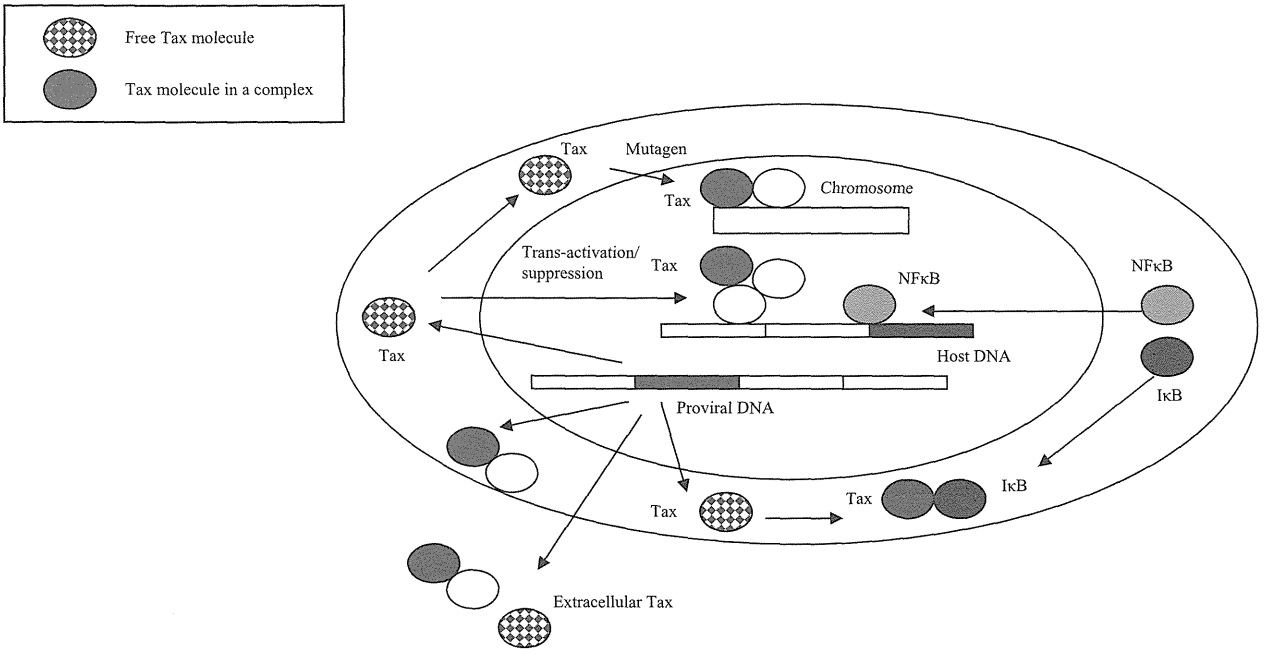

Fig. 6. Present forms of Tax in a HTLV-1 infected T-cell. Tax is synthesized in the cytoplasm based on spliced one of RNAs from the HTLV-1 proviral DNA, often binding other molecules such as IκB in the cytoplasm and cAMP responsive element binding (CREB), DNA, and chromosomes in the nucleus to manifest its pathogenicity. There are at least 2 present forms of Tax, a simple form existing freely or in a simple complex with other molecules, and a complex form forming a complicated complex with other molecules.

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

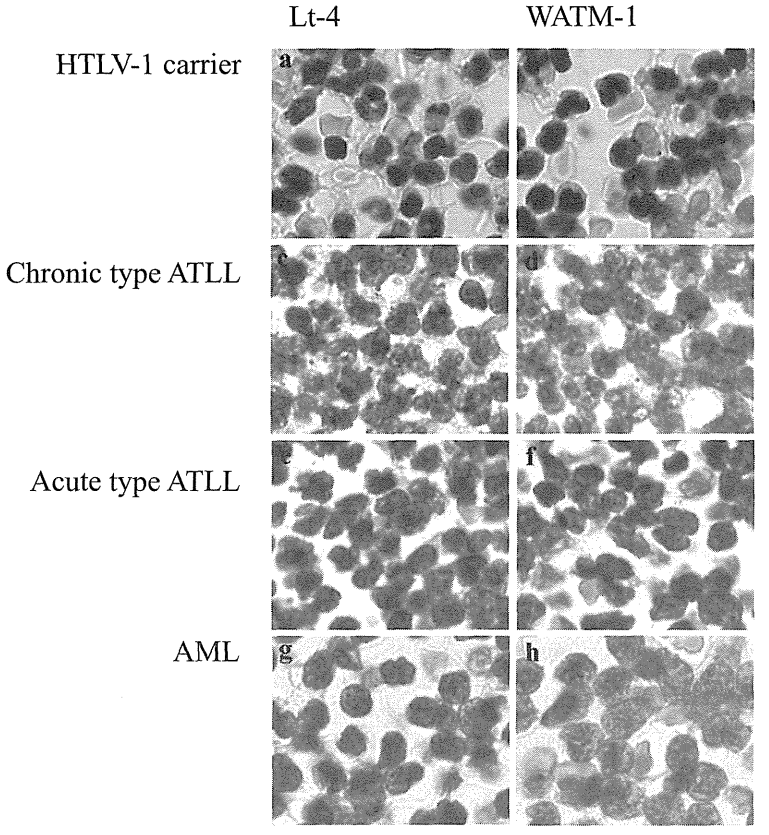

Fig. 7. Tax expression detected by the enzymatic-AR and nsCSA system in peripheral blood tissue specimen (PBTS) (DAB-H_2O_2 reaction and haematoxylin nuclear counterstaining, ×100 oil (×2.5 digital), Olympus BX50, FUJIFILM HC-300). **a** and **b**) HTLV-1 carrier. **c** and **d**) Chronic type ATLL. **e** and **f**) Acute type ATLL. **g** and **h**) Acute myelogenous leukemia (AML). More obvious granular immunostaining is seen in Lt-4 (**a, c** and **e**) than in WATM-1 (**b, d** and **f**) in HTLV-1 carriers, and chronic and acute type ATLL. However, nuclear staining by Lt-4 (**g**) and WATM-1 (**h**) is noted in an AML patient without antibodies against HTLV-1.

Table 5. *Enzymatic-AR and new simplified CSA system of Lt-4 against Tax in PBTS of HTLV-1 carriers, ATLL and the other leukemias*

	Positive cells in PBTS			
	No positive cells	A few positive cells	Some positive cells	Many positive cells
HTLV-1 carrier*	0	0	2	0
Chronic type ATL*	0	0	4	3
Acute type ATL*	0	3	3	0
The other leukemias	2	0	3**	0

*: Kruskal-Wallis test, p=0.0388. **: Nuclear staining in some cells.

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 cytoplasmic 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/

Table 6. *Enzymatic-AR and new simplified CSA system of Lt-4 applied to paraffin sections of malignant lymphomas*

	n	Immunostaining		
		Negative	Positive background cells	Positive lymphoma cells
T/NK-cell neoplasm (TML)	16	0	2	14
ATLL	13	0	0	13
PTCL, NOS	1	0	0	1
EATL	1	0	1	0
Extranodal NKTCL	1	0	1	0
B-cell neoplasm (BML)	12	1	0	11#
FL	7	1	0	6#
DLBCL	3	0	0	3#
LP	1	0	0	1#
B-CLL	1	0	0	1#
Hodgkin lymphoma (HD)				
Mixed cellularity classic HD	0	0	1	0
Total	29	1	3	25 (11#)

[T/NK-cell neoplasm/malignant lymphoma: TML] ATLL: Adult T-cell leukemia/lymphoma. PTCL, NOS: Peripheral T-cell lymphoma, not-otherwise specified. EATL: Enteropathy-associated T-cell lymphoma. Extranodal NKTCL: Extranodal NK/T-cell lymphoma.
[B-cell neoplasm/malignant lymphoma: BML] FL: Follicular lymphoma. DLBCL: Diffuse large B-cell lymphoma. LP: B-cell lymphoplasmacytic lymphoma. B-CLL: B-cell chronic lymphocytic leukemia.
#: Non-specific immunostaining was noted in 11 cases of BML in total. Non-specific nuclear staining was seen in 5 FL and 1 B-CLL. Non-specific nucleolar staining was in 2 DLBCL. Non-specific cytoplasmal fine staining was in 1 FL and 1 DLBCL. Non-specific cytoplasmal dense staining was in 1 LP.

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

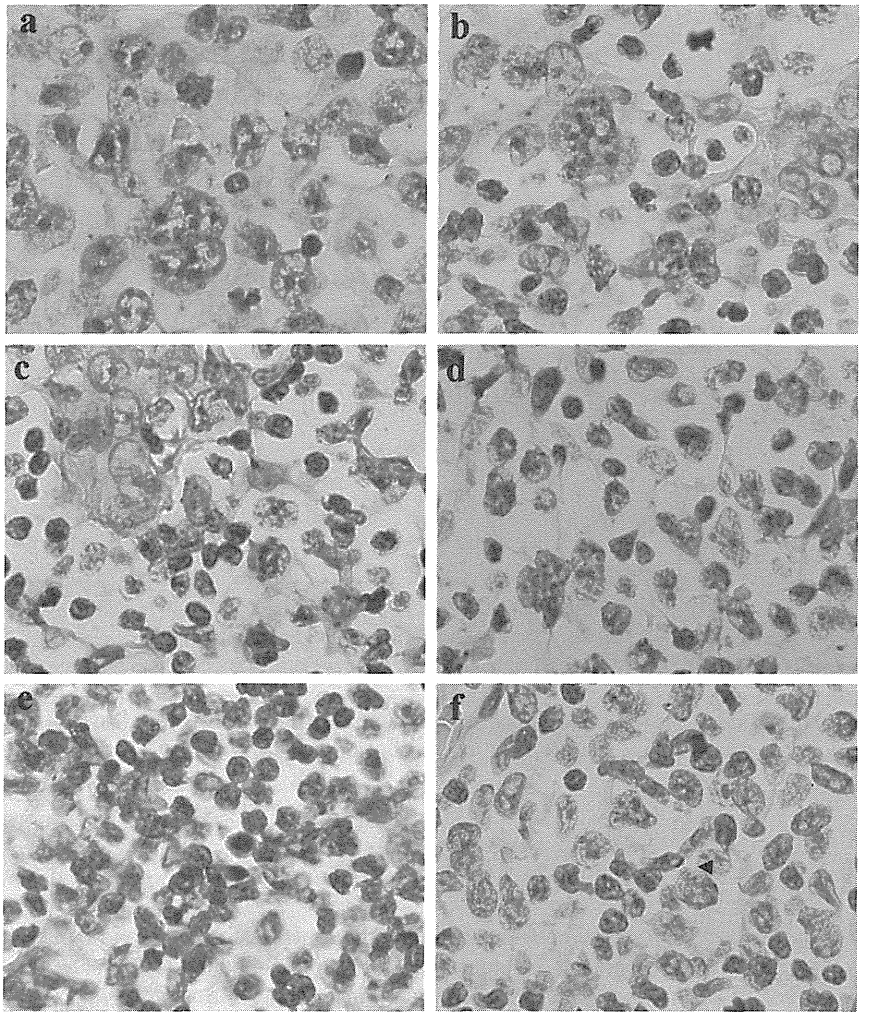

Fig. 8. Tax expression detected by the enzymatic-AR and nsCSA system of Lt-4 in archival paraffin specimens of peripheral T-cell lymphomas with and without clinical information regarding HTLV-1 infection (ATLL and PTCL) (DAB-H_2O_2 reaction and haematoxylin nuclear counterstaining, ×100 oil, Olympus BX50, FUJIFILM HC-300). More or less granular Lt-4 staining is noted in the nucleus and cytoplasm. Variously-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.

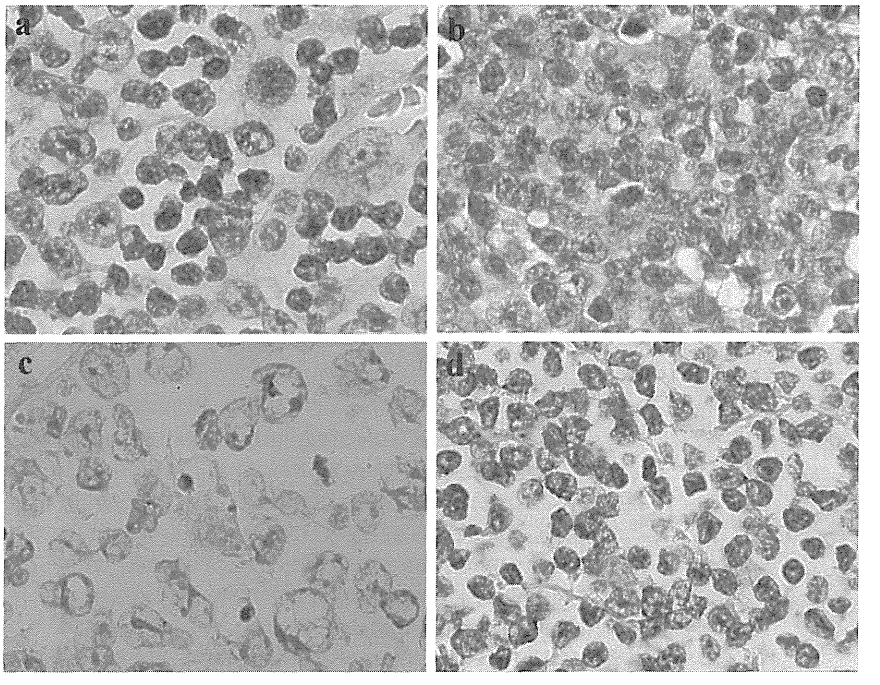

Fig. 9. Non-specific staining in the enzymatic AR and nsCSA system of Lt-4 in archival paraffin specimens of B-cell neoplasms (DAB-H_2O_2 reaction and haematoxylin nuclear counterstaining, ×100 oil, Olympus BX50, FUJIFILM HC-300). **a**) Nuclear staining in follicular lymphoma (FL). **b**) Fine granular staining in diffuse large B-cell lymphoma (DLBCL). **c**) Nucleolar staining in DLBCL. **d**) Dense cytoplasmic staining in lymphoplasmacytic lymphoma/immunocytoma (LP).

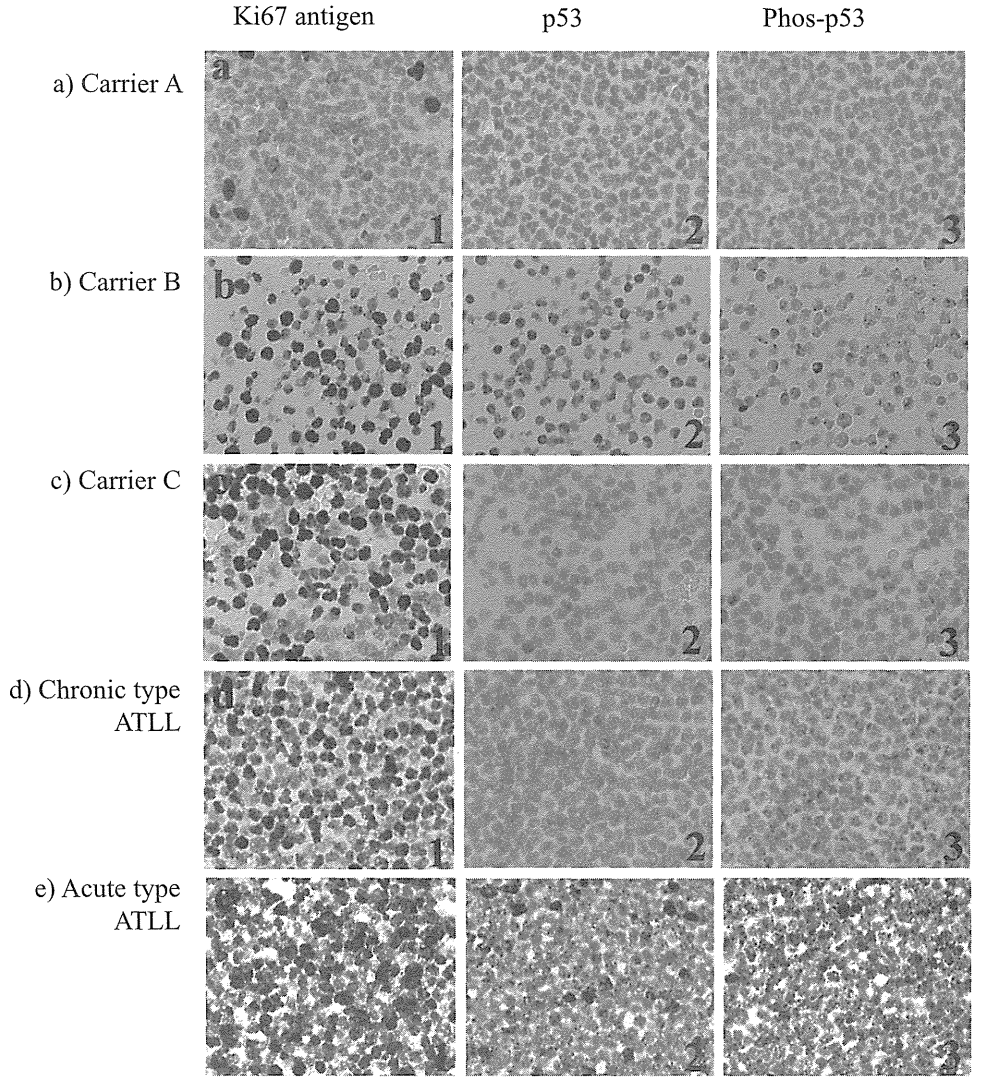

Fig. 10. Expression of Ki67 antigen, p53 protein (p53) and phosphorylated p53 protein (phos-p53) detected by the heating-AR and nsCSA system on the PBTS of HTLV-1 carriers and ATLLs (DAB-H_2O_2 reaction and haematoxylin nuclear counterstaining, ×40 and digital ×2, Olympus BX50, FUJIFILM HC-300). **a–c**) 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 (**c2**) 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 **c2**. Thus, the immunostaining of p53 in **b2** is evaluated as negative.

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, enzyme-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 the p53 protein by phosphorylating it [80], and Tax abrogates p53-induced cell cycle arrest and apoptosis through its CREB/activating transcription factor (ATF)

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 c2) 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 c2 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

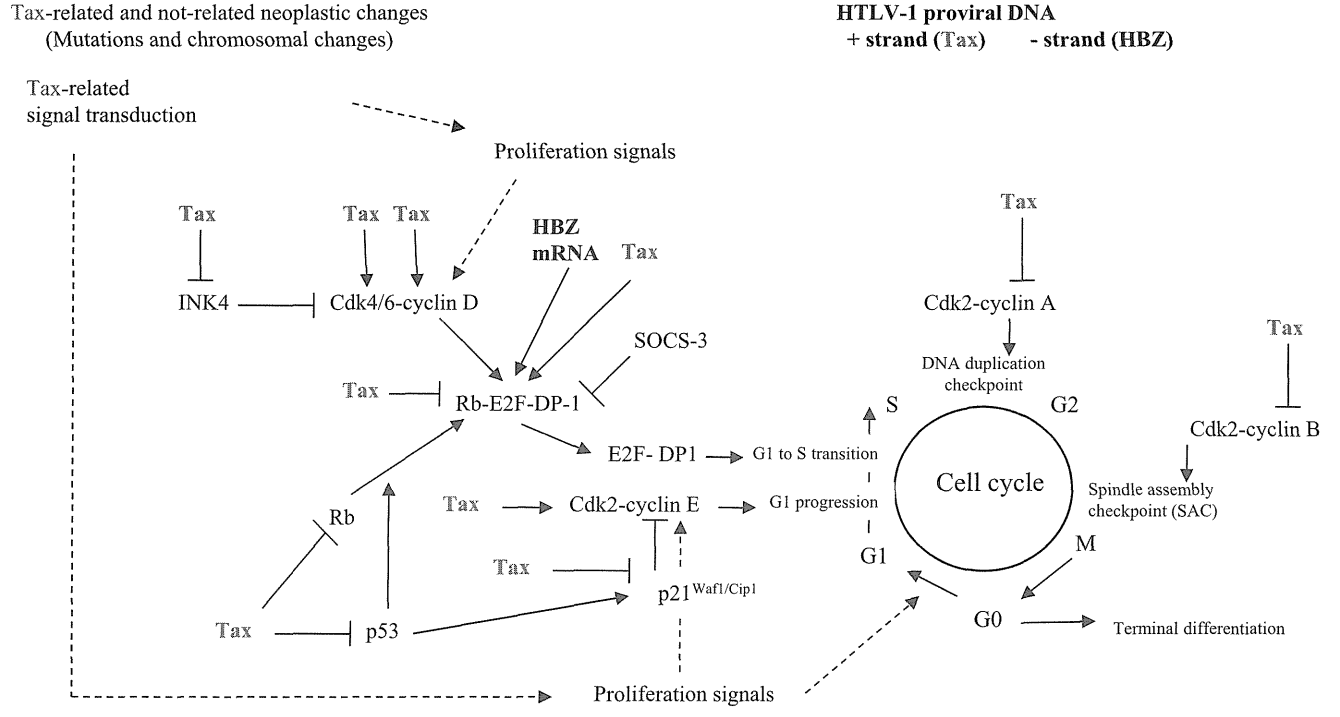

Fig. 11. Effects of Tax and HBZ mRNA molecular mechanism on cell cycle. Tax trans-scripted from the + strand of HTLV-1 proviral DNA trans-activates several genes, targets several molecules in signal transduction as listed in Table 2, and produces proliferation signals to initiate the G1 phase, cyclin 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$^{Waf1/Cip1}$, which is 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-DP-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 transcripted 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 $p21^{Waf1/Cip1}$, which in turn suppresses the Cdk2-cyclin E complex. Further, Tax trans-activates E2F-1 [110], -suppresses Cdk inhibitors such as $p16^{INK4A}$ [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

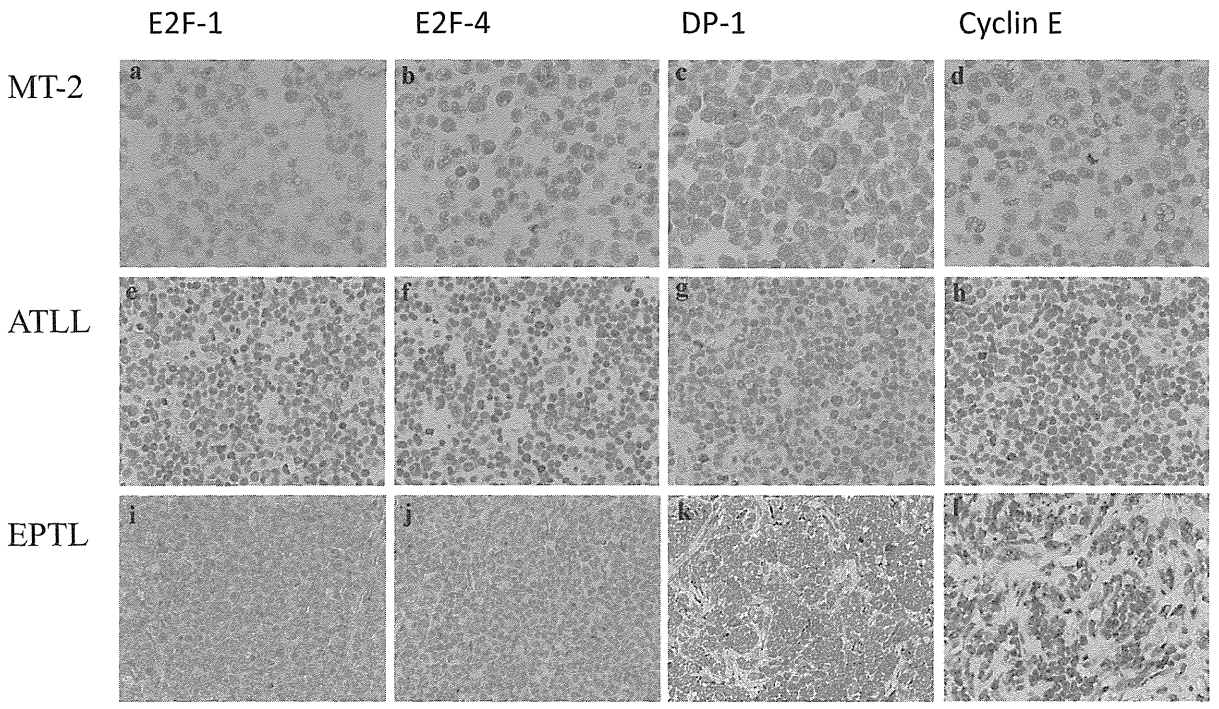

Fig. 12. G1 phase progression and G1–S phase transition detected by a panel of antibodies against E2F-1, E2F-4, DP-1, and cyclin E by the heating-AR and modified ImmunoMax/CSA method (DAB-H_2O_2 reaction and haematoxylin nuclear counterstaining, ×40 Olympus BX50, FUJIFILM HC-300). **a–d**) MT-2. **e–h**) 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.

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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VII. References

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Detection of HTLV-1 in the Labial Salivary Glands of Patients with Sjögren's Syndrome: A Distinct Clinical Subgroup?

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ABSTRACT. Objective. To examine whether patients with Sjögren's syndrome (SS) can be distinguished based on the expression of human T cell lymphotrophic virus type I (HTLV-1) and, if so, whether the subgroups differ in their clinical features and serological measures.

Methods. Polymerase chain reaction (PCR) and nested PCR were used to amplify viral DNA from peripheral blood mononuclear cells (PBMC) in 53 patients with SS, using primers from the HTLV-1 pX, p19, pol, and tax regions. Minor salivary gland biopsy specimens from 33 patients with SS were examined for the presence of HTLV-1 p19 or tax proteins immunohistochemically. The sociodemographic, glandular, and extraglandular manifestations, and laboratory findings including autoantibodies, complement, and immunoglobulin levels, were analyzed.

Results. The HTLV-1 *tax* gene was detected in PBMC samples from 2 of 53 patients (3.8%), whereas the HTLV-1 *pX, p19,* and *pol* genes were not expressed. As well, 100% of PBMC samples from 4 family members of patients in whom the *tax* gene was detected also expressed the *tax* gene. Immunohistochemical staining for HTLV-1 p19 and tax was seen in 10 out of 33 (30.3%) patients with SS each. Overall, 14 (42.4%) patients expressed HTLV-1 p19 or tax proteins, and they had lower rheumatoid factor and C3 levels ($p = 0.015$ and $p = 0.005$, respectively) and higher lymphocyte counts ($p = 0.016$). The prevalence of glandular and extraglandular manifestations did not differ between the HTLV-1-positive and negative patients.

Conclusion. Our findings suggest that HTLV-1 in the salivary glands is involved in the pathogenesis of a subpopulation of SS, and HTLV-1-associated SS might have different immunological patterns than idiopathic SS. (First Release March 1 2012; J Rheumatol 2012;39:809–15; doi:10.3899/jrheum.111075)

Key Indexing Terms:
SJÖGREN'S SYNDROME
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The human T cell lymphotrophic virus type I (HTLV-1) is a human retrovirus associated etiologically with adult T cell leukemia[1]. Individuals infected with HTLV-1 may develop other diseases, including HTLV-1-associated myelopathy/tropical spastic paraparesis[2,3], HTLV-1-associated arthropathy[4], polymyositis[5], and uveitis[6]. HTLV-1 is transmitted vertically from mother to infant and the virus is maintained within ethnic groups[7]. HTLV-1 infection is clustered in Africa, Central and South America, and Japan[8]. Although the prevalence of HTLV-1 in southwest Japan is very high, HTLV-1 is uncommon in neighboring Korea. Recently, one study reported that the HTLV-1 seroprevalence rate was 0.007% in a Korean blood donor population[9].

Sjögren's syndrome (SS) is an autoimmune connective tissue disease characterized by the destruction of salivary and lacrimal glands, resulting in xerostomia and keratoconjunctivitis sicca, and the systemic production of autoantibodies[10]. The pathogenesis of SS remains unclear, but in genetically predisposed individuals, hormonal and environmental factors (i.e., virus) are thought to be capable of triggering this autoimmune exocrinopathy[11]. Among such factors, viral infections including Epstein-Barr virus, Coxsackie virus, human immunodeficiency virus (HIV), hepatitis C virus, and HTLV-1 could prompt epithelial cells to activate the immune system[12].

Retroviruses such as HTLV-1 and HIV infect immune system cells, resulting in the destruction or overstimulation of T cells, and act as potential triggers of autoimmune disease[13,14].

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Several studies have suggested an association between HTLV-1 and SS. For example, transgenic mice possessing the HTLV-1 *tax* gene developed an exocrinopathy resembling SS[15]. Some HTLV-1-infected patients with tropical spastic paresis develop features of SS[16]. A high seroprevalence rate of HTLV-1 and high prevalence of IgA class antibodies to HTLV-1 in the saliva of patients with SS in an area endemic for HTLV-1 has been reported[17,18]. Moreover, the accumulation and expression of HTLV-1 in the salivary glands of patients with SS has been described[19,20,21]. These observations suggest that HTLV-1 plays a role in the pathogenesis of SS.

However, the association of SS with HTLV-1 in regions where HTLV-1 is not endemic remains unclear. In addition, the clinical and serological differences in SS patients with and without this infection are not well characterized. We examined whether SS patients could be distinguished based on the expression of HTLV-1 in salivary glands. We describe the clinical features and serological characteristics of patients with HTLV-1-associated SS.

MATERIALS AND METHODS

Patients. The study group included 53 patients with primary SS. All patients met the diagnostic criteria proposed by the American-European Consensus Group criteria for primary SS[22]. Patients with serious systemic diseases, such as malignancy, viral hepatitis or liver cirrhosis, infection, and endstage renal disease, were excluded. Salivary gland tissues were obtained from minor lip biopsies in 33 patients.

The study was approved by the institutional review board of the Chonnam National University Hospital. All patients provided their written informed consent.

HTLV-1 molecular study. Cellular DNA was isolated from peripheral blood mononuclear cells (PBMC) of all 53 patients. Different regions of the HTLV-1 genome were amplified using polymerase chain reaction (PCR) or nested PCR with specific primer sets (Table 1)[23,24,25]. HTLV-1 proviral DNA *pX* and *p19* genes were detected using PCR, and the *pol* and *tax* genes were detected using nested PCR, as reported[26]. PCR products corresponding to the HTLV-1 tax fragment were purified and sequenced directly using an ABI Prism 3100 genetic analyzer with the BigDye Terminator v3.1 ready reaction kit (Applied Biosystems, Foster City, CA, USA).

Immunohistochemical staining for HTLV-1. Minor salivary gland biopsy specimens were fixed in formaldehyde and embedded in paraffin according to standard protocols. Hematoxylin and eosin staining was performed for histological evaluation. Sections (4 μm thick) were cut for immunohistochemistry. After washing 3 times in phosphate-buffered saline, the sections were incubated with the mouse anti-HTLV-1 p19 monoclonal antibody TP-7 (Abcam, Cambridge, MA, USA) and mouse monoclonal antibody to Tax, Lt-4 at dilutions of 1:100 and 1:250, respectively[27]. Positive immunoreactivity appeared as a brown color.

Clinical manifestations and laboratory tests. For clinical data, we assessed dryness of the eyes, enlargement of the parotid glands, photosensitivity, swollen hand or sclerotic skin, Raynaud's phenomenon (RP), joint pain, lymphadenopathy, renal disease, psychosis, interstitial lung disease, peripheral neuropathy, and autoimmune thyroid disease. Laboratory tests included the antinuclear antibody (ANA) titer, anti-Ro/SSA, anti-La/SSB, rheumatoid factor (RF) titer, C3, C4, IgG, IgA, IgM, peripheral white blood cell, lymphocyte and platelet counts, and hemoglobin level.

Statistical analysis. The Mann-Whitney U test or chi-square test was applied to determine the significance of associations between the presence of p19 or tax antibody in the minor salivary glands and the clinical manifestations of SS. For statistical evaluations of the results, $p < 0.05$ was considered statistically significant. All statistical analyses were conducted using SPSS for Windows v. 17.0 (SPSS, Chicago, IL, USA).

RESULTS

Patient characteristics and clinical manifestations. Fifty-three patients with SS were studied. The median age of the patients was 48 years (range 23–70), and 52 patients (98.1%) were women. Fifty-two patients (98.1%) had ocular symptoms, and dry mouth symptoms were observed in 52 patients (98.1%). A total of 45 of 52 (86.5%) patients had a positive result on Schirmer's I test, and 50 of 52 patients (96.2%) had a positive result on a salivary scan. Parotid gland swelling was observed in 15 (28.3%) patients. The most frequent extraglandular manifestation was arthralgia or arthritis (52.8%), followed by RP (30.2%), peripheral neuropathy (18.9%), lymphadenopathy (17%), and photosensitivity (15.1%; Table 2).

Detection of HTLV-1 DNA in PBMC. We examined the presence of HTLV-1 in DNA samples isolated from PBMC of 53 patients. Four independent regions of the HTLV-1 genome (pX, p19, pol, and tax) were amplified using PCR or 2-step

Table 1. Primer sequences used to identify HTLV-1 DNA.

Amplified Region	Primers	Sequence (5' – 3')	Reaction	Product Size, base-pair
pX	pX1	CCT CCG TCA GCT ACG ACA C	Single PCR	317
	pX2	GGA GCG CCG TGA GCG CAA G		
p19	p19a	CAC CCC TTT CCC TTT CAT TCA CGA	Single PCR	411
	p19b	CCG GCC GGG GTA TCC TTT T		
pol	HL110	CAA GCC TAG CTA CAT AAA C	Nested PCR	188
	HL111	GCG GCT ATT AAG ACC AGG AAG	(outer primer)	
	SK110-2	TCC CCT ACA ATC CAA CCA GCT C	Nested PCR	
	SK111	ATG GGT TTG TTT ATT GCT GAG GG	(inner primer)	
tax	HL43	ATG CTT ATT ATC AGC CCA CTT	Nested PCR	159
	HL44	AGG GTC TTA GAG GTT CTC TGG GT	(outer primer)	
	SK43	CCA GTC TAC GTG TTT GGA GA	Nested PCR	
	SK44	AGC CGA TAA CGC GTC CAT CGA	(inner primer)	

HTLV-1: human T cell lymphotrophic virus type I; PCR: polymerase chain reaction.

Table 2. Baseline characteristics of 53 patients with Sjögren's syndrome.

Median age, yrs (range)	48 (23–70)
Number men:women	1:52
Median followup, yrs (range)	2 (1–11)
Glandular manifestations (%)	
Ocular symptoms	52/53 (98.1)
Oral symptoms	52/53 (98.1)
Positive salivary scan	50/52 (96.2)
Positive findings in minor salivary glands	31/33 (93.9)
Positive Schirmer I test	45/52 (86.5)
Partoid gland swelling	15/53 (28.3)
Extraglandular manifestations (%)	
Arthralgia/arthritis	28/53 (52.8)
Raynaud's phenomenon	16/53 (30.2)
Peripheral neuropathy	10/53 (18.9)
Lymphadenopathy	9/53 (17.0)
Photosensitivity	8/53 (15.1)
Interstitial lung disease	4/53 (7.5)
Autoimmune thyroid disease	3/53 (5.7)
Renal tubular acidosis	3/53 (5.7)
Psychosis	1/53 (1.9)

nested PCR. The only region detected was the HTLV-1 tax region, which was amplified from the PBMC of 2 patients. The PCR product of the *tax* gene was sequenced directly in both the forward and reverse directions and the sequencing result confirmed the HTLV-1 *tax* gene. Therefore, the rate of detection of HTLV-1 tax in the PBMC of patients with SS was 2/53 (3.8%). Subsequently, we tested for HTLV-1 DNA in PBMC from family members of 2 patients who expressed HTLV-1 *tax* gene. Patient 1 was married with 2 sons, and her husband and younger son were studied; both were positive for the HTLV-1 *tax* gene by PCR, but not for the *pX*, *p19*, and *pol* genes. Patient 2 was married with 1 daughter and 1 son, and her husband and daughter were examined; both samples were positive for tax, but negative for the other 3 regions.

Detection of HTLV-1 p19 and tax antigens in the salivary glands. Minor salivary gland biopsies were performed in 33 patients. On salivary gland staining, 10 (30.3%) of 33 patients with SS were positive for HTLV-1 p19 antigen, and 10 (30.3%) patients were positive for tax antigen. Six of the patients (18.2%) expressed both p19 and tax. In total, 14 patients (42.4%) were positive for HTLV-1 p19 or tax antigens. In the positive cases, the p19 and tax antibodies stained the acinar and ductal epithelial cells. A representative case is shown in Figure 1. Of the 2 patients with HTLV-1 PCR-positive PBMC, Patient 1 expressed both p19 and tax antigens in the salivary gland, whereas Patient 2 expressed neither.

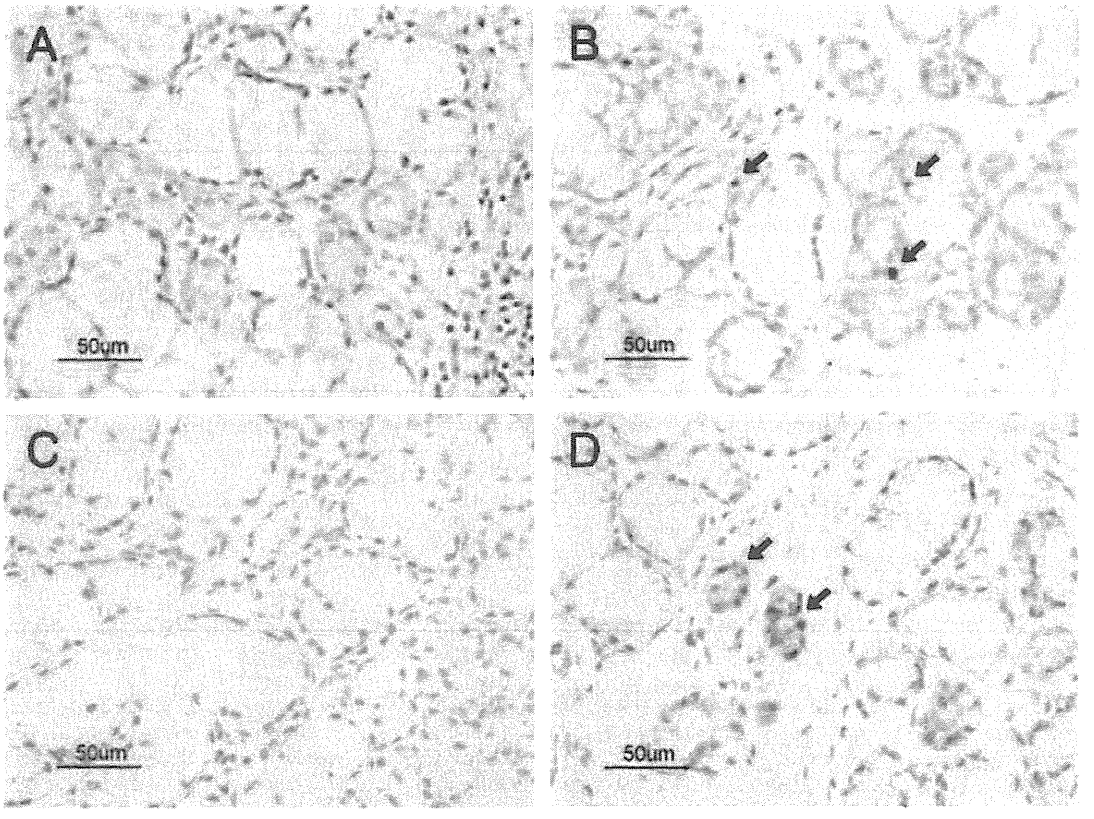

Figure 1. Representative immunohistochemistry for human T cell lymphotrophic virus type I (HTLV-1) antibody in minor salivary gland sections from different patients: (A) negative staining for p19; (B) positive staining for p19; (C) negative staining for tax; and (D) positive staining for tax. HTLV-1 p19 and tax were detected in acinar and ductal epithelial cells (original magnification ×400 for all panels). Arrows indicate antibody expression.

Characterization of HTLV-1-associated SS. Clinical and laboratory features of patients were compared according to the immunohistochemical results (Table 3). The p19-positive patients had lower anti-Ro/SSA titers, RF, and C3 levels ($p = 0.049$, $p = 0.019$, and $p = 0.018$, respectively). The lymphocyte count was significantly higher among the HTLV-1 p19-positive group than the negative group ($p < 0.001$). However, only RF was significantly different ($p = 0.016$) between the patients with and those without tax antibody. When analyzed according to the expression of p19 or tax, the patients who expressed p19 or tax antibodies to HTLV-1 had lower RF and C3 levels ($p = 0.015$ and $p = 0.005$, respectively) and higher lymphocyte counts ($p = 0.016$). The prevalence of glandular and extraglandular manifestations did not differ between the HTLV-1-positive and negative patients (Table 4). The 2 patients who were positive for the *tax* gene in PBMC had a short disease duration (< 1 year), and anti-Ro/SSA, anti-La/SSB, and RF were not detected in the patient who exhibited activity against HTLV-1 p19 and tax in the salivary gland.

DISCUSSION

We studied the presence of HTLV-1 in PBMC and minor salivary glands in patients with SS by PCR and immunohistochemical assays. In PBMC, the HTLV-1 sequence was detected in 2 of 53 (3.8%) patients by PCR analysis. All the samples were positive for tax, but negative for pX, p19, and pol. Using anti-HTLV-1 p19 or tax antibody staining, we found that HTLV-1 proteins were expressed in the minor salivary glands of 42.4% (14/33) of patients with SS. A total of 6 (18.2%) of 33 patients expressed both p19 and tax proteins. Viral expression was detected in acinar and ductal epithelial cells by immunohistochemical staining for HTLV-1 p19 and tax protein.

Table 3. Laboratory findings in patients with Sjögren's syndrome, with and without p19 or tax antibody in the salivary glands.

Characteristic	p19		tax		p19 or tax	
	Positive, n = 10	Negative, n = 23	Positive, n = 10	Negative, n = 23	Positive, n = 14	Negative, n = 19
Antinuclear antibody titer	164.0 ± 125.7	243.5 ± 121.8	220.0 ± 135.0	219.1 ± 125.8	191.4 ± 127.6	240.0 ± 125.1
High anti-Ro titer, > 200 U/ml (%)	4/10 (40)*	18/23 (78.3)	6/10 (60)	16/23 (69.6)	8/14 (57.1)	14/19 (73.7)
Anti-La titer, U/ml	33.1 ± 61.6	44.9 ± 63.6	56.7 ± 77.7	34.0 ± 55.1	41.7 ± 69.3	40.2 ± 58.8
Rheumatoid factor titer, IU/ml	14.8 ± 10.9*	54.6 ± 86.0	19.1 ± 20.1*	51.3 ± 84.5	18.1 ± 17.4*	58.7 ± 91.5
C3, mg/dl	87.6 ± 23.3*	108.7 ± 22.3	94.5 ± 15.3	105.7 ± 27.0	89.1 ± 21.6**	112.0 ± 21.9
C4, mg/dl	18.6 ± 6.2	21.6 ± 7.3	19.8 ± 6.1	21.1 ± 7.5	18.6 ± 6.2	22.3 ± 7.3
IgG, mg/dl	1409.9 ± 902.1	1952.8 ± 1304.8	1549.0 ± 1000.0	1892.3 ± 1297.0	1472.2 ± 965.2	2021.2 ± 1339.4
IgA, mg/dl	336.6 ± 191.9	303.2 ± 117.0	369.1 ± 181.5	289.0 ± 116.4	328.9 ± 168.8	302.2 ± 121.6
IgM, mg/dl	112.3 ± 43.2	167.9 ± 79.9	146.3 ± 70.5	152.8 ± 78.0	138.9 ± 64.1	159.5 ± 82.3
White blood cells, /mm^3	5510.0 ± 1969.5	5356.5 ± 2179.8	5130.0 ± 1566.3	5522.0 ± 2300.0	5421.4 ± 1763.8	5389.5 ± 2347.1
Hemoglobin, g/dl	12.6 ± 1.9	12.7 ± 0.8	12.4 ± 1.3	12.8 ± 1.2	12.7 ± 1.6	12.7 ± 0.9
Platelets, × 10^3/μl	228.3 ± 81.1	344.6 ± 528.0	477.4 ± 799.2	236.3 ± 77.8	404.7 ± 676.2	239.1 ± 82.2
Lymphocytes, /μl	2364.0 ± 948.0***	1228.2 ± 523.0	2014.0 ± 1165.3	1380.4 ± 605.9	2002.9 ± 1027.8*	1255.3 ± 519.5

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Table 4. Clinical manifestations in patients with primary Sjögren's syndrome, with and without p19 or tax antibody in the salivary glands.

Manifestations	p19		tax		p19 or tax	
	Positive, n = 10	Negative, n = 23	Positive, n = 10	Negative, n = 23	Positive, n = 14	Negative, n = 19
Glandular (%)						
Dryness of eyes	9/10 (90)	23/23 (100)	9/10 (90)	23/23 (100)	13/14 (92.9)	19/19 (100)
Dryness of mouth	9/10 (90)	23/23 (100)	9/10 (90)	23/23 (100)	13/14 (92.9)	19/19 (100)
Parotid gland enlargement	2/10 (20)	4/23 (17.4)	1/10 (10)	5/23 (21.7)	2/14 (14.3)	4/19 (21.1)
Extraglandular (%)						
Joint pain	6/10 (60)	16/23 (69.6)	6/10 (60)	16/23 (69.6)	9/14 (64.3)	13/19 (68.4)
Raynaud's phenomenon	4/10 (40)	10/23 (43.5)	4/10 (40)	10/23 (43.5)	7/14 (50)	7/19 (36.8)
Photosensitivity	2/10 (20)	4/23 (17.4)	1/10 (10)	5/23 (21.7)	2/14 (14.3)	4/19 (21.1)
Peripheral neuropathy	3/10 (30)	3/23 (13)	3/10 (30)	3/23 (13)	4/14 (28.6)	2/19 (10.5)
Lymphadenopathy	0/10 (0)	6/23 (26.1)	0/10 (0)	6/23 (26.1)	0/14 (0)	6/19 (31.6)
Interstitial lung disease	1/10 (10)	3/23 (13)	0/10 (0)	4/23 (17.4)	1/14 (7.1)	3/19 (15.8)
Renal tubular acidosis	1/10 (10)	2/23 (8.7)	1/10 (10)	2/23 (8.7)	1/14 (7.1)	2/19 (10.5)
Autoimmune thyroid disease	2/10 (20)	1/23 (4.3)	1/10 (10)	2/23 (8.7)	2/14 (14.3)	1/19 (5.3)
Psychosis	1/10 (10)	0/23 (0)	1/10 (10)	0/23 (0)	1/14 (7.1)	0/19 (0)

Previous studies reported a high prevalence of anti-HTLV-1 antibodies in the peripheral blood of patients with SS (23%–36.7% compared to 3%–8.4% of normal controls) in endemic areas[17,18,28]. However, only a few studies have evaluated the salivary glands of patients with SS in an endemic area. In Ohyama, *et al*[20], HTLV-1 DNA from the salivary glands was amplified by PCR in 100% of HTLV-1-seropositive patients with SS in an area endemic for HTLV-1. In addition, Yamano, *et al*[29] reported that the salivary glands of 47% of patients with SS expressed retroviral antibody p24, and all affected patients were positive for p24 antibody in serum. Regarding the seroprevalence of patients with SS from a region not endemic for HTLV-1, Shattles, *et al*[30] and Mariette, *et al*[19] reported that HTLV-1 antibodies were not detected in the peripheral blood of patients with SS. In contrast, HTLV-1 was detected in the salivary glands in about one-third of these seronegative SS patients[19,30]. In our study, the seroprevalence of HTLV-1 in patients with SS was 3.8% (2/53) in the PCR analysis. The prevalence of HTLV-1 in our SS patients was lower than the prevalence observed in an endemic area such as Japan, but was significantly higher than the rate of 0.007% reported from healthy Korean blood donors[9]. Further, when the seropositivity of the family members of 2 seropositive patients was evaluated, all 4 family members studied were positive for HTLV-1 tax by PCR. Interestingly, in minor salivary gland biopsies, HTLV-1 proteins p19 or tax were detected in 42.4% (14/33) of the patients with SS, including 1 seropositive patient.

Several studies have examined the salivary glands of HTLV-1 seronegative SS patients, with conflicting results. Shattles, *et al*[30] showed that 31% of 39 HTLV-1 seronegative patients with SS expressed p19 antigen in salivary gland biopsies. Sumida, *et al*[31] reported the presence of HTLV-1 tax/rex messenger RNA in labial minor salivary gland samples from 4 (29%) of 14 HTLV-1 seronegative SS patients. Mariette, *et al*[32] detected the *tax* gene, but not the *env* or *pol* genes, in minor salivary gland samples by PCR from 9 (23%) of 40 HTLV-1 seronegative French patients. In contrast, Rigby, *et al*[33] found that none of 49 British patients with SS were positive for the *tax* gene in the salivary glands by PCR. However, our result is consistent with those of several studies that detected HTLV-1 p19 or tax in the salivary glands of seronegative SS patients in nonendemic areas[19,30,31]. The discrepancy between the HTLV-1 seroprevalence and positivity of salivary glands in seronegative patients with SS could be explained in the following ways. First, it has been postulated that HTLV-1 transcription is repressed by a factor in serum or by a product of HTLV-1 itself[34,35], leading to transient HTLV-1 expression by individual cells. Second, a "hit-and-run" mechanism is possible, in which acute infection with a specific virus elicits a chronic pathological response that persists after the original infection has been cleared. Third, the proteins detected in these patients could belong to another yet-unknown exogenous or endogenous retrovirus.

In our study, comparison of the laboratory data of HTLV-1 p19 or tax-positive and negative patients from minor salivary glands showed that autoantibodies including anti-Ro/SSA and RF were lower in HTLV-1-positive patients. Talal, *et al*[36] reported that a subset of patients with SS had serum antibodies to the retroviral proteins (p24 gag), and had lower anti-Ro/SSA and anti-La/SSB autoantibody levels. In addition, Eguchi, *et al*[17] showed that patients with antibodies to HTLV-1 had lower levels of ANA, anti-Ro/SSA, and anti-La/SSB, although the difference was not significant. In contrast, Hida, *et al*[28] found no differences comparing the prevalence of autoantibodies between seropositive and seronegative patients. Although our results differ from some studies[19,20,28], the presence of sicca symptoms in HTLV-1 patients does not seem to depend on the production of autoantibodies. Further, the lymphocyte count in the HTLV-1-positive patients with SS was significantly higher than that in the HTLV-1-negative patients. HTLV-1 infection rapidly induces the activation and proliferation of peripheral blood lymphocytes in patients with HTLV-1-associated myelopathy[37,38], as well as in healthy carriers[39], and this is consistent with the hypothesis that such cells cause the inflammatory lesions that result in tissue damage in the associated diseases. Clinically, there was no significant difference between the HTLV-1-positive and negative patients. Our result concurs with that of Mariette, *et al*[32] and contrasts with data from 2 groups in Japan[17,40], which reported that the frequency of extraglandular manifestations tended to be higher in the HTLV-1-seropositive group than in the HTLV-1-negative group.

Several possible mechanisms have been proposed for the pathogenesis of HTLV-1-associated SS. As we found, HTLV-1 may infect glandular epithelial cells and impair the function of the salivary glands directly. The salivary gland could represent a reservoir in which the virus replicates, and the infection could in turn lead to a cytotoxic immune reaction and cytokine secretion against HTLV-1-infected cells in the salivary glands. In addition, the infiltration of salivary glands by HTLV-1-infected or activated T lymphocytes might contribute to the development of exocrinopathy[20]. In this regard, Sasaki, *et al* reported the accumulation of common T cell clonotypes with an identical TCR *Vß* gene in the salivary glands and PBMC of HTLV-1-associated patients with SS[41].

Our study has some limitations. First, because it is impossible to biopsy normal subjects for ethical reasons, we could not compare the salivary glands of patients with SS to normal control samples. Second, we cannot exclude the possibility that detection of HTLV-1 in the salivary glands of patients with SS represents an incidental presence because of the cross-sectional study design. Third, as several endogenous retroviruses may encode p19 or tax[42], we cannot rule out the possibility that the discovery of HTLV-1 antibodies in the salivary glands involves a cross-reaction of the antibodies with other human endogenous retroviruses.

Our study confirms previous findings and provides addi-

tional evidence that suggests a relationship between HTLV-1 infection and SS. We identified a subset of patients with SS characterized by the presence of HTLV-1 proteins in the labial salivary glands and a relative absence of autoantibodies including anti-Ro/SSA and RF. Larger prospective studies that provide a precise characterization of infected cells are warranted to evaluate the implications of these observations on the pathogenesis of SS.

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REVIEW ARTICLE
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The roles of acquired and innate immunity in human T-cell leukemia virus type 1-mediated diseases

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Human T-cell leukemia virus type 1 (HTLV-1) causes adult T-cell leukemia (ATL) and HTLV-1-associated myelopathy/tropical spastic paraparesis in small subsets of HTLV-1 carriers. HTLV-1-specific T-cell responses play critical roles in anti-viral and anti-tumor host defense during HTLV-1 infections. Some HTLV-1 carriers exhibit selective loss or anergy of HTLV-1-specific T-cells at an asymptomatic stage. This is also observed in ATL patients and may therefore be an underlying risk factor of ATL in combination with elevated proviral loads. HTLV-1-specific T-cells often recognize the viral oncoprotein Tax, indicating expression of Tax protein *in vivo*, although levels of HTLV-1 gene expression are known to be very low. A type-I interferon (IFN) response can be induced by HTLV-1-infected cells and suppresses HTLV-1 expression *in vitro*, suggesting a role of type-I IFN response in viral suppression and pathogenesis *in vivo*. Both acquired and innate immune responses control the status of HTLV-1-infected cells and could be the important determinants in the development of HTLV-1-mediated malignant and inflammatory diseases.

Keywords: adult T-cell leukemia, cytotoxic T lymphocytes, human T-cell leukemia virus type 1, HTLV-1-associated myelopathy/tropical spastic paraparesis, type-I interferon

INTRODUCTION

Human T-lymphotropic virus type I (HTLV-1) causes adult T-cell leukemia (ATL) (Hinuma et al., 1981; Uchiyama, 1997) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP; Gessain et al., 1985; Osame et al., 1986) in a subset of infected individuals. While ATL is a malignant lymphoproliferative disease, HAM/TSP presents as a chronic inflammatory neurodegenerative disease. It is not known how one virus can cause two vastly different diseases. Since no disease-specific difference among HTLV-1 strains have been identified (Daenke et al., 1990; Kinoshita et al., 1991), the different pathogenic consequences must be attributed to host factors. Indeed, the two diseases usually occur in different populations of HTLV-1 carriers. Identification of the determinant factors may allow the prediction of disease risks and also the development of prophylactic and therapeutic strategies.

One of the factors that are known to differ between ATL and HAM/TSP patients is the strength of HTLV-1-specific T-cell responses. T-cell responses are activated in HAM/TSP patients, but are weak in ATL patients, and are thus considered to be one of the most important determinants of the disease manifestation. Many investigators, including us, have been investigating HTLV-1-specific cytotoxic T lymphocyte (CTL) responses, and demonstrated the importance of these CTLs on anti-viral and anti-tumor surveillance in HTLV-1-infected hosts (Jacobson et al., 1990; Bangham and Osame, 2005; Kannagi et al., 2005). Based on these studies we concluded that a reduced HTLV-1-specific T-cell response can be an underlying risk factor for the development of ATL.

Another difference between HAM/TSP and ATL patients is the level of HTLV-1 gene expression. Although HTLV-1 mRNA levels are generally low *in vivo*, they are slightly higher in HAM/TSP patients compared with asymptomatic HTLV-1 carriers (ACs; Yamano et al., 2002). The mechanism causing low levels of HTLV-1 gene expression *in vivo* remains unknown. However, we recently demonstrated that HTLV-1 expression is suppressed by non-lymphoid stromal cells through type-I interferon (IFN), indicating that innate immune responses can be another host determinant for HTLV-1-induced diseases (Kinpara et al., 2009). So far there have only been a limited number of studies reporting a type-I IFN response during HTLV-1 infection.

The status of HTLV-1 expression is critical for host immune responses and viral pathogenesis. In particular, HTLV-1 Tax is a multipotent protein that is a main target of T-cell immunity (Jacobson et al., 1990; Kannagi et al., 1991) and can activate NF-κB, a characteristic transcriptional factor in ATL cells and a strong inducer of inflammatory cytokines (Yoshida, 2001; Jeang et al., 2004; Grassmann et al., 2005). The status of Tax expression *in vivo* has been controversial but would be an extremely important factor to measure in order to understand the mechanism of disease development in HTLV-1 infection. Expression of HTLV-1 basic leucine zipper factor (HBZ) encoded by the minus-strand HTLV-1 genome is also an important factor for viral pathogenesis as HBZ elicits indirect effects on tumor development and inflammation (Satou et al., 2011).

In this review, we aim to understand the conflicting findings that have been reported in regard to HTLV-1 expression *in vivo*. We then describe recent findings that add to the knowledge about well-characterized host T-cell responses, followed by a description of the mechanisms that control viral expression. We finally discuss the relationship between HTLV-1 expression, host immune