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Original Article

Association of High Proliferation in Adult T-cell Leukemia Cells with Apoptosis, and Expression of p53 Protein in Acute Type ATL

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Proliferation, apoptosis and p53 protein expression in adult T-cell leukemia (ATL) cells were investigated. Twenty peripheral blood tissue specimens (PBTS) comprising 7 cases of acute type ATL, 7 cases of chronic type ATL and 6 other leukemias were examined by means of antigen retrieval and the polymer method employing anti-Ki67 antigen (MIB-1), anti-cleaved caspase-3, anti-single stranded DNA and three kinds of anti-p53 protein antibodies including DO7. Most acute and chronic cases of ATL included more than 10% MIB-1-positive proliferating leukemia cells and more than 1% cleaved caspase-3-positive apoptotic cells. Some cells which were positive for both MIB-1 and anti-cleaved caspase-3 antibody were observed in acute type ATL. Nuclear deposition of p53 protein labeled by DO7 was often found in acute type ($p < 0.05$). Within the medium-sized population of ATL cell nuclei, DO7-positive ATL cells had a smaller nuclear area factor (long axis x short axis) than DO7-negative ATL cells. A few proliferating ATL cells entered apoptosis, and the appearance of a subclone of ATL cells with nuclear deposition of p53 protein labeled by DO7 characterized acute type. 〔*J Clin Exp Hematopathol 48(1) : 1-10, 2008*〕

Keywords: adult T-cell leukemia (ATL), peripheral blood tissue specimen (PBTS), proliferation, apoptosis, double immunostaining

INTRODUCTION

Adult T-cell leukemia (ATL) is a neoplastic disease of peripheral T cells infected by human T-lymphotropic virus type-1 (HTLV-1).[1-5] ATL is clinically subcategorized into smoldering, chronic, acute, and lymphoma types.[1]

It is well known that ATL cells in the peripheral blood have so-called flower-shaped nuclei[1] whereas ATL cells in the lymph nodes and the extranodal tissues exhibit various-shaped (pleomorphic) and various-sized (polymorphic) appearances.[6,7] From a cell kinetic analysis of nuclear DNA content of ATL cells in the lymph nodes, it was reported that lymphoma type cases with a high proliferation of ATL cells in the diploid-tetraploid range were associated with leukemia.[8,9] However, the relationship between ATL cells in tissue and in the peripheral blood has not yet been investigated well. In order to investigate leukemia cells in peripheral blood in the same condition of lymphoma cells in tissue, we developed a method to prepare peripheral blood tissue specimens (PBTS).[10] This allowed examination of the immunological phenotype of ATL cells and the expression of HTLV-1-related protein in ATL cells in PBTS.[10]

Because the early detection of a transformation from chronic type to acute type is considered to be clinically important, we aimed to find the difference in proliferation and apoptosis between acute and chronic type ATL cells. We also examined the effects of p53 protein expression in ATL cells.

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MATERIALS AND METHODS

Twenty specimens were examined in this study. Specimens were formalin-fixed and paraffin-embedded PBTS from patients with ATL acute type (n = 7), ATL chronic type

(n = 7), and other leukemias (n = 6). The last group included two patients with B-cell chronic lymphocytic leukemia (B-CLL), two patients with acute myelogenous leukemia (AML), and two patients with chronic myelogenous leukemia (CML).

Clinical data including age, sex, white blood cell count in peripheral blood on the date of PBTS preparation, and status of HTLV-1 infection are shown in Table 1. It was informed for all the leukemia patients to manifest more than 20,000 white blood cells per 1 mm^3 in the peripheral blood in the clinical course.

To determine HTLV-1 infection status, antibodies against HTLV-1 related proteins were examined in a commercial laboratory by the particle agglutination test. Monoclonal integration of HTLV-1 proviral DNA was assessed in Department of Medical Genome Sciences, Laboratory of Tumor Cell Biology, University of Tokyo (Prof. Toshiki Watanabe) in the cohort study of JSPFAD (Joint Study on Predisposing Factors of ATL Development).

This study was performed with the informed consent of all patients and the permission of all ethical committees for clinical studies, as required by Kagoshima University Hospital.

Peripheral blood tissue specimen (PBTS)

The previously reported method to prepare PBTS[10] is shown graphically in Fig. 1. The procedures are briefly explained as follows : 1) Five ml of peripheral blood were sampled by means of a disposable injector. 2) The injector was left undisturbed in a vertical position for more than three hours. 3) The cylinder was removed from the injector, and a buffered 10% formalin solution was poured into the injector. 4) After overnight fixation, a lump of fixed naturally sedimented and coagulated blood cells was removed from the injector and cut along the longitudinal axis, followed by routine processing into a paraffin-embedded specimen.

Table 1. Clinical data for patients providing peripheral blood tissue specimens (PBTS)

No.	Age	Sex	Peripheral WBC count	Anti-HTLV-1 antibodies	Integration of HTLV-1 proviral DNA	Final diagnosis
1	70	F	115, 700	+	Monoclonal	Chronic ATL
2	71	M	22, 200	+	Monoclonal	Chronic ATL
3	57	M	13, 900	+	Monoclonal	Chronic ATL
4	64	M	14, 700	+	Monoclonal	Chronic ATL
5	83	F	19, 500	+	n.t.	Chronic ATL
6	50	F	16, 000	+	n.t.	Chronic ATL
7	44	M	35, 300	+	n.t.	Chronic ATL
8	73	M	175, 500	+	n.t.	Acute ATL
9	69	F	84, 400	+	Monoclonal	Acute ATL
10	56	M	71, 600	+	Monoclonal	Acute ATL
11	74	M	23, 300	+	Monoclonal	Acute ATL
12	68	M	14, 000	+	Monoclonal	Acute ATL
13	70	M	8, 400	+	Oligoclonal	Acute ATL
14	61	F	8, 400	+	Monoclonal	Acute ATL
15	74	F	19, 400	-	n.t.	B-CLL
16	44	M	34, 200	-	n.t.	B-CLL
17	43	M	143, 900	-	n.t.	AML
18	49	F	88, 500	-	n.t.	AML
19	80	M	24, 800	n.t.	n.t.	CMMoL
20	50	M	79, 600	-	n.t.	CML

Peripheral WBC count : The count (/ mm^3) on the date of sampling peripheral blood for PBTS ; Anti-HTLV-1 antibodies : Measured from serum with particle agglutination test. + : positive, - ; negative ; n.t. : not-tested ; Integration of HTLV-1 proviral DNA : DNA extracted from leukemia cells in the peripheral blood was examined with southern blot analysis ; Monoclonal : Monoclonal integration of HTLV-1 proviral DNA was assessed ; Oligoclonal : Oligoclonal integration of HTLV-1 proviral DNA was detected ; Final diagnosis : Final diagnosis was based on hematological, immunological and virological examinations. Acute ATL : Acute type ATL ; Chronic ATL : Chronic type ATL ; B-CLL : B-cell chronic lymphocytic leukemia ; AML : Acute myelogenous leukemia CMMoL : Chronic myelomonocytic leukemia ; CML : Chronic myelogenous leukemia.

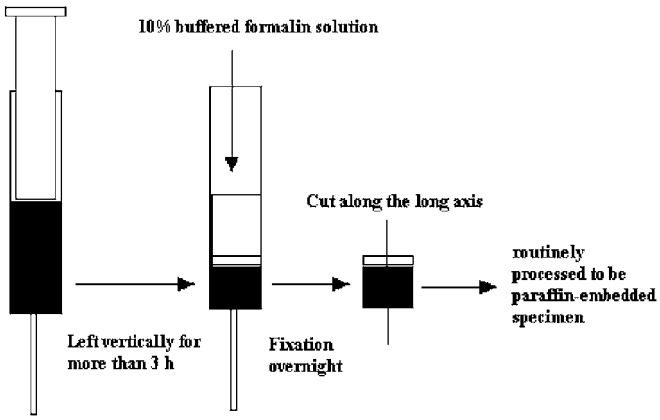

Fig. 1. Procedures for preparing the peripheral blood tissue specimen (PBTS).

Immunohistochemistry

Sections of the prepared PBTS were used. After baking the sections at 60°C for 30 min, the sections were deparaffinized in both xylene and 100% ethanol (3 x 10 min). Endogenous peroxidase in the sections was inactivated by incubating the sections in 0.3% hydrogen peroxide methanol for 20 min. Antigen retrieval (AR) for the antigens other than cleaved caspase-3 and single-stranded DNA (ssDNA) was performed by heating the sections in 0.1M citrate buffer pH 6.0 [Antigen Retrieval Buffer (Dako ChemMate), S2031, Dako, Japan] in an autoclave. The AR for cleaved casapse-3 was done in the same manner in EDTA buffer (Target Retrieval Solution High pH, S3307, Dako). The AR for ssDNA was done by digesting with proteinase K (Code No. 9033, Takara Bio Co, Otsu, Japan, 200 μg/mL in 0.05 M TBS pH7.2) for 10 min. Blocking of non-specific binding of primary antibodies in a casein solution (Non-specific Staining Blocking Reagent, X0909, Dako) was performed before the primary antibody reaction. The primary antibody reaction was done with anti-Ki67 antigen antibody solution [MIB-1, M7240, Dako ; 1 : 50 diluted with Antibody Diluent (Dako ChemMate, S2022, Dako)], anti-p53 protein antibodies solutions (DO7, M7001, 1 : 100 diluted, Dako ; NCL-p53-1801, Novocastra Laboratories Ltd, Newcastle upon Tyne, UK, 1 : 40 diluted ; NCL-p53-PHOS, Novocastra Laboratories Ltd, 1 : 50 diluted) and anti-cleaved caspase-3 rabbit monoclonal antibody solution (Asp175, 5A1, Signaling Technology, Inc. Beverly, MA, 1 : 250 diluted) for 1 hr and with anti-single stranded DNA antibody solution (A4506, Dako, 1 : 100 diluted) for 15 min. The reacted primary antibodies were visualized by means of the polymer method (ChemMate ENVISION, K5007, Dako) and the peroxidase-peroxide-diaminobenzidine tetrahydrochloride (DAB) liquid system (K3468, Dako). After nuclear staining by hematoxylin (Dako ChemMate, S2020, Dako), the sections were dehydrated in ethanol solutions and xylene and mounted in a plastic medium. The processes from the blocking non-specific binding of primary antibody to the nuclear stain were performed by an autostainer (Dako Autostainer).

The immunostaining of the above-mentioned primary antibodies was evaluated by counting the percent of labeled cells in four representative fields on the printed microphotos taken at x40 magnification from the reacted PBTS sections. Fields were graded on a 5-point scale as follows : grade 0 : no labeled cells ; grade 1 : 1% or fewer labeled cells ; grade 2 : 1-10% labeled cells ; grade 3 : 10-50% labeled cells ; grade 4 : more than 50% labeled cells.

Double immunostaining

Double labeling of anti-cleaved caspase-3 rabbit monoclonal antibody (Asp175) with either anti-Ki67 antigen antibody (MIB-1) or anti-p53 protein (DO7) was performed on 7 acute type ATL specimens, 2 chronic type ATL specimens, and 1 AML specimen, according to the method reported previously.[11] After deparaffinization and the inactivation of endogenous peroxidase, the antigen retrieval and immunostaining of anti-cleaved caspase-3 antibody (Asp175) was performed. The immunoreaction products except for DAB deposition were removed by means of glycine treatment.[11] Next, the antigen retrieval and immunostaining with either anti-Ki67 antigen antibody (MIB-1) or anti-p53 protein antibody (DO7) was performed. VIP (Vector VIP substrate kit SK-4600, Vector Laboratories, Burlingame, CA) was employed as a chromogen. After rinsing the sections in water, the sections were air-dried and mounted in VectaMount Permanent Mounting Medium (H-5000, Vector Laboratories, Burlingame, CA).

Morphometry of leukemia cells labeled by anti-p53 protein antibody (DO7)

On the printed microphotos taken at x40 magnification from the PBTS sections reacted with anti-p53 protein antibody (DO7), the nuclear length and width of 50 labeled and unlabeled cells were measured under a loupe (Spiegel pro1000, x10, Germany). An area factor (nuclear length x nuclear width) and a form factor (nuclear length/nuclear width) were then calculated. Differences in area factor and form factor between the labeled and unlabeled cells were tested, using the F-test and student's t-test.

Microphotographs

Microphotos of the specimens in the figures were taken with a digital microscopic camera (Fuji HC-300) attached to a

microscope (BX-50, Olympus Co, Tokyo, Japan). The exact length of the long axis of the microphoto was 215 μm at x40 magnification, and 87 μm at x100 magnification.

RESULTS

Immunostaining of anti-Ki67 antigen, anti-p53 protein, anti-cleaved caspase-3 and anti-ssDNA antibodies in a case of acute type ATL is shown in Fig. 2. An evaluation of the immunostaining in the cases examined is shown in Table 2.

The percentage of proliferating cells labeled by the anti-Ki67 antigen antibody varied from 1 to 94% (Table 2), with more than 10% proliferating cells in many cases. The immunostaining of the anti-Ki67 antigen antibody was dense on many nuclei and tan on some nuclei (Fig. 2a). A few segmented-nuclear leukocyte-like cells were labeled by means

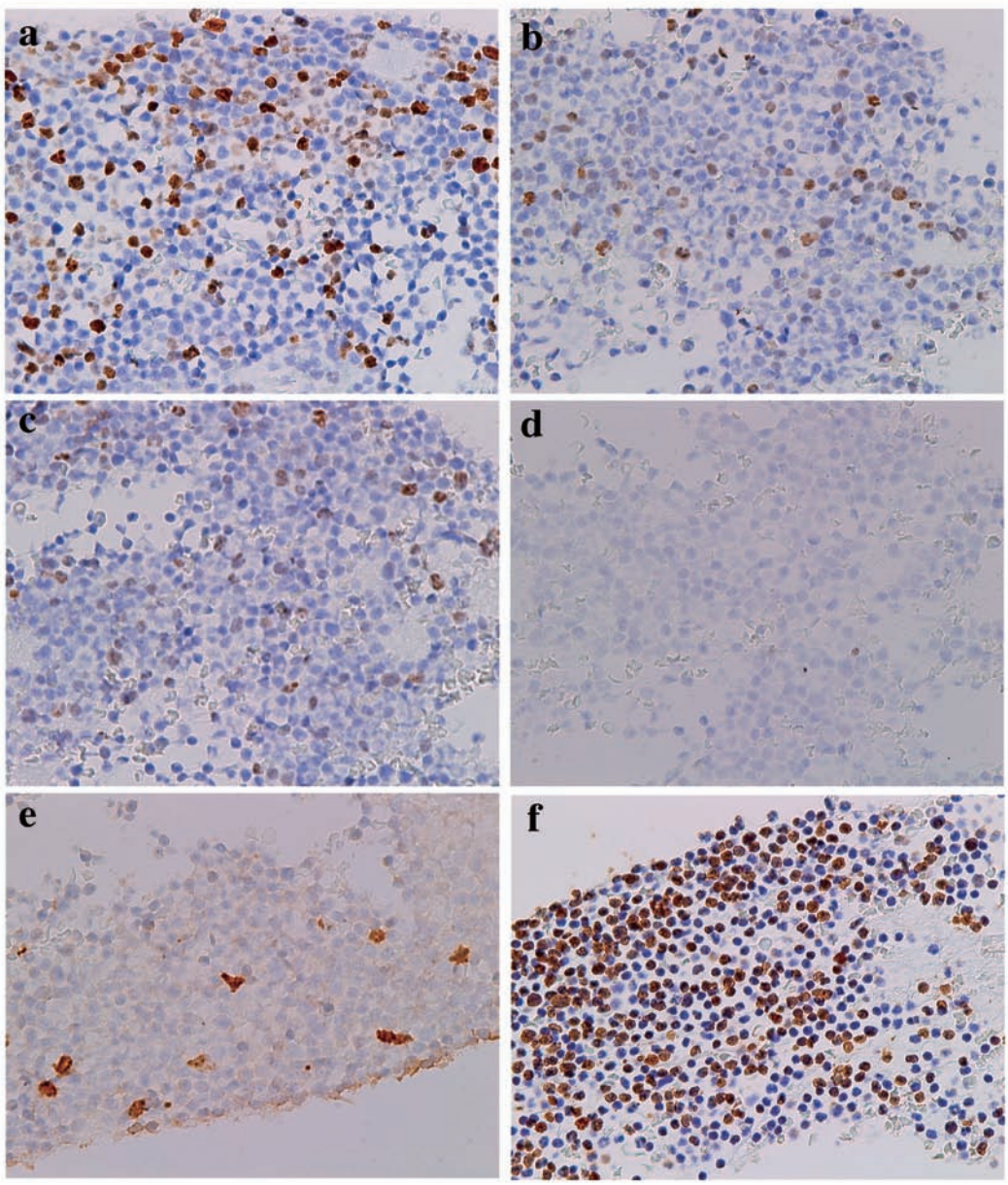

Fig. 2. Immunostaining in PBTS from a case of acute type ATL (Case No. 14). (***2a***) Anti-Ki67 antigen antibody. (***2b***) Anti-p53 protein antibody, DO-7. (***2c***) Anti-p53 protein antibody, 1801. (***2d***) Anti-phosphorylated p53 protein antibody. (***2e***) Anti-cleaved caspase-3 antibody. (***2f***) Anti-ssDNA antibody. Many cells were positive for Ki67 (*2a*), p53-DO7 (*2b*), p53-1801 (*2c*) and ssDNA (*2f*). Some cells were positive for cleaved caspase-3 (*2e*), and a few cells were positive for p53-PHOS (*2d*). Counterstained with hematoxylin, 2a-2f, x40.

Table 2. Evaluation of immunostaining in peripheral blood tissue specimens

Case No	Diagnosis	Evaluation of immunostaining					
		Proliferation	Expression of p53 protein			Apoptosis	
		Ki67 antigen	p53-DO7	p53-1801	p53-Phos	Cleaved caspase 3	ssDNA
1	Chronic ATL	1 (1)	0	0	0	0	4 (52)
2	Chronic ATL	3 (27)	0	0	0	2 (8)	4 (67)
3	Chronic ATL	4 (57)	0	0	0	3 (16)	4 (64)
4	Chronic ATL	4 (58)	2 (3)	2 (2)	1 (1)	2 (3)	4 (54)
5	Chronic ATL	3 (23)	0	0	0	2 (2)	4 (84)
6	Chronic ATL	4 (84)	0	0	0	1 (1)	4 (95)
7	Chronic ATL	4 (72)	0	0	0	2 (4)	4 (53)
8	Acute ATL	3 (17)	2 (2)	2 (2)	0	3 (37)	3 (36)
9	Acute ATL	3 (18)	3 (19)	3 (12)	0	0	3 (32)
10	Acute ATL	4 (52)	3 (19)	2 (5)	1 (1)	3 (34)	3 (28)
11	Acute ATL	4 (55)	2 (2)	0	1 (1)	2 (6)	4 (60)
12	Acute ATL	3 (22)	3 (20)	3 (15)	1 (1)	2 (4)	3 (48)
13	Acute ATL	4 (86)	0	0	0	2 (4)	4 (60)
14	Acute ATL	3 (50)	2 (6)	2 (2)	2 (2)	3 (12)	4 (89)
15	B-CLL	3 (13)	0	0	0	2 (2)	4 (74)
16	B-CLL	2 (8)	0	0	0	1 (1)	4 (92)
17	CML	4 (94)	0	0	0	2 (10)	4 (52)
18	CML	4 (84)	0	0	0	2 (3)	4 (92)
19	AML	2 (3)	0	0	0	1 (1)	1 (1)
20	AML	3 (29)	1 (1)	0	0	2 (8)	4 (98)

Scale for evaluating the immunostaining : Grade 0 : No labeled cells ; Grade 1 : 1% or fewer labeled cells ; Grade 2 : 1-10% labeled cells ; Grade 3 : 10-50% labeled cells ; Grade 4 : More than 50% labeled cells.

( ) : The percentage of positive cells.

Table 3. Expression of p53 protein labeled by the antibody DO7

Diagnosis	Expression of p53 protein labeled by DO7	
	Number of cases in which leukemia cells did not express p53 protein	Number of cases in which leukemia cells expressed p53 protein.
Chronic-ATL #1, #2	6	1
Acute-ATL #1, #3	1	6
Others #2, #3	5	1

#1 : Fisher's exact p = 0.015, #2 : Fisher's exact p = 0.730, #3 : Fisher's exact p = 0.025.

of the polymer method of anti-Ki67 antigen antibody.

The immunostaining of the three antibodies against p53 protein was seen on the nuclei of the leukemia cells (Fig. 2b, 2c and 2d). In most cases revealing p53 protein-positive leukemia cells, p53-DO7 labeled the most cells in the three antibodies (Table 2). Acute type ATL specimens were more likely to include p53-DO7-positive leukemia cells than either the chronic type ATL specimens (p = 0.015) or the other leukemia specimens (p = 0.025) (Table 3). The leukemia cells displayed nuclear length of 5-7 μm, which was the size of the medium-sized lymphoma cells in the tissues,[12,13] and were smaller than those in the smear specimens.[14] DO7 (p53 protein)-positive leukemia cells displayed significantly smaller nuclear area and form factors than those not expressing p53 protein in each case (Table 4), as seen in Fig. 3. However, there were no significant differences in nuclear length, width, area factor and form factor among the cell specimens from chronic type ATL, acute type ATL and the

others.

The anti-cleaved caspase-3 antibody was localized to the cytoplasm of leukemia cells (Fig. 2e). ATL cases showing immunostain grade 3 for cleaved caspase-3 (over 10% leukemia cells labeled) were noted, and two cases of acute type ATL scored 34% and 37% cleaved caspase-3-positive cells (Table 2). Across specimens there was a significant correlation between displaying more than 10% Ki67 antigen-positive proliferating cells and displaying more than 1% of cleaved caspase-3-positive cells in the cases examined ($p = 0.009$, Table 5). But in ATL cases there were two cases indicating 1% and fewer cleaved caspase-3-positive cells. There was no relationship between the expression of p53 protein and the appearance of the cleaved caspase-3.

The immunostaining of the anti-ssDNA antibody was dense and it was also seen on many nuclei (Fig. 2f). In the examined cases, except for one case of AML, many or most nuclei of the leukemia cells were labeled (Table 2). There was no correlation between the appearance of the cells expressing cleaved caspase-3 and the labeling of the anti-ssDNA antibody.

The double immunostaining of the anti-cleaved caspase-3

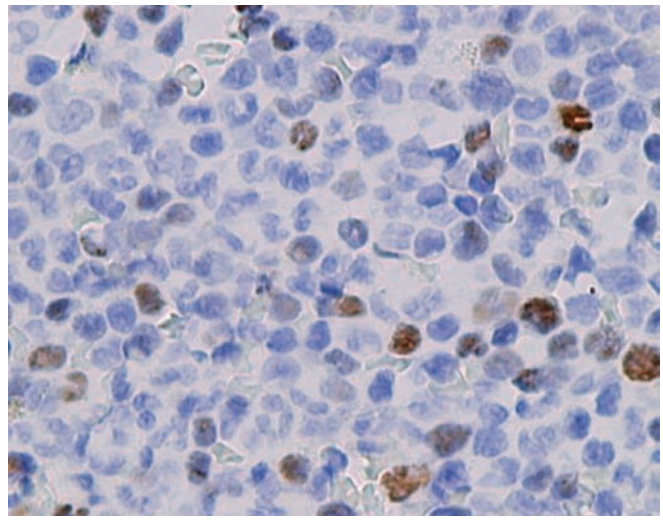

Fig. 3. Immunostaining of anti-p53 protein antibody, DO7 in PBTS from an acute type ATL case (Case No. 14) (x40 original magnification and x4 digital magnification : The long axis of the figure is 108 μm long.). The nuclei labeled by DO7 were somewhat smaller than the non-labeled nuclei. Counterstained with hematoxylin.

Table 4. Nuclear size of leukemia cells expressing p53 protein labeled by DO7

Case No	Diagnosis	Nuclei of leukemia cells not expressing p53 protein				Nuclei of leukemia cells expressing p53 protein			
		Length (μm)	Width (μm)	Area F (μm^2)	Form F	Length (μm)	Width (μm)	Area F (μm^2)	Form F
1	Chronic ATL	5. 9	4. 6	27. 3	1. 32				
2	Chronic ATL	5. 6	4. 5	25. 0	1. 26				
3	Chronic ATL	5. 0	4. 0	20. 3	1. 27				
4	Chronic ATL	6. 3	5. 1	32. 0	1. 26				
5	Chronic ATL	6. 1	4. 8	29. 0	1. 30				
6	Chronic ATL	6. 3	4. 9	31. 0$^{\#1}$	1. 29	5. 7	4. 8	27. 7$^{\#1}$	1. 19
7	Chronic ATL	5. 8	4. 6	26. 6	1. 37				
8	Acute ATL	5. 7	4. 7	26. 7	1. 22	5. 4	4. 3	23. 3	1. 28
9	Acute ATL	6. 1	4. 8	29. 1$^{\#2}$	1. 29$^{\#3}$	4. 8	4. 1	20. 0$^{\#2}$	1. 17$^{\#3}$
10	Acute ATL	5. 6	4. 5	25. 1	1. 26	6. 2	4. 5	27. 7	1. 41
11	Acute ATL	5. 9	4. 6	27. 4$^{\#2}$	1. 31	4. 6	3. 7	17. 6$^{\#2}$	1. 30
12	Acute ATL	6. 2	5. 0	31. 4$^{\#3}$	1. 26$^{\#3}$	4. 8	4. 4	21. 9$^{\#3}$	1. 09$^{\#3}$
13	Acute ATL	5. 1	4. 1	20. 8$^{\#1}$	1. 26	5. 2	4. 3	23. 0$^{\#1}$	1. 21
14	Acute ATL	6. 0	4. 9	29. 5	1. 23				
15	B-CML	5. 9	4. 4	26. 0	1. 39				
16	B-CML	6. 7	5. 3	35. 8	1. 29				
17	B- CLL	6. 0	5. 1	30. 3	1. 19				
18	B-CLL	6. 2	4. 9	30. 2	1. 28				
19	AML	6. 5	5. 6	36. 6	1. 19	6. 1	5. 2	32. 3	1. 18
20	AML	6. 8	5. 6	38. 5	1. 22				

Area F : Area factor = Nuclear length x Nuclear width (μm^2) ; Form F : Form factor = Nuclear length/ Nuclear width ; #1 : F-test ($p < 0.05$) ; #2 : Student's t-test ($p < 0.05$) ; #3 : F and Welch's t-test ($p < 0.05$).

antibody (brown-black DAB) and the anti-Ki67 antigen antibody (purple VIP chromogen) revealed an excellent contrast and staining quality (Figs. 3 and 4) in seven cases of acute type ATL, two cases of chronic type ATL and one case of AML. In many cases of acute type ATL, many leukemia cells having purple nuclei labeled by anti-Ki67 antigen antibody also showed dark brown cytoplasm labeled by anti-cleaved caspase-3 antibody (Figs. 4 and 5). In the high-

Table 5. Relationship between proliferation and apoptosis in leukemia cells

The evaluation of the immunostain of Ki67 antigen	The evaluation of the immunostain of cleaved caspase-3	
	1% or fewer positive cells	More than 1% positive cells
10% or fewer positive cells	3 (0, 1, 2)	0 (0, 0, 0)
more than 10% positive cells	2 (1, 1, 0)	15 (6, 5, 4)
	Statistical significance (Fisher's exact probability)	p = 0.009

(-, -, -) : In parentheses, the numbers of cases with acute ATL, chronic ATL and others.

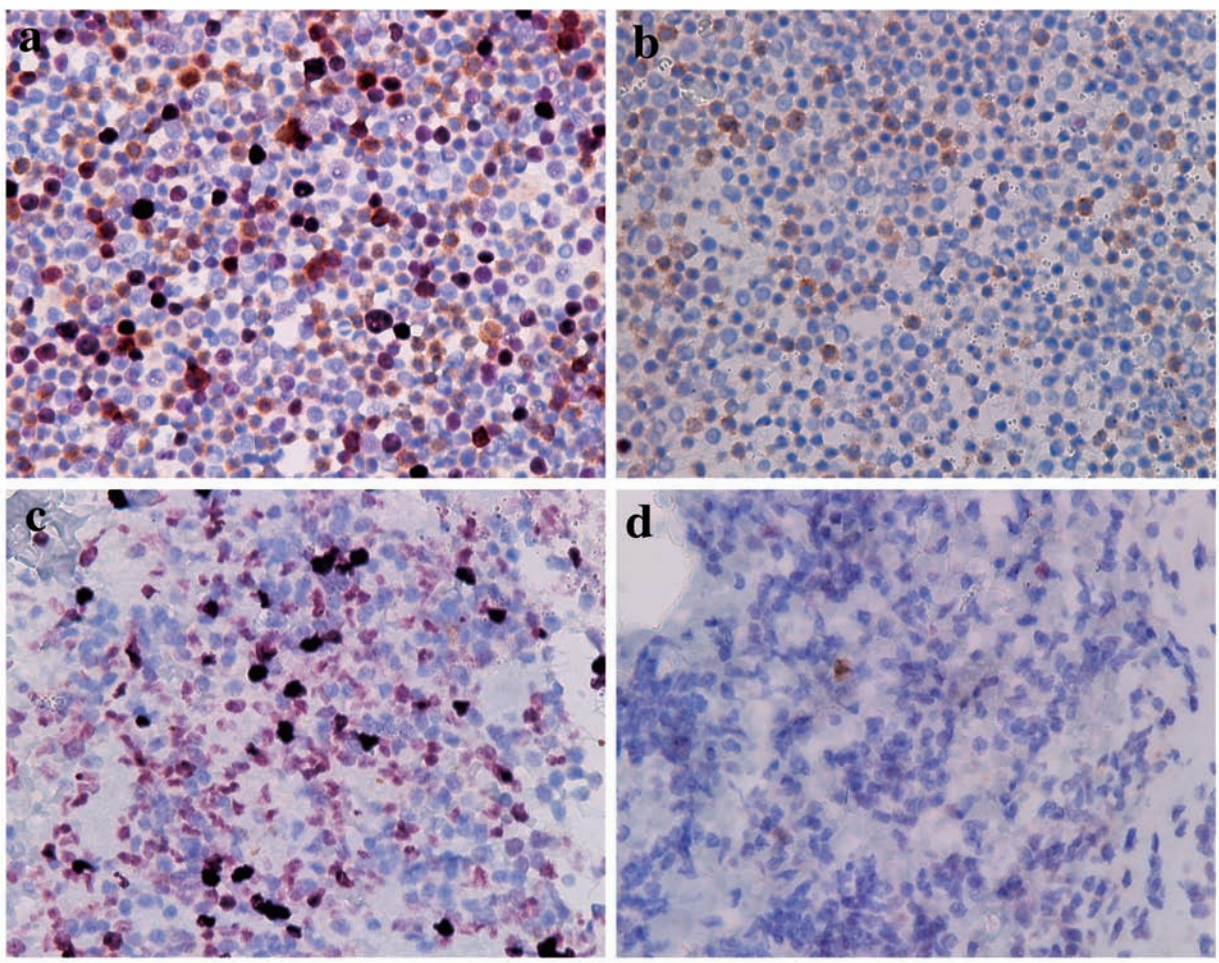

Fig. 4. Double immunostaining of Ki67 antigen/ cleaved caspase-3 and p53 protein/ cleaved caspase-3 in acute and chronic type ATL. (***4a & 4b***) Acute type ATL (Case No. 8). (***4c & 4d***) Chronic type ATL (Case No. 5). (***4a & 4c***) Ki67 antigen (VIP : Purple) and cleaved caspase-3 (DAB : Brown). (***4b & 4d***) p53 protein labeled by DO7 (VIP : Purple) and cleaved caspase-3 (DAB : Brown). Many leukemia cells with purple nuclear staining for Ki67 antigen appeared to have positive dark brown cytoplasmic staining for cleaved caspase-3 in the acute type ATL (*4a*). Rare cleaved caspase-3-positive cells were seen in the chronic type ATL (*4c*). All of the cells with purple nuclear staining for p53 protein were negative for dark brown cytoplasmic staining for cleaved caspase-3 (*4b & 4d*). Counterstained with hematoxylin, 4a-4d, x40.

powered view (Fig. 5), brown-colored apoptotic leukemia cells resembled segmented nuclear leukocytes.

DISCUSSION

It is widely known that the Ki67 antigen is expressed in proliferating cells in the cell cycle phases from late G1 to M.[15] In the present study, we show that many leukemia cells labeled by the anti-Ki67 antigen antibody have the potential to re-enter the proliferation cycle after exiting the peripheral bloodstream.[15] It was previously observed that many leukemia cells labeled by the anti-Ki67 antigen antibody were present in air-dried smear specimens of peripheral blood from ATL patients, suggesting that ATL leukemia cells proliferate even in the peripheral blood. It was also noted that a few segmented-nuclear leukocyte-like cells were labeled by means of the polymer method with the anti-Ki67 antigen antibody, MIB-1. The segmented-nuclear leukocyte-like cells were thought to be apoptotic leukemia cells (Fig. 4) rather than segmented-nuclear leukocytes having a quite small amount of Ki67 antigen in their nuclei under the ATL-derived factor.[16,17]

The p53 protein detected in leukemia cells' nuclei was thought to be mutant because there was no relationship between the expression of p53 protein and the appearance of cleaved caspase-3. It is well accepted that p53 overexpression in nuclei is a result of a p53 mutation.[18] A genetic alteration of the p53 gene in ATL has been reported previously.[19] In this study we report that a significant percentage of acute type ATL specimens showed expression of p53 protein labeled by DO7, suggesting a genetic alteration of the p53 gene in the transition from chronic type to acute type ATL. Phosphorylation of serines at the 15th and 392nd positions of the p53 protein found in ATL is critical for p53 protein-complex formation;[20] however, the p53-Phos antibody, which labels phosphorylation of the serine at the 392th position, labeled only a few cells in merely 5 ATL cases. The physiological expression of the p53 protein with phosphorylation at serine 392 could not be detected by means of the polymer method. Here we also report that leukemia cells expressing p53 protein labeled by p53-DO7 had a significantly smaller nuclear area factor than those without any expression of p53 protein, therefore, the leukemia cells in acute type ATL comprise subclones. A dominant subclone was, however, negative for p53 protein. Our previous microphotometric analyses[12,21,22] showed intermingling atypical lymphocytes to be present in lymphoma type ATL, thus suggesting repeated oncogenic changes in the course of ATL development. The appearance of the smaller leukemia cells with nuclear expression of p53 protein in acute type ATL is probably a part of multistep oncogenesis from chronic type to acute type.

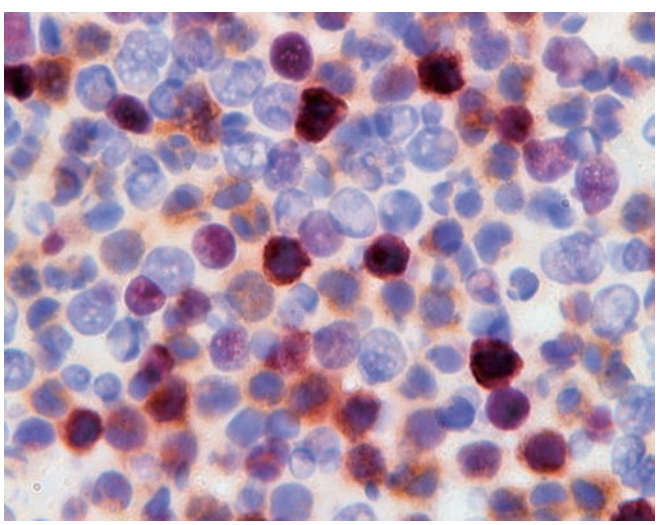

Fig. 5. Double immunostaining of anti-Ki67 antigen antibody (*purple*) and anti-cleaved caspase- 3 (*brown*) in PBTS from a case of acute type ATL (Case No. 8). Counterstained with hematoxylin, x1,000, oil immersion.

Cleaved caspase-3 is an effector caspase and the trigger for the apoptosis cascade,[23] which activates the DNA fragment factors (DFF) which degenerate chromatin.[24] The appearance of the cleaved caspase-3 thus predicts the occurrence of apoptosis. Our results showed no relationship between the expression of p53 protein and the appearance of cleaved caspase-3, thus suggesting that an alternative pathway to activate caspase-3 (other than the p53 protein-related DNA damage-induced pathway) may be located in the leukemia cells. Our results showed a relationship between Ki67 antigen labeling and the expression of cleaved caspase-3 in the leukemias examined, although there were two cases of ATL revealing more than 10% Ki67 antigen labeled cells and 1% or fewer cleaved caspase-3-positive leukemia cells (Table 5). The ATL cells labeled by both anti-Ki67 antigen and anti-cleaved caspase-3 antibodies (Figs. 3 and 4) may be the cells which show the characteristics of the proliferative phase but nevertheless enter apoptosis. Recently, c-jun N-terminal kinase (JNK) has been reported to promote resistance to Fas-mediated apoptosis in a prostate cancer cell line,[25] although JNK is generally a promoter of Fas-mediated apoptosis. Human T-cell lymphotropic virus oncoprotein p40Tax (Tax), in which the expression of ATL leukemia cells was shown to be related to the proliferation of ATL leukemia cells in PBTS,[10] has been reported to induce JNK activity.[26] Therefore, the activation of JNK in ATL leukemia cells may have a bimodal function to propel the proliferation with the other molecular mechanism induced by Tax[2-5] and to induce apoptosis according to cellular condition in ATL leukemia cells.

Single-stranded DNA appears in apoptosis, when it may be detected by a low sensitivity method. This study demonstrates the inability of anti-ssDNA antibody with the polymer

method to label only the nuclei which have fallen into a state of apoptosis, and also shows that ssDNA can detect the degree of degeneration of DNA in the preparation and storage of the formalin-fixed and paraffin-embedded specimen. Especially in PBTS, superoxides from neutrophils may damage DNA strands when high endogenous peroxidase activity is seen.

The major conclusions of this study are as follows : 1) Immunohistochemical analysis of proliferation, p53 expression, and apoptosis could be applied to leukemia cells in PBTS. 2) ATL cells were similar in size to medium-sized lymphoma cells. 3) Acute type ATL was characterized by nuclear p53 protein deposition, detected in ATL cells by the normal antigen-detection sensitivity-method. 4) Acute type ATL cells comprised p53 protein-negative and -positive heterogenous leukemia cells. 5) A relationship between the proliferation and apoptosis of the leukemia cells was observed.

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