Development of Ultra-Super Sensitive Immunohistochemistry and Its Application to the Etiological Study of Adult T-Cell Leukemia/Lymphoma

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Antigen retrieval (AR) and ultra-super sensitive immunohistochemistry (ultra-IHC) have been established for application to archival human pathology specimens. The original ultra-IHC was the ImmunoMax method or the catalyzed signal amplification system (ImmunoMax/CSA method), comprising the streptavidin-biotin complex (sABC) method and catalyzed reporter deposition (CARD) reaction with visualization of its deposition. By introducing procedures to diminish non-specific staining in the original ultra-IHC method, we developed the modified ImmunoMax/CSA method with AR heating sections in an AR solution (heating-AR). The heating-AR and modified ImmunoMax/CSA method visualized expression of the predominantly simple present form of HTLV-1 proviral DNA pX region p40Tax protein (Tax) in adult T-cell leukemia/lymphoma (ATLL) cells in archival pathology specimens in approximately 75% of cases. The simple present form of Tax detected exhibited a close relation with ATLL cell proliferation. We also established a new simplified CSA (nsCSA) system by replacing the sABC method with the secondary antibody- and horse radish peroxidase-labeled polymer reagent method, introducing the pretreatments blocking non-specific binding of secondary antibody reagent, and diminishing the diffusion of deposition in the CARD reaction. Combined with AR treating sections with proteinase K solution (enzymatic-AR), the nsCSA system visualized granular immunostaining of the complex present form of Tax in a small number of ATLL cells in most cases, presenting the possibility of etiological pathological diagnosis of ATLL and suggesting that the complex present form of Tax-positive ATLL cells were young cells derived from ATLL stem cells. The heating-AR and ultra-IHC detected physiological expression of the p53 protein and its probable phosphorylation by Tax in peripheral blood mononuclear cells of peripheral blood tissue specimens from HTLV-1 carriers, as well as physiological and pathological expression of the molecules involved with G1 phase progression and G1–S phase transition (E2F-1, E2F-4, DP-1, and cyclin E) in ATLL and peripheral T-cell lymphoma cells. The ultra-IHC with AR is useful for etiological pathological diagnosis of ATLL since HTLV-1 pathogenicity depends on that of Tax, and can be a useful tool for studies translating advanced molecular biology and pathology to human pathology.

Key words: ultra-super sensitive immunohistochemistry, HTLV-1, p53 protein, cell cycle, adult T-cell leukemia/lymphoma (ATLL)

I. Introduction

Adult T-cell leukemia/lymphoma (ATLL) is a type of peripheral T-cell leukemia/lymphoma (PTCL) that is caused...
by human T-lymphotropic virus type 1 (HTLV-1) [96]. ATLL occurs in the lifetime of 2 to 5% of HTLV-1 carriers [4]. ATLL is subcategorized clinically into smoldering, including cutaneous type, chronic and acute leukemia, and lymphoma types [89]. Most ATLL patients have anti-HTLV-1 antibodies [36] when ATLL cells exhibit monoclonal integration of HTLV-1 proviral DNA [109, 111].

In the natural history of HTLV-1 infection, following latent infection spanning more than 30 years from the initial infection in the perinatal period (Fig. 1) and based on virological, molecular biological and immunological understanding of HTLV-1 infection, early-phase ATLL cells with neoplastic properties dependent on the HTLV-1 proviral DNA pX region p40Tax protein (Tax) [42, 70, 80, 93, 101, 110] appear under the effects of external and internal mutagens [19, 76, 84] following the accumulation of mutations induced by repeated Tax expression [18] in the background of persistent proliferation of HTLV-1-infected T-cells, which is probably induced by HTLV-1 basic leucine zipper (HBZ) mRNA [64, 65]. Then, late-phase ATLL cells with neoplastic properties independent of Tax appear when mutation has progressed in the host cell DNA and HTLV-1 proviral DNA including pX p40Tax DNA sequence [19]. ATLL cells exhibit the CD4+ CD25+ forkhead box protein P3+ (Foxp3+) phenotype of regulatory T-cells [108]. PTCL in a patient with anti-HTLV-1 antibodies or with clinicopathological information of HTLV-1 proviral DNA integration is diagnosed practically as ATLL. On the other hand, etiological pathological diagnosis of ATLL is expected based on immunohistochemistry (IHC) detecting of

Fig. 1. Pathogenicity of human T-lymphotropic virus type 1 (HTLV-1). ATLL is thought to be caused by pathogenicity of the HTLV-1 proviral DNA pX region p40Tax protein (Tax) and effects of external and internal mutagens. Recently, HTLV-1 basic leucine zipper (HBZ) mRNA from the HTLV-1 proviral DNA minus strand is regarded to cause ATLL independently of Tax. A) Latent phase HTLV-1 infection: Tax synthesis is modulated by HTLV-proviral DNA pX region p27Rex protein (Rex). Tax trans-activates and -suppresses host cell genes. HBZ mRNA propels proliferation of the host cells through E2F-1 signaling to the cell cycle. Internal and external mutagens affect host cell genes when APOBEC3G is one of the internal mutagens in activated T-cells. B) Anti-HTLV-1 immunity: Anti-HTLV-1 immunity would eliminate HTLV-1 infected T-cells. C) Early phase ATLL: Neoplastic proliferation of early ATLL cells depends on Tax in the background of the effects of HBZ mRNA, chromosomal changes and mutations. D) Late phase ATLL: Accumulation of chromosomal changes and mutations with and without the effects of Tax, APOBEC3G and the other mutagens confer neoplastic properties to early phase ATLL cells. Tax may disappear and its DNA might be destroyed in most late phase ATLL cells. E) Development of a Tax-independent T-cell neoplasm: Depending on the chromosomal changes and mutations induced by the external and internal mutagens and possibly by HBZ mRNA, HTLV-1 infected T-cells may develop into a Tax-independent T-cell neoplasm.
A. Super sensitive IHC (ordinary-IHC) methods related to ultra-IHC

- (a) Super-sensitive IHC (ordinary-IHC) methods related to ultra-IHC are:
  - Classic enzyme-labeled secondary antibody method,
  - Avidin (streptavidin)-biotin complex method (ABC/sABC method),
  - Horse radish peroxidase (HRP)-labeled streptavidin-biotin complex method (LSAB method),
  - HRP- and secondary antibody-labeled polymer reagent method (Polymer method).

- (b) Ultra-IHC by means of catalyzed reporter deposition (CARD) reaction. The original ImmunoMax/CSA method comprises the sABC method and a CARD reaction with detection of deposited biotinylated tyramide by the LSAB method. Dako CSA II system comprises the enzyme-labeled secondary antibody method and CARD reaction with detection of deposited FITC-labeled tyramide with HRP-labeled anti-FITC antibody. The new simplified CSA system comprises the polymer method and CARD reaction with detection of deposited biotinylated tyramide by the LSAB method or with detection of deposited FITC-labeled tyramide with HRP-labeled anti-FITC antibody.

C. Pretreatments for ultra-IHC by means of CARD reaction

| Pretreatment                | Inactivation of endogenous peroxidase | Suppression of non-specific staining of primary antibody | Masking of endogenous biotin | Suppression of non-specific staining of secondary antibody | Suppression of non-specific staining in CARD reaction |
|-----------------------------|--------------------------------------|--------------------------------------------------------|-------------------------------|---------------------------------------------------------|------------------------------------------------------|
| Original ImmunoMax/CSA method | ○                                    | ○                                                      | ○                             | -                                                       | -                                                   |
| Modified ImmunoMax/CSA method | ○ (2 times)                           | ○                                                      | ○                             | -                                                       | -                                                   |
| Dako CSA II system          | ○                                    | ○                                                      | ○                             | -                                                       | -                                                   |
| New simplified CSA system    | ○ (2 times)                           | ○                                                      | ○                             | -                                                       | ○                                                   |

Fig. 2. Products of super and ultra-super sensitive immunohistochemistry (IHC) and pretreatments for ultra-super sensitive IHC (ultra-IHC). Differences among enzyme-labeled antibody indirect methods can be observed in their products and in each method’s pretreatments. A) Super sensitive IHC (ordinary-IHC) methods related to ultra-IHC are a) classic enzyme-labeled secondary antibody method, b) avidin (streptavidin)-biotin complex method (ABC/sABC method), c) horse radish peroxidase (HRP)-labeled streptavidin-biotin complex method, and d) HRP- and secondary antibody-labeled polymer reagent method (Polymer method). B) Ultra-IHC by means of catalyzed reporter deposition (CARD) reaction. The original ImmunoMax/CSA method comprises the sABC method and a CARD reaction with detection of deposited biotinylated tyramide by the LSAB method. Dako CSA II system comprises the enzyme-labeled secondary antibody method and CARD reaction with detection of deposited FITC-labeled tyramide with HRP-labeled anti-FITC antibody. The new simplified CSA system comprises the polymer method and CARD reaction with detection of deposited biotinylated tyramide by the LSAB method or with detection of deposited FITC-labeled tyramide with HRP-labeled anti-FITC antibody. C) Pretreatment for ultra-IHC with the CARD reaction. The original ImmunoMax/CSA method is equipped with pretreatments for endogenous peroxidase and non-specific binding of the primary antibody. The modified ImmunoMax/CSA method introduces masking of endogenous biotin in itself. The Dako CSA II system is not equipped with additional pretreatments besides those available in the original ImmunoMax/CSA method. The new simplified CSA system is equipped with additional pretreatments for non-specific binding of the secondary antibody and non-specific staining in CARD reaction.
Tax, HTLV-1 proviral DNA pX p27Rex protein (Rex) and HBZ mRNA/protein in PTCL cells. However, the amount of Tax, Rex and HBZ protein in ATL cells is too small to be detected by ordinary super sensitive enzyme-labeled antibody methods of IHC (ordinary-IHC) such as avidin/streptavidin-biotin complex (ABC/sABC, Fig. 2b), labeled streptavidin-biotin complex method (LSAB, Fig. 2c), and polymer methods (Fig. 2d). Therefore, these proteins should be detected by ultra-super sensitive IHC (ultra-IHC) comprising an indirect enzyme-labeled antibody method and catalyzed reporter deposition (CARD) reaction (Fig. 2e). Some ultra-IHC kits are commercially available but suffer from strong non-specific staining. We have succeeded in developing ultra-IHC of Tax and Rex on routine paraffin sections of ATL [30, 31, 35, 62], solving the problems that cause the strong non-specific staining.

This review describes our development of the ultra-IHC and its application, which employs anti-HTLV-1-related antibodies, to the pathological diagnosis of ATL and the analysis of ATL oncogenesis and pathogenesis.

II. Development of Ultra-IHC

Antigen retrieval (AR) heating sections in an AR solution such as 0.01 M citrate buffer, pH 6.0 (heating-AR) heralded the era of so-called paraffin section IHC in the 1990s [86–88]. Currently, many antigens can be detected in archival human pathology specimens by combining heating-AR with ordinary-IHC. In ultra-IHC, heating-AR retrieves larger amounts of antigen based on autoclaving (121°C for 5 min), pressure cooking (for 10 min after boiling) and microwaving (3 times heating for 1 min) but also yields more non-specific staining, whereas heating-AR in 4 M urea solution demonstrated less non-specific staining than heating in 0.01 M citrate buffer [27, 30]. Since the J chain in immunoglobulins A and M and amyloid protein are unmasked by rinsing sections in a solution of highly concentrated denaturing reagent such as urea at 4°C (overnight [17, 71], the heating-AR in 4 M urea is also expected to denature and expose antigens to the antibody. Recently, heating-AR has been performed independently of the AR solution pH [104]. On the other hand, AR treating sections with an enzyme such as proteinase K (enzymatic-AR) is expected to enable visualization of molecules that form complexes or bind with other proteins and DNA, etc. by partly digesting the molecule/tissue complexes obscuring the target molecules [50].

Ultra-IHC was introduced in the field of surgical pathology as the original ImmunoMax/CSA method [67] (Fig. 2e), comprising heating-AR, sABC method (Fig. 2b), CARD reaction (Fig. 2B) and detection of the CARD deposition by LSAB method (Fig. 2c). The ImmunoMax method employed serum solutions such as phosphate buffer saline (PBS) containing 8% horse serum or 8% human AB type serum for blocking non-specific binding (Protein block) of the primary antibody, while CSA system used a serum-free solution such as PBS containing 0.25% casein.

### Table 1. Protocols of ultra-super sensitive immunohistochemistry

| Step | Work |
|------|------|
| a    | Deparaffinization. |
| b    | First inactivation of endogenous peroxidase (IEP) rinsing sections in methanol containing 0.3% H₂O₂ for 20 min. |
| c    | Antigen retrieval |
| d    | Rinse sections in phosphate buffer saline (PBS). |

Set slides to the autostainer.

1. Second IEP rinsing sections in PBS containing 3% H₂O₂ for 5 min.
2–4 3 times Rinse1
5 **Protein block suppressing non-specific binding of primary antibody for 5 min.
6 Primary antibody reaction for 15 min.
7–9 3 times Rinse1
10 Endogenous biotin mask (1) rinsing in avidin solution for 15 min.
11–13 3 times Rinse1
14 Endogenous biotin mask (2) rinsing in biotin solution for 15 min.
15–17 3 times Rinse1
18 **Protein block suppressing non-specific binding of secondary antibody for 5 min.
19 Biotinylated secondary antibody reaction for 15 min.
20–22 3 times Rinse1
23 Horse radish peroxidase (HRP)-labeled streptavidin reaction for 15 min.
24–26 3 times Rinse1
27 ***Protein block suppressing diffusion of catalyzed tyramide in the Catalyzed reporter deposition (CARD) reaction for 1 min.
28 CARD reaction for 15 min.
29–30 2 times Rinse2
31 HRP-labeled streptavidin reaction for 15 min
32–33 2 times Rinse2
34 H₂O₂-Diaminobenzidine (DAB) reaction for 5 min.
35 Rinse with distilled water to stop the DAB reaction.
36 Counterstaining of nuclei
37 Rinse with distilled water.

Remove slides from the autostainer.

Dehydrate slides and mount section with plastic medium.

Rinse1: Rinse with Tris buffer saline containing 0.1% Tween 20 (TBST) warmed at 35°C.
Rinse2: Rinse with TBST warmed at 35°C. But the rinsing buffer is PBS at room temperature in a case when a rinsing buffer can be changed by a semi-autostainer (MicroProbe™, Fisher Scientific Co.).
*: Protein block employs PBS containing 0.25% casein (Dako) with 8% horse serum.
**: Protein block employs PBS containing 0.25% casein (Dako) with 8% goat serum.
***: Protein block employs PBS containing 0.25% casein (Dako) or PBS with 3% BSA.

Steps 18 and 27 were inserted according to the new simplified CSA system (Table 1C).
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Table 1. Continued (1).

B) The protocol of Dako CSA II system, modified according to new simplified CSA system

| Step | Work |
|------|------|
| a    | Deparaffinization. |
| b    | Antigen retrieval |
| c    | Rinse sections in PBS |

Set slides to the autostainer.

1. Inactivation of endogenous peroxidase rinsing sections in methanol containing 0.3% H₂O₂ for 20 min.

2–4 3 times Rinse1
5  *Protein block for 5 min.
6  **Primary antibody reaction for 15 min.
7–9 3 times Rinse1
10 **Protein block for 5 min.
11 CARD reaction of fluorescein isothiocyanate (FITC)-labeled tyramide for 30 min.
12–14 3 times Rinse1
15 ***Protein block for 1 to 5 min.
16 CARD reaction of fluorescein isothiocyanate (FITC)-labeled tyramide for 30 min.
17–18 2 times Rinse2
19 HRP-labeled anti-FITC antibody reaction for 30 min.
20–21 2 times Rinse2
22 H₂O₂-DAB reaction for 5 min.
23 Rinse with distilled water to stop the DAB reaction.
24 Counterstaining of nuclei
25 Rinse with distilled water.

Remove slides from the autostainer.

Dehydrate slides and mount section with plastic medium.

Steps 10 and 15 are added according to the new simplified CSA system (Table 1C).

Rinse2: Rinse with TBST warmed at 35°C. But the rinsing buffer is PBS at room temperature in a case when a rinsing buffer can be changed by a semi-autostainer (MicroProbe™, Fisher Scientific Co.). Rinse: Post-reaction wash with TBST at room temperature.

*: Protein block employs PBS containing 0.25% casein (Dako) with 8% horse serum.
**: Protein block employs PBS containing 0.25% casein (Dako) with 8% goat serum.
***: Protein block suppresses diffusion of depositing FITC-labeled tyramide in the CARD reaction, pre-treating sections with PBS containing 3% polyethylene glycol (PEG) #20000 and 0.1% Tween 20 or with PBS containing 0.3% BSA and 0.1% Tween 20.

for Protein block. As molecular biology developed, the amounts of molecules traditionally targeted by IHC, such as those of signal transduction molecules and phosphorylated functional proteins, became too minute to be visualized by ordinary-IHC. Then, the ultra-IHC was expected to detect much smaller amounts of molecules, amplifying the ordinary-IHC signals a thousand times through the CARD reaction.

Unfortunately, the original ImmunoMax/CSA method required 2 times horse-radish peroxidase (HRP) reaction in methanols containing 0.3% H₂O₂ for 20 min. or with PBS containing 0.3% BSA and 0.1% Tween 20.

Table 1. Continued (2).

C) New simplified CSA system (nsCSA system)

| Step | Work |
|------|------|
| a    | Deparaffinization. |
| b    | First inactivation of endogenous peroxidase (IEP) rinsing sections in methanol containing 0.3% H₂O₂ for 20 min. |
| c    | Antigen retrieval |
| d    | Rinse sections in PBS |

Set slides to the autostainer.

1. Second IEP rinsing sections in PBS containing 3% H₂O₂ for 5 min.

2–4 3 times Rinse1
5  *Protein block for 5 min.
6  **Primary antibody reaction for 15 min.
7–9 3 times Rinse1
10 **Protein block for 5 min.
11 CARD reaction of biotinylated tyramide for 15 min.
12–14 3 times Rinse1 (Go to step15)
15 ***Protein block for 1 min.
16 CARD reaction of biotinylated tyramide for 15 min.
17–18 2 times Rinse2
19 HRP-labeled streptavidin reaction for 15 min.
20–21 2 times Rinse2 (Go to step 22)

In a case employing FITC-labeled tyramide.

15 ****Protein block for 1 min.
16 CARD reaction of FITC-labeled tyramide for 30 min.
17–18 2 times Rinse2
19 HRP-labeled anti-FITC antibody reaction for 30 min.
20–21 2 times Rinse2 (Go to step 22)

22 H₂O₂-DAB reaction for 5 min.
23 Rinse with distilled water to stop the DAB reaction.
24 Counterstaining of nuclei
25 Rinse with distilled water.

Remove slides from the autostainer.

Dehydrate and mount section with plastic medium.

Rinse1: Rinse with Tris buffer saline containing 0.1% Tween 20 (TBST) warmed at 35°C.
Rinse2: Rinse with TBST warmed at 35°C. But the rinsing buffer is PBS at room temperature in a case when a rinsing buffer can be changed by a semi-autostainer (MicroProbe™, Fisher Scientific Co.).

*Protein block: Protein block suppresses non-specific binding of primary antibody, rinsing sections in PBS containing 0.25% casein (Dako) with 8% horse serum or in PBS containing 3% BSA and 8% horse or goat serum

**Protein block: Protein block suppresses non-specific binding of secondary antibody, rinsing sections in PBS containing 0.25% casein (Dako) with 8% goat serum or in PBS containing 3% BSA and 8% goat serum.

***Protein block: Protein block suppresses diffusion of depositing biotinylated tyramide in the CARD reaction, pre-treating sections with PBS containing 0.25% casein (Dako) or PBS with 3% BSA.

****Protein block: Protein block suppresses diffusion of depositing FITC-labeled tyramide in the CARD reaction, pre-treating sections with PBS containing 3% polyethylene glycol (PEG) #20000 and 0.1% Tween 20 or with PBS containing 0.3% BSA and 0.1% Tween 20.
the CARD reaction and dianisobenzidine (DAB)-H₂O₂ reaction for visualization and 2 times biotin-streptavidin binding reaction in the sABC method and in the LSAB method detecting deposited catalyzed tyramide. Moreover, its post-reaction wash appeared incomplete. Therefore, the original ImmunoMax/CSA method (Fig. 2C, Original ImmunoMax/CSA method) amplified an extremely low level of residual activity of endogenous peroxidase, a rather small amount of endogenous biotin, and a trace level of residual reaction reagents into a huge amount of non-specific staining. Thus, the modified ImmunoMax/CSA method (Table 1A) was designed [30] to diminish non-specific staining by introducing double inactivation of endogenous peroxidase before and after AR, endogenous biotin mask treating sections with avidin and biotin solutions between the primary antibody and biotinylated secondary antibody reactions (Fig. 2C, Modified ImmunoMax/CSA method), and the post-reaction wash (PR wash) 3 times in Tris-buffered saline containing 0.5% Tween 20 (TBST) warmed to 35°C [30]. The modified ImmunoMax/CSA method employed PBS containing 8% horse serum and 0.25% casein for Protein block before the primary antibody reaction. Biotinylated antibody deposited in the CARD reaction was washed out by rinsing 3 times in the warmed TBST so the PR wash following the CARD reaction was defined as rinse twice in PBS at room temperature when the PR wash solution could be changed [30]. Finally, the modified ImmunoMax/CSA method comprised 37 steps in an autostainer (Table 1A), where 2 steps of Protein block for the secondary antibody reaction (step 18, Table 1A) and pretreatment for the reaction (step 27, Table 1A) were those employed in the new simplified CSA (nsCSA) system (Table 1C).

However, non-specific staining persisted somewhat in the modified ImmunoMax/CSA method, and varied with each case. The positive staining of the modified ImmunoMax/CSA method was evaluated in comparison with staining performed without the primary antibody reaction (negative control staining). To prevent non-specific staining caused by endogenous biotin, Dako supplied a CSA II system (Table 1B) to replace the sABC method (Fig. 2b) and the biotinylated tyramide-CARD reaction with HRP-labeled secondary antibody method (Fig. 2a) and the fluorescein isothiocyanate (FITC)-labeled tyramide reaction (Fig. 2f) but did not equip the Protein block to suppress the non-specific binding of the secondary antibody and the pretreatment to suppress the diffusion of catalyzed FITC-labeled tyramide (Fig. 2C, Dako CSA II system). Then, non-specific background staining with the Dako CSA II system was quite high. On the other hand, we developed new simplified CSA system (nsCSA system) (Table 1C, Fig. 2g), replacing the sABC method (Fig. 2b) with the HRP- and secondary antibody-labeled polymer reagent method (Fig. 2d), where additional Protein block suppressed non-specific binding of the polymer reagent and pretreatment suppressed diffusion of the catalyzed biotinylated or FITC-labeled tyramide (Fig. 2C, new simplified CSA system) [28, 34].

The reagent for the Protein block was PBS containing 0.25% casein or 3% bovine serum albumin (BSA), diminishing non-specific staining of the primary antibody and polymer reagents in the nsCSA system [28, 34]. It may have been because the blocking reagent can mask most non-specific binding sites, whereas competitive blocking by the blocking reagent in the primary antibody and polymer reagents would mask approximately half of all non-specific binding sites. Catalyzed tyramide rarely diffused to be deposited in areas distant from CARD reaction sites. Pretreatment with biochemically inactive molecules was required to achieve deposition of catalyzed tyramide much nearer to the CARD reaction sites. The pretreatment reagent was PBS containing 0.25% casein or PBS containing 3% BSA and 0.1% Tween 20 for the biotinylated tyramide-CARD reaction, while PBS containing 3% polyethylene glycol (PEG) #20000 and 0.1% Tween 20 or PBS containing 0.3% BSA and 0.1% Tween 20 was employed for the FITC-labeled tyramide-CARD reaction [28]. The nsCSA system of the biotinylated tyramide-CARD reaction was free from non-specific staining of endogenous biotin even in endogenous biotin-rich tissue such as that from the liver [28]. Antigen detection sensitivity was high in the following order: nsCSA system with the biotinylated tyramide-CARD reaction (Table 1C), nsCSA system with the FITC-labeled tyramide-CARD reaction (Table 1C), and the Dako CSA II system (Table 1B) with pretreatments diminishing non-specific staining according to nsCSA system [28] because the polymer reagent method (Fig. 2d) in the nsCSA system was more sensitive than that of the HRP-labeled secondary antibody method (Fig. 2a) in the CSA II system.

Protein block (PBS containing 0.25% casein, Dako) was a powerful reagent in the nsCSA system but the procedure, which exceeded 15 min, prevented antigen-antibody reaction, whereas PBS containing 3% BSA did not [28]. However, ultra-IHC employing PBS containing 3% BSA encountered non-specific staining through the unexpected anti-BSA antibody that had contaminated the secondary antibody reagent. Affinity-purified secondary antibody reagent might be free from such contamination. As mentioned in the following chapter of enzymatic-AR and ultra-IHC of Tax, non-specific staining in B-cell malignant lymphoma cells may be that of a part of an antibody known as the Fc (Fragment, crystallizable) region. This kind of non-specific staining cannot be suppressed by the PBS containing 0.25% casein and the nsCSA system may require additional Protein block with PBS containing 8% horse serum prior to the primary antibody reaction and with PBS containing 8% goat serum prior to the secondary antibody-polymer reagent reaction.

Furthermore, an adequate concentration of CARD reaction-amplification reagent must be determined because the amplification reagent of FITC-labeled tyramide from the CSA II system yielded a larger amount of non-specific staining than that of biotinylated tyramide from the nsCSA system [28].
III. Application of Ultra-IHC of HTLV-1 Tax to Etiological Diagnosis of ATLL and Related Lesions

The pathogenicity of HTLV-1 infection that causes ATLL is thought to be that of Tax, where Rex modulates Tax expression and reproduction of HTLV-1 (Fig. 1) [110].

The molecular mechanisms in Tax pathogenicity have been clarified and the target molecules of Tax are summarized in Table 2 according to Grassmann et al. [23] and Watanabe [106]. Recently, the pathogenicity of HBZ mRNA and protein was analyzed [64, 65]. In addition, Tax, Rex and HBZ proteins are thought to modulate the activation of HTLV-1 proviral DNA and host genes together. Therefore,

| Table 2. Targets and present form of HTLV-1 Tax |
|-----------------------------------------------|
| **Target** | **Effect and present form of Tax** | **References** |
| HTLV-1 LTR | Direct effect, forming a Tax-CREB-LTR’s CRE-motif complex. | [1, 10, 11, 91, 112] |
| Host cells TBP | Direct effect, forming a Tax-CBP complex or a Tax-p300-CAF complex with TBP. | [12, 14] |
| Signal transduction in host cells | | |
| NF-κB | Direct binding, forming a Tax-IKKg (=NEMO) complex and a Tax-NF-κB-CBP complex | [15, 26, 41, 46] |
| IL-2 and IL-2 receptor (IL-2R) | Indirect up-regulation | [3, 7, 9, 22, 38, 66, 68, 81, 85] |
| IL-15 and IL-15 specific receptor (IL-15R) | Indirect activation | [8, 63] |
| IL-13 | Indirect up-regulation | [16, 103] |
| OX40 and OX40L | Indirect up-regulation | [78] |
| Transforming growth factor beta 1 (TGF-β1) | Indirect repression | [6, 54, 56] |
| Kinase-cascades in host cells | | |
| PI3K and its downstream target Akt | Unclear | [60] |
| ERK-JNK cascade | Unclear | [48, 107] |
| Cell cycle dysregulation in host cells | | |
| Cdk4, Cdk6 and Cdk2 | Direct effect, forming a Tax-Cdk2/4/6 complex | [24, 25, 43, 58, 72, 73] |
| Cdk inhibitors (p16INK4A, p15INK4B, p18INK4C, p19INK4D, and p27Kip1) | Direct repression, forming Tax-p16INK4A and -p15INK4B complexes, and indirect suppression (p18INK4C, p19INK4D and p27Kip1) | [2, 43, 61, 93, 94] |
| Cyclin E | Indirect up-regulation | [39, 43, 83] |
| Cyclin D | Indirect up-regulation | [2, 20, 43, 69, 83] |
| Rb protein | Direct effect, forming a Tax-Rb protein complex | [52, 73] |
| E2F | Indirect up-regulation | [57, 75] |
| Cyclin A | Indirect suppression | [53] |
| Chk 1 and Chk 2 | Indirect interaction | [79] |
| APC | Direct effect, forming a Tax-APC complex | [59] |
| Cellular checkpoint protein MAD1 | Direct effect, forming a Tax-MAD1 complex | [44, 47] |
| Tumor suppressors and cellular apoptosis in host cells p53 and p21Waf1/Cip1 | Indirect or direct inactivation, forming a Tax-p530/CBP co-activator complex | [5, 102] |
| hDLG | Direct effect, forming a Tax-hDLG complex | [37, 95] |
| DNA repair and maintaining chromosomes in host cells DNA polymerase β and BER | Indirect suppression | [45] |
| DNA polymerase δ, NER and mismatch repair | Indirect suppression | [49] |
| Bcl-xL that suppresses RAD51 recombination pathway, suppressing homologous recombination. | Induction | [82] |
| hTERT | Indirect induction | [21, 90] |

LTR: Long terminal repeat of HTLV-1 proviral DNA. CREB: Cyclic-AMP-response element binding protein. CRE: Cyclic-AMP responsive. TATA box: Goldberg-Hogness box. TBP: TATA box-bound protein. CBP: Cyclic-AMP-binding protein. CAF: CBP-associated factor. NF-κB: Nuclear factor kappa B. IKKg: I-kappaB-kinase gamma. IKKg=NEMO: NF-κB essential modulator. OX40=CD134: A member of the tumor necrosis factor (TNF)-receptor family and co-stimulatory T-cell molecule. OX40L: OX40 ligand. PI3K: Phosphoinositide 3-kinase. Akt=Protein kinase B (PKB). ERK: Extracellular signal-regulated kinase. JNK: C-Jun NH2-terminal kinase. Cdk: Cyclin-dependent kinase. Rb protein: Retinoblastoma protein. ChK: Checkpoint kinase. APC: Anaphase promoting complex. hDLG: Human DLG, a homologue of the Drosophila discs large PDZ domain-containing tumor suppressor. BER: Base excision repair. NER: Nucleotide excision repair. hTERT: Human telomerase reverse transcriptase.
the minimum target-molecules in ultra-IHC for etiological diagnosis of ATLL were Tax and Rex, while the histochemistry of HBZ mRNA and protein is currently expected to be established.

a) Modified ImmunoMax/CSA method for Tax and Rex on formalin-fixed paraffin-embedded sections

The rat monoclonal antibody against Tax, WATM-1 [100], mouse monoclonal antibody against Tax, Lt-4 [55], and mouse monoclonal antibody against Rex, Rec-6 [30] were supplied by Dr. Y. Tanaka (Table 3). Sections of the HTLV-1-related cell line MT-2 were obtained from formalin-fixed and paraffin-embedded cell pellets. ATLL formalin-fixed paraffin-embedded sections were archival pathology specimens of lymph nodes diagnosed as ATLL. The modified ImmunoMax/CSA method was performed by a semi-autostainer (MicroProbe™, Fisher Scientific Co.) [30, 31, 62]. Heating-AR was performed, heating sections in 4 M urea solution by pressure cooking for 5 min [30]. Negative control staining was that of the primary antibody reaction employing antibody dilution solution containing no primary antibody.

By means of the heating-AR and ordinary sensitive polymer method (Fig. 2d), only Lt-4 showed faint staining in MT-2 while the other antibodies revealed no staining in MT-2 and ATLL. By means of the heating-AR and modified ImmunoMax/CSA method, in comparison with the negative control (Fig. 3a), MT-2 indicated strong expression of WATM-1-labeled Tax (Fig. 3b) and weak expression of Rec-6-labeled Rex (Fig. 3c). In comparison with the negative control (Fig. 3d), ATLL exhibited weak expression of WATM-1-labeled Tax (Fig. 3e) and obvious expression of Rec-6-labeled Rex (Fig. 3f).

It was suggested that it is possible to detect Tax and Rex on archival pathology specimen sections by means of heating-AR and ultra-IHC. Moreover, it was indicated that there was a difference in Tax and Rex expression pattern between MT-2 and ATLL.

b) Application to case study of ATLL and related lesions

We identified HTLV-1-associated non-neoplastic lymphadenopathy (HANNLA) in HTLV-1 carriers [33]. HANNLA demonstrated atypical follicular hyperplasia, follicle-lysis, and enlarging paracortex associated with somewhat atypical lymphocytes [33]. Atypical follicles were also noted in the tonsil of HTLV-1 carriers [97]. Gradual increased expression of Lt-4 and WATM-1-labeled Tax and high expression of Rec-6-labeled Rex was detected by the heating-AR and modified ImmunoMax/CSA method in atypical lymphocytes in the enlarged paracortex of HANNLA, where marked expression of Ia-like antigen was observed, and an increased number of HTLV-1 proviral DNA copies were estimated semi-quantitatively from polymerase chain reaction (PCR) for HTLV-1 proviral DNA and a host cell gene [30]. It was suggested that HTLV-1-infected non-neoplastic T-cells expressed Tax and Rex in the proliferative lesion, possibly related to occurrence of anti-HTLV-1 immunity.

A smoldering type ATLL manifested as erythema in the bilateral dorsal aspects of the upper arms, the fore chest, the back, the abdomen, and the bilateral anterior aspects of the thigh, and as hyperkeratotic erythema in the bilateral aspects of the toes in a 76-year-old Japanese man. Biopsied erythema in the left aspect of the thigh showed epidermotropism of lymphocytes with their intraepidermal microabscess formation (Fig. 4a), their perivascular infiltration in the upper dermis (Fig. 4b) and their infiltration to hair follicles (Fig. 4c). A biopsied lymph node in the left inguinal region demonstrated dermatoPathic lymphadenopathy (DPLA) with hyperplastic lymph follicles and enlarged paracortex (Fig. 4d) where atypical lymphocytes proliferated (Fig. 4e) and comprised CD4+ (Fig. 4f) CD8+ T-cells. Southern blot analysis of DNA extracted from the left inguinal lymph node indicated monoclonal integration of HTLV-1 proviral DNA (Fig. 4j), shown as 1 band in EcoR1 digestion, 3 HTLV-1 proviral DNA bands, and no detection of 2 virus-cellular junction bands in Pst1 digestion. These lesions were diagnosed as that of smoldering ATLL.

The heating-AR and modified ImmunoMax/CSA method showed that some ATLL cells proliferating in the paracortex of the left inguinal lymph node expressed weakly WATM-1-labeled Tax (Fig. 4h) and that many of them expressed Rec-6-labeled Rex (Fig. 4i). It was suggested that proliferating ATLL cells in smoldering type expressed Tax weakly and Rex strongly, identical to that of the ATLL in Figure 3.

ATLL initiating in the right palate tonsil and infiltrating to the cervical and axillary lymph nodes was observed in a 51-year-old Japanese woman. She manifested swelling of the right palate tonsil that showed diffuse proliferation of small to medium-sized cells (Fig. 5a and b) with CD3+ CD4+ T-cell phenotype. One month later, a swollen right palate tonsil and right cervical and axillary lymph nodes (Fig. 5c) revealed infiltrative proliferation of medium-sized to large CD3+ CD4+ CD30- T-cells (Fig. 5d and e) with developing background meshwork of thymidine phosphorylase (TP+) dendritic cells (Fig. 5f). DNA extracted from the right axillary lymph node indicated oligoclonal integration of proviral DNA, shown as 2 bands in EcoR1 digestion, 3 proviral DNA bands, and 3 weakly labeled virus-cellular junction bands in Pst1 digestion (Fig. 5j), which differed from those of a defective HTLV-1 provirus [23, 99]. Rearrangement of T-cell receptor β was noted (Fig. 5k). This case was an early phase of ATLL developing from small- to medium-sized cells to pleomorphic cells with a CD30- activating phenotype. In the heating-AR and modified ImmunoMax/CSA method, most ATLL cells in early phase ATLL in the right axillary lymph node showed obvious expression of WATM-1-labeled Tax (Fig. 5h) and much stronger expression of Rec-6-labeled Rex (Fig. 5i) without obvious staining in the negative control (Fig. 5g), suggesting that early ATLL cells with the activating phenotype expressed Tax in an obvious manner.

ATLL was also observed in Native Americans in Argentina [62]. All 5 Native American ATLL cases demon-
### Table 3. Antibodies that appeared in this review

| Antigen | Antibody | Antigen retrieval | Detection method |
|---------|----------|-------------------|------------------|
| Used for diagnosis of lymphomas | | | |
| CD3 | NCL-CD3-PS1, Novocastra | Heating* | Ordinary-IHC #1 |
| CD4 | NCL-CD4-JF6, Novocastra | Heating* | Ordinary-IHC #1 |
| CD8 | M7103, Dako | Heating* | Ordinary-IHC #1 |
| CD30 (Ber H2) | M0751, Dako | Heating* | Ordinary-IHC #1 |
| Thymidine phosphorylase (TP) | Supplied from Dr. Akiyama S | Heating* | Ordinary-IHC #1 |
| CD5 | Leu 1, Becton Dickinson | Heating** | Ultra-IHC #2 |
| | | | |
| Used for detecting HTLV-1 related proteins | | | |
| HTLV-1 p40Tax | Mouse mAb, L1-4, supplied from Dr. Tanaka Y | Heating*, Enzymatic*** | Ultra-IHC #2,3 |
| HTLV-1 p40Tax | Rat mAb, WATM-1, supplied from Dr. Tanaka Y | Heating*, Enzymatic*** | Ultra-IHC #2,3 |
| HTLV-1 p27Rex | Mouse mAb, Rec-6, supplied from Dr. Tanaka Y | Heating* | Ultra-IHC #2 |
| | | | |
| Used for detecting proliferation, cell cycle and DNA-damaged cell death | | | |
| Ki67 antigen (MB-1) | M7240, Dako | Heating* | Ultra-IHC #3 |
| p53 | NCL-p53-DO7, Novocastra | Heating* | Ultra-IHC #3 |
| p53 phosphorylated | NCL-p53-PHOS, Novocastra | Heating* | Ultra-IHC #3 |
| EZF-1 | sc193, Santa Cruz | Heating** | Ultra-IHC #2 |
| EZF-4 | sc511, Santa Cruz | Heating** | Ultra-IHC #2 |
| DP-1 | sc610, Santa Cruz | Heating** | Ultra-IHC #2 |
| Cyclin E | NCL-CYCLIN E, Novocastra | Heating** | Ultra-IHC #2 |
| | | | |
| Used for detecting autophagy (autophagic vesicle nucleation) | | | |
| Beclin-1 | sc11427, Santa Cruz | Enzymatic*** | Ultra-IHC #3 |
| | | | |
| Used for detecting stem cells, fetal cells, cancer cells and lymphoma cells | | | |
| Survivin | ab469, Abcam | Heating**** | Ordinary-IHC #1 |
| CD117 | NCL-CD117, Novocastra | Heating* | Ultra-IHC #3 |

*: Antigen retrieval heating in 0.01 M citrate buffer pH 6.
**: Antigen retrieval heating in 4 M urea solution by pressure cooking for 5 to 10 min in a draft.
***: Antigen retrieval treating sections by proteinase K solution for 10–30 min at room temperature.
****: Antigen retrieval heating in Diva Decloaker buffer (Biocare Medical) independent from pH of solution.

#1: Ordinary-IHC, super sensitive indirect enzyme-labeled antibody method (enzyme- and secondary antibody-labeled polymer reagent method).
#2: Ultra-IHC (Modified ImmunoMax/CSA method, possibly new simplified CSA system and Dako CSA II system modified according to the new simplified CSA system).
#3: Ultra-IHC (New simplified CSA system).

**Fig. 3.** The modified ImmunoMax/CSA method of Tax and Rex on paraaffin sections of MT-2 and 1 case of lymphoma type ATLL (DAB-H2O2 reaction with methyl green nuclear counterstaining. ×40, Olympus BX50, FUJIFILM HC-300). a–c) MT-2. d–f) ATLL. a and d) Negative control staining performed with antibody dilution fluid containing no primary antibody. b and e) WATM-1 (Tax). c and f) Rec-6 (Rex). In comparison with the negative control staining (a, d) MT-2 cells show stronger Tax staining (b) than ATLL (e) does, although ATLL cells show stronger Rex staining (f) than MT-2 (e) does.
Fig. 4. Tax and Rex Expression in smoldering type ATLL (×40, Olympus BX50, FUJIFILM HC-300). a–e) Haematoxylin & Eosin (H.E.) a–c) The left thigh showed intra-epidermal microabscess formation (a) and infiltration of small atypical lymphocytes around capillaries in the upper dermis (b) and into the hair follicle (c). d–i) The left inguinal lymph node d) Low power view (×10). e) High power view of the enlarged paracortex. f) Ordinary-IHC of CD4 (DAB-H₂O₂ reaction and haematoxylin nuclear counterstaining). The left inguinal lymph node demonstrates dermatopathic lymphadenopathy, of which the enlarged paracortex (d) indicates proliferation of atypical lymphocytes (e) positive for CD4 (f). g–i) The modified ImmunoMax/CSA method (DAB-H₂O₂ reaction with methyl green nuclear counterstaining) for negative control performed without primary antibody (g), WATM-1 for Tax (h), and Rec-6 for Rex (i). In comparison with the negative control staining (g) some cells express Tax (WATM-1) weakly (h) and many cells express Rex (Rec-6) strongly (i). j) Southern-blot analysis of HTLV-1 proviral DNA integration. 1) Positive control (ATLL cell line), 2) Negative control (human placental DNA), and 3) DNA extracted from the left inguinal lymph node. E) EcoR1 digestion. Two DNA sequence (GAATTC) in cellular DNA on both ends of the HTLV-1 proviral DNA are digested. P) Pst1 digestion. The DNA sequence (CTGCAG) in HTLV-1 proviral DNA and in cellular DNA on both ends of the HTLV-1 proviral DNA is digested into 3 proviral DNA bands of 1.2 kb, 1.8 kb, and 2 virus-cellular junction bands. Positive control (1: Monoclonal integration of HTLV-1 proviral DNA in ATLL cell line) shows 1 band labeled by a whole HTLV-1 proviral DNA probe in EcoR1 digestion and 3 proviral DNA bands and 2 virus-cellular junction bands in Pst1 digestion. The DNA extracted from the left inguinal lymph node (3) indicates 1 weak band in EcoR1 digestion (arrow-head) and 3 weak bands of 1.2 kb, 1.8 kb, 2.5 kb in Pst1 digestion (arrows) while 2 virus-cellular junction bands are not recognized, suggesting monoclonal integration of HTLV-1 proviral DNA in some atypical cells.
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Fig. 5. Tax and Rex expression in ATLL that initiated in the right palate tonsil and infiltrated to the cervical and axillary lymph nodes (×10 or ×40, Olympus BX50, FUJIFILM HC-300). a and b) First biopsy of the right palate tonsil (H.E.) showing diffuse proliferation of medium-sized ATLL cells (a, ×10) and their tropism to the tonsillar epithelia (b). c-i) Second biopsy of the right axillary lymph node. c) Diffuse proliferation of pleomorphic ATLL cells can be observed (H.E.). d-f) Ordinary-IHC of CD4 (d), CD30 (e) and thymidine phosphorylase (TP, f) (DAB-H2O2 reaction and haematoxylin nuclear counterstaining). Most of the pleomorphic ATLL cells are CD4 T-cells (d), some of them express CD30 (e), and associate symbiotic growth of TP-positive dendritic cells forming a meshwork. g-i) The modified ImmunoMax/CSA method for the negative control (g), WATM-1 for Tax (h), and Rec-6 for Rex (i) (DAB-H2O2 reaction and haematoxylin nuclear counterstaining). In comparison with the negative control staining (g), expression of Tax (WATM-1) (h) and strong expression of Rex (Rec-6) (i) are detected in the pleomorphic ATLL cells. j) Southern-blot analysis of HTLV-1 proviral DNA integration. 1) Positive control (ATLL cell line), 2) Negative control (human placental DNA), and 3) Sample: DNA extracted from the left axillary lymph node. E) EcoR1 digestion. P) Pst1 digestion. The extracted DNA shows 2 DNA bands from EcoR1 digestion, and 3 proviral DNA bands (arrows) and 3 virus-cellular junction bands (arrow heads) from Pst1 digestion, suggesting oligoclonal integration of proviral DNA in the ATLL cells. k) Southern-blot analysis of T-cell receptor β (TCR β). 1) BamH1 digestion. BamH1 digests the DNA sequence (GGATCC) in cellular DNA on both sides of the TCR β DNA sequence. 2) EcoRV digestion. EcoRV digests the DNA sequence (GAATTC) in the TCR β DNA sequence. A rearranged TCR β band is observed in BamH1 (arrow) and EcoRV digestion (arrow) of the sample, suggesting that these pleomorphic ATLL cells are monoclonal αβ T-cells.
strated obvious Tax and strong Rex expression, respectively, in archival sections of pathology specimens tested by the heating-AR and modified ImmunoMax/CSA method using WATM-1 and Rec-6 [62]. However, 2 of 9 Kagoshima ATLL cases did not demonstrate positive staining of WATM-1-labeled Tax with the heating-AR and modified ImmunoMax/CSA method (Table 4).

Clinically, approximately half of all ATLL cases are leukemia, and the patients’ lymph nodes were usually not examined pathologically. A lump of naturally precipitated and coagulated peripheral blood can be processed into a formalin-fixed and paraffin-embedded specimen, i.e., peripheral blood tissue specimen (PBTS) [35]. Leukemic ATLL cells in the PBTS can be examined in the same manner as those in archival pathology specimens. In PBTS of 6 of 8 leukemia type ATLL cases comprising 4 chronic and 4 acute leukemia type cases, Tax and Rex were detected weakly and strongly, respectively, in a few or some ATLL cells with the heating-AR and modified Immunomax/CSA method using WATM-1 and Rec-6 [35].

HTLV-1-infected T-cells in the proliferative lesion in the paracortex of HANNLA and ATLL cells proliferating in the paracortex of DPLA in smoldering ATLL and in the lymph nodes in early phase lymphoma type ATLL and in lymphoma type ATLL in Native Americans and in Japanese (Kagoshima natives) expressed Tax weakly and Rex strongly with the heating-AR and modified Immunomax/CSA method. Tax expression was correlated to Ki67 antigen+ proliferating cells in PBTS of leukemia type ATLL [35]. A present form of Tax retrieved by heating AR and detected with the modified Immunomax/CSA method of WATM-1 against Tax may be related with ATLL cell proliferation.

c) Enzymatic-AR and ultra-IHC of Tax

Tax is synthesized from the integrated HTLV-1 proviral DNA pX region. As listed in Table 2, Tax forms a complex with cyclic-AMP-response element binding protein (CREB), docks at cyclic-AMP responsive (CRE)-motifs of the HTLV-1 proviral DNA long terminal repeat (LTR) and up-regulates Tax synthesis [1, 10, 11, 42, 91, 112]. On the other hand, Tax binds directly TATA box-bound protein (TBP), recruits CREB-binding protein (CBP), p300 and p300/CBP-associated factor (P/CAF) and forms Tax-CBP-p300 and Tax-P/CAF complexes [12, 14], trans-activating or -suppressing host cells genes. Besides, Tax binds directly I-kappaB-kinase gamma (IKKg, =NEMO: NF-κB essential modulator) and indirectly up-regulation of Nuclear factor kappa B (NF-κB), effecting down-stream
signal transductions and their products [15, 26, 41, 46]. Tax binds directly cyclin-dependent kinases 2, 4, and 6 (Cdk2, Cdk4 and Cdk6) [24, 25, 43, 58, 72, 73] and Cdk inhibitors (p16^{INK4A} and p15^{INK4B}) and indirectly suppresses the other Cdk inhibitors (p18^{INK4C}, p19^{INK4D}, and p27^{Kip1}) [2, 43, 61, 93, 94]. Tax binds also directly retinoblastoma (Rb) protein [52, 73], anaphase promoting complex (APC) [59], cellular checkpoint protein MAD1 [44, 47], and human DLG, a homologue of the Drosophila discs large PDZ domain-containing tumor suppressor [37, 95]. There are at least 2 present forms of Tax, a simple present form (Free Tax molecule, Fig. 6) and a complex present form (Tax molecule in a complex, Fig. 6) that comprises the complicated complexes mentioned above (Fig. 6). The efficiency of AR on antigens in the Tax molecule may depend on its present form in HTLV-1-infected T-cells. Heating-AR in the modified ImmunoMax/CSA method of WATM-1 would expose the simple present form of Tax to the anti-Tax antibody WATM-1. On the other hand, enzymatic-AR digests the simple present form of Tax, some molecules masking Tax in the complicated complexes and tissues around Tax to expose Tax to the primary antibody, as suggested in ultra-IHC of beclin-1 (Table 3) [50, 104]. In order to detect the complex form of Tax, we employed enzymatic-AR treating sections with 200 μg/mL proteinase K solution for 10–30 min at room temperature and nsCSA system (Table 1C, Fig. 2g) [28, 34].

The enzymatic-AR and nsCSA system (Table 1Ca) visualized immunostaining that was more obviously granular with Lt-4 than with WATM-1 in some lymphocytes of HTLV-1 carriers (Fig. 7a and b) and ATLL cells (Fig. 7c–f) in PBTS. Chronic leukemia type ATLL (Fig. 7c and d) exhibited granular Lt-4 immunostaining in more cells than HTLV-1 carriers (Fig. 7a and b) and acute leukemia type ATLL (Fig. 7e and f) (Table 5, Kruskal-Wallis test, p=0.0388). Chronic ATLL cells may depend more on Tax than acute type ATLL cells and HTLV-1-infected cells in HTLV-1 carriers, suggesting the possibility that Tax maintains chronic type ATLL cells. However, non-specific dense nuclear staining with Lt-4 and WATM-1 was observed in an acute myelogenous leukemia (AML) patient without antibodies against HTLV-1 (Fig. 7g and h). Based on hematological diagnosis of peripheral T-cell leukemia, HTLV-1 infection in leukemic cells could be detected in PBTS by the enzymatic-AR and nsCSA system of Lt-4 for Tax. Peripheral T-cell leukemia with Lt-4-labeled leukemia cells has to be diagnosed as ATLL.

The enzymatic-AR and nsCSA system of Lt-4 was applied to paraffin sections of 29 malignant lymphoma cases diagnosed in a hospital’s Department of Pathology. Thirteen of 14 PTCL cases were diagnosed pathologically as ATLL based on clinical information regarding anti-HTLV-1 antibodies in the serum or the integration of HTLV-1 proviral DNA in lymphoma cells (Table 6). As shown in Figure 8, a small number of ATLL cells displayed obvious granular Lt-4 staining in all 13 ATLL cases. Pleomorphic large lymphoma cells of PTCL not otherwise specified (PTCL NOS) displayed obvious granular Lt-4 staining (Fig. 8b). Since the Lt-4 immunostaining provided information regarding HTLV-1 infection, this
PTCL NOS could be diagnosed as ATLL. The enzymatic-AR and nsCSA system of Lt-4 was informative for HTLV-1 infection even in cases dominated by medium-sized cells (Fig. 8e). In an ATLL case, a medium-sized cell exhibited granular Lt-4 immunostaining localized in the cytoplasm, and a large cell facing the medium-sized cells revealed faint Lt-4 immunostaining in the cytoplasm (Fig. 8f). This figure possibly suggested an image of HTLV-1 infection through viral synapse [40]. The positive immunostaining of the enzymatic-AR and nsCSA system of Lt-4 in background cells (Table 6) suggested a possibility of secondary ATLL from other lymphomas [32, 74], especially Hodgkin lymphomas [74].

Non-specific immunostaining in the enzymatic-AR and nsCSA system of Lt-4 was noted in 11 cases of B-cell neoplasm/malignant lymphoma (BML) (Table 6) and could be differentiated from the specific granular Lt-4 immunostaining in ATLL. Nuclear staining was observed in 5 cases of follicular lymphomas (FL, Fig. 9a) and in 1 case of B-cell chronic lymphocytic leukemia (B-CLL), as well as in PBTS of AML (Fig. 7g). Cytoplasmic fine staining was observed in 1 case each of FL and diffuse large B-cell lymphoma (DLBCL, Fig. 9b). Nucleolar staining was observed in 2 DLBCL cases (Fig. 9c). In addition, dense, clustered granule-like cytoplasmatic staining was observed in 1 case of lymphoplasmacytic lymphoma (LP, Fig. 9d).

The non-specific nuclear staining in the enzymatic-AR and nsCSA system of Lt-4 and WATM-1 may have been that of the primary antibody. It may be possible to diminish the nuclear staining by employing Protein block containing 8% horse serum as was done with the modified ImmunoMax/CSA method. However, the modified ImmunoMax/
CSA method of Lt-4 labeled the nuclei of epidermal squamous cells [31]. Since nuclear staining, differing from the granular staining in the nucleus, was often seen in leukemia and lymphoma other than ATLL, such Lt-4 and WATM-1 nuclear staining would not be related to HTLV-1 pathogenicity.

The nucleolar and cytoplasmic non-specific immunostaining in BML was thought to be that of the secondary antibody reagent, although rare HTLV-1-related BML might exist. The secondary antibody reagent is usually goat polyclonal antibodies against immunoglobulin (Ig) G1 from a species of the primary antibody, and often includes a relatively low amount of antibodies against IgG Fc region and BSA (Hapten carrier protein). Nucleolar and cytoplasmic non-specific immunostaining would be diminished if Protein block employing a solution of 8% serum from the secondary antibody species (PBS containing 8% goat serum and 0.25% casein) was carried out prior to the HRP- and secondary antibody-labeled polymer reagent reaction (step 10 in Table 1C). On the other hand, an affinity-purified secondary antibody reagent is also expected to diminish nucleolar and cytoplasmic non-specific immunostaining in the nsCSA system employing the serum-free Protein block.

The obvious granular immunostaining in the enzymatic-AR and nsCSA system of Lt-4 was thought to be pathognomonic for ATLL. Thus, the enzymatic-AR and nsCSA system of Lt-4 was expected to be a useful tool for the etiological pathological diagnosis of ATLL, considering the pathogenicity of Tax.

### IV. Application of Ultra-IHC to Analysis of ATLL Oncogenesis and Pathogenesis

Oncogenesis and pathogenesis of ATLL can be understood conceptually from the viewpoint of HTLV-1 pathogenicity, as shown in Figure 1. HTLV-1 pathogenicity has usually been investigated by means of molecular biology/virology and fruitful results have been reported. It is also studied from a genetic immunological viewpoint because ATLL develops in a host that is immunologically compromised against HTLV-1 infection. It is usually difficult to examine pre-neoplastic phase and early-phase lesions of ATLL pathologically. However, fortunately, as mentioned above, we were able to carry out trials to detect HTLV-1 related molecules by means of ultra-IHC in HANNLA, smoldering ATLL, early phase ATLL and developed/lymphoma type ATLL. Moreover, we developed the PBTS method [35] to prepare sections of peripheral blood mononuclear cells (PBMCs) for IHC. Thus, we were able to examine PBMCs in HTLV-1 carriers and leukemic ATLL cells.

We recognized HANNLA [33] as displaying atypical follicular hyperplasia with irregularly-shaped germinal centers (GCs) and enlarged paracortex. It is possible that some HTLV-1-infected regulatory T-cells were related to the irregularly-shaped GCs [33, 97]. In the enlarged paracortex, atypical lymphocytes expressed Tax weakly and Rex strongly with Ia-like antigen+ cells, while the extracted DNA suggested increased copies of HTLV-1 proviral DNA, suggesting that the enlarged paracortex of HANNLA was...
Fig. 8. Tax expression detected by the enzymatic-AR and nsCSA system of Lt-4 in archival paraffin specimens of peripheral T-cell lymphomas with and without clinical information regarding HTLV-1 infection (ATLL and PTCL) (DAB-H₂O₂ reaction and haematoxylin nuclear counterstaining, ×100 oil, Olympus BX50, FUJIFILM HC-300). More or less granular Lt-4 staining is noted in the nucleus and cytoplasm. Various-sized pleomorphic lymphoma cells in ATLL (a, c–e) and PTCL (b) show dominantly intranuclear granular Lt-4 staining. Medium-sized and large ATLL cells show cytoplasmic Lt-4 staining in cells facing one another (f, arrow head). As Lt-4 staining in PTCL cells (b) provides immunohistochemical information of HTLV-1 infection, this case might be diagnosed pathologically as ATLL.

Fig. 9. Non-specific staining in the enzymatic AR and nsCSA system of Lt-4 in archival paraffin specimens of B-cell neoplasms (DAB-H₂O₂ reaction and haematoxylin nuclear counterstaining, ×100 oil, Olympus BX50, FUJIFILM HC-300). a) Nuclear staining in follicular lymphoma (FL). b) Fine granular staining in diffuse large B-cell lymphoma (DLBCL). c) Nucleolar staining in DLBCL. d) Dense cytoplasmic staining in lymphoplasmacytic lymphoma/immunocytoma (LP).
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The site where occurred anti-HTLV-1 immunity and expansive HTLV-1 infection in the atypical lymphocytes [30] that maintained a viral load in PBMCs. Tax (LT-4 or WATM-1)-positive lymphocytes in the PBTS of HTLV-1 carriers (Fig. 7a and b) may be HTLV-1-infected T-cells circulating from HANNLA-like lesions in the lymphoreticular system.

It is difficult to label individual early neoplastic HTLV-1-infected T-cells but IHC of survivin (Table 3) may succeed in this aspect [104]. Mutagens other than Tax, such as APOBEC3G (apolipoprotein B mRNA-editing, enzymetic-catalytic, polypeptide-like 3G) [19], would precede neoplastic changes such as over-expression of TSLC1 (tumor suppressor in lung cancer 1) [84] independently from Tax in pre-ATLL cells. DNA damages induced by such mutagens may be removed by the p53 protein system when HTLV-1 inactivates p53 by phosphorylating Ser392 in its non-specific DNA binding or oligomerization domains. The immunostaining of p53 in b2 is similar to that of phos-p53 in b3, indicating that phosphorylated p53 is detected by the anti-p53 antibody, and is different from that in e2. Thus, the immunostaining of p53 in b2 is evaluated as negative.

Fig. 10. Expression of Ki67 antigen, p53 protein (p53) and phosphorylated p53 protein (phos-p53) detected by the heating-AR and nsCSA system on the PBTS of HTLV-1 carriers and ATLLs (DAB-H2O2 reaction and haematoxylin nuclear counterstaining, ×40 and digital ×2, Olympus BX50, FUJIFILM HC-300). a–c) HTLV-1 carriers. d) Chronic type ATLL. e) Acute type ATLL. 1) Ki67 antigen. 2) p53. 3) phos-p53. Gradual increase of Ki67 antigen cells in PBTS is noted in HTLV-1 carriers (a1–c1) and most of chronic and acute type ATLLs. Most leukemic ATLL cells were positive for Ki67 antigen (d1 and e1). Physiological expression of p53 in the cytoplasm and nucleus is detected by the heating-AR and nsCSA system in HTLV-1 carriers (e2) and chronic type ATLL (d2), while dense nuclear expression of probable mutated p53 is noted in acute type ATLL (e2), suggesting the possibility of differentiating acute from chronic type ATLL by detecting dense nuclear expression of p53. On the other hand, phos-p53 is observed in HTLV-1 carriers (b3 and c3) and ATLL (d3 and e3), suggesting the possibility of detecting HTLV-1 proviral DNA activity to inactivate p53-mediated DNA damage-induced cell death, since HTLV-1 inactivates p53 by phosphorylating Ser392 in its non-specific DNA binding or oligomerization domains. The immunostaining of p53 in b2 is similar to that of phos-p53 in b3, indicating that phosphorylated p53 is detected by the anti-p53 antibody, and is different from that in e2. Thus, the immunostaining of p53 in b2 is evaluated as negative.
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functional domain [70], prevents repair of damaged DNA by suppressing DNA polymerases β [45] and δ [49] and BclXL [82] (Table 2). It is well known that mutated p53 protein that has accumulated in the nucleus is detected by ordinary-IHC in archival pathology specimens. We wanted to observe physiological expression of p53 protein so that we examined Ki67 antigen (Table 3) for proliferating cells, p53 protein and p53 protein phosphorylated at Ser392 in non-specific DNA binding domain (phos-p53) (Table 3) in PBTS of HTLV-1 carriers and ATLL by means of the heating-AR and nsCSA system (Fig. 10). A gradual increase of Ki67 antigen proliferating cells and gradual enhanced expression of p53 protein and phos-p53 were observed in HTLV-1 carriers. The gradual increase of Ki67 antigen proliferating cells might be due to the effects of Tax and HBZ mRNA [64, 65] in PBMCs of HTLV-1 carriers. Weak detection of p53 protein was dominant in the PBMCs’ cytoplasm of HTLV-1 carriers (Fig. 10 e2) and chronic type ATLL (Fig. 10 d2). This weak cytoplasmic immunostaining of the p53 protein was believed to be its physiological expression. Dense nuclear staining of the p53 protein, probably a mutant p53 protein, was observed in acute type ATLL (Fig. 10 e2) as reported previously [105], suggesting a possible standpoint for bone marrow transplantation therapy in acute type ATLL [77]. After categorizing 8 HTLV-1 carriers in to 3 groups, i.e., p53− phos-p53− (2 cases, Fig. 10 a2 and a3), p53− phos-p53+ (3 cases, Fig. 10 b2 and b3) and p53− phos-p53− (3 cases, Fig. 10 e2 and c3), we determined that there was a significant difference in age among the 3 groups (mean ages: 32, 45 and 50 years, Kruskal-Wallis test p=0.049), indicating physiological expression of the p53 protein (Fig. 10 c2) against accumulation of DNA damages/mutations according to age and inactivation of p53 by HTLV-1 infection [70, 80]. The immunostaining of p53 protein (Fig. 10 b2) was similar to that of phos-P53 (Fig. 10 b3) in PBMCs in the PBTS of carrier B, but differed from that of the p53 protein (Fig. 10 c2 and d2) in carrier C and chronic type ATLL, suggesting that immunostaining of the p53 protein (Fig. 10 b2) was that of phos-p53. In addition, the appearance of phos-p53 in PBMCs might suggest the initial phase of ATLL oncogenesis, since Tax inactivates the p53 protein by phosphorylating it [70, 80]. Further studies on the expression of neoplastic features such as survivin [13] and human telomerase reverse transcriptase (hTERT) [21, 90] and that of mutagens such as APOBEC3G [19] are necessary to evaluate oncogenetic advances in the p53− phos-p53− and p53+ phos-p53+ stages of HTLV-1 carriers. Specific approaches

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**Fig. 11.** Effects of Tax and HBZ mRNA molecular mechanism on cell cycle. Tax transcripted from the + strand of HTLV-1 proviral DNA trans-activates several genes, targets several molecules in signal transduction as listed in Table 2, and produces proliferation signals to initiate the G1 phase, cyclin E related to G1 progression, and cyclin D related to G1–S transition with stimuli from Tax-related and Tax-unrelated neoplastic changes. Tax suppresses p53, which propels for Rb to form a complex with E2F-DP-1, and suppresses p21\[^{Waf1/Cip1}\]cyclin-dependent kinase inhibitor 1 suppressing the Cdk2-cyclin E and Cdk4/6-cyclin D complexes. Furthermore, Tax trans-activates E2F-1, trans-suppresses p16\[^{INK4A}\], and stimulates Cdk4/6 into binding with cyclin D to release the E2F-1 complex from the Rb complex to G1–S transition. On the other hand, HBZ mRNA releases E2F-DP-1 to G1–S transition. Tax also degrades cyclin A to propel over-duplication of DNA in the S phase and disrupts cyclin B to upset spindle assembly checkpoint.
to halting Tax in these stages of HTLV-1 infection seems to be warranted because green tea has the effect of reducing viral load in peripheral blood [92].

Expression of the simple present form of Tax detected by heating-AR and modified ImmunoMax/CSA method of WATM-1 was related with proliferation of probable HTLV-1-infected T-cells in HANNLA and in smoldering type, lymphoma type and leukemic ATLL cells, as mentioned above. The molecular mechanisms of Tax, HBZ mRNA and their effect on the cell cycle are illustrated in Figure 11. Tax transcribed from the + strand of HTLV-1 proviral DNA trans-activates several genes [110], targets several molecules in signal transduction as listed in Table 2, and produces proliferation signals to initiate the G1 phase, cyclin E-related to G1 progression, and cyclin D-related to G1–S transition with stimuli from Tax-related and Tax-unrelated neoplastic changes. Tax suppresses p53 [5, 70, 80, 102], blocking Rb [52, 73] to form a complex with E2F-DP-1, and suppresses p21Waf1/Cip1, which in turn suppresses the Cdk2-cyclin E complex. Further, Tax trans-activates E2F-1 [110], suppresses Cdk inhibitors such as p16\(^{\text{INK4A}}\) [2, 43, 61, 93, 94], and binds Cdk4/6 [24, 25, 43, 58, 72, 73] to bind with cyclin D to release the E2F-DP-1 complex from Rb to G1–S transition, competing with the p53 protein that is inactivated by Tax [70, 80]. DP-1 is stabilized by binding with SOCS-3 in the cytoplasm. On the other hand, HBZ mRNA releases E2F-DP-1 to G1–S transition [64, 65]. Additionally, Tax degrades cyclin A to propel over-duplication of DNA [53] and binds anaphase promoting complex (ABC) to disrupt cyclin B to upset spindle assembly checkpoints [59]. In order to see features in cell cycle in MT-2, 9 cases of lymphoma type ATLL with monoclonal integration of HTLV-1 proviral DNA, and 9 cases of PTCL in Europeans (EPTL) free from HTLV-1 infection, we examined E2F-1 activator and E2F-4 suppressor for G1–S transition, DP-1 [29], and cyclin E with the heating-AR and modified ImmunoMax/CSA method (Table 4). MT-2 expressed E2F-4 in an obvious manner in the nuclei and DP-1 predominantly in the cytoplasm, but not E2F-1 and cyclin E (Fig. 12a–d). ATLL expressed E2F-1 or E2F-4 in the nuclei in spite of weak E2F-1 expression in the cytoplasm of some cells in 1 case (ATLL5 in Table 4), moderate DP-1 expression in the cytoplasm and cyclin E in some cells (Fig. 12g and h, Table 4). In ATLL, Tax expression detected by the heating-AR and modified ImmunoMax/CSA method of WATM-1 revealed correlation with cyclin E expression (Wilcoxon signed-ranks test, \(p=0.0209\)) but did not suggest any relation to the expression of E2F-1, E2F-4, and DP-1 (Table 4), suggesting that Tax expression was correlated with the proliferation of lymphoma type ATLL cells. EPTL expressed both E2F-1 and E2F-4 in some cells weakly, but expressed DP-1 and cyclin E strongly in most cells (Fig. 12i to l, Table 4). The expression of E2F-1 and E2F-4 in EPTL was quite different from that in ATLL and had no correlation with that of cyclin E (Table 4). The molecular mechanism in the G1–S phase

![Fig. 12. G1 phase progression and G1–S phase transition detected by a panel of antibodies against E2F-1, E2F-4, DP-1, and cyclin E by the heating-AR and modified ImmunoMax/CSA method (DAB-H\(_2\)O\(_2\) reaction and haematoxylin nuclear counterstaining, \(\times40\) Olympus BX50, FUJIFILM HC-300). a–d MT-2. e–h ATLL. i–l Peripheral T-cell lymphoma in Europeans (EPTL). a, e, and i E2F-1. b, f, and j E2F-4. c, g, and k DP-1. d, h, and l Cyclin E. The expression pattern of E2F-1 and E2F-4 is different between ATLL and EPTL. The expression pattern of cyclin E is also different between ATLL and EPTL.](image-url)
transition may be damaged or neoplastic in ATLL, where the obscure co-expression of E2F-1 and E2F-4 observed in EPTL was thought to be their physiological expression. The mechanism of HBZ mRNA activating E2F-1 to propel the G1–S phase transition in the cell cycle of ATLL cells [23, 64, 65] was not identified by the heating AR and modified ImmunoMax/CSA method, although the method could visualize physiological expression of E2F-1, E2F-4, DP-1, and cyclin E. Further studies are necessary to elucidate the pathogenicity of HBZ mRNA and protein in ATLL.

Enzymatic-AR and nsCSA system of Lt-4 demonstrated obvious granular staining in a smaller number of ATLL cells than expected, as mentioned above, suggesting that the undigested, probable complex present form of Tax may be a feature of young ATLL cells derived from ATLL stem cells [51]. Since the “stemness” of stem cells allows their original features to be maintained, the dependency of early ATLL cells on Tax would be a feature of ATLL stem cells. Heating-AR and nsCSA system of CD117 [50] (Table 3) might be one way of investigating ATLL stem cells. Further studies are warranted to clarify the features of ATLL stem cells.

V. The Future Prospects of Ultra-IHC

In light of the fact that ultra-IHC has now been established. We introduce ultra-IHC into the field of human archival pathology specimens. Recently, the intercalated antibody-enhanced polymer (IAEP) method comprising the primary mouse monoclonal antibody reaction, secondary anti-mouse Ig rabbit polyclonal antibody, and anti-mouse anti-rabbit Ig- and HRP-labeled polymer reagent reaction, succeeded in detecting the over-expression of ALK-fusion protein that could not labeled by ordinary-IHC [98]. However, it appears that the physiological expression of some antigens is not detected by ordinary-IHC and IAEP methods. Ultra-IHC could label the physiological expression of the tumor-suppressor gene product, p53 protein, and cell cycle-related molecules: E2F-1, E2F-4, DP-1, and cyclin E. We demonstrated through ultra-IHC that the staining of its physiological expression was different from that of inactivated/phosphorylated p53 protein. We also demonstrated the physiological expression patterns of E2F-1, E2F-4, DP-1, and cyclin E in EPTL and their neoplastic and altered expression patterns in ATLL. Therefore, ultra-IHC has the capability to bring IHC of human archival pathology specimens to the next level, where the physiological expression and inactivation of several kinds of molecules can be detected.

The pathogenicity of HTLV-1 was that of Tax, although it is often stated that Tax is not expressed in ATLL. This review reported that ultra-IHC could detect minute amount of simple and complex present forms of Tax in ATLL cells, suggesting that Tax is expressed in ATLL cells. We must keep in mind that extremely small amounts of Tax could be detected by ultra-IHC in most cases of ATLL that is reported to indicate Tax-induced modes of signal transductions. Since atypical lymphocytes in HANNLA and ATLL cells express much less Tax than MT-2 cells [30], as mentioned above and shown in Figure 3, the Tax-induced molecular events listed in Table 2 have to be re-evaluated in the cases of high and low Tax expression in HTLV-1 infected cells probably under constant expression of HBZ mRNA and protein. The molecular events induced by low Tax expression may play roles in ATLL oncogenesis, which spans more than 30 years under anti-HTLV-1 immunity.

In the field of hematopathology, PBTS is a powerful archival pathology specimen, in which PBMCs can be examined through histochemistry including ultra-IHC. Ultra-IHC on PBTS is expected to enable monitoring of various changes in PBMCs in ATLL oncogenesis.

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