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Malignant Lymphomas of Young People under 20 Years of Age in an Endemic Area of Human T-cell Leukemia Virus Type 1

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Introduction

Human T-cell leukemia virus type 1 (HTLV-1) induces adult T-cell leukemia/lymphoma (ATLL) and the other HTLV-1-related diseases. In the HTLV-1 infection routes mother-to-infant infection by breast milk feeding is thought to be the dominant (1). Most of HTLV-1 carriers have anti-HTLV-1 antibodies in their serum. Male-dominance in ATLL patients and HTLV-1 carriers means that ATLL would occur in the HTLV-1 carriers infected at the perinatal period (2). On the other hand, T cells-response to HTLV-1 divides HTLV-1-carriers into high and low immunoresponders (3). ATLL would occur in the latter who have ATL-associated haplotype of HLA (3).

In order to see whether HTLV-1 induced malignant lymphomas (MLs) and HTLV-1-related atypical lymphocytic infiltration (ALI) in the skin in the less than 20-year-old people in the HTLV-1-endemic areas, this study analyzed MLs and skin lesions with ALI in the less than 20 year-old people who would be free at least from HTLV-1 infection in the sexual intercourse (4), employing a statistical method, paraffin-immunohistochemistry and detection of HTLV-1 infection by polymerase chain reaction (PCR) and by in situ hybridization (ISH).

Material and method

Statistical analysis

Data of patients with MLs; Age, sex, site of biopsy and histological diagnosis, were listed from surgical pathology records from 1963 to 1985 in departments of pathology in Kagoshima university, Kagoshima municipal hospital, Kagoshima-shi medical association's hospital, Kagoshima institute of preventive medicine, Imakyure general hospital and Kagoshima citizen's hospital. The ML patients' list was processed by a microcomputer.

Number of patients with MLs diagnosed histopathologically was compared with number of people died from MLs based on the vital statistics of Kagoshima Prefecture (5) to see whether the list of ML patients could show representative features of MLs in Kagoshima.

Age-adjusted and -specific incidence rates of MLs in Kagoshima was calculated, based on number of the ML patients and age-specific population of Kagoshima prefecture in the national census (5). The age-adjusted and -specific incidence rates of MLs in Kagoshima were compared with those in the cancer registries (6) in HTLV-1-endemic and -nonendemic areas in Japan.

Paraffin-immunohistochemistry

Paraffin sections/blocks of 52 of the 104 MLs could be used. Histologically the MLs comprised 6 Hodgkin's disease (HD), 43 non-Hodgkin's MLs (NHMLs) and 3 true histiocytic lymphomas.

Phenotypes of lymphoma cells in the NHMLs were examined by paraffin-immunohistochemistry (7). Employed antibodies were MT-1 and UCHL-1 for T cells, anti-terminal deoxyribnucleotidyl transferase antibody (TdT) for thymocytes (8), MB-1, Mx-pan B, L26, LN-1, -2, -3 and anti-immunoglobulin light chain antibodies for B cells, S100, α 1AT, α 1ACT and anti-muramidase antibodies for histiocytes, and LeuM1 and Ber H2 for activating antigens. The reacted antibodies were visualized by ABC method.

The MLs were categorized according to updated Kiel classification (9).

Detection of HTLV-1 proviral DNA by PCR

A couple of primers SK43 and SK44 PCR for HTLV-1 proviral DNA pX Tax region (10) was employed for this analysis. DNA was extracted from paraffin sections of 39 MLs and 6 ALIs in the skin. Products of the PCR were examined by gel electrophoresis to see whether a band of DNA specific for the primers of PCR was amplified or not. Procedure and method of PCR were reported previously (11).

In situ hybridization of HTLV-I

Highly biotin-labeled concatamer (12) probe for HTLV-1 proviral DNA pX Tax region was produced by a couple of primers SK43 and SK44 PCR with bio-11-dUTP in the reaction solution (13). Procedure of probe-producing was written in the appendix.

Paraffin sections of 20 NHMLs, 5 HDs and 3 ALIs in the skin were examined by ISH.

The paraffin sections were deparaffinized, treated by pepsin solution at pH 2.0 for protein digestion and hydrolysis of DNA. After post-fixation, the sections were incubated in 70% formamid 2x SSC solution at 75℃ for 5 min and cooled rapidly to -30℃ in 95% ethanol for denaturing of DNA in the section. The sections were dehydrolyzed in vacuum chamber. Hybridization reaction with the concatamer probe denatured just before was performed in the moist chamber at 43℃ overnight. After washing the sections for 15 min in 2x SSC and 0.1x SSC at 43℃, hybridized probes were detected by DAKO streptavidine-biotin system and new fucshin system.

Table 1. Incidence rates of MLs in HTLV-1-endemic and -nonendemic areas in Japan

		Age-specific incidence rates(#1) of MLs 1973 - 1977				
	Kagoshima	HTLV-1 endemic			non-endemic	
Age	1985・86	Kagoshima	Fukuoka	Nagasaki	Osaka	Miyagi
Male						
-5	0.80	0.0	0.3	0.0	0.6	1.8
-10	3.00	0.9	0.6	1.1	0.3	0.0
-15	1.40	1.3	0.3	1.1	0.8	0.0
-20	2.50	1.7	0.9	1.1	0.6	0.5
Female						
-5	0.84	0.0	0.0	0.0	0.2	0.0
-10	0.00	0.6	0.0	1.1	0.3	0.0
-15	0.73	1.1	0.3	0.0	0.4	0.0
-20	0.00	0.0	0.3	0.0	0.3	0.3
Age-adjusted incidence rates(#1) of MLs						
Male	10.97	5.8	2.5	6.8	2.6	2.2
Female	6.57	2.8	1.3	5.0	1.3	0.8

#1: Age-specific incidence rates (/100,000 population/year)
Age-adjusted and -specific incidence rates of MLs in Fukuoka, Nagasaki, Osaka and Miyagi were sited in Reference 6.

Result

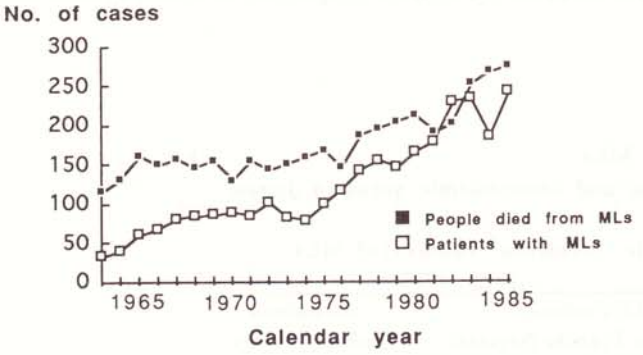
Statistic analysis of the MLs

The list of ML patients counted 2792 patients from 1963 to 1985. Comparing numbers of the ML patients and the people died from MLs based on the vital statistics according to calendar year, the both line-charts were parallel to each other from 1975 to 1985 (Fig. 1). Age-adjusted incidence rate of patients with MLs (Fig. 2) indicated about 9.0 per 100,000 population per one year after 1980.

Less than 20 year-old patients with MLs were 104 in the period, indicating about 1.0/100,000/year incidence rate (Fig. 2).

Age distribution of the ML patients showed a small hill of NHMLs in teenagers (Fig. 3).

Figure 1. Number of ML patients in Kagoshima



From 1975 both numbers of patients with MLs diagnosed histologically and died from MLs in Kagoshima prefecture vital statistics were pararell to each other. A statistical analysis of the former could show representative features of MLs in Kagoshima in the 0period from 1975.

Figure 2. Age-adjusted incidence rates of MLs in Kagoshima

Age-adjusted incidence rate of MLs in Kagoshima increased gradually from 1975 when ATLL was found out and was about 9.0/100,000/year after 1981. In the less than 20-year-old peope the incidence rate of MLs was about 1.0/100,000/year.

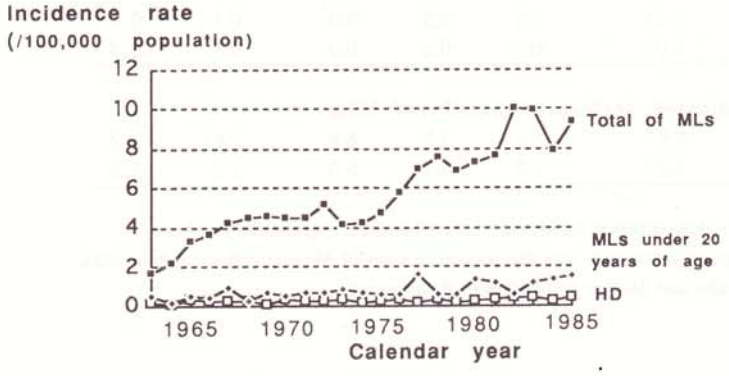
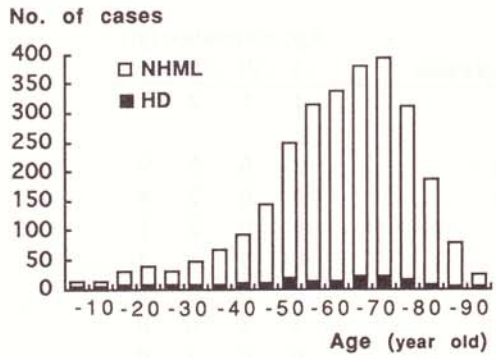


Figure 3. Age distribution of patients with MLs in Kagoshima



A large peak of over 50-year-old patients would include ATLL. A small hill of NHMLs was found in teenagers.

The incidence rates of MLs in Kagoshima were compared with those of MLs registered from 1973 to 1977 in Nagasaki, Fukuoka, Osaka and Miyagi prefecture in Japan in Table 1. Incidence rates of MLs in the less and more than 20-year-old people were higher in HTLV-1-endemic areas (Kagoshima, Nagasaki and Fukuoka) than in HTLV-1-nonendemic areas (Osaka and Miyagi). High incidence rates of MLs in males was found in the both of HTLV-1-endemic and -non-endemic areas.

Paraffin-immunohistochemistry of MLs

As shown in table 2, NHMLs comprised 33 T-cell and 10 B-cell MLs. Most of the MLs were so-called high grade malignant.

T-cell lymphoblastic lymphomas (T-LBs, Fig. 4) dominated in the NHMLs. In 6 cases of T-cell pleomorphic lymphomas (T-Pleos), 2 cases of T-Pleo large cell type (16 year-old male and 17 year-old female) showed peculiar ML cells' figures (Fig. 5). HD cell-like giant cells were also found in one case of T-Pleo medium-sized cell type.

Anti-TdT antibody stained nuclei of the lymphoma cells only in 11 of 20 T-LBs (Fig. 4b). One case of angioimmunoblastic lymphadenopathy with dysproteinemia (AILD) type (14 year-old boy) developed to T-cell Pleo large cell type. Detailed data of this case was reported by Dr. Nakazono (14).

B-cell lymphoblastic lymphoma (B-LB, Fig. 6a) could not be differentiated from T-LB histologically, although starry sky pattern of lymphoma cells' growth was prominent in B-LB. MB-1 reacted with lymphoma cells of B-LB. Comparing with B-LB in table 4, Burkitt type lymphoma (BL, Fig. 6b) showed cohesive growth and reactivity with LN-1 or LN-2. And BL used to be found in the extranodal sites.

Table 2, Malignant lymphomas in the less than 20-year-old people in Kagoshima according to the updated Kiel classification

	No. of caese		Age distribution (yr) -5	-10	-15	-20
Hodgkin's disease	6		1	1	2	2
T-cell MLs	33					
T-LB	22		2	6	5	9
T-Pleo	6		0	0	2	4
medium-sized cell		3	0	0	2	1
large cell		3	0	0	0	3
ALC	2		0	0	0	2
TzML	2		1	1	0	0
AILD type	1		0	0	1	0
B-cell MLs	10					
B-LB	2		0	0	1	1
CB	4		0	0	1	3
BL	3		0	1	1	1
B-IB	1		0	0	0	1
Histiocytic ML	3		1	0	2	0
TTL	52		5	9	15	23

Figure 4. T-cell lymphoblastic lymphoma

PCR- and ISH--postive 17 year-old HTLV-1 carrier male manifested fever, general fatigue, systemic lymph node swelling and leukemia diagnosed as acute lymhocytic leukemia. a) **H.E.** b) **Paraffin-immunohistochemistry of Anti-TdT antibody** without nuclear counterstain. Many lymphoma cells have TdT-positive nuclei. c) **ISH of HTLV-1 (bone marrow)**. A small number of small lymphocytes and few lymphoma cells showed a signal of HTLV-1 proviral DNA.

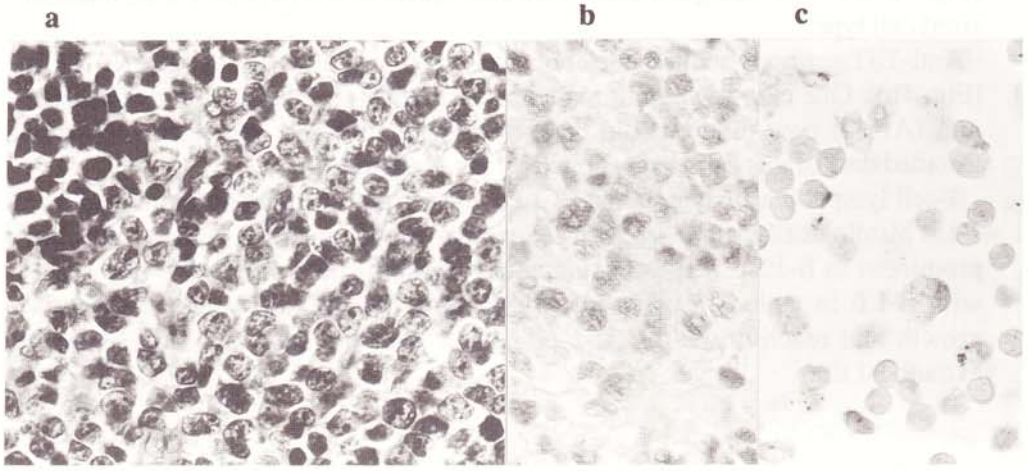


Figure 5. T-cell pleomorphic lymphoma, large cell type
PCR- and ISH--postive 17 year-old HTLV-1 carrier female complained abdominal pain and indicated high fever. a) **H.E.** There are two kinds of lymphoma cells. Large lymphoma cells have rich cytoplasm and irregular-shaped vesicular nuclei with various-sized nucleoli, stippled heterochromatin and irregularly thickened nuclear membrane. Basophilic medium-sized to large lymphoma cells have hyperchromatic nuclei. b) **ISH of HTLV-1.** Some of the large lymphoma cells having rich cytoplasm showed perinucear and nuclear signal of HTLV-1 proviral DNA. The basophilic lymphoma cells showed strong cytoplasmic signal of HTLV-1 proviral DNA. This case was thought to be **the youngest case of ATLL.**

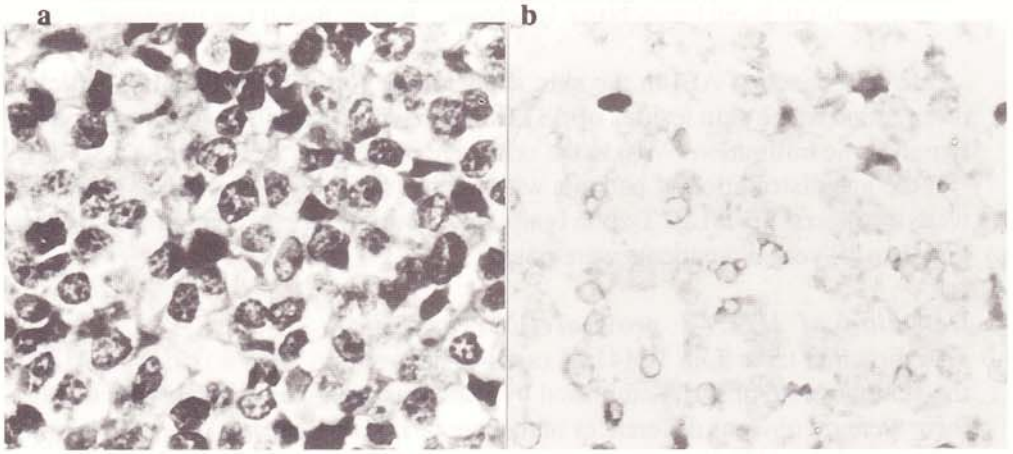


Figure 6. B-cell lymphoblastic (B-LB) and Burkitt type lymphoma (BL)
a) B-LB. H.E. B-LB could not be differentiated histopathologically from T-LB. b) BL. H.E. BL had a little larger nuclei having small nucleoli in the nucleoplasm, different from ML cells of centroblastic lymphoma.

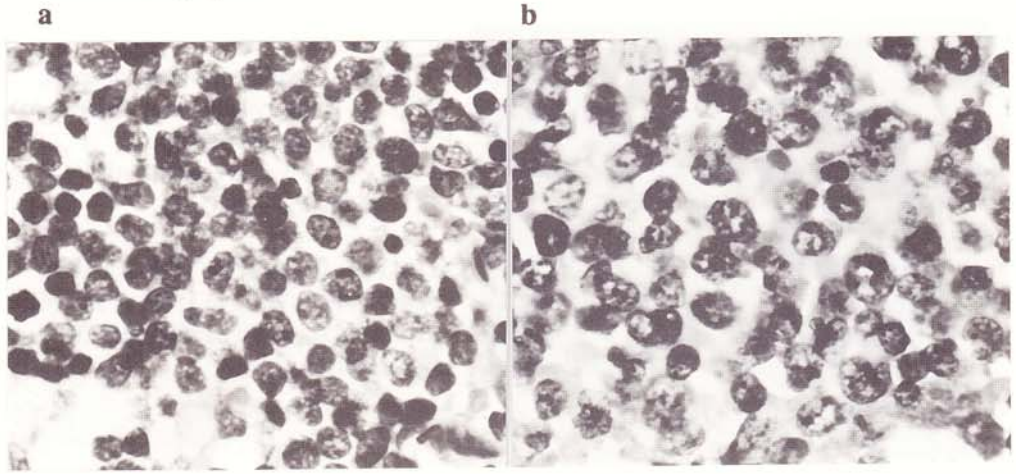


Table 4, Paraffin-immunohistochemistry of B-cell lymphoblastic lymphomas and Burkitt type lymphomas

Case	Age	Sex	Sie	Reaction of lymphoma cells with antibodies MT-1	UCHL-1	MB-1	L26	LN-1	LN-2	LN3
B-LB 1	17	M	Neck LN	-	-	+	n.d.	-	-	-
B-LB 2	13	M	Axiliar LN	-	-	+/-	+	-	-	+/-
Burkitt 1	9	M	Inguinal LN	-	-	+	+	+	+	+
Burkitt 2	16	M	Oral mucosa	-	-	+	+	+	-	-
Burkitt 3	5	F	Retroperitoneum	-	-	+	+	-	+/-	+/-

B-LB: B-cell lymphoblastic lymphoma. Burkitt: Burkitt type lymphoma.

One of 7 cases of ALI in the skin showed hair follicle involvement, which is often found in the skin lesions of ATLL. The atypical lymphocytes in the atypical lymphocytic infiltration (AIL) in the skin were positive for MT-1 and/or UCHL-1.

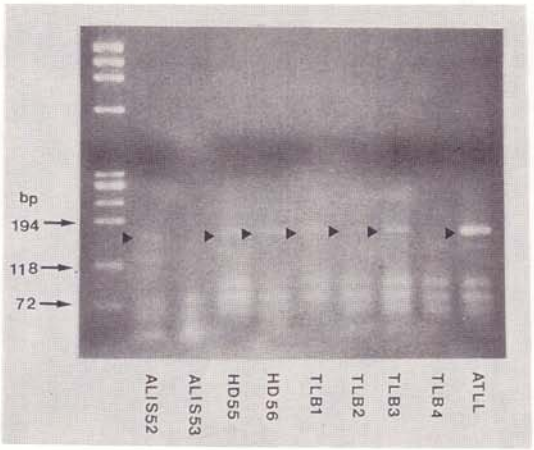
In the age distribution of patients with MLs, 38 (73%) of 52 patients with MLs were teenagers. In T-LB, T-zone lymphoma, Burkitt type and Hodgkin's disease, less than 10 year-old patients were noted.

Detection of HTLV-I proviral DNA by PCR

As shown in table 5, in 16 (41%) cases of MLs and in 5 (83%) cases of ALIs in the skin the band of DNA amplified by PCR was seen. Among subtypes of MLs, there were no obvious differences in the rate of HTLV-1 carriers.

Most of the HTLV-1-carrier patients with MLs and ALIs in the skin were teenagers. Only in T-LB and Hodgkin's disease, 5 year-old girl and 3 year-old boy were found. In ALIs in the skin, one case was 9 year-old boy.

Figure 7. DNA band amplified by PCR



ATLL: Adult T-cell leukemia/lymphoma.
TLB 1 - 4: T-cell lymphoblastic lymphomas.
HD 55, 56: Hodgkin's diseasee.
ALIS 52, 53: Atypical lymphocytic infiltration in the skin.

At the same height of the PCR-amplified DNA of ATLL, TLB1 to 3, HD55 and 56, and ALIS52 showed a band of the PCR-amplified DNA.

Table 5. HTLV-1 infection detected by PCR in MLs and ALI in the skin

	No. of cases with a band of amplified DNA /cases examined	Age distribution -5	-10	-15	-20(yrs)
Malignant lymphoma	16/39 (41%)	1	1	4	10
T-cell type	9/24 (38%)				
T-LB	5/14	0	1	1	3
T-Pleo	2/ 6	0	0	0	2
ALC	1/ 2	0	0	0	1
TzML	0/ 1	-	-	-	-
AILD type	1/ 1	0	0	1	0
B-cell type	4/ 8 (50%)				
B-LB	2/ 2	0	0	1	1
CB	1/ 3	0	0	0	1
BL	1/ 3	0	0	0	1
Hodgkin's disease	3/ 6 (50%)	1	0	1	1
Histiocytic lymphoma	0/ 1 (0%)	-	-	-	-
Atypical lymphocytic infiltration in the skin	5/ 6 (83%)	0	1	1	3

According to calendar year, in a group of cases who belonged to the same birth cohort, HTLV-1 carriers detected by the PCR method appeared with advancing age (Table 6). There were no differences in the HTLV-1 carrier's rate of patients with MLs among 4 periods according to calendar year.

Table 6. Less than 20 year-old HTLV-1 carriers with malignant lymphomas according to calendar year

	No. of HTLV-1 carriers/cases examined			
	Time of diagnosis			
Age(yr.)	-1970	-1975	-1980	-1985
- 5	0/1	-	-	1/1
-10	0/2	0/1	1/2	0/4
-15	1/3	0/2	2/4	1/3
-20	2/2	2/5	1/2	5/7
TTL	3/8 (38%)	2/8 (25%)	4/8 (50%)	7/15 (47%)

ISH with concatamer probe for HTLV-1 proviral DNA pX Tax region

This ISH showed cytoplasmic and perinuclear positive stains ML cells (Fig. 5b) and non-neoplastic intermingling cells such as small lymphocyte (Fig. 4c) and histocytes. When patients with MLs of which any kinds of cells showed ISH-positive stain were treated as HTLV-1 carriers, results of PCR and ISH analysis detecting HTLV-1 infection were compared in 27 cases of MLs and ALIs in the skin (Table 7). A high coincidence rate (21/27: 78%) was gotten in the comparison (p= 0.006).

Table 7. Comparison of PCR and ISH detecting HTLV-1 infection

		Detection by PCR	
		HTLV-1 carriers	non-carriers
Detection by ISH	HTLV-1 carriers	10	5
	non-carriers	1	11

HTLV-1 carriers in ISH: Cases, of which any cells showed ISH-positive stain.
There were 1 PCR-positive and ISH-negative 18-year-old T-LB and 5 PCR-negative and ISH-positive cases comprising 6 year-old T-LB female, 5 year-old BL female, 15 year-old T-Pleo male, 12 year-old HD male and 10 year-old CB male. In the PCR-negative and ISH-positive cases except the CB case, ISH-positive cells were a small number of intermingling lymphocytes/histiocytes among lymphoma cells.

As shown in table 8, there were 13 (52%) **ISH-positive HTLV-1 carriers** in the patients with MLs and 4 (16%) **ISH-positive HTLV-1-related MLs** of which more or less lymphoma cells showed ISH-positive stain.

In one case of T-Pleo large cell type (Fig. 5), basophilic lymphoma cells showed strong ISH-positive stain and large lymphoma cells showed perinuclear ISH-positive stain (Fig. 5b). No obvious rot-like ISH-positive stain could not be seen. In one case of ALC a small number of lymphoma cells showed faint ISH-positive stain. A quite small number of lymphoma cells in 1 T-LB showed cytoplasmic ISH-positive stain (Fig. 4c). In 1 B-cell centroblastic lymphoma (CB, Fig. 7) which was PCR-negative, many lymphoma cells showed rot-like and cytoplasmic ISH-positive stain. In 2 of 3 cases with ALI in the skin small lymphocytes showed ISH-positive stain.

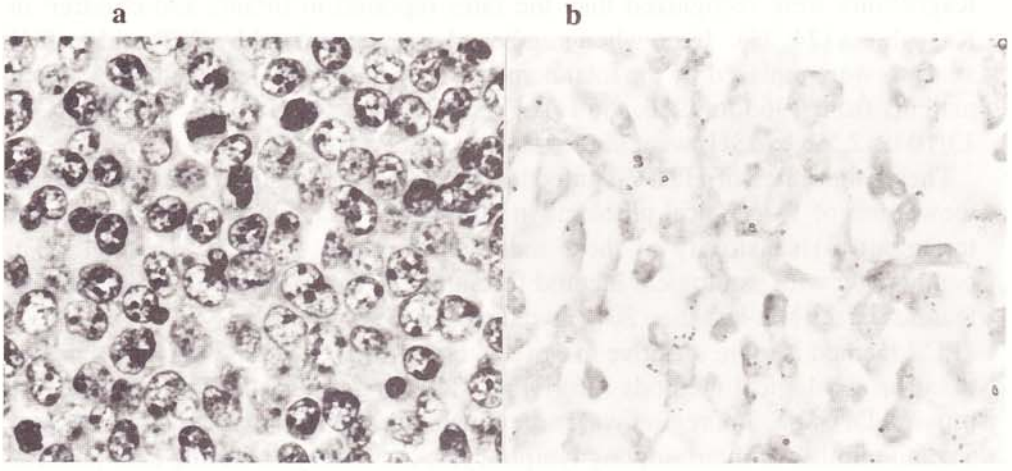
Table 8. ISH with concatamer probe for HTLV-1 proviral DNA pX Tax region in MLs

	NO.	Carrier	Related ML
Total of MLs	25	13(52%)	4(16%)
T-cell MLs	12	5(42%)	3(25%)
T-LB	6	1	1
T-Pleo	4	2	1
ALC	1	1	1
AILD type	1	1	0
B-cell MLs	8	5(63%)	1(13%)
CB	4	3	1
B-IB	1	0	0
Burkitt	3	2	0
Hodgkin's disease	5	3(60%)	0(0%)
ALI in the skin	3	2(67%)	-

Carriers: Cases with malignant lymphoma, of which any cells other than lymphoma cells showed ISH-positive stain.
Related MLs: Cases with malignant lymphomas, of which more or less lymphoma cells showed ISH-positive stain.

Figure 7. Centroblastic lymphoma

PCR-negative and ISH-postive 10 year-old HTLV-1 carrier male manifested splenomegaly and right ingunal lymph node swelling. a) **H.E.** b) **ISH of HTLV-1** Lymphoma cells showed rot-like and cytoplasmic ISH-positive stain.



Discussion

Investigating MLs in the less than 20-year-old people in Kagoshima, which is one of HTLV-1-endemic areas in Japan (15, 16), this study showed higher age-adjusted and -specific ML-incidence rates (about 1.0/100,000/year) in the young generation of Kagoshima than in HTLV-1-nonendemic areas of Japan, an existence of ATLL-like T-cell Pleos in the MLs, a high rate of HTLV-1 infection in the ML patients and an existence of HTLV-1-related MLs.

ML-incidence rates calculated based on the list of the patients with MLs diagnosed histopathologically could be representative in Kagoshima, because numbers of patients with MLs in the list were parallel to those in the vital statics of Kagoshima prefecture. The about 1.0/100,000/year ML-incidence rates in the young generation were found in cancer registry in Nagasaki (6) and reported by Tajima as those in Kyushu (17). Since actual ML-incidence rates in Kagoshima is higher than the calculated ones, the former must be higher than ML-incidence rates reported by cancer registries (6) in HTLV-1-nonendemic areas in Japan.

There were T-Pleos and ALC in the MLs of the Kagoshima's less than 20-year-old people as reported in HTLV-1-nonendemic areas in Japan (18). No obvious differences in histopathological features of childhood lymphomas between HTLV-1-endemic and -nonendemic areas in Japan were reported (19). But at least one case of T-Pleo large cell type showed peculiar basophilic lymphoma cells (Fig. 5a), which were not found in the literature reporting cytological features of T-Pleo including ATLL (7, 9, 20, 21, 22, 23). One case of AILD type T-cell ML, usually found in elder adults, was also found in the MLs (14).

Employing two different methods; PCR and ISH, detecting HTLV-1 infection in paraffin sections of lymphoma tissue, higher HTLV-1 infection rates (16/39; 41% by PCR, 13/25; 52% by ISH) in the MLs-less than 20-year-old patients in Kagoshima were recognized than the rates reported in infants and children in Kagoshima (24, 25). Even when number of cases examined by PCR and/or ISH analysis were replaced by the total number (104) of the ML-less than 20-year-old patients from 1963 to 1985, the HTLV-1 infection rates (16/104; 15% by PCR, 13/104; 12.5% by ISH) were higher than the rates reported.

These high rates of HTLV-1 infection in the MLs should be considered from viewpoints of 1) technical problems in PCR and ISH, 2) blood transfusion in the treatment, 3) sensitivity of these methods to detecting HTLV-1 infection in comparison with serological method for anti-HTLV-1 infection, and 4) peculiar features of the ML-less than 20-year-old people in Kagoshima.

PCR method is more sensitive in early detection of HTLV-1 infection in neonates than the serological methods (26). This study suggested that ISH for HTLV-1 proviral DNA pX Tax region was more sensitive than PCR method, showing that a small number of intermingling lymphocytes or histiocytes having ISH-positive stain were not detected by PCR method (Table 7). But a poor procedure in the

both methods was indicated by an existence of PCR-negative and ISH-positive CB and PCR-positive and ISH-negative T-LB (Table 7). In PCR-negative and ISH-positive CB (Fig. 7), the extracted DNA from the section of lymphoma tissue would be lost in DNA collection processes or the concatamer DNA amplified in PCR (12) was ignored on the gel. In the PCR-positive and ISH-negative T-LB, a quite small number of intermingling lymphocytes/histiocytes with ISH-positive stain were not recognized in the microscopic evaluation.

The transmission rate of blood transfusion from HTLV-1 carriers was reported to be 60% and the seropositive HTLV-1 carrier rate in the blood donors in Kyushu was 8% (17). Because the infection rate of HTLV-1 was calculated as 4.8% (8 x 0.6), the HTLV-1 infection rates in the ML-less than 20-year-old patients in Kagoshima were not explained by the transmission of HTLV-1 by blood transfusion.

As it was suggested that ATLL would occur in the low immunoresponders recognized by HLA haplotype (3), only peculiar features of the ML-less than 20-year-old patients in Kagoshima explained the high HTLV-1 infection rates indicated in this study.

But the appearance of HTLV-1 carriers with advancing age in the young ML-patients suggested that an immunotolerance against HTLV-1 may exist in the infant -child period of these patients. And in this period the latent HTLV-1 infection may be not able to be recognized even by PCR and ISH methods. Further studies employing serological, PCR and ISH methods must be performed to see whether such immunotolerance exists in the natural history of vertical HTLV-1 infection.

No obvious differences in the HTLV-1 detection rates among the periods of diagnosis (Table 6) suggested that a high HTLV-1 infection rate in the candidates for young ML-patients would continue in Kagoshima, different from the situation reported in Okinawa (27) and Tsushima (28).

The high rates of HTLV-1 infection in the ML-less than 20-year-old patients suggested a close relation between HTLV-1 infection and ML occurrence in the generation and explained the reason why the incidence rates of MLs were higher in HTLV-1-endemic areas than in HTLV-1-nonendemic areas even in the young generation.

By ISH with a probe for HTLV-1 pX Tax region, this study showed the youngest patients (17 year-old female) with T-Pleo having a close relation to HTLV-1 infection (Fig. 5). But it was not determined whether this T-Pleo was ATLL or not, because ISH analysis could not show monoclonal integration of HTLV-1 in lymphoma cells. We knew and will report in other papers that ATLL type T-cell lymphomas (7) would be differentiated by many lymphoma cells with ISH-positive stains and that there were T-cell and B-cell NHMLs and HDs of which more or less lymphoma cells had ISH-positive stains. Probably this case was the youngest case of ATLL, showing that its basophilic lymphoma cells (Fig. 5) might be apoptotic cells revealing a strong ISH-positive stain.

As for ISH with concatamer probe for HTLV-1 pX Tax region in this study, the pretreatment of the sections was designed to be applied for DNA-DNA and RNA-DNA hybridization. Thus, this ISH could detect all kinds of HTLV-1 RNAs (29), proviral DNA, viral itself and viral DNA genome, although the cytoplasmic ISH-positive stain may suggest a strong signal of HTLV-1 pX Tax region (29).

Besides, the ALI in the skin in the 20-year-old people in Kagoshima may be one of HTLV-1-related condition. Molecular histopathological differential diagnosis of the ALI in the skin from smoldering ATL (30) should be established in the future.

At last, the transmission routes and rates of HTLV-1reported by serological studies of HTLV-1 carriers and patients with HTLV-1-related diseases must be studied again by PCR and ISH methods.

Summary

Studying malignant lymphomas (MLs) in the less than 20-year-old people in Kagoshima, HTLV-1-endemic area in Japan, by means of a statistical analysis, paraffin-immunohistochemistry, polymerase chain reaction (PCR) analysis for HTLV-1 proviral DNA pX Tax region, and in situ hybridization (ISH) analysis with concatamer probe synthesized by PCR for HTLV-1 proviral DNA pX Tax region, the following features of the MLs and the patients could be shown. 1) A higher incidence rate (about 1.0/100,000/year) of MLs in the young people in Kagoshima was indicated than in HTLV-1-nonendemic areas in Japan. 2) There were a small number of peculiar T-cell pleomorphic lymphoma and AILD type T-cell ML in Kagoshima. 3) A high HTLV-1 infection rate in the young patients with MLs in Kagoshima was indicated by the PCR and ISH analysis, not explained by technical problems of the methods and HTLV-1 transmission by blood transfusion in their treatment. Only peculiar features of the young ML patients may explain the high rate of HTLV-1 infection. The high rate HTLV-1 infection in the candidates of the young ML-patients would continue in Kagoshima. 3) There were a 17 year-old female patients with T-Pleo with peculiar lymphoma cells and a relation to HTLV-1 infection. Besides, HTLV-1-related T-cell and B-cell MLs (17 year-old T-LB, 16 year-old ALC and 10 year-old CB males) were found. 4) An existence of HTLV-related atypical lymphocytic infiltration (ALI) in the skin was indicated. Consequently HTLV-1 induced MLs and ALI in the skin lesion in the less than 20-year old people in Kagoshima.

Key words: Malignant lymphoma, adult T-cell leukemia/lymphoma, children, statistics, paraffin-immunohistochemistry, HTLV-1, polymerase chain reaction, in situ hybridization

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Appendix

Production of ISH probe for HTLV-1 proviral DNA pX Tax region

Producing highly biotin-labeled probe by 2 times PCR, the following experiments were performed. Template DNA was extracted from ATL cell line KUT2. Two kinds of PCR protocols (No.1 and No.2) were designed (Table A). A couple of primers were SK43 and SK44.

A smear of product (Concatamer) of the second PCR was gotten (12), when the first PCR products were used as the template (Fig. A).

When DNA extracted from the band at 159bp on the gel was employed as a template, the 2nd PCR according to protocol No.2 yielded also a concatamer. Diluting the DNA extracted from the band at 159bp on the gel as a template, PCR according to protocol No.1 with 1:1,000,000 diluted DNA solution as a template yielded a band at 159bp (Fig. B4). With DNA solutions diluted less than 1:1,000,000 as a template, a concatamer appeared as a band of DNA at 159bp melted (Fig. B1 to 3, 1:10, 1: 100, 1:10,000).

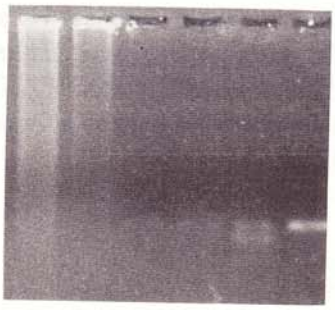
The 2nd PCR with biotin-11-dUTP in the solution (13) could synthesize a band of DNA (probe) at 159bp and a smear of DNA longer than 159bp (**concatamer probe**), depending the concentration of the template DNA. These probes were detected by streptavidine-biotin system and new fucshin system (Fig. C1,2). Biotinylated SK45 annealed the both not-biotinylated probes (Fig. C3,4).

In an experimental ISH with the both probes, the concatamer probe showed much stronger ISH stain in small lymphocytes than the other. Then, this study employed the highly biotinylated concatamer probe.

Table A. Protocols of PCR

		Reaction time	
	Temperature	No.1 protocol	N0.2 protocol
denaturing	94℃	15 s	2 min.
annealing	55℃	30 s	2 min.
extension	72℃	30 s	3 min.
No. of cycles		30	50

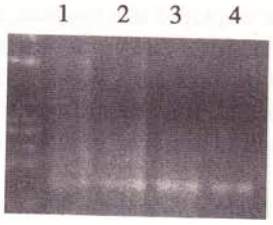
Figure A. Products of the first PCR and the second PCR



According to the protocol No.1, the first PCR yielded a band of DNA at 159 bp (1). After heat denaturing followed by rapid cooling, the band was divided to two bands of double- and single-stranded DNA (2). After the heat denaturing followed by gradual cooling, the band of single-stranded DNA was lost (3). DNA extracted by ethanol sedimentation from the PCR product showed also the same band (4).

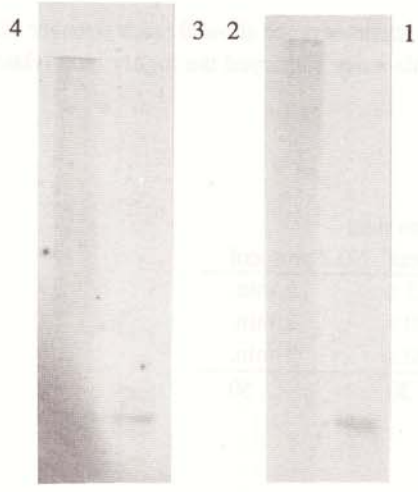
According to the protocol No.2, the second PCR showed a smear (concatamer in ref. 12) of DNA, employing 5 (5) and 0.5 micron l (6) of the first PCR product as the template.

Figure B. The second PCR products, employing DNA extracted from a band on the gel as a template according to protocol No. 1



PCR according to protocol No.1 with 1:1,000,000 diluted DNA solution as a template yielded a band at 159bp (4). With DNA solutions diluted less than 1:1,000,000 as a template, a smear of DNA appeared, as a band of DNA at 159bp melted (1 to 3, 1:1, 1: 100, 1:10,000).

Figure C. Detection of incorporated biotin in the probes and annealing of the not-biotinylated probes with biotinylated SK45



Probes synthesized by the second PCR with Bio-11-dUTP were loaded on the gel and transfered to membrane by a vacuum transfer method. The biotinylated probe (1) and concatamer probe (2) were detected. The non-biotinylated probe (3) and concatamer probe (4) were annealed with biotinylated SK45.

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