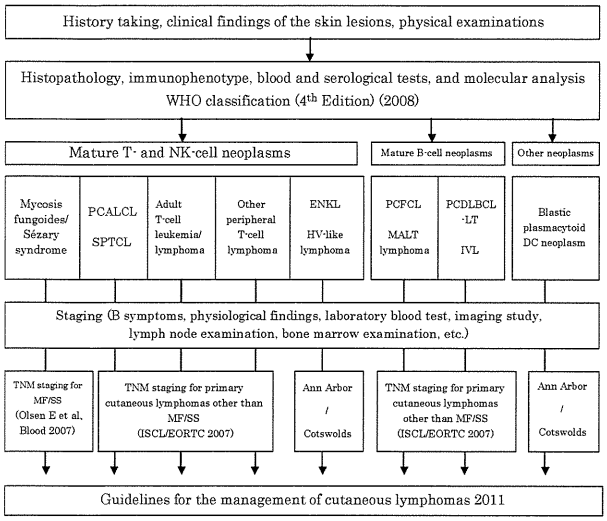

Figure 1. Diagnostic and staging algorithm for cutaneous lymphomas. DC, dendritic cell; ENKL, extranodal T/NK-cell lymphoma, nasal type; HV, hydroa vacciniforme; IVL, intravascular large B-cell lymphoma; MALT lymphoma, extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue type; MF/SS, mycosis fungoides/Sézary syndrome; PCALCL, primary cutaneous anaplastic large cell lymphoma; PCDLBCL-LT, primary cutaneous diffuse large B-cell lymphoma, leg type; PCFCL, primary cutaneous follicle center lymphoma; SPTCL, subcutaneous panniculitis-like T-cell lymphoma; TNM, tumor–node–metastasis; WHO, World Health Organization.

count of $\geq$1000/μL with a positive clone). Additional parameters that meet the B_2 criteria include the following: CD4/CD8 ratio of 10 or more, $CD4^+CD7^-$ of 40% or more, and $CD4^+CD26^-$ of 30% or more.[3,12,13] Cases with erythroderma who meet the B_2 criteria are defined as SS, or stage IVA_1 (Table S3 and Fig. S1). Erythrodermic MF of the B_0 or B_1 category is classified as stage IIIA or IIIB.

If lymphoma cells replace all or large portions of the lymph node structure, the condition is diagnosed as N_3 and is classified as stage IV_2 (Table S3). Even if the lymph node is infiltrated by atypical cells, a diagnosis of N_3 is not made as long as the foci are small and nodal architecture is preserved.[3,12]

TNM classification of cutaneous lymphoma other than MF/SS (ISCL/EORTC 2007)

No TNM classification appropriate for the evaluation of cutaneous lesions was available for primary cutaneous lymphoma categories other than MF/SS. In 2007, the ISCL and EORTC proposed a new TNM classification system (Table S4).[4] Although the TNM classification reflect the extent of lesions, an adequate staging system has not been established yet. Moreover, the classification does not indicate prognoses for some disease types.[14] The category of "non-MF/SS" covers many types of cutaneous lymphoma, and new staging systems are needed for each disease type, based on the collected clinical data and prognostic analysis.

Table 2. Classification of cutaneous lymphomas

Cutaneous T/NK cell lymphoma
Mycosis fungoides: MF
Variants
Folliculotropic MF
Pagetoid reticulosis
Granulomatous slack skin
Sézary syndrome: SS
Adult T-cell leukemia/lymphoma
Primary cutaneous $CD30^+$ T-cell lymphoproliferative disorders
Primary cutaneous anaplastic large cell lymphoma
Lymphomatoid papulosis
Subcutaneous panniculitis-like T-cell lymphoma
Extranodal NK/T-cell lymphoma, nasal type
Hydroa vacciniforme-like lymphoma
Primary cutaneous γδ T-cell lymphoma
Primary cutaneous $CD8^+$ aggressive epidermotropic cytotoxic T-cell lymphoma*
Primary cutaneous $CD4^+$ small/medium T-cell lymphoma*
Peripheral T-cell lymphoma, not otherwise specified
Cutaneous B-cell lymphomas
Extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue
Primary cutaneous follicle center lymphoma
Primary cutaneous diffuse large B-cell lymphoma, leg type
Intravascular large B-cell lymphoma
Hematological precursor cell neoplasm
Blastic plasmacytoid dendritic cell neoplasm

*Provisional. Representative clinicopathological features of MF/SS, anaplastic large cell lymphoma, adult T-cell leukemia/lymphoma, subcutaneous panniculitis-like T-cell lymphoma, extranodal NK/T cell lymphoma, hydroa vacciniforme-like lymphoma, blastic plasmacytoid dendritic cell neoplasm have been shown in Figs S1–S7.

Staging of other cutaneous lymphomas and hematopoietic malignancies

Shimoyama and colleagues have provided a widely-used classification of adult T-cell leukemia/lymphoma (ATLL): acute, lymphoma, chronic and smoldering types.[15] According to Shimoyama's criteria, ATLL patients with cutaneous lesions only are usually classified into the smoldering group. It is not appropriate to stage ATLL patients with the TNM system proposed by Kim *et al.*[4] because of the presence of minimal hematological disease. Furthermore, for other hematological malignancies such as ENKL, nasal type, and blastic plasmacytoid dendritic cell neoplasm, the Ann Arbor or Cotswolds staging (Table S5)[16] has been widely adopted in Japan because of hematological and extracutaneous spreading of the illness.

EPIDEMIOLOGY OF CUTANEOUS LYMPHOMA

In line with the WHO classification (3rd edn), the incidence of all types of lymphomas was reported by pathologists in Japan.[17] The data were distinct from those in Western countries and similar in several ways to other data from Asia, although the relatively high rate of ATLL was attributed to the geographical difference in the etiologic factor, human T-lymphotropic virus

type 1 (HTLV-1). The JSCS – Lymphoma Study Group has conducted a nationwide survey of cutaneous lymphoma annually since 2007 (www.okayama-hihuka.jp/pdf/kekka2010.pdf). MF/SS account for approximately 51% of all cutaneous lymphomas, followed by ALCL and ATLL at approximately 9–8% each. B-cell lymphoma accounts for approximately 15% of all cutaneous lymphoma in Japan, so it is less frequent than in Europe or North America. ENLK, nasal type, accounts for only approximately 2%, which is nearly always associated with Epstein–Barr virus (EBV) infection. The NK-cell type is dominant in Japan.

PROGNOSTIC ANALYSIS

Prognostic analyses of patients with cutaneous lymphoma are limited.[18–21] In the present guidelines, we have highlighted the prognoses of MF/SS, ATLL, and ENLK, nasal type, the latter two of which preferentially occur in Japan. For the other types of cutaneous lymphoma, we have used reports from other countries (Table 3).[22–26]

MF/SS

Previous researchers already contributed to disease staging and prognostic analysis for MF/SS.[27] Since the new staging was advocated in 2007, prognostic analyses have been reported from Japan and the UK (Table 3).[18,22] The survival rates of Japanese patients with MF/SS were similar to those shown in previous studies conducted in the USA and Europe. The prognoses of patients with skin tumor (stage IIB) and extracutaneous involvement (stage IV) were significantly worse than those of patients with early-stage disease (stages IA–IIA). Erythrodermic MF patients without blood involvement (stage IIIA) showed excellent survival. Independent prognostic factors in multivariate analyses were higher age and the presence of either skin tumor or extracutaneous disease.[18] Although findings in Japan showed the prognosis for stage IIIA to be quite favorable, a British analysis indicated that it was similar to the prognosis for stage IIb,[22] this may have occurred because the two reports did not use the same diagnostic criteria for erythrodermic lymphoma, resulting in differences in patient characteristics.

ATLL

A recent observation in Japan indicated that the patch and plaque types of ATLL were associated with better survival rates.[19] Multivariate analysis demonstrated that the hazard ratios of the erythrodermic and nodulotumoral types were significantly higher than that of the patch type, and that the eruption type is an independent prognostic factor for ATLL. The overall survival worsened as the T stage became more advanced: the multipapular type and T2 were comparable, and the purpuric type had a significantly poorer prognosis than T1 (Fig. S3).[19]

ENKL

Suzuki *et al.*[20] have reported the prognosis of a total 150 patients with ENKL, nasal type, consisting of 123 nasal and 27 extranasal (16 cutaneous, nine hepatosplenic, one intestinal and one nodal) lymphomas. We focused on patients with the cutaneous type of ENKL, and re-examined their prognoses. Patients with stage I disease (determined by the Ann Arbor staging system) showed a favorable prognosis in 5-year overall survival of 75%, but the prognoses deteriorated in the advanced stages (Table 3). Unlike a previous study on $CD56^+$ hematological neoplasms with or without EBV infection in Europe,[28] our data highlighted that ENKL is usually associated with EBV infection, and assessed the prognoses of "nasal" and "cutaneous" ENKL separately.

TREATMENT GUIDELINES

Treatment guidelines for MF/SS

Mycosis fungoides/Sézary syndrome is the oldest defined form of cutaneous lymphoma, and is more common than other primary cutaneous lymphomas (Tables 4–11). At present, no treatment based on high-level evidence is available for this condition. In many cases, the clinical course may extend for 10 years or more. Therefore, the success or failure of therapeutic intervention may be difficult to determine. Moreover, ethical issues may complicate the implementation of randomized placebo-controlled studies. Only four randomized studies have compared the effectiveness of different treatment methods[29–32] and only one randomized placebo-controlled study has been conducted.[33] These guidelines give substantial weight to consensus among the committee members. The "B" recommendation level has been given to first-line therapies for daily clinical practice.

An additional problem is that far fewer treatment options are available for MF/SS in Japan than in Western countries. In the present guidelines, we have included information on treatment modalities that have not been approved by the Japanese National Health Insurance system. Experimental therapies not yet approved overseas or in Japan have been omitted from these guidelines.

CQ1: Is monitoring the clinical course without treatment recommended for MF?
Degree of recommendation: C1 (stage IA only), C2 (other than stage IA).
Recommendation: In stage IA of MF, one acceptable option is to monitor the clinical course without treatment. For stages beyond IA, monitoring the clinical course without treatment is generally not recommended (Data S1).

CQ2: Are topical steroids recommended for MF/SS?
Degree of recommendation: B.
Recommendation: Topical steroid therapy is recommended at all stages of MF/SS (Data S1).

CQ3: Is topical chemotherapy recommended for MF/SS?
Degree of recommendation: C1.
Recommendation: Mechlorethamine/nitrogen mustard (HN2) or carmustine (BCNU) topical chemotherapy is currently used in Europe and North America, and is recommended for early-stage MF (stage IA through IIA). These agents are not yet approved or available in Japan. Nimustine hydrochloride (ACNU) is currently used topically in some facilities in Japan,

Table 3. Survival rates of various cutaneous lymphomas and hematological neoplasms

Disease	Stage	5y-OS	5y-DSS	Median survival time (months)	References
MF/SS	IA	94–100	98–100	426	18, 22
	IB	84–89	89–95	258	
	IIA	78–87	87–89	190	
	IIB	47–73	56–88	56–78	
	IIIA	47–100	54–100	56	
	IIIB	40	48	41	
	IVA1	0–37	0–41	23–46	
	IVA2	18–33	23–50	25–46	
	IVB	0–18	0–18	13–17	
ATLL	T1	82.5	82.5	192.6*	19, 23
	T2	27.3	27.3	47.9	
	T3	0	0	17.3	
	T4	0	0	3	
	Multi-papular type	42.1	47.1		
	Purpuric type	40.0	40.0		
ALCL	T1	85	93		24
	T2	81	93		
	T3	63	77		
	Leg (–)	86	100		
	Leg (+)	53	67		
SPTCL	HPS (–)	91			25
	HPS (+)	45			
		Total 82			
Nasal ENKL	I	55 (4 years)		59.8	20
	II	33 (4 years)		11.2	
	III	31 (4 years)		33.1	
	IV	10 (4 years)		5.3	
		Total 36 (4 years)		Total 12.9	
Cutaneous extranasal ENKL	I	75 (2 years)		Not reached	20
	II	0 (2 years)		6.2	
	III	Not reached†		75.5†	
	IV	14 (2 years)		4	
		Total 33 (2 years)		Total 6.8	
BNKL	BM/blood (–)	0	25.3 (2 years)	17.1	21
	BM/blood (+)	19.6	46.4 (2 years)	20.4	
	Skin (–)	0	21 (2 years)	24.2	
	Skin (+)	20	48 (2 years)	22.2	
Extranodal MZL of MALT		94–97			26, 27
PCFCL		87–96			
PCDLBCL		37–73			

*Mean survival time. †One case. ALCL, anaplastic large cell lymphoma; ATLL, adult T cell leukemia/lymphoma; BM, bone marrow; BNKL, blastic NK-cell lymphoma; DSS, disease-specific survival; ENKL, extranodal NK/T-cell lymphoma; HPS, hemophagocytic syndrome; MF, mycosis fungoides; MZL of MALT, marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue; OS, overall survival; PCDLBCL, primary cutaneous diffuse large B-cell lymphoma; PCFCL, primary cutaneous follicle center lymphoma; SPTCL, subcutaneous panniculitis-like T-cell lymphoma; SS, Sezary syndrome.

and can be considered for small skin lesions or for short-term use (Data S1).

CQ4: Is ultraviolet (UV) light therapy recommended for MF/SS?
Degree of recommendation: B.
Recommendation: Oral psoralen plus UV-A therapy (PUVA) therapy or narrow-band UV-B therapy is recommended for early-stage MF (stage IA through IIA) (Data S1).

CQ5: Is PUVA therapy with concomitant retinoid or interferon (IFN) therapy recommended for MF/SS?
Degree of recommendation: B.
Recommendation: PUVA with concomitant oral etretinate (RePUVA) or PUVA with concomitant IFN is recommended for MF/SS (Data S1).

CQ6: Is radiation therapy recommended for MF/SS?
Degree of recommendation: B.
Recommendation: Localized radiation therapy is recommended as a palliative treatment for skin lesions in MF, regardless of disease stage. Total skin electron beam therapy is recommended for MF (stage IB through IIA) (Data S1).

CQ7: Are oral retinoids recommended for MF/SS?
Degree of recommendation: B-C1.

Table 4. Summary of clinical questions and degree of recommendation for mycosis fungoides/Sézary syndrome

Clinical question	Degree of recommendation
CQ1: Is monitoring the clinical course without treatment recommended for mycosis fungoides?	C1 (stage IA) C2 (other than stage IA)
CQ2: Are topical steroids recommended for mycosis fungoides/Sézary syndrome?	B
CQ3: Is topical chemotherapy recommended for mycosis fungoides/Sézary syndrome?	C1
CQ4: Is ultraviolet light therapy recommended for mycosis fungoides/Sézary syndrome?	B
CQ5: Is psoralen plus ultraviolet A therapy with concomitant retinoid or interferon therapy recommended for mycosis fungoides/Sézary syndrome?	B
CQ6: Is radiation therapy recommended for mycosis fungoides/Sézary syndrome?	B
CQ7: Are oral retinoids recommended for mycosis fungoides/Sézary syndrome?	B-C1
CQ8: Is interferon therapy recommended for mycosis fungoides/Sézary syndrome?	B-C1
CQ9: Is extracorporeal photochemotherapy recommended for mycosis fungoides/Sézary syndrome?	B (erythroderma) C1 (non-erythroderma)
CQ10: Are molecular-targeted therapies recommended for mycosis fungoides/Sézary syndrome?	B-C1
CQ11: Is chemotherapy recommended for mycosis fungoides/Sézary syndrome?	B (refractory, extracutaneous lesions) D (early stage)
CQ12: Is hematopoietic stem cell transplantation recommended for mycosis fungoides/Sézary syndrome?	C1 (allogeneic) C2 (autologous)

Table 5. (MF/SS-1) Topical therapy of first choice recommended for stages I and IIA*

Treatment	Degree of recommendation	CQ
Monitoring the clinical course without treatment	C1 (stage IA only)/C2	CQ1
Topical steroid therapy†	B	CQ2
ACNU topical therapy‡	C1	CQ3
BB-UVB†	B	CQ4
NB-UVB	B	CQ4
PUVA	B	CQ4
Localized radiation therapy§	B	CQ6

*If the patient does not respond to the topical therapy selected for initial treatment, before proceeding to a second-line therapy recommended for stage I through IIA (Table 6 MFSS-2), consider the use of other first-line topical therapies. †Stage IA/IB. ‡Small area, short-term use. §Radical radiation therapy for "minimal" stage IA unilesional mycosis fungoides, or where multiple lesions are localized within the same radiation field or multiple field in close proximity, and palliative radiation for infiltrated plaques resistant to topical therapy other than radiation. ACNU, nimustine hydrochloride; BB, broad-band; NB, narrowband; PUVA, psoralen plus ultraviolet A therapy; UVB, ultraviolet B.

Table 6. (MF/SS-2) Second-line therapy recommended for stages I and IIA

Treatment	Degree of recommendation	CQ
TSEB*	B	CQ6
Etretinate†,‡	B-C1	CQ7
IFN-α†,§	B-C1	CQ8
IFN-γ†	B-C1	CQ8
RePUVA†	B	CQ5
IFN-α + PUVA †,§	B	CQ5
IFN-γ + PUVA †	B	CQ5
Chemotherapy¶	D/B¶	CQ11

*TSEB can be used as first-line therapy for stage IB/IIA (T2) with intense subjective symptoms accompanied by extensive highly infiltrated plaques and histopathological confirmation of folliculotropic mycosis fungoides or large cell transformation. †Can be a first-line treatment if systemic therapy is required (B1 or histopathological confirmation of folliculotropic mycosis fungoides or large cell transformation). BRM therapy (etretinate, IFN-α, IFN-γ) can be used as monotherapy or in concomitant administration with PUVA, and its concomitant use can also be investigated with topical therapies other than PUVA. ‡Duration of response to oral etretinate is usually short; consider for use as concomitant therapy. §IFN-α therapy has been used in only a few cases in Japan. ¶Third-line therapy for stage IB/IIA disease resistant to skin-targeted therapy and BRM therapy. BRM, biological response modifiers; IFN, interferon; PUVA, psoralen plus ultraviolet A therapy; TSEB, total skin electron beam.

Recommendation: Oral etretinate can be useful in the treatment of MF/SS (Data S1).

CQ8: Is IFN therapy recommended for MF/SS?
Degree of recommendation: B-C1.

Recommendation: IFN-α therapy is recommended in early-stage MF/SS (stage IA–IIA) if systemic therapy is required, and in advanced disease (stage IIB–IVA1). This treatment option has not yet been approved in Japan. IFN-γ, which has been used for the treatment of MF in Japan, is considered as effective as IFN-α, and may prove useful (Data S1).

CQ9: Is extracorporeal photochemotherapy (ECP) recommended for MF/SS?
Degree of recommendation: B (erythrodermic MF/SS), C1 (non-erythrodermic disease).

Recommendation: ECP/photopheresis is recommended for stage T4 erythrodermic MF and SS. It may also be considered in cases of refractory non-erythrodermic MF. ECP is

Table 7. (MF/SS-3) First-line therapy recommended for stage IIB*

Treatment	Degree of recommendation	CQ
Concomitant use of the following forms of BRM therapy and topical therapy		
BRM therapy		
Etretinate	B-C1	CQ5,7
IFN-α†‡	B-C1	CQ5,8
IFN-γ‡	B-C1	CQ5,8
Topical therapy		
PUVA ± localized radiation therapy§	B	CQ4,5,6
Localized radiation therapy§	B	CQ6
TSEB¶	B	CQ6

*If the patient does not respond to initial treatment, before proceeding to a second-line therapy recommended for refractory stage IIB (Table 8 MFSS-4), consider other first-line topical therapies. †Concomitant therapy with IFN-α and PUVA: degree of recommendation = B. IFN-α therapy has been used in only a few cases in Japan. ‡IFN-α monotherapy or IFN-γ monotherapy can be used as first-line therapy. §Palliative radiation for localized tumors. ¶If lesions extend over <10% of body surface area, TSEB monotherapy can be used as first-line therapy. BRM, biological response modifiers; IFN, interferon; PUVA, psoralen plus ultraviolet A therapy; TSEB, total skin electron beam.

Table 8. (MF/SS-4) Treatment methods recommended for refractory stage IIB/III or stage IV mycosis fungoides

Treatment	Degree of recommendation	CQ
Chemotherapy*	B	CQ11

*Consider concomitant use of topical therapy appropriate for T classification.

Table 9. (MF/SS-5) First-line therapy recommended for stage III *

Treatment	Degree of recommendation	CQ
ECP ± IFN-α†	B	CQ9
TSEB + ECP†‡	B	CQ6
Concomitant use of the following forms of BRM therapy and topical therapy		
BRM therapy		
Etretinate	B-C1	CQ5,7
IFN-α†§	B-C1	CQ5,8
IFN-γ§	B-C1	CQ5,8
Topical therapy		
PUVA	B	CQ4,5
TSEB§	B	CQ6

*If the patient does not respond to initial therapy, before proceeding to a therapy recommended for refractory stage III (Table 8 MFSS-4), consider other first-line therapies. †ECP and IFN-α therapy have been used in only a few cases in Japan. ‡TSEB monotherapy can be used as first-line therapy for stage IIIA disease. §IFN-α monotherapy or IFN-γ monotherapy can be used as first-line therapy. BRM, biological response modifiers; ECP, Extracorporeal photochemotherapy; IFN, interferon; PUVA, psoralen plus ultraviolet A therapy; TSEB, total skin electron beam.

Table 10. (MF/SS-6) Recommended therapy for Sézary syndrome (stage T4, IVA1-IVB)*

Treatment	Degree of recommendation	CQ
ECP ± IFN-α†	B	CQ9
TSEB + ECP†	B	CQ6
Chemotherapy ± IFN-α†	B	CQ11

*For stage IVA1 Sézary syndrome with a low Sézary cell count, initial therapy selection may be the same as for stage IIIB (Table 9 MF/SS-5). †ECP and IFN-α therapy have been used in only a few cases in Japan. ECP, extracorporeal photochemotherapy; IFN, interferon; TSEB, total skin electron beam.

Table 11. (MF/SS-7) Treatment to be considered for refractory stage IV disease

Treatment	Degree of recommendation	CQ
Allogeneic hematopoietic stem cell transplantation	C1	CQ12
Autologous hematopoietic stem cell transplantation	C2	CQ12

not yet approved by the Japanese National Health Insurance system, and currently almost no Japanese medical institutions perform the procedure (Data S1).

CQ10: Are molecular-targeted therapies recommended for MF/SS?
Degree of recommendation: B-C1.
Recommendation: Treatment with denileukin diftitox, vorinostat or romidepsin may be useful in recurrent or refractory MF/SS. Vorinostat is the only drug in this category that is approved for coverage by Japanese health insurance (Data S1).

CQ11: Is chemotherapy recommended for MF/SS?
Degree of recommendation: B (if disease is refractory or accompanied by extracutaneous lesions), D (early-stage MF).
Recommendation: Chemotherapy is not recommended as a first line of treatment in early-stage MF (stage IA–IIA). Chemotherapy is recommended for MF/SS stage IB–IIIB that is resistant to topical therapy or biological response modifier therapy, and for MF/SS stage IVA1–IVB accompanied by extracutaneous lesions (Data S1).

CQ12: Is hematopoietic stem cell transplantation recommended for MF/SS?
Degree of recommendation: C1 (allogeneic hematopoietic stem cell transplantation), C2 (autologous hematopoietic stem cell transplantation).
Recommendation: Autologous hematopoietic stem cell transplantation with concomitant high-dose chemotherapy is not generally recommended for MF/SS. In young patients with advanced disease, allogeneic hematopoietic stem cell transplantation may be considered in the context of a clinical study (Data S1).

Cutaneous T/NK-cell lymphoma other than MF/SS (non-MF/SS)

Cutaneous T/NK cell lymphomas other than MF/SS are classified by WHO–EORTC into two broad categories: relatively aggressive lymphomas with poor prognosis (aggressive group), and indolent lymphomas with favorable prognosis (indolent group) (Table 12).[1,34–39] In patients with aggressive lymphomas including primary cutaneous $CD8^+$ aggressive epidermotropic cytotoxic T-cell lymphoma, primary cutaneous γδ T-cell lymphoma, and peripheral T-cell lymphoma, not otherwise specified, the 5-year survival rates are less than 20%. However, the clinical course is not uniform, and patients whose symptoms are limited to cutaneous lesions may live for much longer.

For patients who present with cutaneous lesions only, without general symptoms or notable laboratory test findings, skin-directed therapies used for MF/SS might be chosen as a first-line treatment. Systemic chemotherapy may be considered for patients with tumor infiltration into the lymph nodes or visceral organs. However, the best treatment option must be explored for each individual patient, based on that patient's conditions. Clinical questions (CQ) are not defined in this category because uniform guidelines are difficult to develop. In contrast, CQ have been defined in each lymphoma in the indolent group (primary cutaneous anaplastic large cell lymphoma, subcutaneous panniculitis-like T-cell lymphoma, and primary cutaneous $CD4^+$ small/medium T-cell lymphoma).

The MF/SS staging classifications are not applicable to cutaneous T/NK cell lymphomas other than MF/SS because of differences in disease progression. In 2007, the ISCL and EORTC jointly advocated the TNM classification system for cutaneous lymphomas other than MF/SS.[4] Because the prognostic impact of this classification system has not yet been validated, it might be premature to establish guidelines based on it. However, no other applicable classification systems are available at the present time. In order to obtain clinical information based on common criteria, we have adopted the TNM classification in the present guidelines.

Primary cutaneous anaplastic large cell lymphoma.

CQ13: Are localized therapies such as radiation therapy or surgical resection recommended for primary cutaneous anaplastic large cell lymphoma?
Degree of recommendation: B.
Recommendation: Remission can be induced by radiation therapy or surgical resection in many patients, so these methods are recommended where feasible (Data S1).

CQ14: Is chemotherapy recommended for primary cutaneous anaplastic large cell lymphoma?
Degree of recommendation: B (for lymph node lesions and visceral organ infiltration), C1 (symptoms limited to cutaneous lesions only).
Recommendation: For patients with cutaneous lesions only, if those lesions are resistant to topical treatment such as radiotherapy and surgical excision, or if they have multiple lesions, chemotherapy may be considered. Chemotherapy is recommended for lymph node lesions and for infiltration in the visceral organs (Data S1).

Subcutaneous panniculitis-like T-cell lymphoma.

CQ15: Is radiation therapy recommended for subcutaneous panniculitis-like T-cell lymphoma?
Degree of recommendation: C1.
Recommendation: Radiation therapy can provide control of localized lesions within the irradiated area. Radiation can be considered as initial therapy for skin lesions within a localized area (T1, T2) without systemic symptoms (Data S1).

CQ16: Are oral steroids recommended for subcutaneous panniculitis-like T-cell lymphoma?
Degree of recommendation: B.
Recommendation: Steroid monotherapy has been reported to relieve systemic symptoms such as pyrexia and abnormal hepatic function and to induce remission in some cases; oral steroids are recommended for subcutaneous panniculitis-like T-cell lymphoma (Data S1).

Table 12. Summary of CQ and degree of recommendation for cutaneous T-/natural killer cell lymphoma (non-MF/SS)

Clinical question	Degree of recommendation
CQ13: Are localized therapies such as radiation therapy B or surgical resection recommended for primary cutaneous anaplastic large cell lymphoma?	B
CQ14: Is chemotherapy recommended for primary cutaneous anaplastic large cell lymphoma?	B (extracutaneous lesions) C1 (cutaneous lesions only)
CQ15: Is radiation therapy recommended for subcutaneous panniculitis-like T-cell lymphoma?	C1
CQ16: Are oral steroids recommended for subcutaneous panniculitis-like T-cell lymphoma?	B
CQ17: Is combination chemotherapy recommended for subcutaneous panniculitis-like T-cell lymphoma?	B-C1
CQ18: Is radiation therapy recommended for primary cutaneous $CD4^+$ small/medium T-cell lymphoma?	B
CQ19: Is chemotherapy recommended for primary cutaneous $CD4^+$ small/medium T-cell lymphoma?	C1

CQ17: Is combination chemotherapy recommended for subcutaneous panniculitis-like T-cell lymphoma?
Degree of recommendation: B-C1.
Recommendation: Combination chemotherapy may be considered if the condition is resistant to steroid therapy. Prognosis is poor for patients complicated by hemophagocytosis; combination chemotherapy is recommended in such cases (Data S1).

Primary cutaneous CD4$^+$ small/medium T-cell lymphoma.

CQ18: Is radiation therapy recommended for primary cutaneous CD4$^+$ small/medium T-cell lymphoma?
Degree of recommendation: B.
Recommendation: Radiation therapy can induce remission in many cases, and survival rates are relatively good. Radiation therapy is recommended for single and localized lesions (T1, T2) (Data S1).

CQ19: Is chemotherapy recommended for primary cutaneous CD4$^+$ small/medium T-cell lymphoma?
Degree of recommendation: C1.
Recommendation: Chemotherapy can also be considered for primary cutaneous CD4$^+$ small/medium T-cell lymphoma with multiple lesions (Data S1).

ATLL (disease type limited to cutaneous lesions)

Adult T-cell leukemia/lymphoma is a form of T-cell lymphoma caused by HTLV-1 which occurs in a variety of organs (Table 13). Three major findings required for diagnosis: (i) appearance of morphologically abnormal T lymphocytes (typically CD4$^+$ and CD25$^+$); (ii) seropositivity for anti-HTLV-1 antibody; and (iii) Southern blot confirmation for monoclonal integration of HTLV-1 provirus into tumor cells.[15,40] For cutaneous symptoms to be diagnosed as eruptions specific to ATLL, histological confirmation is required for (i) and (iii). In particular, (iii) is required for a differential diagnosis to exclude other cutaneous lymphomas such as MF. The overall treatment guidelines for ATLL must involve cooperation and coordination with other departments, including departments of hematology and oncology. Thus, we limit these guidelines to instances in which only cutaneous lesions are detected. However, no uniform diagnostic criteria exist for the conventionally advocated concept of "cutaneous" ATLL.[40–43] The present guidelines cover ATLL cases, where systemic treatments such as chemotherapy and transplantation are not indicated.

Table 13. Summary of CQ and degree of recommendation for adult T-cell leukemia/lymphoma (ATLL) with cutaneous lesions only

Clinical question	Degree of recommendation
CQ20: Is ultraviolet light therapy recommended for ATLL with cutaneous lesions only?	B-C1
CQ21: Is radiation therapy recommended for ATLL with cutaneous lesions only?	B
CQ22: Are oral retinoids recommended for ATLL with cutaneous lesions only?	C1
CQ23: Is interferon therapy recommended for ATLL with cutaneous lesions only?	C1
CQ24: Is single-agent chemotherapy recommended for ATLL with cutaneous lesions only?	B-C1

Eruptions specific to ATLL are defined as cutaneous symptoms in cases seropositive for anti-HTLV-1 antibody and where cutaneous histology shows monoclonal integration of HTLV-1. In the present guidelines, we have provisionally considered "ATLL with cutaneous lesions only" to be "cases in which ATLL cells account for <5% of all peripheral blood cells, excluding the acute, lymphoma, and chronic types".[19,40]

CQ20: Is UV light therapy recommended for ATLL with cutaneous lesions only?
Degree of recommendation: B-C1.
Recommendation: PUVA therapy can induce remission in ATLL with cutaneous lesions only, and may be useful. Regardless of whether extracutaneous lesions are present, PUVA can be expected to relieve cutaneous symptoms. However, beneficial effects of PUVA on extracutaneous lesions or the prognosis of patients have not been confirmed (Data S1).

CQ21: Is radiation therapy recommended for ATLL with cutaneous lesions only?
Degree of recommendation: B.
Recommendation: Radiation therapy can be expected to provide symptomatic relief in ATLL with cutaneous lesions only, and is recommended. However, beneficial effects on the prognosis of patients have not been confirmed (Data S1).

CQ22: Are oral retinoids recommended for ATLL with cutaneous lesions only?
Degree of recommendation: C1.
Recommendation: Retinoids can induce remission in ATLL with cutaneous lesions only, and may be considered for use (Data S1).

CQ23: Is IFN therapy recommended for ATLL with cutaneous lesions only?
Degree of recommendation: C1.
Recommendation: IFN-γ can relieve symptoms in ATLL with cutaneous lesions only, and may be considered for use. Beneficial effects on extracutaneous lesions or the prognosis of patients have not been confirmed (Data S1).

CQ24: Is single-agent chemotherapy recommended for ATLL with cutaneous lesions only?
Degree of recommendation: B-C1.
Recommendation: Single-agent chemotherapy can be useful for disease refractory to skin-direct therapy in cases where combination chemotherapy is not indicated. However, beneficial effects on the prognosis of patients have not been confirmed (Data S1).

Other T/NK-cell lymphomas

In addition to ENKL, the WHO classification for hematopoietic malignancies, revised in 2008, has listed hydroa

vacciniforme-like lymphoma as an independent disease (Table 14).[2] This condition has been reported in Asia, including Japan, in Mexico, and in Peru. Hydroa vacciniforme-like lymphoma is a form of T-cell lymphoma that is associated with EBV. It occurs most frequently in children and adolescents, and is often accompanied by photosensitivity and hypersensitivity to insect bites. Prognosis, although varied, is poor if complicated by systemic conditions such as hemophagocytosis. There have been no reports of treatment for this condition alone, but a few reports are available on treatment of chronic active EBV infection and on EBV^+ T/NK-cell lymphoproliferative diseases. Treatment has been attempted with antiviral therapy using the antiviral agents acyclovir and ganciclovir, immunotherapy using agents such as IFN-α and interleukin 2, and chemotherapy using corticosteroids and etoposide.[44] However, the reports involve a very small number of cases, insufficient even for descriptive research, so findings cannot be considered conclusive.

Blastic plasmacytoid dendritic cell neoplasm is a rare disease formerly designated as $CD4^+/CD56^+$ hematodermic neoplasm.[45] Most patients usually respond to initial polychemotherapy, but the relapse rate is high. The prognosis is dismal, with a median overall survival of 12–14 months.

ENKL, nasal type.

CQ25: Is CHOP (cyclophosphamide, adriamycin, vincristine, prednisone) chemotherapy recommended for ENKL, nasal type?
Degree of recommendation: C2.
Recommendation: ENKL, nasal type, generally responds poorly or only temporarily to CHOP therapy; this treatment is not recommended (Data S1).

CQ26: Is combination radiation therapy and chemotherapy recommended for ENKL, nasal type?
Degree of recommendation: B.
Recommendation: For localized lesions, radiation therapy with simultaneous or subsequent DeVIC (dexamethasone, VP16, ifosfamide, carboplatin) chemotherapy is recommended (Data S1).

Blastic plasmacytoid dendritic cell neoplasm.

CQ27: Is chemotherapy recommended for blastic plasmacytoid dendritic cell neoplasm?
Degree of recommendation: C1.
Recommendation: No standard treatment has been established for blastic plasmacytoid dendritic cell neoplasm. Multidrug chemotherapy may be considered. However, such treatment provides only temporary effectiveness, and almost all patients die within a few years (Data S1).

Hydroa vacciniforme-like lymphoma.

CQ28: Is allogenic hematopoietic stem cell transplantation recommended for hydroa vacciniforme-like lymphoma?
Degree of recommendation: B-C1.
Recommendation: Allogenic hematopoietic stem cell transplantation may be useful in the treatment of hydroa vacciniforme-like lymphoma (Data S1).

Cutaneous B-cell lymphoma

The WHO–EORTC classification of 2005 lists the following subtypes within the category of cutaneous B-cell lymphoma:[1] primary cutaneous marginal zone B-cell lymphoma (PCMZL); primary cutaneous follicle center cell lymphoma (PCFCL), primary cutaneous diffuse large B-cell lymphoma, leg type (PCDLBCL, leg type); PCLBCL, other; and intravascular large B-cell lymphoma (IVL) (Table 15). In the 2008 revision of the WHO classification of hematopoietic malignancies, the nomenclature, the PCMZL was replaced by "extranodular marginal zone B-cell lymphoma (MALT lymphoma)".[2] The term, PCDLBCL, leg type, was entered as a subcategory of "diffuse large B-cell lymphoma, not otherwise specified". The term "primary cutaneous diffuse large B-cell lymphoma, other" was removed from the list. Disease type is an important prognostic factor for cutaneous B-cell lymphoma. Both PCFCL and PCMZL are indolent-type lymphomas with a favorable prognosis, while prognosis is poor in PCDLBCL and IVL. In the following discussion, cutaneous B-cell lymphoma is divided into two groups: the indolent group and diffuse large cell group.

No randomized clinical trials have been conducted in these disease groups, and research has been limited primarily to descriptive studies. However, in 2008, the EORTC and ISCL published guidelines for the treatments of cutaneous B-cell lymphoma, based on previous reports.[46] Most of the reported treatment methods for topical therapy involved radiation and/or surgical resection. Most of the methods for systemic therapy involved chemotherapy and the administration of rituximab. However, a few reports were found on topical administration of IFN-α and on the use of photodynamic therapy.

Table 14. Summary of CQ and degree of recommendation for other natural killer (NK)/T-cell lymphomas and related diseases

Clinical question	Degree of recommendation
CQ25: Is CHOP (cyclophosphamide, adriamycin, vincristine, prednisone) chemotherapy recommended for extranodal NK/T-cell lymphoma, nasal type?	C2
CQ26: Is combination radiation therapy and chemotherapy recommended for extranodal NK/T-cell lymphoma, nasal type?	B
CQ27: Is chemotherapy recommended for blastic plasmacytoid dendritic cell neoplasm?	C1
CQ28: Is allogenic stem cell transplantation recommended for hydroa vacciniforme-like lymphoma?	B-C1

Table 15. Summary of CQ and degree of recommendation for primary cutaneous B-cell lymphoma (indolent type: primary cutaneous follicle center lymphoma and extranodal marginal zone lymphoma)

Clinical question	Degree of recommendation
CQ29: Is radiation therapy recommended for indolent-type primary cutaneous B-cell lymphoma?	B
CQ30: Is surgical resection recommended for indolent-type primary cutaneous B-cell lymphoma?	B
CQ31: Is rituximab monotherapy recommended for indolent-type primary cutaneous B-cell lymphoma?	B-C1
CQ32: Is combination chemotherapy recommended for indolent-type primary cutaneous B-cell lymphoma?	C1
CQ33: Is combination chemotherapy recommended for primary cutaneous diffuse large B-cell lymphoma?	B
CQ34: Is rituximab monotherapy recommended for primary cutaneous diffuse large B-cell lymphoma?	B
CQ35: Are surgical resection and radiation therapy recommended for diffuse large B-cell lymphoma?	C1

CQ29: Is radiation therapy recommended for indolent-type primary cutaneous B-cell lymphoma?
Degree of recommendation: B.
Recommendation: Radiotherapy is recommended for diseases in the indolent group (PCMZL and PCFCL) (Data S1).

CQ30: Is surgical resection recommended for indolent-type primary cutaneous B-cell lymphoma?
Degree of recommendation: B.
Recommendation: Surgical resection is recommended for resectable lesions of diseases in the indolent group (PCMZL and PCFCL) (Data S1).

CQ31: Is rituximab monotherapy recommended for indolent-type primary cutaneous B-cell lymphoma?
Degree of recommendation: B-C1.
Recommendation: Rituximab may be useful for the treatment of diseases in the indolent group (PCMZL and PCFCL), particularly in cases of multiple lesions (Data S1).

CQ32: Is combination chemotherapy recommended for indolent-type primary cutaneous B-cell lymphoma?
Degree of recommendation: C1.
Recommendation: Combination chemotherapy may be considered for diseases in the indolent group that are refractory to other treatment regimens, and for advanced extracutaneous disease (Data S1).

CQ33: Is combination chemotherapy recommended for PCDLBCL?
Degree of recommendation: B.
Recommendation: Combination chemotherapy, and particularly the concomitant use of rituximab, is recommended for PCDLBCL, leg type, and for IVL (Data S1).

CQ34: Is rituximab monotherapy recommended for PCDLBCL?
Degree of recommendation: B.
Recommendation: Rituximab monotherapy is recommended for the treatment of PCDLBCL in cases where combination therapy may be poorly tolerated, such as in the elderly and in patients with severe complications (Data S1).

CQ35 Are surgical resection and radiation therapy recommended for PCDLBCL?
Degree of recommendation: C1.
Recommendation: In patients who cannot tolerate rituximab combination chemotherapy, such as the elderly and patients with severe complications, surgical resection and radiation therapy may be considered (Data S1).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Data S1. References used for treatment recommendations (CQ1–CQ35).

Table S1. Terminology for clinical features of mycosis fungoides/Sézary syndrome.

Table S2. Tumor, lymph nodes, metastasis, blood (TNMB) classification for mycosis fungoides/Sézary syndrome.

Table S3. TNMB staging for mycosis fungoides/Sézary syndrome (International Society for Cutaneous Lymphomas/European Organization for Research and Treatment of Cancer, Cutaneous Lymphomas Task Force).

Table S4. Tumor–node–metastasis classification for primary cutaneous lymphomas other than mycosis fungoides/Sézary syndrome.

Table S5. Ann Arbor/Cotswold staging.

Figure S1. Clinicopathological features of mycosis fungoides/Sézary syndrome.

Figure S2. Clinical features of anaplastic large cell lymphoma.

Figure S3. Clinical features of adult T-cell leukemia/lymphoma.

Figure S4. Clinicopathological features of subcutaneous panniculitis-like T-cell lymphoma.

Figure S5. Clinical features of extranodal natural killer/T-cell lymphoma, nasal type.

Figure S6. Clinicopathological features of hydroa vacciniforme-like lymphoma.

Figure S7. Clinical features of blastic plasmacytoid dendritic cell neoplasm.

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Proviral loads of human T-lymphotropic virus Type 1 in asymptomatic carriers with different infection routes

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High human T-lymphotropic virus Type 1 (HTLV-1) proviral DNA load (PVL) has been reported to be one risk factor for the development of adult T-cell leukemia/lymphoma (ATL). ATL is also believed to develop in HTLV-1 carriers who acquire infection perinatally. ATL cells have been reported to frequently harbor defective provirus. In our study, PVLs for three different regions of HTLV-1 provirus (5′LTR-*gag*, *gag* and *pX*) were measured in 309 asymptomatic carriers with different infection routes. PVLs for the *pX* region in 21 asymptomatic carriers with maternal infection was significantly higher than in 24 carriers with spousal infection. Among 161 carriers with relatively high *pX* PVLs (equal to or greater than 1 copy per 100 peripheral blood mononuclear cells), 26 carriers (16%) had low *gag* PVL/*pX* PVL (less than 0.5) and four (2%) had low 5′LTR-*gag* PVL/*pX* PVL (less than 0.5). Low *gag* PVL/*pX* PVL ratio, which reflects deficiency and/or polymorphism of HTLV-1 proviral DNA sequences for the *gag* region, was also associated with maternal infection. These data suggest that HTLV-1 carriers with maternal infection tend to have high PVLs, which may be related to provirus with deficiency and/or the polymorphism of proviral DNA sequences. In addition, there is a possibility that this ratio may be used as a tool to differentiate the infection routes of asymptomatic HTLV-1 carriers, which supports the need for a large scale study.

Human T-lymphotropic virus Type 1 (HTLV-1) is the causative agent of adult T-cell leukemia/lymphoma (ATL) and a progressive neurological disease known as HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP).[1–4] Major routes of HTLV-1 infection have been reported as mother to child infection at infancy, sexual contact between spouses and blood transfusion.[5–7] The majority of HTLV-1 carriers are asymptomatic, and only a fraction of carriers develop ATL after a long latent period.[8,9] It has been reported that approximately 4% of HTLV-1 carriers develop ATL eventually.[10] Studies of the mothers of patients with ATL have reported most of them to be HTLV-1 carriers.[11,12] Therefore, ATL is believed to develop in HTLV-1 carriers who acquire infection perinatally. However, there has been no method of identifying the infection route of HTLV-1 positive individuals without information on family HTLV-1 status.

When an individual is infected by HTLV-1, the virus randomly integrates into the genome of affected T-cells in the form of provirus.[13] HTLV-1 infection drives the proliferation of T-cells, leading to the clonal expansion of HTLV-1 infected cells.[14–16] Recently, it was reported that HTLV-1 clonal expansion *in vivo* is favored by orientation of the provirus in the same sense as the nearest host gene.[17] We have reported that the clonality of HTLV-1 infected cells in adult seroconverters who were newly infected from HTLV-1 carrier spouses is more heterogeneous and less stable than that of long-term carriers who acquired infection from their mothers at infancy.[18] The selective maintenance of certain clones is supposed in the latter. Recently, we reported that clonal expansion of HTLV-1 infected cells was found in a certain population of asymptomatic carriers and that these carriers had high proviral DNA loads (PVLs).[19] High PVLs have been reported to be a risk factor for developing ATL.[20,21] In another study, we analyzed the PVLs of 13 pairs of HTLV-1 seroconverters and their spouses.[22] Although seroconverters and their spouses shared the same HTLV-1, PVLs in both individuals in a couple were not always equivalent. These findings suggested that host-related factors play an important role to determining the PVL in each carrier. However, it was

Key words: HTLV-1, defective virus, infection route, proviral DNA loads

Abbreviations: ATL: adult T-cell leukemia/lymphoma; HTLV-1: human T-lymphotropic virus type 1, LTR: long-terminal repeat, PBMCs: peripheral blood mononuclear cells, PCR: polymerase chain reaction; PVLs: proviral DNA loads

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not clear in that study whether HTLV-1 carriers who acquired infection from their mothers at infancy have more PVLs than the carriers who acquired infection from their spouses in adulthood.

Defective provirus has frequently been detectable in patients with ATL.[23–27] The complete HTLV-1 provirus is approximately 9 kb and contains the coding regions for core protein (*gag*), protease (*pro*), polymerase (*pol*), envelope protein (*env*), regulatory proteins, such as Tax and Rex, and some accessory molecules between 5′ and 3′ long-terminal repeats (LTRs).[8,28] Tamiya *et al.*[23] reported two types of genome deletion in defective provirus. One form retains both LTRs and lacks internal sequences, such as the *gag* and *pol* regions. The other form has the 3′ LTR, and the 5′ LTR and its flanking internal sequences are preferentially deleted. HTLV-1 infected cells harboring the latter defective virus were frequently found in patients with ATL.[26] Both types of defective provirus were suspected of being harbored by the clonally expanded HTLV-1 infected cells in asymptomatic carriers.[19] The polymorphism of the proviral genome was also found in asymptomatic carriers in that study; however, we could not show how commonly the deficiency or polymorphism of the proviral genome was detectable.

These questions prompted us to investigate HTLV-1 PVLs in asymptomatic carriers with different infection routes. In addition, to clarify whether the defective provirus and/or polymorphism of the proviral genome affected PVLs, we tested PVLs for three different regions (5′LTR-*gag*, *gag* and *pX*) of provirus in each individual and compared them among the carriers with different infection routes in our study.

Material and Methods

Samples

Samples of peripheral blood mononuclear cells (PBMCs) were obtained from 309 HTLV-1 carriers (103 men and 206 women, median age: 67 years), who had no symptoms or laboratory data suggesting HTLV-1 related disease, in the Miyazaki Cohort Study.[29] Infection routes were investigated by family HTLV-1 status and history of HTLV-1 seroconversion.[18,22] An HTLV-1 carrier with HTLV-1 positive mother/HTLV-1 negative spouse or with HTLV-1 positive siblings/HTLV-1 negative spouse or with HTLV-1 seroconverter was defined as infected by his/her mother. An HTLV-1 carrier who was a HTLV-1 seroconverter with HTLV-1 positive spouse or with HTLV-1 negative mother/HTLV-1 positive spouse was defined as infected by his/her spouse. Carriers with history of blood transfusion were excluded from the analysis of family status. As a result, 21 and 24 carriers were defined as infected by their mothers and by their spouses, respectively. Infection routes could not be determined in 264 carriers. Informed consent was obtained from the study participants and the study protocol was approved by the institutional review board at University of Miyazaki.

Real-time polymerase chain reaction

PVLs for three different proviral regions (5′LTR-*gag*, *gag* and *pX*) were determined by real-time polymerase chain reaction (PCR) using Light Cycler 2.0 (Roche Diagnostics, Mannheim, Germany). Genomic DNA was isolated from PBMCs of asymptomatic HTLV-1 carriers by sodium dodecyl sulfate-proteinase K digestion, followed by phenol-chloroform extraction and ethanol precipitation. Approximately 100 ng genomic DNA was used as the template. The nucleotide position number of HTLV-1 provirus was according to Seiki *et al.*[30] (accession no. J02029). The primers and probes for real-time PCR were designed to minimize the differences of the melting points 5′LTR-*gag*, *gag* and *pX* and were as follows: 5′LTR-*gag*: the forward primer (5′LTR-SDS-F 5′-AAGTACCGGCGACTCCGTTG-3′: positions 700–719), the reverse primer (HTLV-gag-LTR-R2 5′-GGCTAGCGCTACGGGAAAAG-3′: positions 854–835) and the FAM-labeled probe (5′-FAM-CGTCCGGGATACGAGCGCCCCTT-TAMRA-3′: positions 788–810); *gag*: the forward primer (HTLV-gag-F5 5′-ACCCTTCCTGGGCCTCTATC-3′: positions 1,602–1,621), the reverse primer (HTLV-gag-R5 5′-TCTGGCAGCCCATTGTCAAG-3′: positions 1,695–1,676) and the FAM-labeled probe (HTLV-gag-P5 5′-FAM-ACCACGCCTTCGTAGAACGCCTCAAC-TAMRA-3′: positions 1,644–1,669); *pX*: the forward primer (HTLV-pX2-S 5′-CGGATACCCAGTCTACGTGTT-3′: positions 7,359–7,379), the reverse primer (HTLV-pX2-AS 5′-CAGTAGGGCGTGACGATGTA-3′: positions 7,458–7,439) and the FAM-labeled probe (HTLV-pX2-Probe 5′-FAM-CTGTGTACAAGGCGACTGGTGCC-TAMRA-3′: positions 7,386–7,408).[18,26] A coding region for albumin (*Alb*) was used to measure the copy number of human genome. The primers and the probe for the *Alb* were as follows: The forward primer (Alb-S2 5′-TGTCATCTCTTGTGGGCTGT-3′), the reverse primer (Alb-AS2 5′-GGTTCTCTTTCACTGACATCTGC-3′) and the FAM-labeled probe (Alb-probe 5′-FAM-CCTGTCATGCCCACACAAATCTCTCC-TAMRA-3′). A plasmid containing PCR products for HTLV-1 5′LTR-*gag*, *gag*, *pX* regions and *Alb* was constructed using pGEM T-Easy Vector (Promega Corporation, Madison, WI) and was used as a control template for real-time PCR. PVLs of each region of HTLV-1 provirus were measured in a duplicate manner and were shown as copies per 100 PBMCs.

Detection of provirus with deletion of HTLV-1 internal sequence by long PCR

To detect the provirus with large deletion of HTLV-1 internal sequence, long PCR, which amplifies provirus maintaining both 5′ and 3′ LTR, was performed as described previously.[19] The primers were as follows: 5′LTR (HTLV-0647F 5′-GTTCCACCCCTTTCCCTTTCATTCACGACTGACTGC-3′: positions 647–682) and 3′LTR (HTLV-8345R 5′-GGCTCT AAGCCCCCGGGGGATATTTGGGGCTCATGG-3′: positions

8,345–8,310).[26] Long PCR was performed using LA Taq Hot start version (Takara Bio, Shiga, Japan). Genomic DNA containing 200 copies of HTLV-1 provirus for the *pX* region was used for this assay. To ensure that the same amount of provirus was used in each reaction, PCR for the *pX* region was performed as an internal control. Primers for this PCR were as follows: the forward primer (HTLV-7396F 5′-GGCGACTGGTGCCCCATCTCTGGGGGACTATGTTCG-3′: positions 7,396–7,431) and the reverse primer described above (HTLV-8345R). The PCR products were electrophoresed on 0.8% agarose gel and visualized by ethidium bromide staining.

Detection of provirus with deletion of 5′LTR and its flanking internal sequence by inverse long PCR

As described in results, both *gag* PVL/*pX* PVL ratio and 5′LTR-*gag* PVL/*pX* PVL ratio were low at less than 0.5 in two carriers (C20 and 21) and they were suspected of having provirus with deletion of 5′LTR and its flanking internal sequence. Inverse long PCR (IL-PCR) was used to amplify the genomic DNA adjacent to the 3′LTR of HTLV-1 provirus according to the method described previously with slight modifications.[15] In brief, the genomic DNA was digested with *Kpn* I, *Hind* III, *Sal* I or *Spe* I, and then self-ligated by T4 ligase following digestion with *Mlu* I. Amplification of the resultant DNA was performed using the LA Taq Hot start version. The primers used in this analysis were as follows; a forward primer in the U5 region of the LTR (5′-TGCCTGACCCTGCTTGCTCAACTCTACGTCTTTG-3′: positions 8,856–8,889) and a reverse primer, HTLV-7002R (5′-AGTATTTGAAAAGGAAGGAAGAGGAGAAGGCA-3′: positions 7,002–6,971). Subcloning of the amplified fragments of IL-PCR were subjected to sequencing assay according to the protocol of the Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) using ABI Prism 310 DNA Sequencer (Applied Biosystems) and the human genomic sequence downstream of the HTLV-1 provirus was obtained. The human genomic sequence upstream of the provirus was assumed based on this information by BLAT search (http://genome.ucsc.edu/cgi-bin/hgBlat).[31] The primers for human genomic sequence upstream of the provirus were designed and long PCR was performed using a forward primer (5′-GTGATCCATGGTGTTTGTCCACCTGAAAGC-3′) and a reverse primer HTLV-7002R in C20, and a forward primer (5′-TCCAAGTGGGATGTCACGGCCACTTCTC-3′) and a reverse primer HTLV-7002R in C21. To determine the upstream junction sequence between host genome and provirus, the PCR products were subjected to direct sequencing using the Big Dye Terminator v1.1 Cycle Sequencing Kit.

Statistical Analysis

Mann-Whitney's U test was used to compare *pX* PVLs, *gag* PVL/*pX* PVL or 5′LTR-*gag*/*pX* PVL ratios among the groups of asymptomatic HTLV-1 carriers with different infection routes. Spearman's correlation coefficient by rank was used to determine the relationship between *pX* PVL and *gag* PVL/*pX* PVL or 5′LTR-*gag* PVL/*pX* PVL ratio.

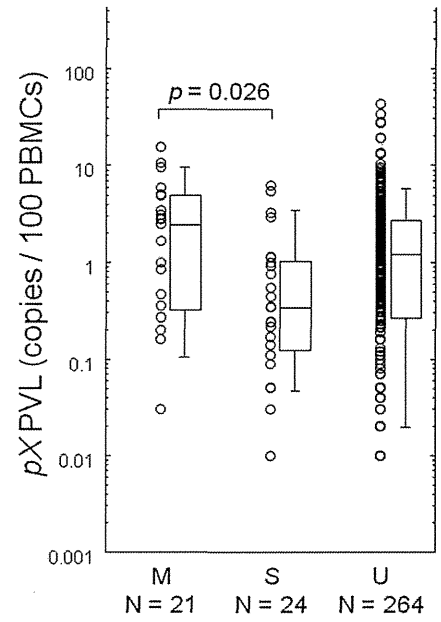

Figure 1. *PX* PVLs in HTLV-1 carriers with different infection routes M: Carriers with infection from mothers; S: Carriers with infection from spouses; U: Carriers with undetermined infection routes.

Results

pX PVLs in HTLV-1 carriers with different infectious routes

PVLs for the 5′LTR-*gag*, *gag* and *pX* regions in each individual were measured in 309 asymptomatic HTLV-1 carriers. Because the *pX* region has been reported to be conserved in the HTLV-1 provirus, *pX* PVL was considered to represent total PVLs.[23,25] As shown in Figure 1, median *pX* PVL (2.49 copies/100 PBMCs) in 21 asymptomatic carriers, who were infected by their mothers, was significantly higher than that (0.34 copies/100 PBMCs) in 24 carriers who were infected by their spouses ($p = 0.026$). Median *pX* PVL in 264 asymptomatic carriers, whose infection routes were undetermined, was between these values (1.24 copies/100 PBMCs).

PVLs for 3 different proviral regions (5′LTR-gag, gag and pX) of HTLV-1

To determine whether PVLs for three different proviral regions (5′LTR-*gag*, *gag* and *pX*) of HTLV-1 were equal in asymptomatic carriers, PVLs for the 5′LTR-*gag* and *gag* regions were measured and compared to PVLs for the *pX* region. Because 100 ng of genomic DNA, which is derived approximately 15,000 PBMCs, was used for the template for real time-PCR, 148 carriers with *pX* PVL, which was less than 1 copy/100 PBMCs, were not provided for further analysis to avoid unstable result due to the small number of proviral copies in each reaction. The results of our study were

shown as the ratio of PVLs for the 5′LTR-*gag* or *gag* regions to PVL for the *pX* region in each individual (Fig. 2). The median 5′LTR-*gag* PVL/*pX* PVL ratio of 161 HTLV-1 carriers tested was 0.97. Therefore, HTLV-1 proviral sequence for 5′LTR-*gag* PVL was considered to be conserved in the majority of asymptomatic carriers. The median *gag* PVL/*pX* PVL ratio, however, was 0.61.

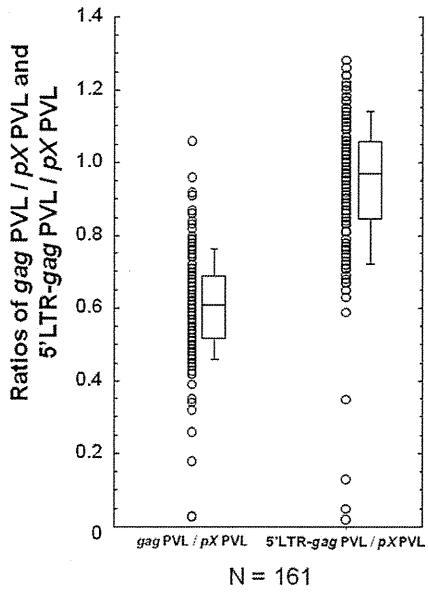

Figure 2. The ratios of PVLs for the 5′LTR-*gag* or *gag* regions to PVL for the *pX* region in 161 asymptomatic HTLV-1 carriers, whose *pX* PVLs were equal to or greater than 1 copy/100 PBMCs.

Detection of provirus with deletion of HTLV-1 internal sequence by long PCR

To determine whether the provirus with deletion of HTLV-1 internal sequence accounted for low *gag* PVL/*pX* PVL ratio, long PCR was performed. For this analysis, we chose 26 carriers with low *gag* PVL/*pX* PVL ratios of less than 0.5; however, adequate DNA sample for long PCR was available in only 17 of the 26 subjects. All subjects except C1 showed a band of 7.7 kb, which was considered to be derived from complete provirus, and some additional smaller bands suggesting defective provirus (Fig. 3*a*). C1 showed only a dense band of 4.5 kb. C1 was analyzed in our previous study and a large deficiency (3.2 kb, positions 1,203–4,368) of internal sequence was shown.[19] Additional four carriers (C3, 4, 11 and 13) showed dense bands equal to or stronger than the band for complete provirus (arrows in Fig. 3*a*). Cloning and DNA sequencing of these dense bands showed large deficiencies of internal sequences (4.9 kb, positions 1,368–6,286 in C3; 0.9 kb, positions 1,413–2,284 in C4; 4.8 kb, positions 1,009–5,763 in C11 and 4.8 kb, positions 1,133–5,974 in C13).

Four carriers (C18–21) had low 5′LTR-*gag* PVL/*pX* PVL ratios of less than 0.5. Long PCR of C18 and 19 showed dense bands of 7.7 kb, which were considered to be derived from complete provirus, and some additional smaller bands (Fig. 3*b*). Polymorphism of proviral DNA sequence of the sites for primers and/or probe for 5′LTR-*gag* PVL was suspected in these two cases, and cloning and DNA sequencing of the PCR products were performed. The polymorphisms of DNA sequence for the annealing site of the forward primer (708 G > A and 709 C > G in C18; 712 C > T in C19) were consistently found, and these polymorphisms were

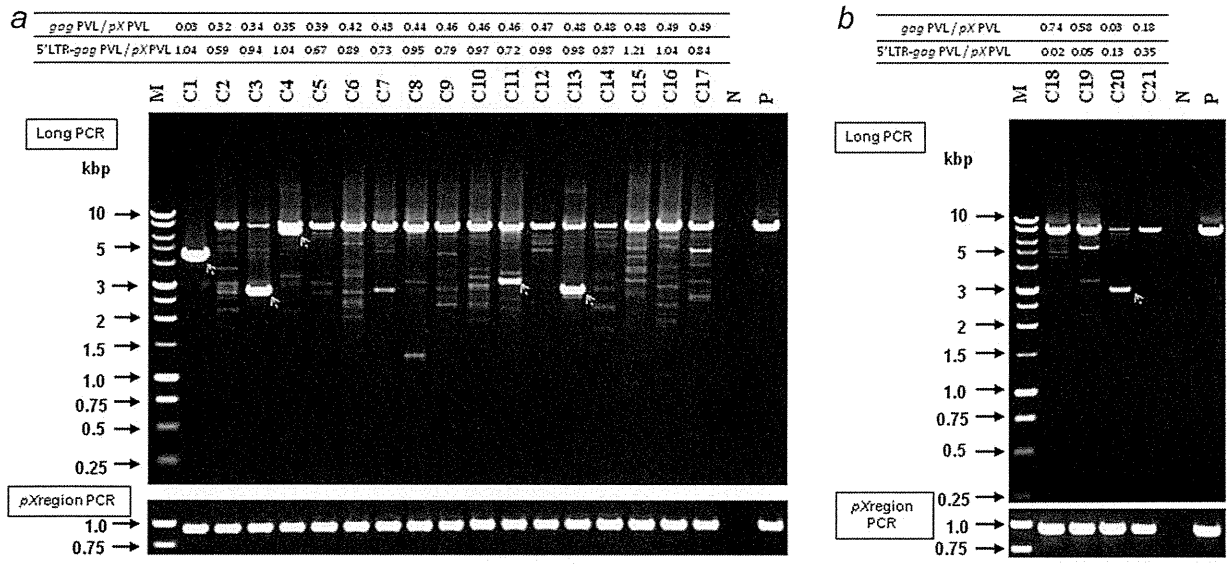

Figure 3. Detection of defective provirus by long PCR. (*a*) Asymptomatic HTLV-1 carriers with low *gag* PVL/*pX* PVL ratios less than 0.5. (*b*) Asymptomatic HTLV-1 carriers with low 5′LTR-*gag* PVL/*pX* PVL ratios less than 0.5. Arrows indicate PCR products for HTLV-1 provirus lacking large internal sequence. M: Molecular weight marker; N: HTLV-1-negative subject; P: HTLV-1-positive cell line, ED-40515(-).

considered to account for the decreased efficacy of real time-PCR for 5′LTR-*gag* PVL.

Detection of provirus with deletion of 5′LTR and its flanking internal sequence by IL-PCR

Both *gag* PVL/*pX* PVL ratio and 5′LTR-*gag* PVL/*pX* PVL ratio were low at less than 0.5 in the additional two carriers (C20 and 21). Long PCR showed a weak band of 7.7 kb for complete provirus and a stronger band of 2.9 kb in C20 (Fig. 3*b*). In the case of C21, only a weak band for complete band was observed (Fig 3*b*). These data suggested defective provirus, which had not been detected by long PCR, existed in C20 and C21. Because these proviruses were suspected of lacking 5′LTR and its flanking internal sequence, we attempted to identify them by IL-PCR. First, the genomic DNA of C20 and C21 were digested with *Kpn* I, *Hind* III, *Sal* I or *Spe* I, and resultant DNA was provided for IL-PCR as a template. In C20, approximately 1.1 kb of PCR product was obtained in digestion with *Kpn* I alone (Fig. 4*a-1*). No IL-PCR product was obtained using other restriction enzymes (data not shown). When this PCR product was digested with *Kpn* I, two major bands appeared, as expected (Fig. 4*a-1*). Cloning and sequencing revealed that this product consisted of HTLV-1 provirus (*Kpn* I site at position: 6,141 to the end of 3′LTR) and its flanking genomic DNA of human chromosome 2 (2q13). Based on the information obtained, a forward primer to anneal the upstream human genome adjuncted to the provirus was prepared and clone-specific PCR was performed. Cloning and sequencing of this clone-specific PCR product revealed that it lacked 5′LTR and its internal flanking sequence (until position 5,999; Fig. 4*a-2*). In the case of C21, IL-PCR product was obtained in digestion with *Hind* III alone. Following the same procedure as in C20, it was revealed that a provirus integrated in human chromosome 18 (18p11.32), and that it lacked 5′LTR and its internal flanking sequence (until position 4,976) (Figs. 4*b-1* and 4*b-2*).

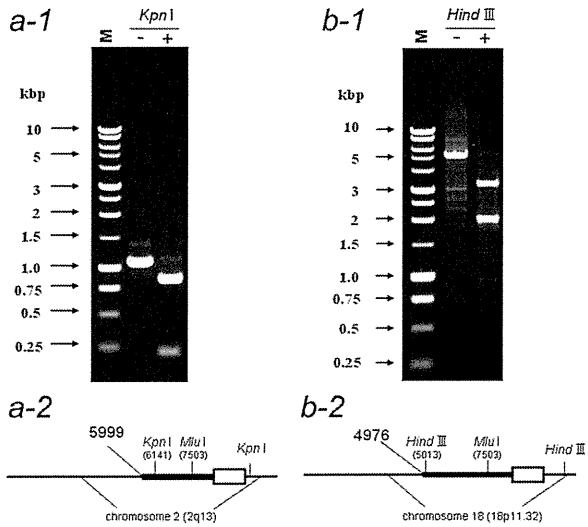

Figure 4. Detection of provirus with deletion of 5′LTR and its internal flanking sequence by IL-PCR. (*a-1*) Long PCR products from an asymptomatic HTLV-1 carrier, C20, with or without *Kpn* I digestion. (*a-2*) Scheme of the structure of defective provirus in C20. (*b-1*) Long PCR products from an asymptomatic HTLV-1 carrier, C21, with or without Hind III digestion. (*b-2*) Scheme of the structure of defective provirus in C21.

Relationship between pX PVL and gag PVL/pX PVL or 5′LTR-gag/pX PVL ratios

To determine whether the HTLV-1 PVLs correlated with the number of provirus with deficiency and/or polymorphism of the *gag* or 5′LTR-*gag* regions, the relationship between *pX* PVL and *gag* PVL/*pX* PVL or 5′LTR-*gag*/*pX* PVL ratios was analyzed. As shown in Figure 5*a*, there was a negative

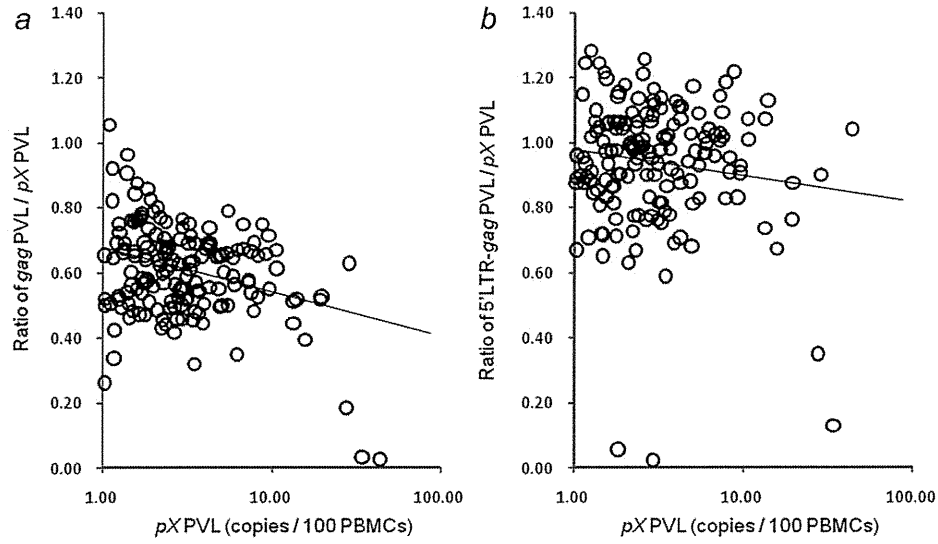

Figure 5. Relations of *pX* PVL and *gag* PVL/*pX* PVL or 5′LTR-*gag* PVL/*pX* PVL ratios in 161 asymptomatic carriers. (*a*) Relation of *pX* PVL and *gag* PVL/*pX* PVL. (*b*) Relation of *pX* PVL and 5′LTR-*gag* PVL/*pX* PVL.

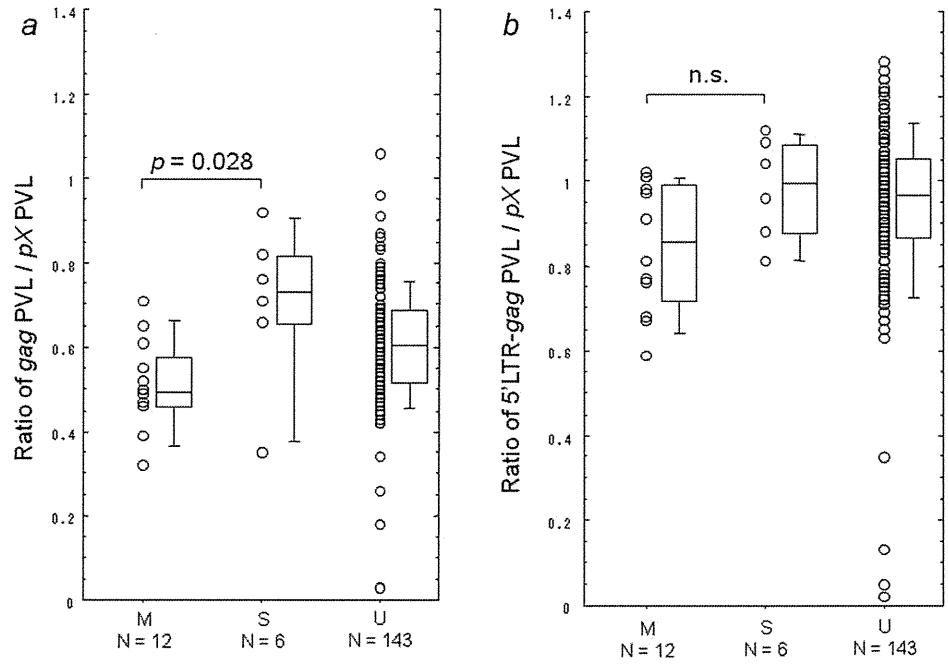

Figure 6. The ratios of *gag* PVL/*pX* PVL or 5′LTR-*gag* PVL/*pX* PVL in HTLV-1 carriers with different infection routes in 161 asymptomatic carriers. (*a*) The ratio of *gag* PVL/*pX* PVL. (*b*) The ratio of 5′LTR-*gag* PVL/*pX* PVL. M: Carriers with infection from mothers; S: Carriers with infection from spouses; U: Carriers with undetermined infection routes.

correlation between *pX* PVL and the *gag* PVL/*pX* PVL ratio ($r = -0.46$, $p = 0.02$). Therefore, HTLV-1 infected cells harboring provirus with deficiency and/or polymorphism of the *gag* region were considered to be more prevalent in asymptomatic carriers with high PVL. In the case of 5′LTR-*gag*/*pX* PVL ratio, the trend was not obvious (Fig. 5*b*) ($r = -0.20$, $p = 0.94$). However, variability of the 5′LTR-*gag*/*pX* PVL ratio was greater than that of *gag* PVL/*pX* PVL ratio. This may have been the result of technical inadequacies in the measurement of 5′LTR-*gag* PVL.

The ratios of gag PVL/pX PVL and 5′LTR-gag PVL/pX PVL in HTLV-1 carriers with different infection routes

Next, the relationships between infection routes and the *gag* PVL/*pX* PVL or 5′LTR-*gag*/*pX* PVL ratios were analyzed. The median ratio of *gag* PVL/*pX* PVL in 12 HTLV-1 carriers with maternal infection (0.50) was significantly lower than that in six carriers with spousal infection (0.74) ($p = 0.028$) (Fig. 6*a*). The median *gag* PVL/*pX* PVL ratio of 143 carriers with undetermined infection route (0.62) was between these. The 5′LTR-*gag* PVL/*pX* PVL ratio did not reveal a significant difference between the carriers with maternal infection and spousal infection (Fig. 6*b*). Therefore, the carriers with maternal infection were considered to have a greater number of HTLV-1 infected cells harboring provirus with deficiency and/or polymorphism of the *gag* region. In addition, when a *gag* PVL/*pX* PVL ratio of 0.65 was used as cut-off value, 11 of 12 (92%) carriers with maternal infection, against only one of six (17%) carriers with spousal infection, showed lower values.

Discussion

First, HTLV-1 PVLs in asymptomatic carriers with different infection routes were analyzed. *PX* PVL in 21 asymptomatic carriers with maternal infection was significantly higher than that in 24 carriers with spousal infection. These results agreed with data reported by Roucoux *et al.*[32] showing that PVLs in index HTLV-1 positive carriers were higher than those of their newly infected partners. Asymptomatic carriers whose infection routes were undetermined showed values between these. Previously, we analyzed the PVLs of HTLV-1 seroconverters and their spouses and showed that PVLs were not equivalent between them.[22] Because HTLV-1 in a seroconverter and in his/her spouse is identical, the host factor was considered important in the determination of HTLV-1 PVL. The results of our study suggest that infection route and/or time of infection are factors in the determination of PVL in HTLV-1 carriers. We also reported that HTLV-1 carriers who developed ATL had high PVLs even before they developed the disease.[20] Recently, Iwanaga *et al.*[21] also tested the PVLs of 1,218 HTLV-1 carriers and found that HTLV-1 carriers that developed ATL had high PVLs. These data suggest that high HTLV-1 PVL is a risk factor for developing ATL. In our study, HTLV-1 carriers with maternal infection tended to have high PVLs. This may account for why perinatal infection is a risk factor of ATL at least in part.

Because the frequent detection of defective provirus in patients with ATL has been reported, we examined provirus with deficiencies and/or polymorphism of proviral sequence in asymptomatic HTLV-1 carriers. The *pX* region has been

reported to be conserved in HTLV-1 provirus, and PCR for this region was used to measure total PVL.[23,25] Ohshima *et al.*[25] reported that variation of DNA sequence is frequently detected in the *gag* region of HTLV-1 provirus in patients with ATL. Kamihira *et al.*[24] also reported that most of deficient provirus in patients with ATL lacked part of the *gag* region in the proviral regions of HTLV-1 tested. HTLV-1 provirus with deletion of the 5′LTR, and its flanking internal sequences was also found in patients with ATL.[26] In our study, therefore, we tried to find provirus with deficiencies and/or polymorphism of DNA sequence in the asymptomatic carriers by measuring PVLs for the *gag* and 5′LTR-*gag* regions as ratios to *pX* region PVLs. As a result, median 5′LTR-*gag* PVL/*pX* PVL and *gag* PVL/*pX* PVL ratios of 161 HTLV-1 carriers with relatively high *pX* PVL (equal to or greater than one copy per 100 PBMCs) were 0.97 and 0.61, respectively. Our interpretation of this result was that many HTLV-1 infected cells in asymptomatic carriers harbor provirus with deficiency and/or polymorphism of DNA sequences for the sites of primers and/or probe for *gag* real time-PCR.

Long PCR analysis was performed on 17 carriers with low *gag* PVL/*pX* PVL ratios. Five of 17 carriers (29%) were shown to have the provirus with large deletions of internal DNA sequence including the *gag* region. The clonal expansion of HTLV-1 infected cells harboring defective provirus in these five carriers was most likely. In fact, clonal expansion of HTLV-1 infected cells in C1 was already shown in our previous study.[19] The reason for the low *gag* PVL/*pX* PVL ratios in the other 12 carriers was not clear. Contribution of the sum total of HTLV-1 infected cells with defective provirus, which did not reveal dense bands, was possible. Alternatively, polymorphism of the proviral DNA sequence for the *gag* region may have decreased the efficiency of real time-PCR for *gag* PVL. However, cloning and DNA sequencing of the sites for primers and probes for real time-PCR for *gag* PVL in these carriers did not show consistent polymorphism of the proviral DNA (data not shown). This may be because there is high diversity of proviral DNA sequence in the *gag* region of HTLV-1 and it was not possible to prepare cloning primers to work for all of them.

The other two (C20 and 21) showed low ratios not only of 5′LTR-*gag* PVL/*pX* PVL but also of *gag* PVL/*pX* PVL. Our previous study showed that they had high PVLs and clonal expansion of HTLV-1 infected cells with defective provirus.[19] We could not identify the type of defective provirus in the previous study. In our study, however, we found provirus lacking 5′LTR and its internal flanking region existed in these carriers.

In our study, the provirus with deficiency and/or polymorphism of the *gag* region was commonly found in asymptomatic HTLV-1 carriers. Few carriers had provirus lacking 5′LTR and its flanking sequence. Carriers with provirus with deficiency and/or polymorphism of the *gag* region were found frequently among asymptomatic carriers with high PVLs. These infected cells may not express certain HTLV-1 proteins. This change may make it possible for the HTLV-1 infected cells to avoid attack by cytotoxic T-lymphocytes.[33] Therefore, there is a possibility that provirus with deficiency and/or polymorphism of HTLV-1 provirus contributes to the survival of HTLV-1 infected cells. Indeed, our previous study showed that C1, 20 and 21 had clonal expansion of HTLV-1 infected cells.[19]

Low *gag* PVL/*pX* PVL ratio was found to be associated with maternal infection. The reason carriers with maternal infection have a greater number of HTLV-1 infected cells harboring provirus with deficiency and/or polymorphism of the *gag* region was not clear in our study. The replication of HTLV-1 infected cells in long-term infected carriers may account for this. Alternatively, a low level of new cell to cell infection *in vivo* can contribute to the creation of deficiency and/or polymorphism in proviral genome.

Maternal infection has been considered to be a risk factor for the development of ATL in asymptomatic carriers. However, there has been no method to identify infection route in the absence of information on family HTLV-1 status. The results of our study suggest the possibility that *gag* PVL/*pX* PVL ratio can be used as a tool to differentiate the infection routes of asymptomatic HTLV-1 carriers. Due to the fact that only a small number of HTLV-1 carriers with known infectious routes were analyzed in our study, further study with a larger number of subjects is necessary.

A major limitation of our study is that the subjects were elderly individuals, whose median age was 67 years old. The average age at onset of ATL was reported as 60 years.[34] Therefore, it is not clear whether the same result would be obtained from an analysis of younger HTLV-1 asymptomatic carriers. In addition, carriers with low *pX* PVL (less than 1 copy/100 PBMCs) were not provided for the analysis of deficiency and/or polymorphism of HTLV-1 proviral sequence because of technical limitations. Further analysis of carriers with low PVLs using improved methodology is necessary.

In conclusion, our study showed that *pX* PVL in carriers with maternal infection was significantly higher than that in carriers with spousal infection. Low *gag* PVL/*pX* PVL ratio reflecting deficiency and/or polymorphism in proviral genome was associated with high PVLs and maternal infection. These data suggest that development of ATL in carriers with maternal infection may be due in part to high PVL, which can be related to provirus with deficiency and/or polymorphism in proviral genome. In addition, *gag* PVL/*pX* PVL ratio has potential for use as a tool to differentiate infection routes of asymptomatic HTLV-1 carriers. Further study is necessary to clarify the mechanism of deficiency and/or polymorphism in HTLV-1 proviral genome and its implications in ATL development.

Acknowledgements

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