Expression of E2F Transcription Factors in Adult T-cell Leukemia/ Lymphoma cells in vivo; A Highly Sensitive Immunohistochemical Study

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Expression of E2F Transcription Factors in Adult T-cell Leukemia/ Lymphoma cells in vivo; A Highly Sensitive Immunohistochemical Study

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Introduction

Human T-cell leukemia virus type 1 (HTLV-1) induces adult T-cell leukemia/ lymphoma (ATLL). Molecular mechanism in HTLV-1-related ATLL leukemogenesis has been clarified by Yoshida M et al (1-4). Based on the pX mRNA of the integrated proviral DNA of HTLV-1, p40Tax (Tax) and p27Rex (Rex) are synthesized. Tax activates the integrated HTLV-1 proviral DNA(1, 2), trans-activates or -depresses host cell genes and interacts with several bioactive proteins such as IκB (1,3) to induce proliferation or apoptosis of host cells and to make host cells to produce cytokines or to express abnormal phenotypes. On the other hand, Rex suppresses the activation of HTLV-1 proviral DNA and modulates the activation to reproduce HTLV-1 (1). Then, Tax is thought to play important roles in ATLL leukemogenesis.

Introducing highly sensitive immunohistochemistry (ImmunoMax) from the laboratory of Prof. Feller AC (Institute of Pathology, Luebeck Medical College, Germany) (5) to the immunohistochemistry of Tax and Rex, and employing rat monoclonal antibodies, WATM-1 against Tax and Rec-6 against Rex, we succeeded to visualize Tax and Rex in HTLV-1-infected lymphocytes (6) and ATLL cells in vivo (7). In most cases of ATLL, more or less amount of Tax could be recognized in ATLL cells in lymph nodes and in the other organs such as skin (7). Cytoplasmic Tax could be recognized, but intranuclear Tax is of too small amount to be visualized obviously even by the ImmunoMax. ATLL cells in lymph nodes showed various amount of cytoplasmic Tax and a relatively large amount of Rex, which reduces the amount of cytoplasmic Tax. On the other hand, MT-2 cells, HTLV-1-related cell line cells, have a large amount of cytoplasmic Tax and a small amount of Rex.

Molecular biology of proliferation signal transduction, DNA damage and cell cycle has been developing (Fig. 1). Interaction of Tax with molecles that play important roles in the molecular reactions has been clarified. In cell cycle there are checkpoints in G1, S, G2 and M phases and in mitotic spindle body formation. Abnormalities in S, G2 and M phases would induce arrest in

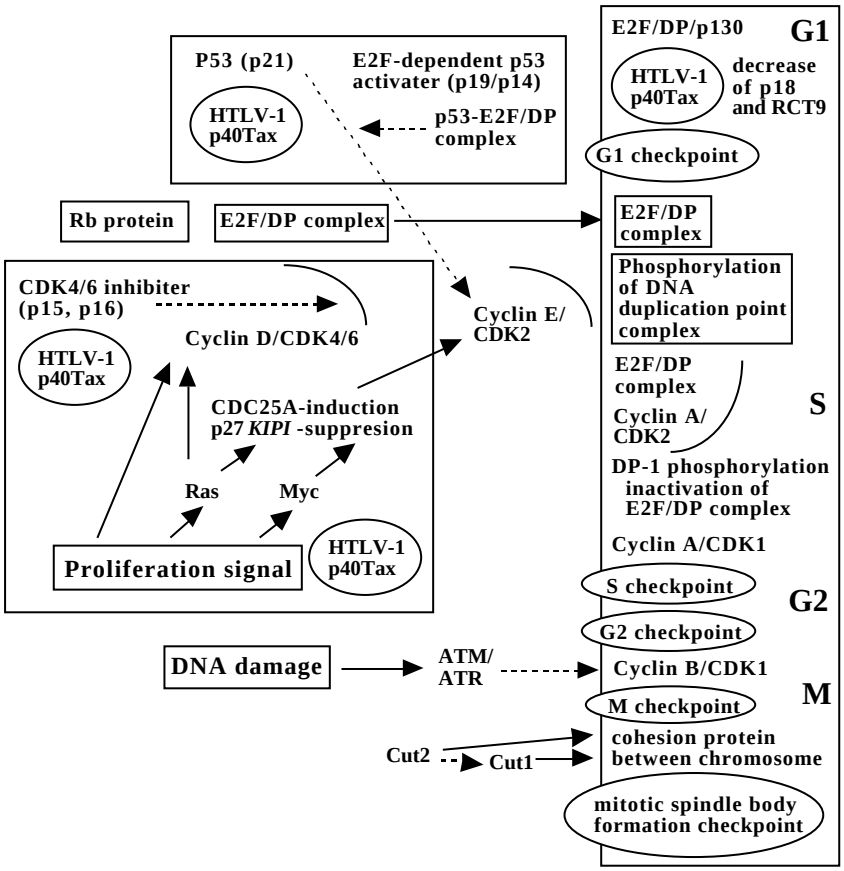

Figure 1. Molecular mechanism of ATLL leukemogenesis under HTLV-1 proviral DNA pX p40Tax from viewpoints of proliferation signal transduction, DNA damage and cell cycle

In normal proliferation signal transduction, the signals, which originate in several kinds of receptors or in onco-/proliferative gene activation, end to phosphorylate a complex of retinoblastoma (Rb) protein and E2F transcriptional factors (E2F)-DP-1 complex to release the E2F-DP-1 complex from the Rb protein, and phosphorylate DNA replication point complex by activated cyclin E/CDK2. The both positive regulation systems of DNA replication in G1/S transition by the E2F-DP-1 complex and the cyclin E/CDK2 promote cell cycle. HTLV-1 p40Tax (Tax) trans-suppresses CDK 4/6 inhibitors, p15 and p16, trans-activates cell surface receptors such as interleukin 2 receptor (IL2R) and onco-/proliferative genes such as Ras and Myc. Cyclin E/CDK2 inhibitor p53-related p21 is not transcribed because of combination of Tax and p53. E2F-dependent negative feedback of the positive regulation of DNA replication would be disturbed by combining of Tax with E2F-dependent p53 activator, p19/p14. Then, Tax activates the both positive regulation of DNA replication in G1/S transition and suppresses its negative regulation. Furthermore, Tax suppresses G1 cell cycle suppressers, p18 and RCT9, although the molecular mechanism is not clarified. On the other hand, from a viewpoint of cell cycle, many chromosomal abnormalities in ATLL cells suggest that HTLV-1-related proteins effect on M phase checkpoint and on mitotic spindle body formation checkpoint.

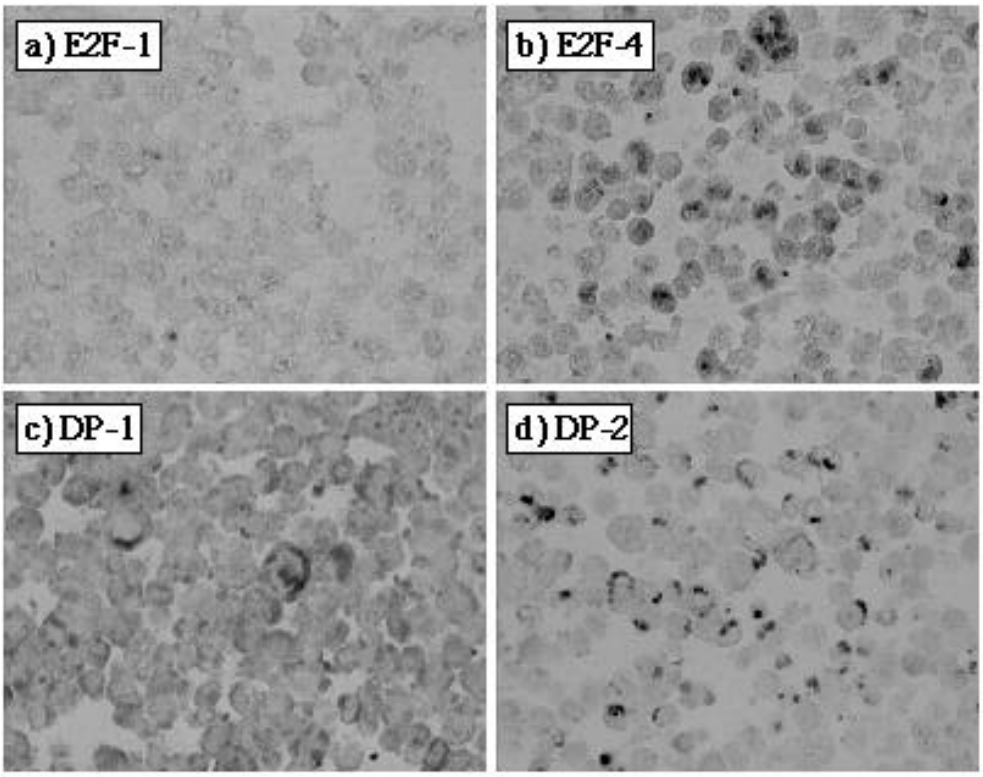

Figure 2. High sensitive immunohistochemistry of E2F-1 (KH95) and-4 (D3) and DP-1 (K-20) and -2 (C-20) in MT-2 cells

E2F-1 is not labeled in nuclei of MT-2 cells (a). E2F-4 is recognized in nuclei of some MT-2 cells (b). DP-1 appeared in a few large cells of MT-2 cells, indicating cytoplasmic stain (c). DP-2 is labeled in cytoplasm of some of MT-2 cells, revealing granular stain (d).

cell cycle that ends in apoptosis. Overriding these checkpoints with abnormalities would yield abnormal clones. Overriding of abnormalities in the mitotic spindle body formation in metaphase-anaphase transition induces chromosomal instability. Effects of Tax in a molecular level in the G1/S checkpoint have been clarified by Yoshida M et al (4). Tax transactivates host cell oncogenes and induces abnormal phenotypes, which are parts of proliferation signal transduction system. The proliferation signal induces phosphorylation of retinoblastoma (Rb) protein-E2F transcription factor (E2F)-DP-1 complex by a complex of cyclin D/cyclin-dependent kinase (CDK) 4/6 in one side, and suppresses $p27^{KIPI}$ and induces CDC25A to phosphorylate DNA replication beginning point complex by cyclin E/CDK2 complex in the other side. The E2F-DP-1 complex released from Rb protein to nucleus and the phosphorylation of the DNA replication beginning point complex make

Table 1. Expression of E2F-1 and -4 and DP-1 and -2, detected by highly sensitive immunohistochemistry

	E2F-1 KH95	E2F-4 D3	DP-1 K-20	DP-2 C-20
MT-2	-	N++	C++	C+
Lymph follicle	C++	-	C++	C+
γ / δ T-cell lymphoma	-	C+	C+/-	-
1, ATLL	C+/-	-	C+/-	-
2, ATLL	C+	-	C++	C+/-
3, ATLL	N+	-	C+	-
4, ATLL	-	C+/-	C+	C+
5, ATLL	-	N+/-	-	-
6, ATLL	C+	N++	C+	-
7, ATLL	-	-	C+	-
8, ATLL	-	-	-	-
9, ATLL	-	- (NC++)#	C+(C++)#	-
10, ATLL	-	- (NC++)#	C+(C++)#	-

C: Cytoplasmic stain N: Nuclear stain
( )#: Evaluation in intermingling lymphocytes
Evaluation of the stain
-: No stain
+/-: Weakly stain
+: Positve stain
++: Strongly positive stain

cell to enter in S phase. Several negative control systems in the molecular events in G1/S transition can be blocked by Tax. CDK 4/6 inhibitors (p16) are trans-suppressed by E-box combined with Tax (4, 8) Tax combines p53 (9, 10) that suppresses activation of cyclin E/CDK2 complex. E2F-dependent p53 activator (p19/p14) is thought to combine with Tax and to lose its effect, because p14 has the exons 2 and 3 same as those of p16. G1 cell cycle suppressers, p18 and RCT9, are reduced by Tax in G1 phase, although its mechanism is unknown (8). Then, Tax induces release of E2F-DP-1 complex to propel G1/S transition in cell cycle. The overriding of the molecular reaction abnormalities that induced by Tax is thought to be of the oncogenic changes in ATLL leukemogenesis.

This study aimed to see whether abnormal expression of E2F-DP-1 complex is common in ATLL cells in vivo.

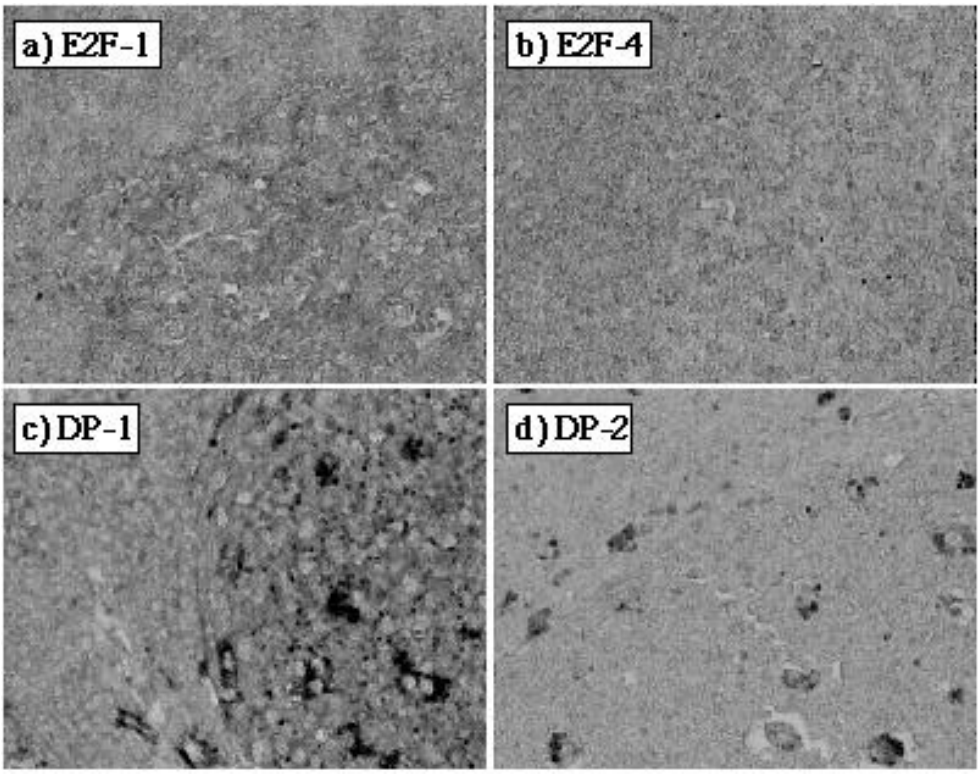

Figure 3. High sensitive immunohistochemistry of E2F-1 (KH95) and -4 (D3) and DP-1 (K-20) and -2 (C-20) in human tonsillar tissue

Mantle zone lymphocytes, centrocytes and centroblasts show obscure cytoplasmic satin of E2F-1 (a) and DP-1 (c). A few centroblasts reveal obscure nuclear stain (a). In germinal center, dendritic cells and tingible body macrophages show obvious cytoplasmic stain of DP-1 (c) and -2 (d).

Material and Method

Materials employed in this study were paraffin sections of MT-2 cell block, human tonsil tissue, one gamma/delta T-cell lymphoma and 10 cases of ATLL. Monoclonal integration of HTLV-1 proviral DNA in neoplastic cells was proved in all the cases of ATLL.

Employed antibodies were anti-E2F-1 antibody (Ab) (KH95, monoclonal (Mo), Santa Cruz Biotechnology Inc.), anti-E2F-4 antibody (D3, Mo, Santa Cruz Biotechnology Inc.), anti-DP-1 antibody (K-20, polyclonal (Po), Santa Cruz Biotechnology Inc.), and anti-DP-2 antibody (C-20, Po, Santa Cruz Biotechnology Inc.).

Optimal antigen retrieval was determined, comparing retrieval solutions; 4M urea solution, 0.01M citrate pH 6.0 and 0.01M citrate buffer pH 6.0, and

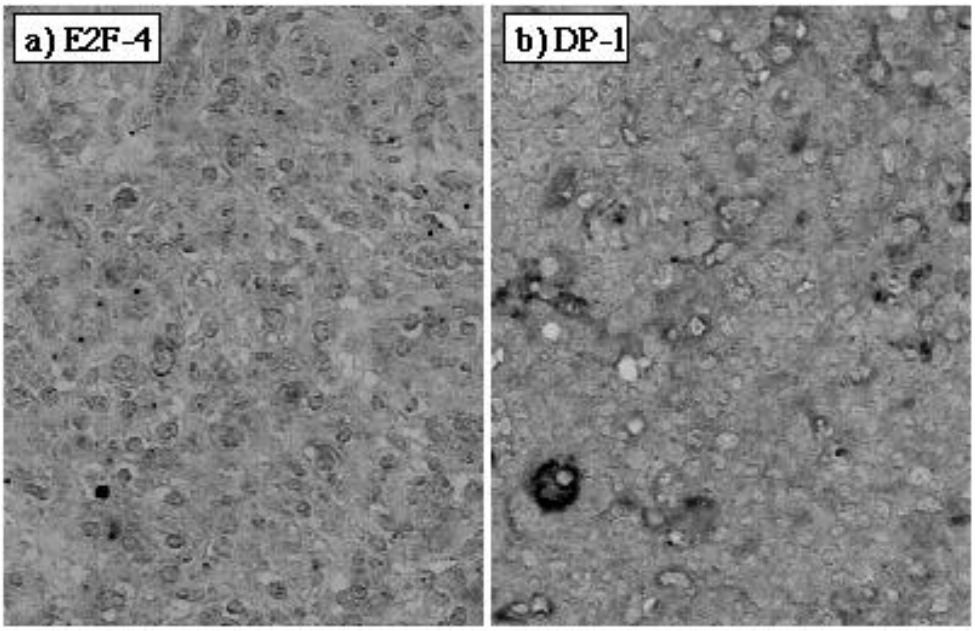

Figure 4. Appearance of E2F-4 (D3) and DP-1 (K-20) in lymphoma cells of gamma/delta T-cell lymphoma.

A few large lymphoma cells show nuclear stain of E2F-4 (a). Many lymphoma cells show obscure cytoplasmic stain of E2F-4 (a) and DP-1 (b), although the cytoplasmic stain of DP-1 is stronger than that of E2F-4.

methods; high pressure cookpot method and autoclave method.

Sections dewaxed were retrieved by means of the optimal antigen retrieval method and incubated in non-fat milk solution including animal serum (80 micron liters horse serum in 1 ml non-fat milk solution for Mo Ab, gout serum for Po Ab). Primary antibody solutions were applied on the sections at 4℃ overnight. The reacted primary antibodies were visualized by means of tyramine signal amplification (TSA) system and DAB-H_2O_2 reaction.

Result

The both E2F-1 (KH95) and -4 (D3) antibodies labeled nuclei of MT-2 cells in the optimal antigen retrieval in 0.01M citrate buffer pH 6.0 by means of autoclave heating. By blocking non-specific binding of antibody by pre-incubating sections in non-fat milk solution including animal serum, as shown in Fig. 2a and b, only E2F-4 antibody (D3) labeled nuclei of MT-2 cells.

The evaluation of the highly sensitive immunohistochemistry of E2F-1, and

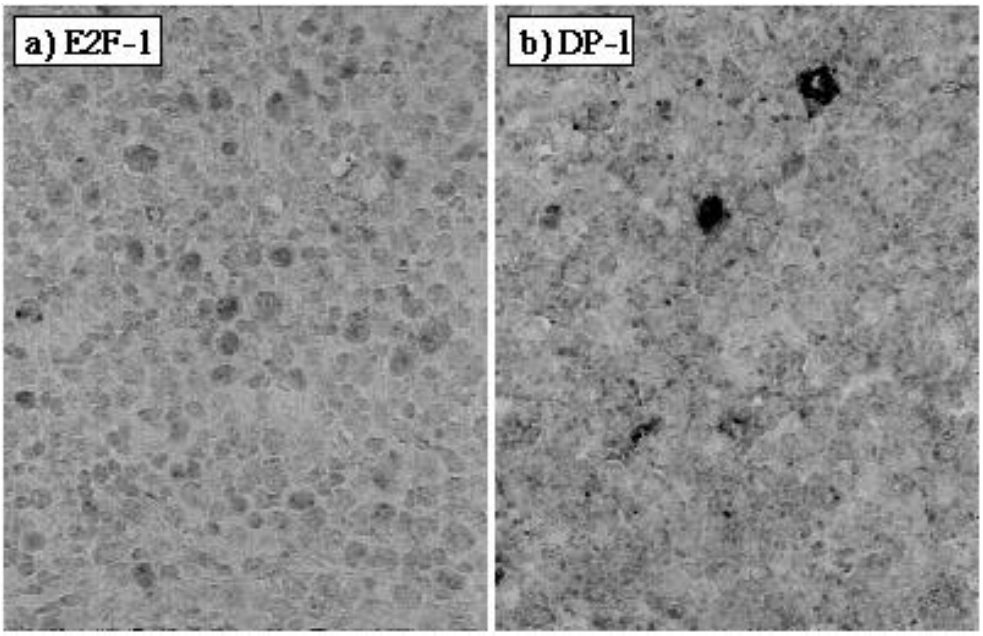

Figure 5. Appearance of E2F-1 (KH95) and DP-1 (K-20) in Case 3 of ATLL
Obvious nuclear stain of E2F-1 is seen in many lymphoma cells (a). Stronger nuclear stain of E2F-1 is recognized in larger ATLL cells. Cytoplasmic DP-1 is seen (b).

-4, and DP-1 and -2 is shown in table 1.

MT-2 cells (Fig. 2) showed nuclear stain of E2F-4 and cytoplasmic stain of DP-1 and DP-2.

Human tonsil tissue (Fig. 3) showed cytoplasmic stain of E2F-1 and DP-1 in mantle lymphocytes, centrocytes and centroblasts, although the stain of E2F-1 was weaker than that of DP-1. A few centroblasts showed obscure nuclear stain of E2F-1. Some follicular dendritic cells and tingible body macrophages revealed strong cytoplasmic stain of DP-1 and DP-2.

A small number of gamma/delta T-cell lymphoma cells showed dominantly obscure cytoplasmic stain of E2F-4 (Fig. 4a), but a few large lymphoma cells revealed nuclear stain of E2F-1. On the other hand, many lymphoma cells revealed cytoplasmic stain of DP-1 (Fig. 4b).

ATLL cells showed obvious nuclear stain of E2F-1 (Fig. 5a) or -4 (Fig. 6a) in three cases and cytoplasmic stain of E2F-1 or -4 in other three cases (table 1). Cytoplasmic stain of DP-1 was recognized in many ATLL cells in the all cases except one case, of which ATLL cells revealed nuclear stain of E2F-4 but did not show stain of DP-1. In two cases, small to medium-sized intermingling lymphocytes revealing strong cytoplasmic and nuclear stain of E2F-4 and DP-1 (Fig. 7), when ATLL cells did not show stain of E2F-4.

Cytoplasmic stain of DP-2 was seen only in two cases of ATLL (Table 1).

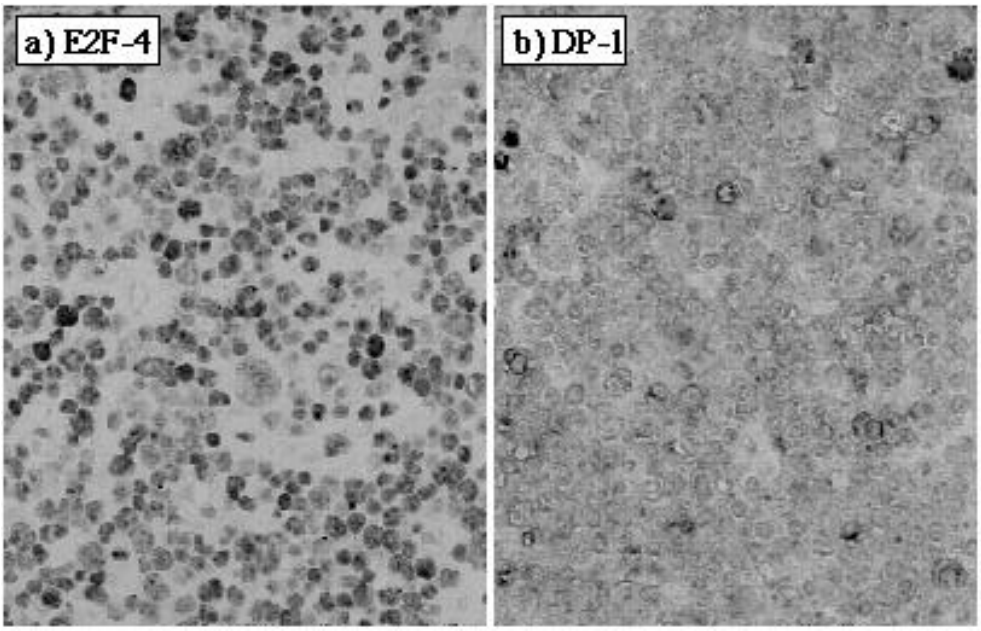

Figure 6. Appearance of E2F-4 (D3) and DP-1 (K-20) in Case 6 of ATLL.
Obvious nuclear stain of E2F-4 (a) and cytoplasmic stain of DP-1 (b) are recognized in most lymphoma cells. Stronger nuclear stain of E2F-4 is seen in larger ATLL.

Discussion

Various molecules that play important roles in cell cycle appear in a peculiar phase of cell cycle. E2F-DP-1 complex is released from Rb protein, appears in nucleus in G1/S transition, and is inactivated immediately through phosphorylation by cyclin A/CDK 2 complex (11). Then, the intranuclear appearance of E2F-DP-1 complex represents the G1/S transition of cell cycle. The quite obscure stain of E2F-1 in a few centroblasts in germinal center of human tonsil (Fig. 3a) was thought to be a physiological amount of E2F-1 that appears in the G1/S transition. DP-1 increases in cytoplasm of G0 and G1 phase cells. Therefore, cytoplasmic DP-1 in lymphocytes of human tonsil (Fig. 3c) may be a mixture of E2F-DP-1 complex and the other complex forms of DP-1. Because nuclear stain of DP-1 could not be recognized, the amount of E2F-DP-1 complex or the reacted amount of anti-DP-1 polyclonal antibody, K-20, was thought to be less than that can be detected by the highly sensitive immunohistochemistry employed in this study (12). On the other hand, some MT-2 cells indicated E2F-4 in nucleus (Fig. 2b), and a small number of MT-2 cells showed DP-1 in cytoplasm (Fig. 2c). MT-2 cells were thought to present a larger amount of E2F-4 and DP-1 in the G1/S transition

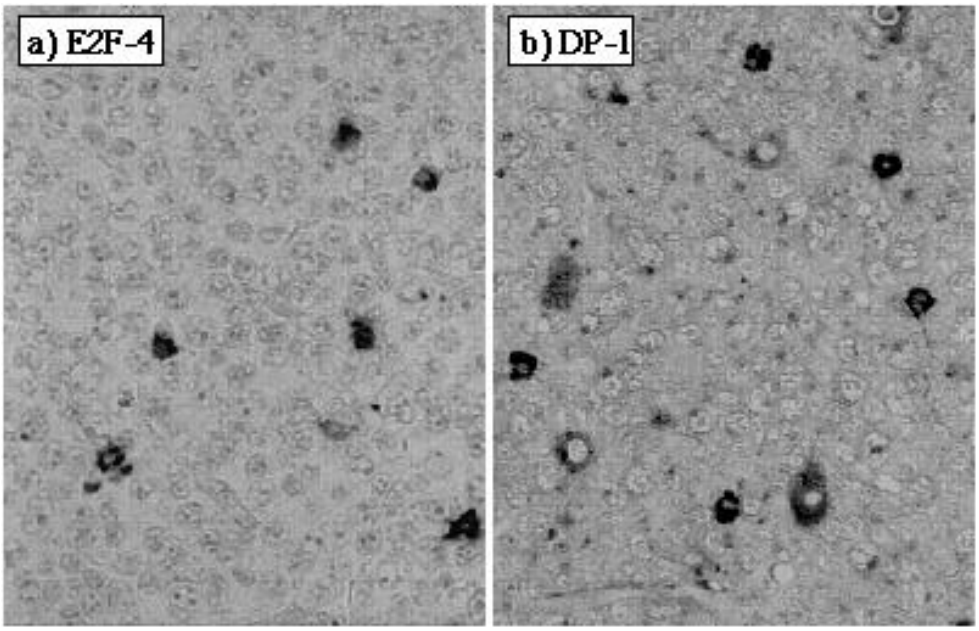

Figure 7. Appearance of E2F-4 (D3) and DP-1 (K-20) in intermingling lymphocytes in Case 9 of ATLL.

The intermingling lymphocytes that are candidates for ATLL cells showed strong stain of E2F-4 (a) and DP-1 (b). The lymphoma cells indicated heterochromatin-like fine deposition-like weak stain. Fine deposition-like weak stain in lymphoma cells might be of intranuclear E2F-4.

than the lymphocytes in human tonsil. A small number of large lymphoma cells in gamma/delta T-cell lymphoma showed intranuclear stain of E2F-4, suggesting that the lymphoma cells maintained physiological appearance of E2F-4.

From a viewpoint of checkpoints in cell cycle, oncogenic progression is thought to override several checkpoints in spite of abnormal cell conditions that must make cell cycle arrest at each checkpoint. Many ATLL cells in three cases revealed nuclear stain of E2F-1 or -4, although larger ATLL cells showed stronger stain than smaller ATLL cells. Constant release of E2F-DP-1 complex from Rb protein or failure in the inactivation of E2F-DP-1 complex through phosphorylation by cyclin A/CDK2 can explain the appearance of E2F-1 or -4 in many ATLL cells, although overexpression of E2F in cytoplasm can be induced by Tax (13). But, it has not yet proved how HTLV-1 Tax effects on various molecules that concern with cell cycle, although strong cell cycle suppressers, p18 and RCT9, were reported to be suppressed in ATLL cells (8). Co-operative G1/S transition promoters, cyclin E/CDK2 and E2F-DP-1 complex are activated by Tax that combines p53 to suppress tran-

scription of p21 and transactivates oncogenes such as Ras and Myc, of which signal induces CDC25A and suppresses $p27^{KIPI}$. Thus, cyclin E/CDK2 might be overexpressed in the cases of ATLL that did not show nuclear stain of E2F-1 or -4, although the E2F families other than E2F-1 and -4 might be overexpressed or the expression of E2F-1 or -4 is quite low but enough to propel G1/S transition.

There were two cases of ATLL, of which ATLL cells did not show stain of E2F-1 or -4 and the intermingling lymphocytes revealed strong stain of E2F-4 and DP-1. Because the intermingling lymphocytes in ATLL have irregular-shaped nuclei and can be seen as candidates of ATLL cells, overexpression of E2F-DP-1 complex induced by Tax is thought to occur in several phases of ATLL leukemogenesis.

On the other hand, methylation in several suppresser genes such as CDK 4/6 inhibiters, p15 and p16 genes, has been reported to be seen in non-Hodgkin lymphomas (14-20) including ATLL (21). Then, the oncogenic processes to suppress CDK 4/6 inhibiters, p15 and p16 genes, may be a common feature in the leukemogenesis of ATLL and in not-ATLL non-Hodgkin lymphomas. After suppression of p15 and p16 genes by Tax in ATLL leukemogenesis (8) p15 and p16 genes would be methylated so that p15 and p16 genes in ATLL cells are methylated (21). Even long terminal repeat (LTR) of HTLV-1 is reported to be mutated in some ATLL cases (22). Probably, in ATLL leukemogenesis, suppression of suprresser genes and HTLV-1 proviral DNA after their activation may follow the overriding of the abnormal proliferation condition of cell cycle in lymphoma cells and in dysplastic lymphocytes. The suppression of such genes and HTLV proviral DNA might be methylated or mutated after the overriding. Then, ATLL and the other lymphomas have the same leukemogenesis porcesses, although Tax induces abnormal expression of molecules that prepare abnormal condition for the ATLL leukemogenesis.

Expression of DP-2 was recognized in MT-2 cells. But only two cases of ATLL revealed DP-2 in cytoplasm of ATLL cells. Probably, overexpression of DP-2 would not be included in a major process in ATLL leukemogenesis.

Consequently, overexpression of E2F or DP-1 was recognized in 9 of 10 cases of ATLL, although the expression could not be explained in the same way from viewpoints of cell cycle and of the overriding of checkpoints associating abnormalities. Probably Tax effects on various aspects of the proliferation signal transduction system (8) so that the expression of the related molecules in ATLL cells varies case to case.

At last, many chromosomal abnormalities, which would be induced by the disturbance in M phase checkpoint and in mitotic spindle body formation checkpoint of cell cycle, are seen in ATLL. It must be studied whether HTLV-1-related proteins effect on host cell genes (23), on cohesion protein between chromosomes, and on separation of chromosomes by Cut 1 and Cut 2 proteins (Fig. 1). Because integration of HTLV-1 proviral DNA is a kind of DNA damage for the HTLV-1-infected cells, abnormal expression of the

DNA damage-related proteins and genes, such as ataxia teleangiectasia-mutated gene (ATM)/ataxia teleangiectasia- and rad3-related gene (ATR) and cyclin B/CDK 1 that suppress G2/M transition, must be studies. Although it is well known that the proliferation signal transduction system in the Tax-related ATLL leukemogenesis has been clarified by Yoshida M et al, further studies of the ATLL leukemogenesis must be performed from viewpoints of cell cycle and DNA damage.

Summary

It has been clarified that HTLV-1 Tax effects on proliferation signal transduction, which ends to release E2F-DP-1 complex from Rb protein to nucleus. This study aimed to see how E2F-1 or -4 and DP-1 and -2 were expressed in ATLL cells in vivo, by employing antigen-retrieval and highly sensitive immunohistochemistry (TSA). MT-2 cells showed E2F-4 in nuclei of some cells and DP-1 in cytoplasm of a few cells, indicating E2F-DP-1 complex in G1/S transition in spite of no intranuclear stain of DP-1. In human tonsil, lymphocytes of lymph follicle showed E2F-1 and DP-1 in G1/S transition. Only a few large lymphoma cells of gamma/delta T-cell lymphoma showed nuclear stain of E2F-4, maintaining physiological appearance of E2F in G1/S transition. Many ATLL cells of three cases showed nuclear stain of E2F-1 or -4 and cytoplasmic stain of DP-1, indicating overriding G1/S transition with a high level expression of E2F-1 or -4 in phases of cell cycle other than G1/S transition. In other six cases of ATLL, cytoplasmic stain of E2F-1 or -4 and/or cytoplasmic stain of DP-1 was recognized, suggesting expression of E2F-DP-1 complex in spite of a small amount of intranuclear E2F, which could not be visualized well by means of the antigen-retrieval and TSA method employed in this study. In two cases of ATLL, of which ATLL cells showed only cytoplasmic stain of DP-1, intermingling lymphocytes showed strong stain of E2F-4 and DP-1, suggesting overexpression of E2F-DP-1 complex in various phases of ATLL leukemogenesis. Consequently, expression of E2F-DP-1 complex that is an end process in the proliferation signal transduction system activated under HTLV-1 Tax is suggested to be common in ATLL leukemogenesis.

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