent of ATL, several studies have demonstrated that viral gene expression is rare, except for the expression of the HTLV-1 antisense gene product HBZ (Gaudray et al., 2002). Mounting evidence has shown that ATL does not contain the somatic mutant genes that can explain its aggressiveness. However, it is evident that ATL cells possess multiple deregulations of genome and gene regulation, namely the molecular hallmarks of ATL, which should be targeted (Figure 1). We believe that normal cells acquire a succession of molecular hallmarks as they progressively evolve into a

neoplastic state and that the multistep process of human pathogenesis can be rationalized by the need of incipient cancer cells for acquiring traits that enable them to become leukemogenic and ultimately malignant. In this review, we first summarize the essence of each molecular hallmarks of ATL cells. Basic molecular analyses and next-generation global analyses of ATL samples have revealed the molecular traits of ATL. We address new developments that broaden the scope of the conceptualization and describe recent advances of science to acquisition of the molecular mechanistic underpinnings. Finally, we discuss the possibility and future direction of treating ATL.

MOLECULAR HALLMARKS CONTROLLED BY TAX AND HBZ

Since 1985, numerous excellent studies have identified signaling abnormalities in HTLV-1-associated cells, mainly induced by HTLV-1 Tax. In general, T cell disorders include several deregulations of cellular processes that regulates the cell cycle, cell proliferation, and cell survival. Tax has been shown to disrupt all these cellular processes. The classical oncogenic function of Tax was first demonstrated in a study of cell cycle regulation. Tax was found to inhibit the cyclin-dependent kinase (CDK) inhibitor (CKI) CDKN2A (p16^{INK4A}) by physical interaction (Suzuki et al., 1996). Several subsequent studies also revealed the mitogenic activity of Tax exerted through the stimulation of G1 to S phase transition (Akagi et al., 1996; Neuveut et al., 1998; Schmitt et al., 1998;



Suzuki et al., 1999; Iwanaga et al., 2001; Haller et al., 2002; Liang et al., 2002). Tax also affects a cohort of cell cycle-related proteins, including CDKs, CDKN1A, CDKN1B, and CDKN2A, by regulating their expression or by physical interaction. Tax also participates in genetic damage (Jeang et al., 1990; Ressler et al., 1997; Kao and Marriott, 1999). Because of particular functions of Tax that can interact with many host factors (Interactome; Boxus et al., 2008; Simonis et al., 2012), Tax can activate several signaling pathways and lead to abnormal gene expression (a Tax-dependent molecular hallmark) and overproduction of several cytokines. Especially, IL-2 and its receptor are important for T cell activation. IL-2 signals through its receptor are primarily delivered by two molecular families, the Janus tyrosine kinases (JAKs) and signal transducers and activators of transcription (STATs), whose activation leads to lymphocyte proliferation. Tax can also activate the NF-κB and NFAT pathways responsible for the predominant expression of IL-2 and the IL-2 receptor (Ballard et al., 1988; Ruben et al., 1988; Hoyos et al., 1989; McGuire et al., 1993; Good et al., 1996). These findings implicate the IL-2–IL-2 receptor autocrine loop in ATL; however, several studies have shown that alterations of the loop alone are not sufficient to ensure the maintenance and proliferation of ATL cells because most Tax- or HTLV-1-immortalized T cells still require exogenous IL-2 and do not express detectable amounts of either IL-2 mRNA or protein (Akagi and Shimotohno, 1993; Chung et al., 2003; Chen et al., 2010). Similarly, IL-15 and its receptor and IL-13 and its receptor have been associated with Tax-expressing cells because similar to IL-2, they activate the STAT pathways (Azimi et al., 1999; Mariner et al., 2001; Chung et al., 2003; Wäldele et al., 2004). IL-6 is also transduced by Tax through the NF-κB pathway (Villiger et al., 1991; Muraoka et al., 1993). Because IL-6 mainly participates in inflammatory signaling, HTLV-1 infection can induce cytokine-dependent inflammation, which is frequently observed in ATL as well as HTLV-1-associated myelopathy/Tropical spastic paraparesis (HAM/TSP; Oh et al., 2011). OX40, a member of the TNF-receptor superfamily, is

specifically expressed in HTLV-1-infected cells, whose expression is induced by the Tax-NF-κB pathway. Tax may play a role in leukemic cell infiltration in addition to cell adhesion *in vivo* (Imura et al., 1997). Tax affects not only the abovementioned signaling pathways but also the TGF-β pathway (Kim et al., 1990; Höllsberg et al., 1994; Arnulf et al., 2002; Lee et al., 2002). It has been recently shown that TGF-β signaling is activated by HBZ by binding with Smad 2/3 (Zhao et al., 2011). TP53 is the master regulator of the cell cycle that guards against DNA damage by inducing the transcription of several genes. Tax can inhibit TP53 functioning in multiple ways (Grassmann et al., 2005).

Strong NF-κB activation is the outstanding hallmark provided by Tax. NF-κB represents a family of inducible transcription factors that regulate diverse biological processes, including the growth and survival of both T cells and non-lymphoid cells. Transcriptional activation of genes such as several cytokines and apoptosis-resistance factors plays an important role in immunity. Tax acts as an intracellular stimulator of IKK by physical interaction, leading to persistent activation of NF-κB-mediated transcription. The Tax/IKK complex formation relies on the physical interaction between Tax and the IKK regulatory subunit IKKγ. The Tax/IKKγ interaction is required for recruiting Tax to the IKK catalytic subunits and for Tax-mediated IKK activation (Sun and Yamaoka, 2005). Recent studies have identified cellular proteins that are important for Tax-mediated NF-κB activation, such as NRP/Optineurin and TAX1BP1 (Journo et al., 2009; Shembade et al., 2011), and the ubiquitin-specific peptidase USP20 (Yasunaga et al., 2011). Subcellular localization of Tax also predominantly controls Tax-mediated NF-κB activation (Fryrear et al., 2009). Given that NF-κB governs the expression of a large array of cellular genes that control various cellular functions, the phenotypes of HTLV-1-infected cells are dominated by Tax-mediated abnormal activation.

Tax also activates several signaling pathways through key transcriptional factors such as CREB, SRF, and AP-1. It does not directly

bind to promoter or enhancer DNA, however, disruption of these pathways causes serious gene expression disorders (Grassmann et al., 2005).

It should be also noted that HTLV-1 antisense product HBZ seems to be involved in leukemogenesis; its expression is sustained in leukemic cells. *In vitro* and *in vivo* studies have demonstrated that the growth-promoting activity of HBZ RNA may play an important role in oncogenesis by HTLV-I (Satou et al., 2006). Furthermore, transgenic expression of HBZ in CD4+ T cells induces T cell lymphomas and systemic inflammation in mice. HBZ directly induces *Foxp3* gene transcription, and the increased CD4+Foxp3+ T_{reg} cells in HBZ transgenic mice are functionally impaired, suggesting that the expression of HBZ in CD4+ T cells may be a key mechanism of HTLV-1-induced neoplastic and inflammatory diseases (Satou et al., 2011).

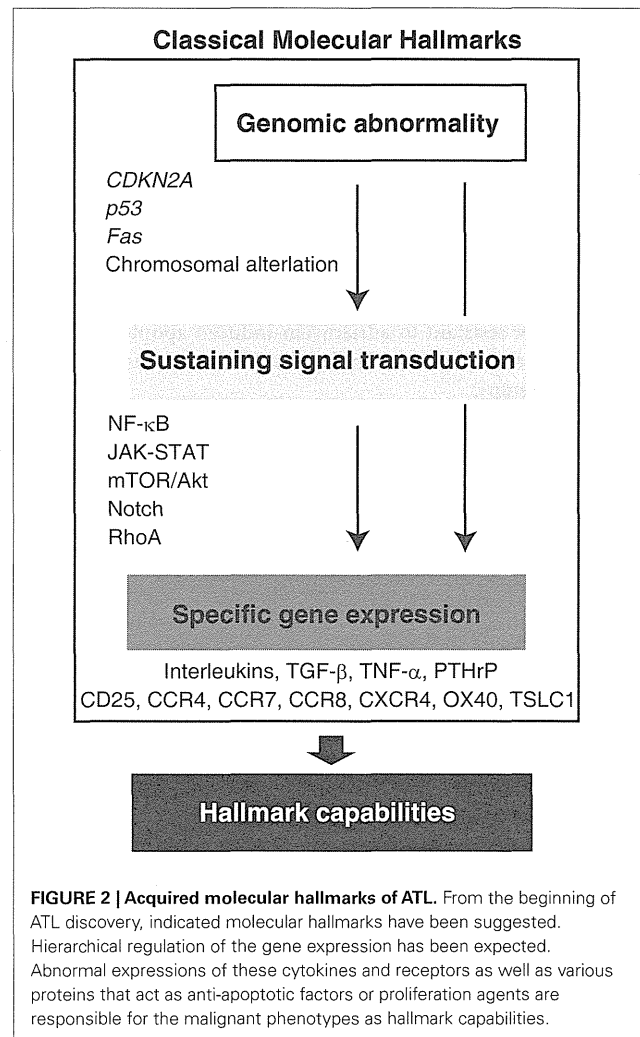
Taking together with these mounting evidences, Tax and HBZ undoubtedly contribute to leukemogenesis in HTLV-1-infected T cells. However, as a low rate of incidence, clinical observation implies that HTLV-1 itself does not have a strong capacity of leukemogenesis in contrast with other animal leukemia viruses.

CHROMOSOMAL CHANGES AND GENE ALTERATIONS IN ATL

Tax is not expressed in most ATL cases because HTLV-1 provirus is substantially silenced by proviral defect and/or epigenetic mechanism (Tamiya et al., 1996; Koiwa et al., 2002; Taniguchi et al., 2005). However, leukemic cells possess very similar traits to Tax-expressing cells (Figure 1). The paradoxical truth, i.e., memory of Tax, still remains to be elucidated. Investigation of established ATL cell lines and primary ATL samples has led to the identification of the molecular hallmarks of leukemic cells, which may partially explain their malignant characteristics.

From 1980s, chromosomal analyses of clinical cases were reported. Therefore, we know that ATL is characterized by various abnormal chromosomes. Kamada et al. (1992) reported that 96% of ATL cases had an abnormal chromosome pattern, suggesting that a genomic catastrophe underlies the clinical and molecular characteristics of ATL, which is consistent with all other cancers. In 2000s, global analysis has been available and whole genomic analysis could be challenged. Comparative genomic hybridization (CGH) revealed the genomic distinctions between the clinical subtypes; patients with aggressive ATL (acute and lymphoma) have a higher number of chromosomal imbalances, losses, and gains than those with indolent ATL (chronic and smoldering). Thus, genomic abnormalities are prognostic factors (Tsukasaki et al., 2001a). High-resolution analyses based on microarrays unveiled the genomic properties of ATL (Oshiro et al., 2006) and shed light on the genomic characteristics that affect the gene expression pattern (Figure 2).

In cancer cells, genetic mutations and deletions are generally observed in genes important for cell cycle regulation, cell survival, and cell proliferation. In ATL, the accumulation of genetic deletions in the gene encoding the cell cycle regulator *CDKN2A* have been reported (Hatta et al., 1995; Uchida et al., 1996). Southern blotting analyses of samples from large cohort studies (114 patients) also demonstrated that the *CDKN2* family (*p15* and *p16*) is lost in more frequently observed in the acute type (33.8%) than in the chronic type of ATL (5.4%; Yamada et al., 1997). Patients



with these genetic deletions showed reduced survival compared with those without deletions. *CDKN2A* transcription is also regulated in an epigenetic manner. Abnormal DNA methylation has been during ATL progression (Nosaka et al., 2000).

Alterations of other cell cycle-regulating genes are unusual. Mutations of *CDKN1B* (*p27^{Kip1}*) is rare in non-Hodgkin's lymphoma and ATL (Morosetti et al., 1995). Mutations of *Rb* gene have also been observed in a few ATL patients (Hatta et al., 1997). Extensive analyses have revealed that 26–40% of ATL cases have a *p53* gene mutation, which is much less than that observed in other cancers (Nagai et al., 1991; Cesarman et al., 1992; Sakashita et al., 1992; Nishimura et al., 1995). Gene expression analysis of ATL samples showed that *CDKN1A* (*p21^{Cip1}*) is commonly down regulated in ATL (Watanabe et al., 2010). In contrast, Tax activates *p21^{Cip1}* expression in HTLV-1-infected cells, which may be an important mechanism for stopping the host at the G1/S boundary and repairing damaged DNA before entering the S phase entry (Cereseto et al., 1996; de La Fuente et al., 2000). Although *p21^{Cip1}* transcription may be activated by the Tax-CREB pathway in infected cells,

it seems to be epigenetically silenced in several cancers, including ATL. Thus, these gene alterations and related expression patterns are important molecular hallmarks of ATL as well as other cancers. Of note, no ATL cell has been shown to have both mutations, suggesting that either *p16* or *p53* mutation may be sufficient to promote the more aggressive phenotype of leukemic cells (Tawara et al., 2006). In addition, as many ATL patients carry a wild-type *p53*, TP53 activation by antagonists such as Nutlin-3a may be a promising strategy for ATL therapy (Hasegawa et al., 2009). The ability to resist apoptotic cell death is conferred by *Fas* gene mutation (Tamiya et al., 1998; Maeda et al., 1999). Indeed, *Fas*-negative ATL cells are resistant to adriamycin-induced apoptosis *in vitro*, which is consistent with the finding that ATL in this case is resistant to chemotherapy.

Single nucleotide polymorphism (SNP) is another possible cause for developing ATL. TNF- α polymorphism may be associated with increased susceptibility to development of ATL in HTLV-1 carriers (Tsukasaki et al., 2001b). An immunological study also suggested that the HLA haplotype is related to ATL progression (Yashiki et al., 2001). Currently, whole SNP can be detected by next-generation sequencing and other array-based techniques. Global understanding of SNPs in ATL patients may provide us with the genetic basis of the familial clustering of ATL.

By utilizing global survey techniques for analyzing the gene copy number, several research groups have detected specific gene copy number patterns in ATL. CGH methods first demonstrated gains at 14q32 and 2p16-22 in ATL cell lines (Ariyama et al., 1999). Fluorescence *in situ* hybridization (FISH) analysis with several yeast artificial chromosome (YAC), BAC, and PAC clones also mapped the breakpoints in ATL cell lines with 6q aberrations (Tagawa et al., 2002). Clinical subtype-specific genomic alterations in aggressive ATL were also identified by array-based CGH (Oshiro et al., 2006). The lymphoma type of ATL had significantly more frequent gains at 1q, 2p, 4q, 7p, and 7q and losses at 10p, 13q, 16q, and 18p, whereas the acute type showed a gain of 3/3p. *CARMA1* is also a possible target gene for 7p22 amplification in the lymphoma type but not in the acute type of ATL. In contrast, *BCL11B* is expressed in the acute type of ATL, regardless of 14q32 gain/amplification; however, there is no or low expression of the gene in the lymphoma type of ATL. Taken together, these findings suggest that the acute and lymphoma types of ATL are genomically distinct. The physiological molecular hallmarks of ATL have been identified by integrating the gene copy number and gene expression analyses findings, and these are described below.

GLOBAL GENE EXPRESSION ANALYSES OF ATL

Genetic alterations and other modulations enhance specific gene expression signatures. Classically, based on a comparison between normal T cells and ATL cell lines as well as primary leukemic cells, ATL has been shown to have a distinct gene expression pattern that may reflect its clinical pathogenesis. In addition, global expression analyses, mainly based on microarray, have identified emerging molecular hallmarks that can possibly be targeted in ATL therapies.

First, Harhaj et al. (1999) performed gene array analysis of HTLV-1-immortalized cells and reported up- and downregulated genes. Ruckes et al. (2001) performed suppressive subtractive

hybridization that enabled the isolation of novel sequences derived from unknown genes. They identified a number of genes linked to Tax transformation and ATL leukemogenesis. Systematic comparison of gene expression between cultured cells from patients with acute ATL and that of stimulated peripheral T lymphocytes revealed 346 cDNA clones, which included the genes encoding IL-2 receptor α chain and p21^{Cip1}. They also included a dual-specific protein phosphatase (PAC1), an interferon-inducible factor (ISG15), a basic helix–loop–helix transcription factor (DEC-1), and the secreted anti-apoptotic chemokine I-309. Pise-Masison et al. (2002) also reported the results of analyses performed using an Affymetrix Hu6800 GeneChip. They identified 763 genes with differentially regulated expression in HTLV-1 cell lines.

Tsukasaki et al. (2004) directly analyzed the gene expression patterns of fresh ATL samples (three acute types and one chronic type) using high-density oligonucleotide DNA arrays. A total of 203 genes that included ribosomal proteins, proteasome subunits, translation factors, immunophilins, heat shock proteins, and genes important for DNA replication were found to be upregulated in ATL cases, and 91 genes were downregulated. Sasaki et al. (2005) also determined the expression profiles of more than 12,000 genes in eight cases of the acute type of ATL using microarray analysis, and they found that 192 genes were upregulated more than twofold compared with healthy CD4+ and CD4+/CD45RO+ T cells. In particular, the expression of tumor suppressor in lung cancer 1 (*TSLC1*), caveolin 1, and prostaglandin D2 synthase was increased by more than 30-fold. Interestingly, comparison of gene expression between established cell lines with or without HTLV-1 infection led to the identification of the specific HTLV-1-related cell surface marker CD70, which is also expressed in freshly isolated leukemic cells (Baba et al., 2008).

Integrated analyses of the genome and its expression revealed more detailed data for the acquisition of molecular and physiological information. Choi et al. (2007) isolated CD4+ T cells from ATL patients and profiled their gene expression using DNA microarrays containing >44,000 probe sets. Changes in the chromosomal copy number were examined for 24 cell specimens using microarrays harboring approximately 50,000 probe sets. Coordinated analysis revealed a novel molecular target, the HGF–MET pathway. Ligation of MET by increased plasma HGF levels may confer a growth advantage on ATL cells through the phosphatidylinositol 3-kinase (PI3K) and Ras pathways.

We performed further global integrated analyses of samples from ATL patients using mRNA expression array ($n = 52$), microRNA (miRNA) microarray ($n = 40$), and DNA copy number analysis using SNP-based array ($n = 168$; Yamagishi et al., 2012). Each analysis revealed many novel molecular characteristics of ATL (data available in the Gene Expression Omnibus database). Analysis of this massive dataset derived from primary ATL samples led to the conclusion that genetic and epigenetic imbalances are involved in ATL leukemogenesis through miRNA and the signaling pathways responsible for leukemic cell survival, proliferation, and invasiveness. Taken together, these findings clearly indicate that disrupted gene expression is a molecular hallmark of ATL. Diverse abnormalities have been found in each of these comprehensive studies; however, several gene alterations and other critical events have been commonly implicated as determinants of the gene

expression pattern. Abnormal expression of different cytokines, their receptors, and various proteins that act as anti-apoptotic factors or proliferation agents is responsible for malignant phenotypes as hallmark capabilities (Figure 2). From the spreading intelligence, we have to acquire the genuine molecular targets harboring in the bare bones of ATL.

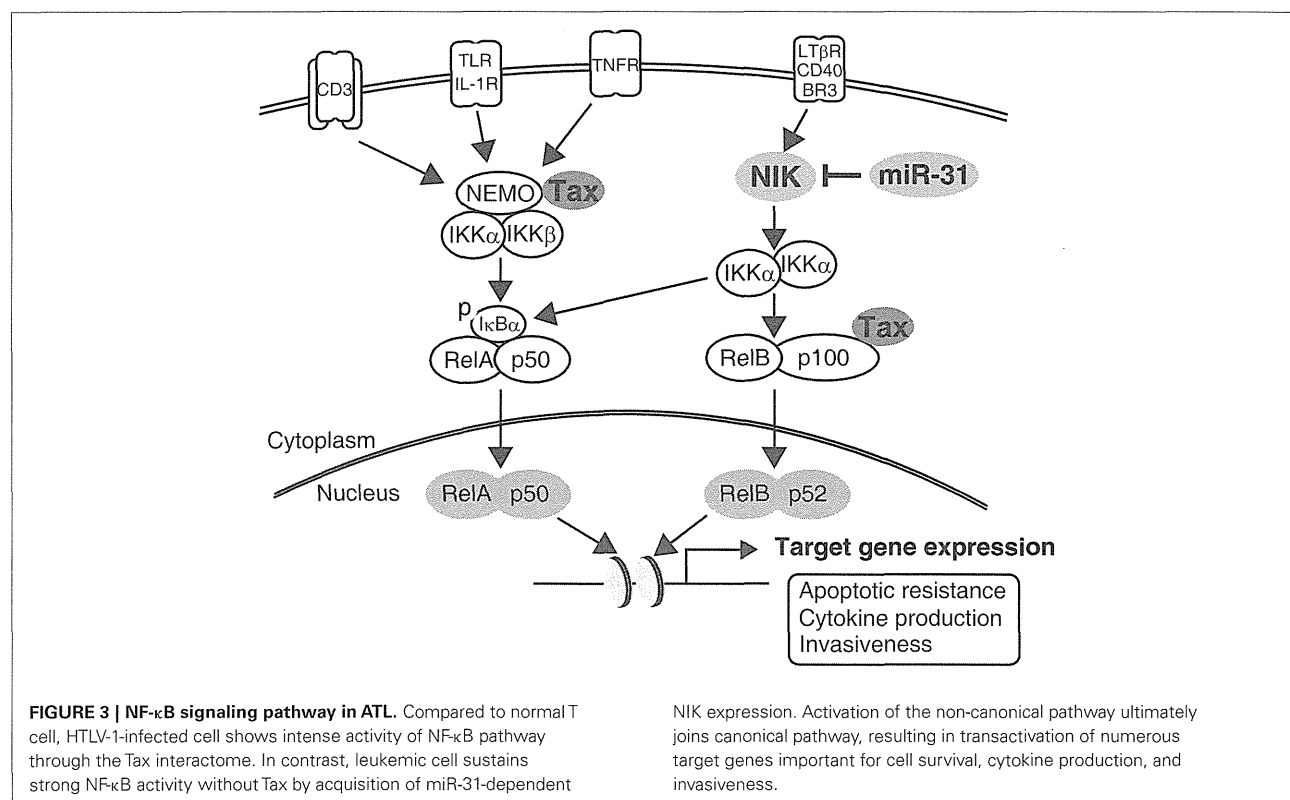
SIGNALING NETWORKS IN ATL: MAIN DRIVING FORCES

The cellular complexity of signaling networks confers robustness to specific gene expression and biological functions. This is supported by the fact that the inhibition of a signaling pathway can strictly suppress abnormal gene expression. The functional characteristics of ATL cells, such as chronic proliferation, abnormal survival, and penetrating multiple organs, is supported by aberrant gene expression, as described above.

Activation of NF- κ B signaling is the most outstanding hallmark of both HTLV-1-infected cells and leukemic cells. Abnormal activation and deficient negative control of the NF- κ B pathway strongly contributes to prolonged survival, proliferation, and invasiveness, all of which are observed in ATL cells. Ruben et al. (1988) found that Tax can activate IL-2 receptor gene expression in a NF- κ B-dependent manner. Following this, several studies clearly demonstrated that Tax can activate NF- κ B activity through a multistep (Sun and Yamaoka, 2005). Strong and persistent activation of NF- κ B signaling was then demonstrated in leukemic cell lines that did not express Tax (Mori et al., 1999). In transformed ATL cells, both the canonical and non-canonical NF- κ B pathways are persistently activated because NF- κ B inducing kinase (NIK) is aberrantly expressed in ATL (Saitoh et al., 2008). NIK plays a central role in

non-canonical NF- κ B signaling by IKK α phosphorylation (Thu and Richmond, 2010). Very recently, we found a novel linkage between NF- κ B activation and miRNA deregulation. Comprehensive gene expression analysis and *in vitro* experiments revealed that miRNA-31 (miR-31) can regulate NIK expression through the 3' untranslated region (UTR). In ATL cells, miR-31 expression is genetically and epigenetically silenced, which in turn induces constitutive NF- κ B activation through NIK expression (Figure 3; Yamagishi et al., 2012). Because current evidence clearly indicates that miR-31 dominates NF- κ B activity in T cells, manipulating cellular miR-31 levels may be a novel molecular approach to reduce NF- κ B activity and induce apoptosis.

Numerous efforts have demonstrated that NF- κ B inhibitory agents may be suitable for treating ATL. Inhibitors such as bortezomib (Satou et al., 2004), Bay 11-7082 (Mori et al., 2002), DHMEQ (Watanabe et al., 2005), ACHP (Sanda et al., 2006), and IMD0354 (Uota et al., 2012) have the ability to kill leukemic cells. Fludarabine, a purine analog that has significant activity in B cell malignancies, also inhibits the NF- κ B activity in ATL (Nishioka et al., 2007). Tax degradation by arsenic trioxide and IFN- α treatments is also useful for inhibiting NF- κ B in HTLV-1-infected cells (El-Sabban et al., 2000). In the near future, gene manipulation for example by siRNA targeting NIK may be a promising strategy for inhibiting NF- κ B activity with strict specificity (Yamagishi et al., 2012). Because NF- κ B prevention showed good results in a xenograft model of cell lines with or without Tax (Dewan et al., 2003; Watanabe et al., 2005; Ohsugi et al., 2007a), molecular targeting therapy based on the NF- κ B pathway is a promising new ATL treatment. Of note, NF- κ B inhibition by DHMEQ can also remove



virus-carrying cells from carrier peripheral blood mononuclear cell (PBMC) samples (Watanabe et al., 2005).

Why is NF- κ B important for ATL cell survival? One of the target genes is BCL-XL, which is expressed when NF- κ B is stimulated. In HTLV-1-infected cell lines, BCL-XL is expressed through the Tax-NF- κ B pathway. Interestingly, fresh ATL samples show BCL-XL overexpression (Nicot et al., 2000). Given that BCL-XL is a principal anti-apoptotic protein as a BCL-2 family member, persistent BCL-XL expression is one of the molecular capability of apoptotic resistance, which may contribute to clinical chemoresistance. Indeed, inhibition of the NF- κ B pathway by NIK depletion leads to impaired BCL-XL levels and apoptotic cell death in ATL (Yamagishi et al., 2012). In addition, a recent study has implicated that both BFL1 (BCL2A1) and BCL-XL are responsible for ATL cell survival. BFL1 expression is regulated by Tax and HBZ through the NF- κ B and AP-1 pathways, respectively (Macaire et al., 2012).

The JAK-STAT pathway, which is universal and essential to cytokine receptor signaling, is also one of the best understood signal transduction cascades in several cell types, including ATL cells. The JAK-STAT pathway mediates signaling by cytokines, which control cell survival, proliferation, and differentiation. Constitutive JAK activation leads to persistent activation of STAT transcription factors, and several cancers exhibit constitutive STAT activation in the absence of JAK- or STAT-activating mutations. JAK-STAT activation in HTLV-1-infected cells was first reported in 1995 (Migone et al., 1995). Takemoto et al. (1997) demonstrated that STAT-3 and STAT5 are activated in freshly isolated ATL cells. However, functional redundancy was also reported. Blockade of the JAK3-STAT5 pathway by AG490 failed to inhibit the proliferation of HTLV-1-transformed T cell lines, despite activation of the pathway (Kirken et al., 2000). Recently, a JAK2-STAT5 inhibitor, AZ960, was shown to have an anti-proliferative effect on HTLV-1-infected and ATL cell lines (Yang et al., 2010). However, which gene is transactivated by STAT remains unknown. The biological relevance of the JAK-STAT pathway in ATL remains to be elucidated.

EMERGING SIGNALING PATHWAYS AND INHIBITORY AGENTS

In addition to the abovementioned pathways, several signaling pathways, including the AP-1, NFAT, and CREB pathways, may be involved in the functional characteristics of ATL (Hall and Fujii, 2005). These are activated by Tax. Leukemic cells may have certain activities in each of these pathways.

The RhoA family also participates in Tax-mediated immortalization. A recent study employing a proteomic approach to identify Tax-binding proteins in a HTLV-1-infected T cell line identified direct interactions between Tax and several small GTPases, including RhoA, Rac1, and Cdc42, all of which are involved in a wide variety of cellular processes, including cytoskeleton organization and transcriptional activation (Wu et al., 2004). Recently, our global gene expression analysis as well as miRNA study strongly suggested that RhoA is overexpressed in ATL cells. A decreased miR-31 level may contribute to RhoA expression and migration activity (Yamagishi et al., 2012). The gene expression signature determined from patient samples could identify the additional signaling pathways involved in leukemogenesis (authors' unpublished data).

Recently, several studies have demonstrated that ATL shows emerging signaling activation, which is well established as oncogenic signaling in other types of cancer. Typically, the Notch pathway, which is the signaling pathway implicated in T cell acute lymphoblastic leukemia (T-ALL), is also activated in ATL (Pancewicz et al., 2010). More than 30% of ATL cases show activating mutations in *NOTCH1*, leading to reduced *CDC4/Fbw7*-mediated degradation and stabilization of the intracellular cleaved form of Notch1 (ICN1). Inhibition of Notch signaling by a γ -secretase inhibitor reduced tumor cell proliferation and tumor formation in ATL-engrafted mice. It is therefore suggested that activated Notch may be important for ATL pathogenesis.

TGF- β signaling is also reportedly important. Increased TGF- β production was observed in freshly isolated ATL cells, and this was at least partially mediated by AP-1 (Niitsu et al., 1988; Kim et al., 1990). Nevertheless TGF- β could inhibit the growth of normal T lymphocytes, HTLV-1-infected cells were resistant to this inhibition (Höllsberg et al., 1994). In ATL cells, transcription factor 8 (TCF8) is frequently disrupted by several mechanisms, primarily by epigenetic dysregulation. TCF8 mutant mice frequently develop invasive CD4+ T cell lymphomas *in vivo*. Downregulation of TCF8 expression in ATL cells *in vitro* is associated with resistance to TGF- β 1, suggesting that escape from TGF- β 1-mediated growth inhibition is important in the pathogenesis of ATL (Hidaka et al., 2008). The same research group also reported that ZEB1 downregulation and Smad7 overexpression contribute to resistance to TGF- β 1-mediated growth suppression in ATL (Nakahata et al., 2010). ZEB1 plays a critical role in regulating the epithelial-mesenchymal transition (EMT) in several solid cancers. The general function of ZEB1 in lymphocytes, particularly in association with leukemogenesis and TGF- β signaling, is a very intriguing issue.

If a chemical drug that can inhibit cellular signaling can induce apoptosis specifically, it is probable that leukemic cell survival is supported by the targeted signaling. Recent studies of inhibitors have indicated that mTOR signaling may be involved in ATL. mTOR is a serine/threonine-specific protein kinase that is located downstream of the PI3K/Akt pathway. Deregulation of mTOR signaling is implicated in a range of cancers due to its roles in cell survival and proliferation, protein synthesis and breakdown, membrane trafficking, and protein kinase C signaling (Zoncu et al., 2011). Ikezoe et al. (2007) used several inhibitors to show that the PI3K/Akt/mTOR pathway is activated in ATL cell lines as well as fresh ATL cells. In addition, a recent study reported that the mTOR complex 1 (mTORC1) inhibitor everolimus has a dramatic inhibitory effect on the growth of HTLV-1-positive and HTLV-1-negative malignant T cells, whereas normal resting or activated T cells are resistant to it (Darwiche et al., 2011). Furthermore, in addition to HTLV-1-infected cells in which Tax activates the Akt pathway (Liu et al., 2001), transformed leukemic cells in which Tax is not expressed may have some Akt activity. The most important point is how these pathways are constitutively activated in ATL. The functional importance of this pathway and its therapeutic potential will be addressed in the near future.

In the context of inhibitory agents, a higher response rate following azidothymidine/interferon α (AZT/IFN α) treatment of ATL patients has been reported in several human trials (Gill et al., 1995; Bazarbachi et al., 2010). AZT treatment of ATL patients

inhibits telomerase activity and results in progressive telomere shortening and increased p14^{ARF} expression. A functional relationship between p53 and sensitivity to AZT has also been suggested (Datta et al., 2006). Ritonavir, developed as a protease against HIV-1, also has an anti-ATL effect. Ritonavir decreases NF- κ B activity linked to the inhibition of I κ B α phosphorylation and induces the apoptosis of ATL cells. In addition, it very efficiently prevents tumor growth and leukemic infiltration in various organs of NOG mice when administered at the same dose as that used in the treatment of patients with AIDS (Dewan et al., 2006). Together, several signaling networks are deregulated in leukemic cells, and these are a specific molecular feature of ATL (Figure 2). At present, signal interception is the most effective strategy to treat ATL. At the same time, the occurrence of incidental side effects should be carefully considered because these signaling pathways are essential for normal cell function and survival.

CYTOKINES PRODUCTION AND ATL

CD4+ T cells play a central role in the immune response by controlling cells such as B cells, dendritic cells, and cytotoxic cells and their responses through various cytokines. Deregulation of the associated signaling pathways leads to abnormal gene expressions, including that of several cytokines. ATL has been implicated in the production of various cytokines, including IL-1 (Wano et al., 1987), TGF- β (Niitsu et al., 1988), TNF- α , IFN- γ , GM-CSF (Yamada et al., 1996), and PTHrP (Watanabe et al., 1990). Especially, an elevated serum C-terminal PTHrP level is a characteristic marker of the HTLV-1 carrier status, and the determination of this level in ATL patients could be useful for assessing the prognosis (Yamaguchi et al., 1994). SCID mice model of ATL also showed clearly elevated serum levels of calcium and C-terminal PTHrP, resulting in the development of hypercalcemia (Takaori-Kondo et al., 1998). The high frequency of hypercalcemia is one of the notable clinical characteristics of ATL, in particular, the aggressive types of ATL (Kiyokawa et al., 1987). Besides PTHrP, which plays an important role in bone resorption by stimulating osteoclasts, abnormal expression of the RANK ligand (RANKL) has also been demonstrated in ATL with hypercalcemia (Nosaka et al., 2002). Recent studies have revealed that a central region of HTLV-1 gp46 acts as an antagonist for osteoprotegerin and leads to hypercalcemia (Sagara et al., 2007, 2009).

SCID mice engrafted with cells from Tax-transgenic mice that develop lymphoma produced TNF- α , PDGF-BB, sICAM-1, and sVCAM-1 as factors that may contribute to high levels of organ infiltration (Watters et al., 2010). PDGF, in particular, the BB isoform, is a well-known potent osteotropic factor that stimulates the osteoclasts and osteoblasts functions (Yi et al., 2002). High IL-2 production was not observed in previous ATL studies or in our microarray data (Yamagishi et al., 2012). Nevertheless, IL-2 is an HTLV-1-induced cytokine associated with the NF- κ B pathway (Hoyos et al., 1989). In contrast, receptor subsets for IL-2 (IL2Rs) are generally overexpressed in ATL cells.

Interestingly, smoldering/chronic ATL PBMCs spontaneously proliferate *ex vivo* in an IL-12-, IL-9-, and IL-15-dependent manner, whereas acute type ATL PBMCs do not proliferate or proliferate independent of cytokines. Furthermore, purified leukemic cells from indolent ATL cases produce IL-2/IL-9

and the downstream JAK-STAT pathway is activated. Thus, autocrine/paracrine cytokine stimulation of leukemic cell proliferation may occur in patients with smoldering/chronic ATL (Chen et al., 2010).

CELL SURFACE MARKERS AND THEIR FUNCTIONS

IL-2 receptor α (CD25) was the first marker of ATL and HTLV-1-infected cells. CD25 expression is dependent on NF- κ B activity (Ruben et al., 1988). Global gene expression analysis has also validated the high CD25 mRNA level in ATL patient samples (Yamagishi et al., 2012).

Chemokines and their receptors mainly function in the migration and tissue localization of lymphocytes. The expression of the following ATL-specific chemokine receptors has been identified: CCR4 (Yoshie et al., 2002), CCR7 (Hasegawa et al., 2000), CCR8 (Ruckes et al., 2001), and CXCR4 (Twizere et al., 2007). In addition, other cell surface proteins such as OX40 (Imura et al., 1997) and TSLC1 (Sasaki et al., 2005) are highly expressed in ATL cells and are thus the molecular hallmarks of ATL; they may also participate in leukemogenesis. For example, TSLC1, a well-known tumor suppressor in various carcinomas, is overexpressed in ATL. The cytoplasmic domain of TSLC1 directly interacts with the PDZ domain of TIAM1 and induces the formation of lamellipodia through Rac activation in HTLV-1-transformed and ATL cell lines. TIAM1 may integrate signals from TSLC1 to regulate the actin cytoskeleton through Rac activation (Masuda et al., 2010).

Some ligands are also expressed by ATL cells; therefore, autocrine/paracrine stimulation is implicated. Tax develops a strategy based on the activation of the SDF-1 α /CXCR4 axis in infected cells (Twizere et al., 2007). In Tax-transgenic mice and their transplantation model, AMD3100, a CXCR4 antagonist, inhibits the infiltration of lymphomatous cells into tissues *in vivo*, indicating the involvement of the SDF-1 α /CXCR4 interaction in leukemic cell migration (Kawaguchi et al., 2009). CCR4 expression is clinically considerable. The defucosylated anti-CCR4 monoclonal antibody KW-0761 induces CCR4-specific antibody-dependent cellular cytotoxicity (ADCC) against CCR4-positive ATL cells. In view of its molecular functions, CCR4 expression may also account for the frequent infiltration of ATL cells into the skin and lymph nodes (Yoshie et al., 2002). Specific surface markers are therefore worthy of attention to identify concentrations of leukemic cells as well as minor infected cells in asymptomatic carriers. A recent study reported the development of a new method for concentrating leukemic cells by multi-color flow cytometry. The majority of leukemic cells are included in the CD4+, CD3-dim, and CD7-low subpopulations (Tian et al., 2011). Consequently, characteristic expression of cytokines and their receptors is clearly required for leukemic cell behavior, which in turn may be used as landmarks and/or therapeutic motifs.

NEW PARADIGM FROM miRNA

According to the summary of previous ATL studies described above, we can fight ATL to a certain extent. However, we cannot cure ATL because of relapse with multidrug resistance, immunodeficiency, and strong invasiveness. In addition to the previously proposed molecular hallmarks (Figure 2), we urgently need a conceptual advance that can promote understanding of the source of

disrupted gene expression. Indeed, in the course of our remarkable progress in researching ATL and other malignancies, new observations have helped in clarifying and modifying the original formulations of the hallmark capabilities.

One of the most significant recent advances in biomedical research has been the discovery of the 22-nt-long class of non-coding RNA designated miRNA that posttranscriptionally regulates gene expression by binding to the target mRNAs. miRNA is expressed by all metazoans and plants, as well as by several DNA viruses; it regulates cellular processes such as development, differentiation, growth, homeostasis, stress responses, apoptosis, and immune activation (Esquela-Kerscher and Slack, 2006). In ATL field, some studies have been reported, and several miRNA aberrations have been identified in HTLV-1-infected cells and ATL samples.

Pichler et al. (2008) first identified abnormal miRNA expression in HTLV-1-infected cells. They explored the interconnections between HTLV-1 and cellular miRNAs by using several HTLV-1-transformed cell lines. miR-21, miR-24, miR-146a, and miR-155 were found to be upregulated and miR-223 was found to be deregulated in HTLV-1-infected cells. In particular, miR-146a expression was directly stimulated by Tax through the NF- κ B pathway. *In silico* analysis predicts that many candidate genes may be deregulated by miRNA changes (Pichler et al., 2008).

Yeung et al. (2008) performed miRNA microarray analysis of 327 well-characterized human miRNAs in 7 HTLV-1-related cell lines and four acute ATL patient samples. They found that miR-18a, miR-93, and miR-130b were overexpressed in ATL samples. Of note, these miRNAs were also upregulated by PHA-mediated T cell activation. Tumor protein p53 inducible nuclear protein 1 (TP53INP1) is a gene targeted by one of miR-93 and miR-130b, and reduced TP53INP1 expression mediated by miRNA upregulation contributes to cell proliferation and survival (Yeung et al., 2008).

Bellon et al. (2009) also reported the result of miRNA array analysis of 7 ATL samples and normal PBMC and CD4+ T cells and revealed that miR-150, miR-155, miR-223, miR-142-3p, and miR-142-5p are upregulated, whereas miR-181a, miR-132, miR-125a, and miR-146b are downregulated in ATL. They discussed that miRNAs involved in normal hematopoiesis and immune responses are profoundly deregulated in ATL tumor cells *ex vivo* (Bellon et al., 2009).

Each of these studies has identified interesting miRNAs that are deregulated in ATL-related cells; however, no identical miRNA patterns have been observed. The amount of cellular miRNAs may be susceptible to various environmental conditions such as transcriptional activity, maturation processing, and epigenetic regulation. The end results appear to be affected by the methodology employed and the conditions and types of samples used. Very recently, we established global gene expression analyses of a large cohort ATL study that included analyses of mRNA expressions, miRNA levels, and genomic copy number (Yamagishi et al., 2012). A strict threshold ($p < 1 \times 10^{-5}$) and two-dimensional hierarchical clustering analysis revealed 61 miRNAs with significantly altered expression levels in ATL cells ($n = 40$) compared with control CD4+ T cells ($n = 22$). It is most important that primary ATL samples show global miRNA downregulation, similar to

observations in other cancer researches (Lu et al., 2005; Gaur et al., 2007). Fifty-nine of the 61 miRNA (96.7%) showed decreased expression in ATL. The amount of cellular miRNA may be susceptible to various environments such as transcriptional activity, maturation processing, and also epigenetic regulation. Among them, miR-31 is the most profoundly repressed miRNA in all ATL individuals (fold change, 0.00403). It is a known tumor suppressor that may also be associated with metastatic breast cancer (Valastyan et al., 2009). Other downregulated miRNAs found in ATL patients may also be involved in the hallmark capabilities of ATL, since they are uniformly decreased in tested ATL samples and each miRNA may regulate a large number of genes.

Several predictions and experimental approaches have defined a novel miR-31 target gene, MAP3K14 (also called NIK), which is a persistent NF- κ B activator in various malignancies, including B cell lymphoma (Pham et al., 2011), multiple myeloma (Annunziata et al., 2007), breast cancer (Yamamoto et al., 2010), pancreatic cancer (Nishina et al., 2009), and ATL (Saitoh et al., 2008). Interestingly, all these malignancies have low miR-31 levels. Manipulation of the miR-31 level clearly indicated that the miR-31 level was negatively correlated with cellular NF- κ B activity. Importantly, enforced miR-31 expression in B cells attenuated both BAFF and CD40L-mediated NIK accumulation and subsequent canonical and non-canonical NF- κ B signaling. As discussed above, NF- κ B activity dominates the regulation of apoptosis and subsequent cell survival. Induced miR-31 expression or NIK knockdown reduces apoptotic resistant proteins such as BCL-XL and XIAP, resulting in strong apoptosis in ATL cell lines as well as in primary leukemic cells from ATL patients (Yamagishi et al., 2012). Several lines of evidence definitively support two notions: (1) miR-31 acts as a tumor suppressor in T cells and (2) NIK-regulated NF- κ B is of pivotal importance to cancer cell survival (Uribesalgo et al., 2012).

The fact that deregulated miRNA expression predominates NF- κ B activity is a conceptual advance. Regulation of global miRNA downregulation and each regulatory network may shed light on our understanding of the next-generation molecular hallmarks of ATL and of molecules suitable for therapeutic targeting (Figure 4). Since a single miRNA can regulate the expression of multiple genes, pleiotropic miRNA may have potential as molecular therapy. Profound miR-31 loss is a characteristic of ATL; however, decreased miR-31 expression seems to be commonly observed in various malignancies. The regulatory mechanism of miR-31 had not been identified until our discovery. In general, down modulation of gene expression is coordinated by some contents of transcriptional factors and an epigenetic regulatory mechanism.

EPIGENETIC DEREGULATION OBSERVED IN ATL

Technological advances in genomics and epigenomics have supplied new methods to distinguish one cell type from another. The epigenetic code consists of the combined on-off states of hundreds of genes, which coordinately dictate cellular identity and function. Increasing attention is being paid to global regulatory factors and molecular mechanisms by which control gene transcription. This genome programming operates fundamentally through DNA methylation, histone chemical modification, and protein complex binding in these environments.

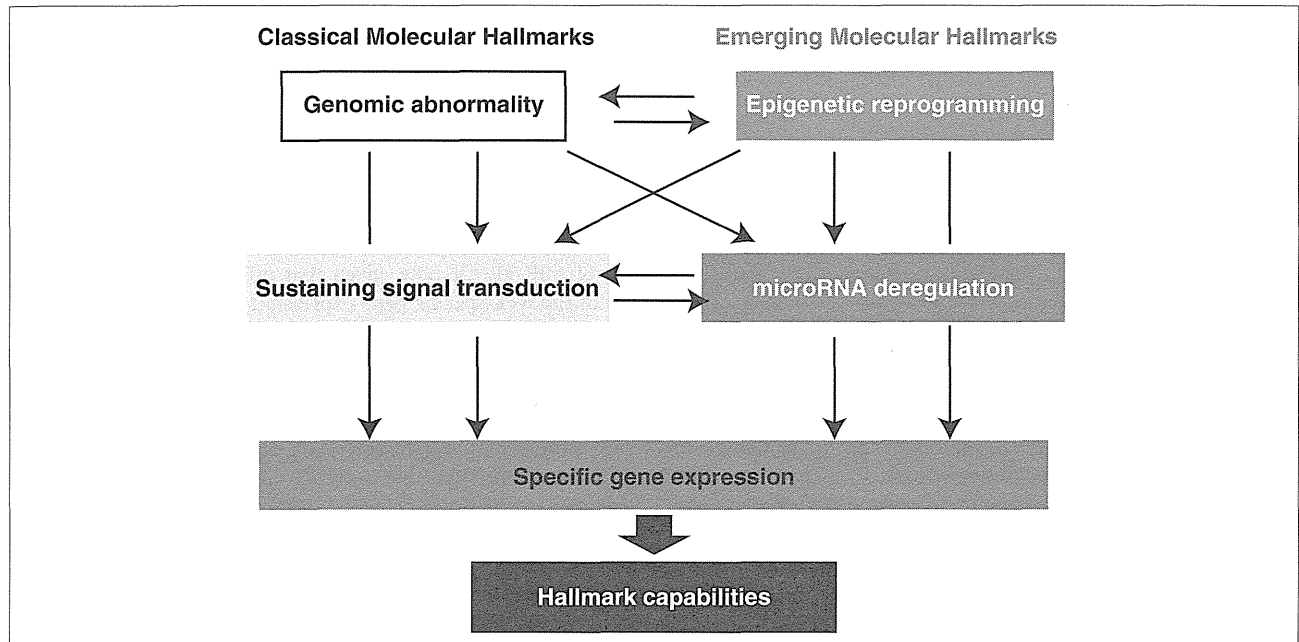


FIGURE 4 | Emerging molecular hallmarks, microRNA deregulation and epigenetic reprogramming. An increasing body of research suggests that two additional molecular hallmarks are involved in the pathogenesis of ATL. In addition to the genomic abnormality, epigenetic imbalance widely governs the

downstream molecular capabilities. Deregulation of the cellular miRNA levels directly influence hundreds of genes expression. Importantly, cross talking among each category can attain more complex gene regulatory network that is indispensable for exercise of various functions at appropriate timing.

Cancer-associated epigenetic reprogramming has been suggested because DNA methylation is a transcriptional regulator. In ATL, no attempt has been made to determine global epigenetic statements that can explain deregulated gene expression. Analysis of epigenetic factors such as DNA methylation and related gene silencing has been reported, particularly in some tumor suppressor genes such as the CDK inhibitor family.

The CpG island of *CDKN2A* gene is more frequently methylated in fresh tumor cells isolated from patients with acute ATL (47%) or lymphoma type ATL (73%) than in fresh tumor cells isolated from patients with chronic (17%) and smoldering (17%) ATL, which are relatively less malignant (Nosaka et al., 2000). No *CDKN2A* gene is methylated in asymptomatic carriers or uninfected individuals. A possible inverse correlation between *CDKN2A* mRNA expression and gene methylation status is suggested. Methylation-specific polymerase chain reaction (MSP) also suggested the presence of an additional DNA methylation in *CDKN2B* gene (20%; Hofmann et al., 2001). In addition to the cell cycle regulators, multifunctional factors involved in cell proliferation, differentiation, and apoptosis, e.g., bone morphogenetic protein (BMP) is deregulated by aberrant DNA methylation in malignant lymphomas (Daibata et al., 2007) and also ATL (Taniguchi et al., 2008). Above all, the BMP6 promoter is hypermethylated in ATL: acute (96%), lymphoma (94%), chronic (44%), and smoldering (20%). BMP6 promoter methylation seems to be a common epigenetic event at later stages of ATL. The adenomatous polyposis coli (APC) gene is also a tumor suppressor, and its mRNA level is at least partially regulated by DNA methylation

(Tsuchiya et al., 2000). In ATL, 48% of primary samples have methylated promoter DNA in the APC region (Yang et al., 2005).

The methylated CpG island amplification/representational difference analysis method revealed 53 aberrantly hypermethylated DNA sequences in ATL (Yasunaga et al., 2004). Among them, kruppel-like factor 4 (KLF4) and early growth response 3 (EGR3) were found to be responsible for apoptotic resistance in ATL cell lines, implicating that DNA methylation is involved in leukemogenesis. Abnormal DNA demethylation may also be involved. MEL1S, an alternatively spliced form of MEL1, is frequently expressed in ATL cells because of DNA hypomethylation at an alternative transcriptional start site. Aberrant MEL1S expression is associated with dysregulation of TGF- β -mediated signaling (Yoshida et al., 2004). Thus, altered DNA methylation pattern, including DNA demethylation, is one of the molecular hallmarks of ATL linking leukemogenesis to gene transcription control.

Histone modifications such as histone acetylation and specific methylations confer dynamic exchanges of transcription. Although a global survey of histone modification in ATL (such as by ChIP-on-chip analysis) has not been reported, experimental evidence with epigenetic drugs strongly suggests that epigenetic reprogramming is the background of the molecular hallmarks of ATL. Histone deacetylase (HDAC) inhibitors effectively inhibit the proliferation of several cancers (Spiegel et al., 2012) as well as that of HTLV-1-infected cell lines and primary ATL samples (Nishioka et al., 2008). Analysis of signaling cascades suggested that HDAC inhibition can block nuclear translocation of NF- κ B components. Paradoxically, another study implicated that the HDAC inhibitors

can actively modulate the NF- κ B pathway through RelA acetylation (Chiechio et al., 2009). Anyway, abnormal histone deacetylation may be involved in cell survival and cell cycle regulation in ATL cells.

Histone acetylation and DNA methylation actually cooperate in regulating a cohort of genes during multiple processes of leukemogenesis. For example, thioredoxin-binding protein-2 (TBP-2) expression is lost during the transformation step in HTLV-1-infected T cells (Nishinaka et al., 2004). TBP-2 seems to play a crucial role in the growth regulation of T cells. Sequential treatment with a DNA methylation inhibitor, 5-Aza-dC, and an HDAC inhibitor can restore the TBP-2 expression, suggesting that loss of TBP-2 expression is caused by both DNA methylation and histone deacetylation in transformed infected cell lines (Ahsan et al., 2006).

Besides acetylation, the N-terminus of histone proteins contains several residues that can be methylated. Integrated histone modification consequently decides the degrees of chromatin condensation and subsequent transcriptional sensitivity. Trimethylation of the histone H3 Lys27 (H3K27me3) mark plays a central role in the repression of transcription, mainly in the euchromatin region. The Polycomb family is a master regulator of the H3K27me3 level by inducing and maintaining the histone mark. Progress over the past decade has defined two main protein complexes: Polycomb repressive complex 1 (PRC1) and PRC2, with fundamental roles in Polycomb-mediated gene silencing (Schuettengruber et al., 2007). PRC2 methylates the histone histone 3 lysine 27 (H3K27). PRC1 is commonly viewed as an important, direct executor of silencing at target genes. Although H3K27 methylation is a key chromatin mark, there is ongoing debate about its molecular consequences. In the context of cancer research, deregulation by the Polycomb family confers a specific gene expression pattern responsible for chronic proliferation, survival, peculiar development, and cancer-associated stemness in various cancer types, including ATL (Sparmann and van Lohuizen, 2006).

The involvement of the Polycomb family in ATL was first revealed by global gene expression analysis. Significantly higher levels of enhancer of zeste homolog 2 (EZH2) as well as RING1 and YY1 binding protein (RYBP) transcripts with enhanced H3K27me3 levels were found in ATL cells compared with normal CD4⁺ T cells (Sasaki et al., 2011). EZH2 serves as the catalytic subunit in the PRC2 and mediates gene silencing by catalyzing the trimethylation of H3K27 at the promoters of target genes. EZH2 is highly expressed in many cancer types, including breast and prostate cancer and lymphomas, and it is often correlated with advanced stages of tumor progression and a poor prognosis. Importantly, EZH2 inhibition by 3-deazaneplanocin A and the HDAC inhibitor panobinostat showed a synergistic effect in killing the ATL cell lines. Because the Polycomb family generally contributes to silencing of tumor suppressor genes, e.g., the *CDKN2* family, the genes silenced in ATL should be addressed to elucidate the functional significance of the Polycomb family in the leukemogenic process.

We recently identified a notable gene silenced by Polycomb. A human gene that encodes miR-31, *hsa-miR-31*, is located at 9p21.3, which is adjacent to clusters of the *CDKN2* and *IFNA*

families. In addition to the genetic loss (12.5% of ATL cases), transcription of the miR-31 precursor is completely lost in ATL cells. Computational predictions and experimental evidence clearly demonstrated that an assembly of YY1 binding motifs upstream of the miR-31 region is responsible for the occupancy of the Polycomb family at the target region, which leads to H3K27me3-dependent transcriptional repression. Overexpression of EZH2 and suppressor of zeste 12 (SUZ12) homolog, components of PRC2, in ATL cells can induce and maintain the epigenetic silencing of miR-31. Of note, given that miR-31 is a master regulator of the ATL-specific gene expression pattern described above, Polycomb-mediated loss can influence gene expression downstream of miR-31 (Figure 4). Indeed, the amount of EZH2 and SUZ12 directly strengthens cellular miR-31 depletion, which in turn activates the NF- κ B pathway through NIK induction and confers anti-apoptotic features to T cell (Yamagishi et al., 2012). It is noteworthy that the molecular and biological interconnections between Polycomb–miR-31–NF- κ B are conserved in breast cancer cells and B lymphocytes. By organizing the new principle, various cell types may realize the more complex gene regulatory network required for maintenance and execution of cellular functions. Imbalance of this network probably switches the cell fate from one to another.

The origin of epigenetic reprogramming observed in ATL cells remains elusive. In addition to self-dysfunction of the epigenetic machinery, a possible mechanism is viral hijacking; HTLV-1 Tax can physically associate with the key histone modifiers HDAC1 (Ego et al., 2002), SUV39H1 (Kamoi et al., 2006), and SMYD3 (Yamamoto et al., 2011). However, at present, the possible influence of the Tax-epigenetic association on gene regulation is unknown. Governing the epigenetic system by Tax may disrupt gene expression, leading to chronic proliferation and abnormal survival of HTLV-1-infected cells. In the context of viral gene regulation, epigenetic changes, mainly DNA methylation, in the HTLV-1 provirus may facilitate ATL cell evasion of the host immune system by suppressing viral gene transcription (Koiwa et al., 2002; Taniguchi et al., 2005). Recent studies using *in vivo* models strongly suggested that Tax and also other viral proteins are directly linked to leukemogenesis, despite viral gene expression being rare in circulating leukemic cells in patients (Hasegawa et al., 2006; Ohsugi et al., 2007b; Banerjee et al., 2010; Satou et al., 2011). Furthermore, not only histone methylation but also other histone modifications such as phosphorylation and ubiquitination are intriguing for understanding the molecular and physiological hallmarks of ATL.

THERAPEUTIC TARGETING OF ATL

To establish more effective molecular-targeted therapies for ATL, we need to understand the exact molecular underpinnings of ATL. In addition to classical molecular characteristics, the emerging hallmarks of miRNA deregulation and epigenetic reprogramming broaden the scope of conceptualization of the responsible molecular mechanism (Figure 4). As highlighted in this review, ATL possesses six molecular hallmarks: genomic abnormality, specific changes in gene expression, sustaining activated signaling, producing cytokines, miRNA deregulation, and epigenetic reprogramming. These molecular hallmarks confer robustness to leukemic

cell hallmark capabilities: resisting cell death, promoting cell cycle, invasiveness, chronic proliferation, replicating immortality, and drug resistance (Figure 5). Consideration of hallmark principles should aid in developing future therapeutics. Several studies with inhibitory agents have clearly indicated that blockade of signaling drivers appears to be both practical and feasible for inducing leukemic cell apoptosis. However, common clinical traits of ATL include relapse and drug resistance. Importantly, each of the core hallmark capabilities is regulated by a partially redundant signaling pathways. Consequently, a targeted therapeutic agent that inhibits only one key pathway in ATL may not completely shut off another hallmark capability, allowing some ATL cells to survive with residual function until they or their progeny eventually adapt to the selective pressure imposed by the therapy. In this case, given that the number of parallel signaling pathways supporting a given hallmark is limited, it may become possible to target all of these supporting pathways therapeutically, thereby preventing the development of adaptive resistance. However, it is possible that this could involve critical side effects. Alternatively,

most upstream elements that can act pleiotropically in leukemic cells, e.g., miRNA and epigenetics, may be heralded as one of the fruits of remarkable progress into understanding the ATL mechanism. Moreover, selective co-targeting of multiple core and emerging molecular hallmarks in mechanism-guided combinations therapies will result in more effective and durable therapies for aggressive ATL.

CONCLUSION AND FUTURE DIRECTION

We have explored our present understanding of the molecular aspects of ATL to refine and extend the six specific traits, the molecular hallmarks of ATL, which have provided a useful conceptual framework for understanding the complex biology of ATL (Figure 5).

Other areas are currently in rapid flux. In recent years, the biological importance of several elaborate ATL models, including the Tax-transgenic model (Hasegawa et al., 2006), HBZ transgenic model (Satou et al., 2011), and HTLV-1-infected humanized SCID mice (Banerjee et al., 2010), has been proposed. ATL-initiating

