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ORIGINAL ARTICLE

Factors predisposing to HTLV-1 infection in residents of the greater Tokyo area

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Abstract Human T-cell leukemia virus type 1 (HTLV-1) is the etiological agent for adult T-cell leukemia. The geographic distribution of HTLV-1 carriers is quite uneven in Japan and the greatest prevalence is in southwestern Japan. Because many people move from endemic areas to the greater Tokyo area, the geographic distribution might have changed. Therefore, we investigated the factors predisposing to HTLV-1 infection, including birthplace, for 88 HTLV-1-infected individuals in greater Tokyo who visited our outpatient clinic. Of these, 39.5% were born in endemic areas, which include Kyushu/Okinawa, south Shikoku, Kii, Tohoku, and Hokkaido, whereas 38.3% were born in greater Tokyo and the proportion is presumed to be increasing. Half of the HTLV-1 infected individuals in greater Tokyo came from endemic areas, whereas around half of the remaining half was presumed to be involved in sexual transmission from a spouse from an endemic area. Overall, they constituted approximately 70% of the HTLV-1 carriers in greater Tokyo. These migration effects may increase the prevalence of HTLV-1 in the greater Tokyo area; nationwide surveillance is warranted.

Keywords Breast-feeding · Geographic distribution · HTLV-1 carrier · Sexual transmission · Tokyo

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1 Introduction

Human T-cell leukemia virus type 1 (HTLV-1) is the etiological agent for adult T-cell leukemia (ATL) [1] and other diseases, including HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [2] and HTLV-1 uveitis/HTLV-1-associated uveitis (HU/HAU) [3]. In HTLV-1 transmission, breast-feeding plays a major role in mother-to-child transmission and some cases are infected through sexual transmission [4–7]. There are several endemic regions in the world, including Japan [8], specifically southwestern Japan, including Okinawa, Kyushu, and southern Shikoku, although the geographic distribution is quite uneven [9]. A previous nationwide survey of Japan found that 77.9% of ATL patients were born in southern Japan (Kyushu, Kii, and South Shikoku), whereas 9.5% were born in Tohoku and Hokkaido, in northern Japan, which is another endemic area. Only 2.9% were from Kanto district which includes Tokyo, and metropolitan Tokyo was not regarded as an endemic region [9]. The geographic distribution of HTLV-1 carriers might be changing as people continue to move to metropolitan Tokyo from endemic regions. From the perspective of public health, it is important to evaluate the prevalence and distribution of HTLV-1 infection accurately, although no study has examined the geographic distribution of ATL patients in Japan since 1983. Therefore, we investigated the

factors predisposing to HTLV-1 infection, including birthplace, in HTLV-1-infected individuals in metropolitan Tokyo who visited our outpatient clinic. These data are useful for determining the future prevalence of HTLV-1 infection in this area.

2 Subjects and methods

Between March 2006 and December 2007, 107 HTLV-1 infected individuals visited the outpatient clinic at The Research Hospital, The Institute of Medical Science, The University of Tokyo, for a consultation regarding their concerns about HTLV-1 infection or to join a cohort study of HTLV-1 carriers [Joint Study of Predisposing Factors for ATL Development (JSPFAD), supported by the Ministry of Education, Culture, Sports, Science, and Technology of Japan]. Of the 107 individuals, 88 resided in greater Tokyo, which includes Metropolitan Tokyo and Kanagawa, Chiba, Saitama, Tochigi, Gunma, and Ibaraki Prefectures. We investigated the birthplace and other predisposing factors that might be related to HTLV-1 infection in these cases and their families by reviewing their medical records and an inquiry form. We defined Kyushu, south Shikoku, Kii, Tohoku and Hokkaido as endemic areas in Japan [9]. Approval was obtained from the institutional review board. Informed consent was obtained according to the Declaration of Helsinki.

3 Results

3.1 Subject characteristics (Table 1)

Of the subjects, 73 individuals were asymptomatic HTLV-1 carriers, whereas six had HAM, and one had HU. Eight individuals had already developed ATL: three had the smoldering type, three had the chronic type, one had the lymphoma type, and one had the acute type. The sex distribution showed female dominance, with a male/female ratio of 3:5. The majority of cases were in their 30s to 50s and 13 cases (14.8%) were >60 years old.

The most common reason HTLV-1-infected individuals were checked for anti-HTLV-1 antibody was a blood donation at a Japanese Red Cross Blood Center; 36 individuals (40.9%) were identified as anti-HTLV-1 antibody (+) when they donated blood. The next most common reasons were pregnancy ($n = 18$; 20.5%) and having an HTLV-1-infected spouse or relative ($n = 17$; 19.3%). Twelve cases (13.6%) were diagnosed with HTLV-1 infection only when they developed HTLV-1-associated disease. Considering only individuals <40 years old, the proportions diagnosed as HTLV-1 carriers on blood donation and pregnancy increased to 50.0 and 35.7%, respectively (data not shown).

Table 1 Characteristics of visitors to the outpatient clinic for HTLV-1-infected individuals residing in the greater Tokyo area

Characteristic	Category	Number of individuals (total $n = 88$)
Age (years)	21–30	4
	31–40	24
	41–50	26
	51–60	21
	61–70	9
	71–80	4
Sex	Male	32
	Female	56
HTLV-1 disease status	Carrier	73
	HAM	6
	HU	1
	ATL	
	Smoldering	3
	Chronic	3
	Lymphoma	1
	Acute	1
Reason for anti-HTLV-1 antibody testing	Blood donation	36
	Pregnancy	18
	HTLV-1-infected spouse or relative	17
	Developed HTLV-1-associated disease	12
	Visited hospital for a disease unrelated to HTLV-1	4
	Unknown	1

3.2 Birthplaces of HTLV-1 infected individuals and their mothers in greater Tokyo

We examined the geographic distribution of the birthplaces of the subjects and their mothers (Tables 2, 3). Thirty-two cases (39.5%) of HTLV-1-infected individuals in greater Tokyo had moved from endemic areas, and 31 cases (38.3%) were born in the greater Tokyo area. More subjects' mothers were born in endemic areas than the subjects. Forty-five (57.0%) of the mothers of HTLV-1-infected individuals in greater Tokyo were born in endemic areas, whereas only 13 (16.5%) mothers were born in greater Tokyo. The number of subjects' mothers born in greater Tokyo was less than half the number of subjects who were born greater Tokyo.

Table 2 Birthplaces of HTLV-1-infected individuals in the greater Tokyo area

Area of birth	Subjects (%)
Hokkaido/Tohoku	9 (11.1)
Tokyo metropolitan	31 (38.3)
Chubu	10 (12.3)
Hokuriku	1 (1.2)
Kinki	3 (3.7)
Chugoku/north Shikoku	2 (2.5)
Kii/south Shikoku	3 (3.7)
Kyushu	20 (24.7)
Other	2 (2.5)
Total	81 (100)
Unknown	7

Hokkaido/Tohoku, Kii/South Shikoku and Kyushu were defined as endemic

Table 3 Birthplaces of mothers of HTLV-1-infected individuals residing in the greater Tokyo

Area of birth	Mothers (%)
Hokkaido/Tohoku	13 (16.5)
Tokyo metropolitan	13 (16.5)
Chubu	12 (15.2)
Hokuriku	0 (0.0)
Kinki	2 (2.5)
Chugoku/north Shikoku	5 (6.3)
Kii/south Shikoku	3 (3.8)
Kyushu	29 (36.7)
Other	2 (2.5)
Total	79 (100)
Unknown	9

3.3 Factors predisposing to HTLV-1 infection in individuals born in greater Tokyo

We also analyzed the mothers, relatives, and spouses of the 31 cases born in greater Tokyo to identify factors that might be related to HTLV-1 infection (Table 4; Fig. 1). Thirteen cases (41.9%) had mothers from endemic areas and five of these mothers were HTLV-1 infected. The rest of the mothers were not examined for anti-HTLV-1 antibodies, although one HTLV-1-infected subject with one of these mothers who were not examined had an HTLV-1 infected sibling. A total of four cases, including this one, had HTLV-1-infected siblings (Fig. 1).

Two cases had husbands who were HTLV-1-infected and were born in endemic areas; one case had a husband who was infected but not born in an endemic area; and three cases had husbands who were born in endemic areas

Table 4 Predisposing factors in 31 HTLV-1-infected individuals born in the greater Tokyo area (see Fig. 1)

Factor	Number of cases (%)
Mother from an endemic area	13 (41.9)
HTLV-1-infected mother	5[a] (16.1)
HTLV-1 infected sibling	4[b] (12.9)
Father from an endemic area	5[c] (16.1)
Husband from an endemic area	5[c] (16.1)
HTLV-1-infected husband	3[d] (9.7)
No factors/Unknown	10 (32.2)

[a] All of the mothers were from endemic areas

[b] Three of the cases had mothers from endemic areas and two of these three mothers were HTLV-1-infected

[c] Two of the five cases with fathers from endemic areas also had mothers from endemic areas, and one also had a mother and a husband from endemic areas

[d] Two of the cases were from endemic areas, and the birthplace was unknown in one case

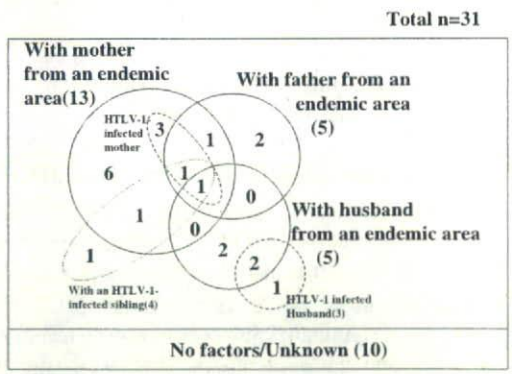

Fig. 1 Schematic presentation of the predisposing factors for 31 HTLV-1-infected individuals born in greater Tokyo. Thirteen cases born in greater Tokyo had mothers from endemic areas, five of whom were HTLV-1 infected. One more case was presumed to have an HTLV-1-infected mother because he had an HTLV-1-infected sibling. In seven cases, a father or a husband from an endemic area or an HTLV-1-infected husband was the only factor predisposing to HTLV-1 infection

and not examined for HTLV-1 antibodies. Among the latter three cases, one case had both a father and mother from an endemic area, and the mother was HTLV-1-infected. Thus, six cases (19.4%) had husbands who were HTLV-1 infected and/or were born in endemic areas (Fig. 1), and in five of these cases (16.1%), the only factor predisposing to HTLV-1 infection was a husband who was HTLV-1 infected or was born in an endemic area.

Two cases had a father and mother from endemic areas, and one had a father, mother, and husband from endemic areas. Two of these three mothers were HTLV-1 infected. Two cases had only a father from an endemic area, making

Table 5 HTLV-1-infected subjects categorized by their and their mother's birthplaces

Birthplace of subjects	Birthplace of their mothers	Number of cases
Endemic area	Endemic area	26
	Non-endemic area	4
	Unknown	2
Non-endemic area	Endemic area	15
	Non-endemic area	30
	Unknown	4
Unknown		7

that the only factor predisposing to HTLV-1 infection in those two cases (Fig. 1). Overall, seven of 17 individuals born in the greater Tokyo area who did not have a mother from an endemic area or an HTLV-1 infected sibling had a husband who was from an endemic area or HTLV-1 infected or a father from an endemic area as the only predisposing factor.

3.4 Factors predisposing to HTLV-1 infection in individuals from non-endemic areas

To further estimate predisposing factors in HTLV-1-infected individuals from non-endemic areas, including greater Tokyo, we analyzed HTLV-1-infected individuals born in non-endemic areas whose mothers were also born in non-endemic areas. As shown in Table 5, the total number of HTLV-1-infected subjects who were from non-endemic areas and whose mothers were also from non-endemic areas was 30 subjects, 15 of whom were born in the greater Tokyo area. [In the 31 HTLV-1-infected individuals born in the greater Tokyo area, 13 individuals had mothers who were born in endemic areas and three had mothers whose birthplaces were unknown (Table 4 and data not shown).] Seven (23.3%) of the 30 cases had HTLV-1-infected husbands, and one case (3.3%) had a father with ATL (Table 6; Fig. 2). Three individuals had a husband from an endemic area, one of which was diagnosed with ATL, and one had a husband whose mother was from an endemic area. Five cases had fathers from endemic areas, and one of these five also had an HTLV-1-infected husband. A father with ATL was from an endemic area and was included in this category. As a result, eitght individuals had an HTLV-1-infected husband or father and five more individuals had a husband or father from an endemic area. One individual had a husband whose mother was from an endemic area (Fig. 2). These cases represent 46.7% of the 30 subjects from non-endemic areas whose mothers were also from non-endemic areas.

Table 6 Predisposing factors in 30 HTLV-1-infected individuals born in non-endemic areas and with mothers from non-endemic areas (see Fig. 2)

Factor	Number of cases (%)
HTLV-1-infected husband	7 (23.3%)
Husband from an endemic area	3[a] (10.0%)
Husband with mother from an endemic area	1 (3.3%)
Father with ATL	1[b] (3.3%)
Father from an endemic area	5[c] (16.7%)
No factors/unknown	16 (53.3%)

[a] One husband was HTLV-1 infected

[b] This father was from an endemic area

[c] One case also had an HTLV-1-infected husband

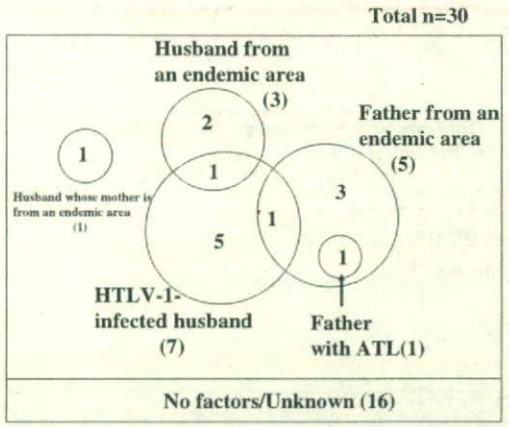

Fig. 2 Schematic presentation of the predisposing factors for 30 HTLV-1-infected individuals born in non-endemic areas with mothers from non-endemic areas. Eight cases had an HTLV-1-infected husband or father. One case had a husband with a mother from an endemic area. There were five more cases who had a husband or a father from an endemic area as the only factor predisposing HTLV-1 infection

4 Discussion

Adult T-cell leukemia is one of the most incurable lymphoid malignancies. The mean survival time of the most commonly used combination of chemotherapies is <1 year [10–13], although a recent prospective randomized study showed some improvement in survival [14, 15]. Several studies have reported promising results of hematopoietic stem cell transplantation, but the study population was small, and the procedure's efficacy remains to be elucidated [16–19]. Given the poor prognosis of ATL, preventing HTLV-1 infection is the most effective strategy for reducing mortality from the disease. In HTLV-1 transmission, breast-feeding plays a major role, and not breast-feeding is shown to be effective in preventing mother-to-child transmission [4, 5]. In endemic areas such

as Kyushu, pregnant women are advised to refrain from breast-feeding, especially in Nagasaki prefecture. Nevertheless, because of the uneven distribution of HTLV-1 carriers in Japan, pregnant women in other areas have received insufficient information on the virus, so it is important from the perspective of public health to determine whether the disease distribution has changed.

Previous nationwide surveys of HTLV-1 carriers based on the rate of anti-HTLV-1 antibody -positive volunteer blood donors [20] and numbers of ATL patients [9] indicated several endemic areas in Japan, including Kyushu and southern Shikoku. In these studies, approximately 55% of HTLV-1 carriers resided in these areas. By contrast, the estimated HTLV-1 prevalence for greater Tokyo was 0.7% [20], which was approximately one-tenth of that for Kyushu, based on the seropositive rate in volunteer blood donors. From these data, greater Tokyo was considered to be a non-endemic area. However, there has been no nationwide survey in the 25 years since these studies, and there is little information on whether the distribution of HTLV-1 carriers has changed with the migration of people to greater Tokyo from endemic areas.

This study investigated the factors predisposing to HTLV-1 infection including the birthplaces of HTLV-1 infected individuals living in greater Tokyo who visited our outpatient clinic to estimate the effect of migration to greater Tokyo. Of 88 HTLV-1-infected individuals in greater Tokyo, approximately 40% of the cases (32 cases) came from endemic areas, whereas almost the same number of individuals were born in greater Tokyo (Table 2). As the number of subjects' mothers born in greater Tokyo was less than half of subjects who were born in greater Tokyo (Table 3), it was assumed that about half of the HTLV-1-infected subjects born in greater Tokyo had mothers from other areas, especially endemic areas. In fact, 41.9% (13 cases) of the 31 subjects born in the greater Tokyo area had mothers from endemic areas (Table 4) and were the offspring of settlers from endemic areas. Of these 13 mothers, 5 were proven to be HTLV-1 infected, and one more mother was very likely HTLV-1 infected because the HTLV-1-infected individual having this mother had an HTLV-1 infected sibling. No mother was proven to be negative for anti-HTLV-1 antibodies. These results make it highly probable that these mothers from endemic areas were HTLV-1 carriers. Fifteen subjects born in non-endemic areas including the 13 cases born in greater Tokyo, had mothers from endemic areas (Table 5).As a result, 47 cases were from endemic areas or had mothers from endemic areas (Table 5) and they constituted 53.4% of the HTLV-1-infected individuals in the greater Tokyo area. These data suggest that more than half of HTLV-1 carriers in greater Tokyo consist of individuals who moved from endemic areas or their offspring. In the fourth nationwide survey [9], 11% of ATL patients resided in Tokyo, although only 2.9% were born there. From these results, we can estimate that 26.3% (2.9/11) of the HTLV-1 carriers in greater Tokyo were born there, which was less than our result (41.9%); this suggests that the second generation of HTLV-1 carriers from endemic areas is increasing in greater Tokyo.

Of the remaining HTLV-1-infected individuals in greater Tokyo, namely cases born in non-endemic areas and with mothers from non-endemic areas (Table 6; Fig. 2), seven cases had HTLV-1-infected husbands and one had an HTLV-1-infected father. This father and one of the husbands were from endemic areas, and the birthplaces of the other four husbands were unknown. There was no HTLV-1-infected husband from greater Tokyo. It is very probable that these cases were infected via sexual transmission from their husbands or involved in mother-to-child transmission from sexually infected mothers because they had no other predisposing factor. Other than these cases, two more husbands were from an endemic area, one other husband had a mother from an endemic area, and three more cases had fathers from endemic areas. As these cases also had no other factors predisposing to HTLV-1 infection, it is probable that they or their mothers were infected via sexual transmission from their spouse, although none of these husbands and fathers was examined for anti-HTLV-1 antibodies. Overall, these cases of possible sexual transmission represent 46.6% (14 cases) of the HTLV-1-infected individuals from non-endemic areas whose mothers were also from non-endemic areas (Fig. 2) and correspond to 15.9% of the HTLV-1-infected individuals in the greater Tokyo area. Combined with the cases who were from endemic areas or had mothers from endemic areas, we estimate that roughly 70% of the HTLV-1 carriers in greater Tokyo came from endemic areas or were infected via sexual transmission from spouses, many of whom were from endemic areas.

Considering only individuals born in greater Tokyo, the only factor predisposing to HTLV-1 infection was a husband or father from an endemic area or an HTLV-1-infected husband in seven cases (Fig. 1), and these cases were probably involved in sexual transmission. They constituted 38.8% of the cases who did not have a mother from an endemic area. Therefore, over 40% of the HTLV-1-infected individuals born in greater Tokyo were assumed to be the offspring of HTLV-1 carrier mothers who moved from an endemic area (Table 4) and around 40% of the remainder were thought to have resulted from sexual transmission. The proportion of possible sexual transmission in cases born in greater Tokyo paralleled that in cases from non-endemic areas with a mother from a non-endemic area, as described above.

Several studies have reported a decline in the prevalence of HTLV-1 [5, 21]. This was explained by the effects of refraining from breast-feeding or lifestyle changes in Japan

such as shortening the period of breast-feeding or having fewer children. Nevertheless, the migration of HTLV-1 carriers from endemic areas could increase the prevalence in the greater Tokyo area, which is regarded as a non-endemic area. In greater Tokyo, most of the HTLV-1 carriers, especially younger ones are diagnosed by chance (Table 1), and they can obtain little information about HTLV-1. An accurate evaluation of the present distribution of the prevalence of HTLV-1 is needed.

Among the subjects of this study, the sex distribution showed female dominance, and the percentage of individuals over 60 years old was relatively low. One possible reason for the female dominance is that female carriers included 18 cases checked for anti-HTLV-1 antibody during pregnancy. Another reason may be that unemployed female carriers such as housewives can visit the outpatient clinic more readily than male carriers. One reason for the relatively low percentage of elderly may be that most of the individuals who visited our hospital had obtained information about our outpatient clinic for HTLV-1 carriers or the JSPFAD study through the Internet, with which elderly individuals are generally unfamiliar. These subject characteristics may have influenced on our results.

In this analysis birthplaces were defined by prefecture. As the distribution of HTLV-1 carriers is uneven, even within a prefecture in some districts, endemic and non-endemic areas are admixed in a prefecture. It is necessary to investigate the birthplace more precisely in order to accurately estimate migration effects.

In conclusion, we surveyed the factors predisposing to HTLV-1 infection in individuals living in the greater Tokyo area and found an effect of migration from endemic areas. Because the distribution of HTLV-1 carriers in Japan has not been explored recently, a nationwide survey of HTLV-1 carriers is warranted.

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SPECIAL ARTICLE

Definition, Prognostic Factors, Treatment, and Response Criteria of Adult T-Cell Leukemia-Lymphoma: A Proposal From an International Consensus Meeting

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ABSTRACT

Adult T-cell leukemia-lymphoma (ATL) is a distinct peripheral T-lymphocytic malignancy associated with a retrovirus designated human T-cell lymphotropic virus type I (HTLV-1). The diversity in clinical features and prognosis of patients with this disease has led to its subclassification into the following four categories: acute, lymphoma, chronic, and smoldering types. The chronic and smoldering subtypes are considered indolent and are usually managed with watchful waiting until disease progression, analogous to the management of some patients with chronic lymphoid leukemia (CLL) or other indolent histology lymphomas. Patients with aggressive ATL generally have a poor prognosis because of multidrug resistance of malignant cells, a large tumor burden with multiorgan failure, hypercalcemia, and/or frequent infectious complications as a result of a profound T-cell immunodeficiency. Under the sponsorship of the 13th International Conference on Human Retrovirology: HTLV, a group of ATL researchers joined to form a consensus statement based on established data to define prognostic factors, clinical subclassifications, and treatment strategies. A set of response criteria specific for ATL reflecting a combination of those for lymphoma and CLL was proposed. Clinical subclassification is useful but is limited because of the diverse prognosis among each subtype. Molecular abnormalities within the host genome, such as tumor suppressor genes, may account for these diversities. A treatment strategy based on the clinical subclassification and prognostic factors is suggested, including watchful waiting approach, chemotherapy, antiviral therapy, allogeneic hematopoietic stem-cell transplantation (alloHSCT), and targeted therapies.

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DEFINITION

Adult T-cell leukemia-lymphoma (ATL) is a distinct peripheral T-lymphocytic malignancy associated with a retrovirus designated human T-cell leukemia virus type 1 or human T-cell lymphotropic virus type 1 (HTLV-1).[1-3] We recommend following the WHO classification of ATL published in 2001.[4]

PROGNOSTIC FACTORS

Major prognostic indicators[5-8] for ATL have been elucidated in 854 patients; advanced performance status (PS), high lactic dehydrogenase (LDH) level, age ≥ 40 years, more than three involved lesions, and hypercalcemia[5] are prognostic factors that have been identified by multivariate analysis. These factors were used to construct a risk model.[5] Additional factors associated with poor prognosis include thrombocytopenia,[9] eosinophilia,[10] bone marrow involvement,[11] high interleukin-5 serum level,[12] C-C chemokine receptor 4 expression,[13] lung resistance–related protein,[14] *p53* mutation,[15] and *p16* deletion.[9] For the chronic type of ATL, high LDH, high blood urea nitrogen, and low albumin levels have been identified as poor prognostic factors by multivariate analysis.[6] Univariate analysis has revealed that neutrophilia,[11] *p16* deletion,[9] and chromosomal deletion detected by comparative genomic hybridization[16] are associated with poor prognosis in chronic ATL. In contrast, chronic lymphoid leukemia (CLL)–like morphology of ATL cells was associated with longer transformation-free survival of chronic ATL.[17] Primary cutaneous tumoral type, although generally included among smoldering ATL, was a poor prognostic factor by univariate analyses.[18] A combination of these and more novel prognostic factors may be superior to elucidate better risk ATL groups for stratification of treatment decision than the Shimoyama criteria, which stratify

ATL into four clinical subtypes or risk groups, although these factors have not been evaluated simultaneously by a multivariate analysis.[5,19] Of note, these prognostic factors may not have to be applied when considering new therapeutic strategies (eg, antiretroviral therapies).

There are limited data comparing Japanese patients with those in the other countries, and there are no prospective studies addressing this issue.[18,20-22] In a retrospective review of 89 patients predominantly of Caribbean origin, the median age at diagnosis was 50 years, whereas in the Japanese population, it is 57 years.[20] In addition, survival times according to the Shimoyama subclassification in both Caribbean and Japanese populations seem to be comparable (acute: 4 *v* 6 months; lymphomatous: 9 *v* 10 months; chronic: 17 *v* 24 months; and smoldering: 34 months *v* > 5 years, respectively). Although patients of Caribbean origin with less aggressive subtypes fared worse, it is not clear that this is statistically significant.

CLINICAL SUBCLASSIFICATION

Criteria

We recommend following the Shimoyama criteria on ATL clinical subtype classification published in 1991.[19]

Required Evaluation

Involved organ examination: peripheral blood. The diagnosis of ATL requires detection of ATL cells in peripheral blood in patients with acute, chronic, or smoldering type with leukemic manifestations.[4,19] Typical ATL cells have markedly polylobated nuclei with homogeneous and condensed chromatin, small or absent nucleoli, and agranular and basophilic cytoplasm. These so-called flower cells are considered pathognomonic. However, the diversity of recognized ATL cell morphology is considerable.[17,23] Even in patients with extremely unusual morphology, a small percentage of prototype ATL cells have always been seen in blood films, leading to a suspected diagnosis of ATL. This should be confirmed by mature T-cell phenotype, HTLV-1 serology, and monoclonal HTLV-1 provirus in all patients.[17] Five percent or more of abnormal T lymphocytes in peripheral blood confirmed by cytology and immunophenotyping are required to diagnose ATL in patients without histologically proven tumor lesions.[19]

Bone marrow examination. A bone marrow aspiration or biopsy is generally not required to make the diagnosis of ATL. Nevertheless, assessment of the bone marrow may add useful information regarding the normal bone marrow elements before therapy. Furthermore, bone marrow involvement is an independent poor prognostic factor for ATL, similar to that found in peripheral T-cell lymphoma unspecified.[11,24]

Radiologic imaging and endoscopy. Computed tomography (CT) scans of the neck, thorax, abdomen, and pelvis are mandatory to detect sites of nodal and extranodal ATL disease. Upper GI tract endoscopy, with biopsy, should be considered because GI tract involvement is frequent in aggressive ATL.[25] These imaging modalities may detect complicated opportunistic infections including pneumonia, abscess formation, and intestinal infections such as strongyloidiasis and cytomegalovirus.[19] CNS evaluation by radiologic imaging and/or lumbar puncture for cerebral/meningeal ATL involvement or opportunistic infections should be considered for patients in the setting of altered consciousness without hypercalcemia.[26]

Biopsy. When the diagnosis of ATL is not obtained by peripheral-blood examination or when a new lesion appears during watchful waiting for indolent ATL, biopsy of suspicious lesion should be performed. Frequently involved tissues include lymph nodes, skin, liver, spleen, lung, GI tract, bone marrow, bone, and CNS.[4-8,11,25,26] As in other types of lymphomas, an excisional biopsy is recommended, instead of core needle biopsy, for lymph nodes. Whenever possible, sufficient sample should be obtained both for histopathologic examination and molecular analyses, including Southern blotting or other (eg, linker-mediated polymerase chain reaction) analysis of HTLV-1 provirus integration.

Tumor marker. Similar to serum LDH reflecting disease bulk/activity, the soluble form of interleukin-2 receptor α-chain is elevated in aggressive ATL patients, indolent ATL patients, and HTLV-1 carriers compared with normal individuals, perhaps with better accuracy than LDH.[27] These serum markers are useful to detect acute transformation of indolent ATL as well as to detect early relapse of ATL after therapy. Serum thymidine kinase levels have also been reported as a promising tumor marker for ATL.[28] However, in the current general practice for the management of ATL patients, only LDH level is required.

Immunophenotype. In most patients, ATL cells exhibit the phenotype of mature $CD4^+$ T cells and express CD2, CD5, CD25, CD45RO, CD29, T-cell receptor $\alpha\beta$, and HLA-DR.[4] Most ATL cells lack CD7 and CD26 and exhibit diminished CD3 expression. Most ATL cells are CD52 positive, but occasionally, patients are negative, and this may correlate with coexpression of CD30. Immunophenotypic analysis of CD3, CD4, CD7, CD8, and CD25 is the minimum requirement for an ATL diagnosis.

Cytogenetics. Karyotypic abnormalities revealed by conventional cytogenetics or comparative genomic hybridization are more common and complex in the acute and lymphoma types compared with the chronic type, with aneuploidy and several hot spots such as 14q and 3p.[16,29] More sensitive array-comparative genomic hybridization revealed that the lymphoma type had significantly more frequent gains at 1q, 2p, 4q, 7p, and 7q and more losses of 10p, 13q, 16q, and 18p, whereas the acute type showed a gain of 3/3p.[30] Currently, outside of clinical trials, cytogenetic analysis is not required.

Molecular biology of HTLV-1. Monoclonal integration of HTLV-1 proviral DNA is found in all cases of ATL as described in the WHO classification.[4] Integration of defective HTLV-1 into ATL cells is observed in approximately one third of ATL patients and is associated with clinical subtypes and prognosis.[31] It is recommended to perform molecular analysis of HTLV-1 integration when possible. Either Southern blotting or polymerase chain reaction for HTLV-1 can be used to identify the presence of viral integration, whereas the latter can be used for quantitative purposes. Seronegativity for HTLV-1 is quite useful to differentiate T-cell lymphomas from ATL, although HTLV-1 is not detected in lymphoma cells other than ATL. Clinically, the diagnosis of ATL is made based on seropositivity for HTLV-1 and histologically and/or cytologically proven peripheral T-cell malignancy, although rare cases of T-cell lymphomas other than ATL developing in HTLV-1 carriers have been observed.[6,8]

Molecular biology of host genome. Mutation or deletion of tumor suppressor genes, such as *p53* or $p15^{INK4B}/p16^{INK4A}$, is observed in approximately half of ATL patients and is associated with clinical subtypes and prognosis.[9,15] These new molecular markers may

help guide therapeutic decisions between conventional chemotherapy, combination of zidovudine (AZT) and interferon alfa (IFN-α), and alloHSCT. In addition to *p53* mutations when considering AZT and IFN-α combination, IRF-4 may be predictive of response.[32]

TREATMENT

Criteria for Treatment Decisions

Treatment decisions should be based on the ATL subclassification and the prognostic factors at onset and response to initial therapy (Table 1). The prognostic factors include clinical factors, such as PS, LDH, age, number of involved lesions, and hypercalcemia, and molecular factors, such as Ki-67 expression, alteration of *p53* or $p15^{INK4B}$/$p16^{INK4A}$, and overexpression of IRF-4.[5,6,8,9,15,19,33-35]

Current Treatment Options

Chemotherapy. The results of a phase III study suggest that, at the expense of higher toxicities, the vincristine, cyclophosphamide, doxorubicin, and prednisone (VCAP); doxorubicin, ranimustine, and prednisone (AMP); and vindesine, etoposide, carboplatin, and prednisone (VECP) regimen is superior to biweekly cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) in newly diagnosed acute, lymphoma, or unfavorable chronic types of ATL.[36] The rate of complete response (CR) was higher in the VCAP-AMP-VECP arm than the biweekly CHOP arm (40% *v* 25%, respectively; $P = .020$). Overall survival (OS) at 3 years was 24% in the VCAP-AMP-VECP arm and 13% in the CHOP arm ($P = .085$). However, the median survival time of 13 months still compares unfavorably to other hematologic malignancies. The superiority of VCAP-AMP-VECP to biweekly CHOP may be explained by the more prolonged, dose-dense schedule of therapy in addition to four more drugs. In addition, agents such as carboplatin and ranimustine that are not affected by multidrug resistance–related genes, which are frequently expressed in ATL cells at onset, were incorporated.[14,36] Intrathecal prophylaxis, which was incorporated in both arms of the phase III study, should be considered for patients with aggressive ATL even in the absence of clinical symptoms because a previous analysis revealed that more than half of relapses at a new site after chemotherapy occurred in the CNS.[37]

IFN-α and AZT. Numerous small phase II studies using AZT and IFN-α have shown responses in ATL patients.[38-42] High-doses of both agents are recommended (6 to 9 million units of IFN-α in combination with daily divided AZT doses of 800 to 1,000 mg/d). However, only patients with wild-type *p53* and low IFN regulatory factor 4 expression seem to exhibit long-term responses to AZT/IFN-α therapy.[32,43,44]

The results of a recent worldwide meta-analysis on the use of AZT/IFN for ATL in 209 patients treated from 1994 to 2006 were presented at the 13th International Conference on Human Retrovirology: HTLV and at the 49th Annual Meeting of the American Society of Hematology.[21,22] One hundred patients received first-line AZT/IFN-α therapy. In these patients, the response rate was 66%, including 43% of patients achieving CR. In patients treated with first-line AZT/IFN-α, the median survival time was 24 months, and the 5-year OS rate was 50%, whereas these values were 7 months and 20%, respectively, in 84 patients who received first-line chemotherapy. The

Table 1. Recommended Strategy for the Treatment of ATL

Smoldering- or favorable chronic-type ATL
Consider inclusion in prospective clinical trials
Symptomatic patients (skin lesions, opportunistic infections, and so on): consider AZT/IFN-α or watch and wait
Asymptomatic patients: consider watch and wait
Unfavorable chronic- or acute-type ATL
Recommend: inclusion in prospective clinical trials
If outside clinical trials, check prognostic factors (including clinical and molecular factors if possible):
Good prognostic factors: consider chemotherapy (VCAP-AMP-VECP evaluated by a randomized phase III trial against biweekly CHOP) or AZT/IFN-α (evaluated by a retrospective worldwide meta-analysis)
Poor prognostic factors: consider chemotherapy followed by conventional or reduced-intensity allogeneic HSCT (evaluated by retrospective or prospective Japanese analyses, respectively)
Poor response to initial therapy with chemotherapy or AZT/IFN-α: consider conventional or reduced-intensity allogeneic HSCT
Lymphoma-type ATL
Recommend: inclusion in prospective clinical trials
If outside clinical trials, consider chemotherapy (VCAP-AMP-VECP)
Check prognostic factors and response to chemotherapy (including clinical and molecular factors if possible):
Favorable prognostic profiles and good response to initial therapy: consider chemotherapy
Unfavorable prognostic profiles or poor response to initial therapy with chemotherapy: consider conventional or reduced-intensity allogeneic HSCT
Options for clinical trials (first line)
Test the effect of up-front allogeneic HSCT
Test promising targeted therapies such as arsenic trioxide + IFN-α, bortezomib + chemotherapy, or antiangiogenic therapy
Consider a phase II global study testing pegylated IFN and AZT
Options for clinical trials (relapse or progressive disease)
Test the effect of promising targeted therapies such as arsenic trioxide and IFN-α, bortezomib, a purine nucleotide phosphorylase inhibitor, histone deacetylase inhibitors, monoclonal antibodies, antiangiogenic therapy, and survivin, β-catenin, syk, and lyn inhibitors, etc.
Consider conventional or reduced-intensity allogeneic HSCT when possible

Abbreviations: ATL, adult T-cell leukemia-lymphoma; AZT, zidovudine; IFN-α, interferon alfa; VCAP-AMP-VECP, vincristine, cyclophosphamide, doxorubicin, and prednisone; doxorubicin, ranimustine, and prednisone; and vindesine, etoposide, carboplatin, and prednisone; CHOP, cyclophosphamide, doxorubicin, vincristine, and prednisone; HSCT, hematopoietic stem-cell transplantation.

median survival times of patients with acute-type ATL treated with first-line AZT/IFN-α and chemotherapy were 12 and 9 months, respectively. However, achievement of CR with first-line AZT/IFN-α therapy resulted in a prolonged survival time of more than 10 years in 70% of the study population and 75% of the acute-type ATL subgroup. Patients with lymphoma-type ATL did not benefit from AZT/IFN-α therapy; the median survival times of these patients treated with first-line AZT/IFN-α and chemotherapy were 12 and 15 months, respectively. Finally, first-line AZT/IFN-α therapy in chronic- and smoldering-type ATL resulted in 100% OS at a median follow-up time of 5 years. Although the results for AZT/IFN-α in indolent ATL seem to be promising compared with the results seen with watchful waiting until disease progression recently reported from Japan,[45] the possibility of selection bias cannot be ruled out. In conclusion, these results suggest that treatment of ATL using AZT/IFN-α results in high response and CR rates particularly in acute, chronic, and smoldering types of ATL, resulting in prolonged survival in a significant proportion of patients. Although this is a retrospective analysis, the results seem to be promising, and further studies comparing AZT/IFN-α and chemotherapy in acute ATL are warranted.

alloHSCT. alloHSCT is now considered a promising treatment of young patients with aggressive ATL. Despite higher treatment-related mortality in a retrospective multicenter analysis, the estimated 3-year OS rate of 45% is promising, possibly reflecting a graft-versus-ATL effect.[46] A phase I trial of alloHSCT with reduced-intensity conditioning for ATL also revealed promising results. Minimal residual disease after alloHSCT detected by proviral load was much less compared with that after chemotherapy or AZT/IFN-α therapy, suggesting the presence of a graft-versus-ATL effect as well as graft-versus-HTLV-1 activity.[47] It remains uncertain which type of alloHSCT (myeloablative or reduced-intensity conditioning) is most suitable for the treatment of ATL. However, myeloablative alloHSCT, but not reduced-intensity conditioning alloHSCT, might be considered for the treatment of patients with progressive disease (PD) at relapse as well as at onset. Furthermore, selection criteria with respect to response to previous treatments, sources of stem cells, and HTLV-1 viral status of the donor remain to be determined.

Required Pretreatment Evaluation

The diagnosis of ATL is based on HTLV-1 seropositivity and histologically and/or cytologically proven peripheral T-cell malignancy as described in the WHO classification.[4] In uncertain cases, Southern blot hybridization for monoclonal integration of HTLV-1 provirus is useful for the diagnosis, although the sensitivity is to detect the presence of approximately 5% or more monoclonal ATL cells in peripheral-blood mononuclear cells or fresh biopsy.[6]

Traditionally, patients with indolent ATL (ie, the chronic or smoldering type) have been managed similarly to patients with CLL, with a watchful waiting policy until disease progression.[6,8,9] In the consecutive trials for aggressive ATL by Japan Clinical Oncology Group (JCOG)–Lymphoma Study Group, previously untreated patients with aggressive ATL (ie, acute-, lymphoma-, or unfavorable chronic-type ATL) were eligible for participation.[36] Unfavorable chronic-type ATL was defined by at least one of the following three factors: a low serum albumin, high LDH, or high blood urea nitrogen concentration. Unfavorable chronic-type ATL had an unfavorable prognosis similar to acute- or lymphoma-type ATL when treated with chemotherapy.[6] In those trials, other eligibility criteria included no prior chemotherapy, age of 15 to 69 years, and Eastern Cooperative Oncology Group PS of 0 to 3 or 4 as a result of hypercalcemia.[6,36] Eligibility criteria for organ function were also described.[6,36]

Supportive Care

Sulfamethoxazole-trimethoprim and antifungal agents were recommended for the prophylaxis of *Pneumocystis jiroveci* pneumonia and fungal infections, respectively, in the JCOG trials.[6,36] Although cytomegalovirus infection commonly occurs in ATL patients, ganciclovir is not routinely recommended for prophylaxis. In addition, in patients not receiving chemotherapy, antifungal prophylaxis may not be critical. Prophylaxis with anti-*Strongyloides* agents, such as ivermectin or albendazole, should be considered to avoid systemic infection in patients with a history of past and/or present exposure to the parasite in the tropics. Treatment with corticosteroids and proton pump inhibitors may precipitate fulminant *Strongyloides* infestation and warrants testing before these agents are used in endemic areas. It is suggested that *Strongyloides* infection may increase the risk of subsequent development of ATL. Therefore, in HTLV-1 carriers, although not yet demonstrated, prophylaxis of *Strongyloides* may reduce the risk of ATL development.[48-50] Hypercalcemia associated with aggressive ATL should be managed with treatment of the disease, hydration, and bisphosphonate therapy.[6,8]

RESPONSE CRITERIA

The complex presentation of ATL, often with both leukemic and lymphomatous components, makes response assessment difficult; however, response criteria are mandatory to ensure uniform interpretation of clinical trials (Table 2). Most current ATL trials use response criteria proposed by JCOG that have been applied since 1991.[6,36] At the international consensus meetings, a modification of the JCOG criteria was suggested, reflecting the criteria for CLL and NHL that had been published later (Table 2).[51,52] CR was defined as disappearance of all clinical, microscopic, and radiographic evidence of disease. Specific lymph node requirements include that all nodes must have regressed to normal size (≤ 1.5 cm in their greatest transverse diameter) and previously involved nodes that were 1.1 to 1.5 cm must have decreased to ≤ 1.0 cm.[51] Because HTLV-1 carriers frequently have a small percentage of abnormal lymphocytes with polylobated nuclei, so-called flower cells, in peripheral blood, provided that less than 5% of such cells remained, CR was judged to have been attained if the absolute lymphocyte count, including flower cells, was less than 4×10^9/L.[36,52] A designation of unconfirmed CR was adopted to include patients with a ≥ 75% reduction in tumor size but with a residual mass after treatment, as previously reported for NHL.[47] These patients must also have an absolute lymphocyte count, including flower cells, of less than 4×10^9/L. Partial response (PR) was defined as a ≥ 50% reduction in the sum of the products of the greatest diameters of measurable disease without the appearance of new lesions. In addition, PR was required to satisfy a 50% or greater reduction in absolute abnormal lymphocyte counts in peripheral blood. PD in peripheral blood was defined by a ≥ 50% increase from nadir in the count of flower cells and an absolute lymphocyte count, including flower cells, of ≥ 4×10^9/L. PD or relapsed disease in the other lesions was defined as a ≥ 50% increase from nadir in the sum of the products of measurable disease or the appearance of new lesions excluding skin. Stable disease

Table 2. Response Criteria for Adult T-Cell Leukemia-Lymphoma

Response	Definition	Lymph Nodes	Extranodal Masses	Spleen, Liver	Skin	Peripheral Blood	Bone Marrow
Complete remission*	Disappearance of all disease	Normal	Normal	Normal	Normal	Normal†	Normal
Uncertified complete remission*	Stable residual mass in bulky lesion	≥ 75% decrease‡	≥ 75% decrease‡	Normal	Normal	Normal†	Normal
Partial remission*	Regression of disease	≥ 50% decrease‡	≥ 50% decrease‡	No increase	≥ 50% decrease	≥ 50% decrease	Irrelevant
Stable disease*	Failure to attain complete/partial remission and no progressive disease	No change in size	No change in size	No change in size	No change in size	No change	No change
Relapsed disease or progressive disease	New or increased lesions	New or ≥ 50% increase§	New or ≥ 50% increase§	New or ≥ 50% increase	≥ 50% increase	New or ≥ 50% increase‖	Reappearance
Not assessable							

*Require each criterion to be present for a period of at least 4 weeks.
†Provided that < 5% of flower cells remained, complete remission was judged to have been attained if the absolute lymphocyte count, including flower cells, was $< 4 \times 10^9$/L.
‡Calculated by the sum of the products of the greatest diameters of measurable disease.
§Defined by ≥ 50% increase from nadir in the sum of the products of measurable disease.
‖Defined by ≥ 50% increase from nadir in the count of flower cells and an absolute lymphocyte count, including flower cells, of $> 4 \times 10^9$/L.

was defined as failure to attain CR/PR or PD. CR, unconfirmed CR, PR, and stable disease require each criterion for a period of at least 4 weeks.

Recently, revised response criteria were proposed for lymphoma. New guidelines were presented incorporating positron emission tomography (PET), especially for assessment of CR.[53] It is well known and described in the criteria that several kinds of lymphoma, including peripheral T-cell lymphomas, are variably [^{18}F]fluorodeoxyglucose avid.[53] No report described the PET results in response assessment of ATL until now. The usefulness of PET or PET/CT should be evaluated in response assessment of ATL in a prospective study. Meanwhile, PET or PET/CT should be used for evaluation of response when the tumorous lesions are fluorodeoxyglucose avid at diagnosis.

ISSUES FOR FUTURE INVESTIGATIONS IN ATL

Targeted Therapy

Several new agents against ATL are now under investigation. A promising targeted therapy for ATL is the combination of arsenic trioxide and IFN-α, which targets both Tax and the nuclear factor-κB pathway.[54-56] This combination exhibits clinical efficacy in relapsed/refractory ATL patients[57] and is currently being evaluated in untreated patients. Monoclonal antibodies against several molecules expressed on the surface of ATL cells and other lymphoid malignant cells, such as CD25, CD2, CD52, and chemokine receptor 4, have been promising in recent clinical trials. Histone deacetylase inhibitors such as vorinostat (suberoylanilide hydroxamic acid), romidepsin, and panobinostat (LBH589) have also been promising in preclinical and/or clinical studies against T-cell malignancies including ATL. Pralatrexate, a novel antifolate, and forodesine, a purine nucleotide phosphorylase inhibitor, are potential new agents with potent preclinical activity in T-cell malignancies including ATL. Other potential therapies for ATL under investigation include the combination of the proteasome inhibitor bortezomib with high-dose CHOP chemotherapy[58] and antiangiogenic therapy, such as anti–vascular endothelial growth factor monoclonal antibodies[59] or antitransferrin receptor.[60] Microarray analysis has identified survivin, β-catenin, syk, and lyn as potential targets for therapy.[61]

Prevention

Two steps should be considered for the prevention of HTLV-1–associated ATL. The first step is the prevention of HTLV-1 infection. This has been established in some HTLV-1 endemic areas in Japan by screening for HTLV-1 among blood donors and refraining from breast feeding among pregnant women who are carriers. The second step is the prevention of ATL development among HTLV-1 carriers. This has not been established partly because only approximately 5% of HTLV-1 carriers develop the disease in their lifetime and the risk factors remain unknown. Therefore, a cohort study of HTLV-1 carriers (Joint Study of Predisposing Factors for ATL Development) is ongoing nationwide in Japan.

Clinical Trials

Clinical trials have been paramount to the recent advances in ATL treatment, including assessment of chemotherapy, AZT/IFN-α, and alloHSCT, as described earlier. We have proposed a strategy for ATL treatment stratified by subclassification and prognostic factors. However, future clinical trials should be incorporated to ensure that the consensus is continually updated to establish evidence-based practice guidelines.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

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Identification of human minor histocompatibility antigens based on genetic association with highly parallel genotyping of pooled DNA

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Minor histocompatibility (H) antigens are the molecular targets of allo-immunity responsible both for the development of antitumor effects and for graft-versus-host disease (GVHD) in allogeneic hematopoietic stem cell transplantation (allo-HSCT). However, despite their potential clinical use, our knowledge of human minor H antigens is largely limited by the lack of efficient methods of their characterization. Here we report a robust and efficient method of minor H gene discovery that combines whole genome association scans (WGASs) with cytotoxic T-lymphocyte (CTL) assays, in which the genetic loci of minor H genes recognized by the CTL clones are precisely identified using pooled-DNA analysis of immortalized lymphoblastoid cell lines with/without susceptibility to those CTLs. Using this method, we have successfully mapped 2 loci: one previously characterized (*HMSD* encoding ACC-6), and one novel. The novel minor H antigen encoded by *BCL2A1* was identified within a 26 kb linkage disequilibrium block on chromosome 15q25, which had been directly mapped by WGAS. The pool size required to identify these regions was no more than 100 individuals. Thus, once CTL clones are generated, this method should substantially facilitate discovery of minor H antigens applicable to targeted allo-immune therapies and also contribute to our understanding of human allo-immunity. (Blood. 2008;111: 3286-3294)

Introduction

Currently, allogeneic hematopoietic stem cell transplantation (allo-HSCT) has been established as one of the most effective therapeutic options for hematopoietic malignancies[1] and is also implicated as a promising approach for some solid cancers.[2] Its major therapeutic benefits are obtained from allo-immunity directed against patients' tumor cells (graft-versus-tumor [GVT] effects). However, the same kind of allo-immune reactions can also be directed against normal host tissues resulting in graft-versus-host disease (GVHD). In HLA-matched transplants, both GVT and GVHD are initiated by the recognition of HLA-bound polymorphic peptides, or minor histocompatibility (H) antigens, by donor T cells. Minor H antigens are typically encoded by dichotomous single nucleotide polymorphism (SNP) alleles, and may potentially be targeted by allo-immune reactions if the donor and recipient are mismatched at the minor H loci. Identification and characterization of minor H antigens that are specifically expressed in hematopoietic tissues, but not in other normal tissues, could contribute to the development of selective antileukemic therapies while minimizing unfavorable GVHD reactions, one of the most serious complications of allo-HSCT.[3,4] Unfortunately, the total number of such useful minor H antigens that are currently molecularly characterized is still disappointingly small, including HA-1,[5] HA-2,[6] ACC-1[Y] and ACC-2,[7] DRN-7,[8] ACC-6,[9] LB-ADIR-1F,[10] HB-1,[11] LRH-1,[12] and 7A7-PANE1,[13] limiting the number of patients eligible for such GVT-oriented immunotherapy.

Several techniques have been developed to identify novel minor H antigens targeted by CTLs generated from patients who have undergone transplantation. Among these, linkage analysis based on the cytotoxicity of the CTL clones against panels of lymphoblastoid cell lines (B-LCLs) from large pedigrees was proposed as a novel genetic approach,[14] and has been successfully applied to identify novel minor H epitopes encoded by the *BCL2A1* and *P2RX5* genes.[7,12] Nevertheless, the technology is still largely limited by its resolution, especially when large segregating families are not available. Linkage analysis using B-LCL panels from the Centre d'Etude du Polymorphisme Humain (CEPH) could only localize minor H loci within a range of 1.64 Mb to 5.5 Mb, which still contained 11 to 46 genes,[7,12,14] thus requiring additional selection procedures to identify the actual minor H genes.

On the other hand, clinically relevant minor H antigens might be associated with common polymorphisms within the human

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population, and therefore could be ideal targets of genetic association studies, considering recent advances of large-scale genotyping technologies and the assets of the International HapMap Project.[15,16] In this alternative genetic approach using the extensive linkage disequilibrium (LD) found within the human genome, target loci can be more efficiently localized within relatively small haplotype blocks without depending on limited numbers of recombination events, given the large number of genotyped genetic markers.[17] Moreover, since the presence of a target minor H allele in individual target cells can be determined by ordinary immunologic assays using minor H antigen-specific CTLs, the characterization of minor H antigens should be significantly more straightforward than identifying alleles associated with typical common complex diseases, for which typically weak-to-moderate genetic effects have been assumed.[18]

In this report, we describe a high-performance, cost-effective method for the identification of minor H antigens, in which whole genome association scans (WGASs) are performed based on SNP array analysis of pooled DNA samples constructed from cytotoxicity-positive (CTX^+) and cytotoxicity-negative (CTX^-) B-LCLs as determined by their susceptibility to CTL clones. Based on this method, termed WGA/CTL, we were able to map the previously characterized ACC-6 minor H locus to a 115-kb block containing only 4 genes, including *HMSD*.[9] Moreover, using the same approach, a novel minor H antigen encoded by the *BCL2A1* gene was identified within a 26-kb block containing only *BCL2A1* on chromosome 15q25. Surprisingly, the pool size required to identify these regions was no more than 100 individuals. Thus, this WGA/CTL method has significant potential to accelerate the discovery of minor H antigens that could be used in more selective, and thus more effective, allo-immune therapies in the near future.

Methods

Cell isolation and cell cultures

This study was approved by the institutional review board of the Aichi Cancer Center and the University of Tokyo. All blood or tissue samples were collected after written informed consent was obtained in accordance with the Declaration of Helsinki. B-LCLs were derived from allo-HSCT donors, recipients, and healthy volunteers. B-LCLs were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate.

Generation of CTL lines and clones

CTL lines were generated from peripheral blood mononuclear cells (PBMCs) obtained after transplantation by stimulation with irradiated (33 Gy) recipient PBMCs harvested before HSCT, thereafter stimulated weekly in RPMI 1640 supplemented with 10% pooled human serum and 2 mM L-glutamine. IL-2 was added on days 1 and 5 after the second and third stimulations. CTL clones were isolated by standard limiting dilution and expanded as previously described.[7] CTL-1B9 was isolated from PBMCs harvested on day 30 after transplantation from a patient receiving a marrow graft from his HLA-identical sibling (HLA A11, A24, B39, B51, Cw7, Cw14), and CTL-2A12 has been described recently.[9]

Chromium release assay

Target cells were labeled with 0.1 mCi (3.7 MBq) of ^{51}Cr for 2 hours, and 10^3 target cells/well were mixed with CTL at the effector-to-target (E/T) ratio indicated in a standard 4-hour cytotoxicity. All assays were performed at least in duplicate. Percent specific lysis was calculated as follows: ((Experimental cpm − Spontaneous cpm) / (Maximum cpm − Spontaneous cpm)) × 100.

Immunophenotyping by enzyme-linked immunosorbent assay

B-LCL cells (20 000 per well, which had been retrovirally transduced with restriction HLA cDNA for individual CTLs, if necessary) were plated in each well of 96-well round-bottomed plates, and corresponding CTL clones (10 000 per well) were added to each well. After overnight incubation at 37°C, 50 μL supernatant was collected and released IFN-γ was measured by standard enzyme-linked immunosorbent assay (ELISA).

Construction of pooled DNA and microarray experiments

Genomic DNA was individually extracted from immunophenotyped B-LCLs. After DNA concentrations were measured and adjusted to 50 μg/mL using the PicoGreen dsDNA Quantitation Reagent (Molecular Probes, Eugene, OR), the DNA specimens from CTX^+ and CTX^- B-LCLs were separately combined to generate individual pools. DNA pools were analyzed in pairs using Affymetrix GeneChip SNP-genotyping microarrays (Affymetrix, Tokyo, Japan) according to the manufacturer's protocol,[19,20] where 2 independent experiments were performed for each array type (for more detailed statistical analysis for generated microarray data, see Document S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article).

Estimation of LD blocks

LD structures of the candidate loci were evaluated based on empirical data from the International Hap Map Project (http://www.hapmap.org/).[15] LD data for the relevant HapMap panels were downloaded from the HapMap web site and further analyzed using Haploview software (http://www.broad.mit.edu/mpg/haploview/).[21]

Transfection of 293T cells and ELISA

Twenty thousand 293T cells retrovirally transduced with HLA-A*2402 were plated in each well of 96-well flat-bottomed plates, cultured overnight at 37°C, then transfected with 0.12 μg of plasmid containing full-length *BCL2A1* cDNA generated from either the patient or his donor using Trans IT-293 (Mirus, Madison, WI). B-LCLs of the recipient and his donor were used as positive and negative controls, respectively. Ten thousand CTL-1B9 cells were added to each well 20 hours after transfection. After overnight incubation at 37°C, 50 μL of supernatant was collected and IFN-γ was measured by ELISA.

SNP identification by direct sequencing

Complementary DNA prepared from B-LCLs was polymerase chain reaction (PCR) amplified for the coding region of *BCL2A1* using the following primers: sense: 5′-AGAAGATGACAGACTGTGAATTTGG-3′; antisense: 5′-TCAACAGTATTGCTTCAGGAGAG-3′.

PCR products were purified and directly sequenced with the same primer and BigDye Terminator kit (version 3.1) by using ABI PRISM 3100 (Applied Biosystems, Foster City, CA).

Confirmatory SNP genotyping

Genotyping was carried out using fluorogenic 3′-minor groove binding (MGB) probes in a PCR assay. PCR was conducted in 10-μL reactions containing both allelic probes, 500 nM each of the primers, 1 × TaqMan Universal PCR Master Mix (Applied Biosystems), and 1 μL (100 ng) DNA. PCR cycling conditions were as follows: predenature, 50°C for 2 minutes, 95°C for 10 minutes, followed by 35 cycles of 92°C for 15 seconds and 60°C for 1 minute in a GeneAmp PCR System 9700 (Applied Biosystems). The PCR products were analyzed on an ABI 7900HT with the aid of SDS 2.2 software (Applied Biosystems).

Epitope reconstitution assay

The candidate *BCL2A1*-encoded minor H epitope and its allelic counterpart (DYLQYVLQI) peptides were synthesized by standard Fmoc chemistry. ^{51}Cr-labeled CTX^- donor B-LCLs were incubated with graded concentrations of the peptides and then used as targets in standard cytotoxicity assays.

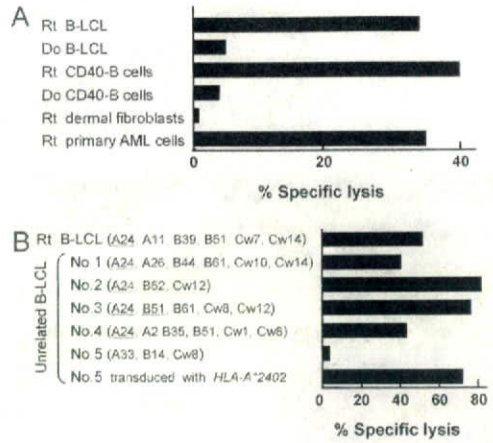

Figure 1. Specificity of CTL-1B9 against hematopoietic cells and its restriction HLA. (A) The cytolytic activity of CTL-1B9 was evaluated in a standard 4-hour ^{51}Cr release assay (E/T ratio, 20:1). Targets used were B-LCL, CD40-activated (CD40-B) B cells, dermal fibroblasts, and primary acute myeloid leukemia cells from the recipient (Rt), and B-LCL and CD40-B cells from his donor (Do). Rt dermal fibroblasts were pretreated with 500 U/mL IFN-γ and 10 ng/mL TNF-α for 48 hours before ^{51}Cr labeling. (B) Cytolytic activity of CTL-1B9 against a panel of B-LCLs derived from unrelated individuals, each of whom shared 1 or 2 class I MHC allele(s) with the recipient from whom the CTL-1B9 was generated. The shared HLA allele(s) with the recipient are underlined. B-LCLs (no. 5) which did not share any HLA alleles with the recipient, were retrovirally transduced with HLA-A*2402 cDNA and included to confirm HLA-A*2402 restriction by CTL-1B9. Results are typical of 2 experiments and data are the mean plus or minus the standard deviation (SD) of triplicates.

Results

CTL-based typing and SNP array analysis of pooled DNA

CTL-2A12 and CTL-1B9 are CTL clones established from the peripheral blood of 2 patients with leukemia who had received HLA-identical sibling HSCTs. Each clone demonstrated specific lysis against the B-LCLs of the recipient but not against donor B-LCLs, indicating recognition of minor H antigen (Figure 1A and Kawase et al[9]). The minor H antigen for CTL-2A12 had been previously identified by expression cloning[9]; on the other hand, the target minor H antigen for the HLA-A24–restricted CTL-1B9 clone, which was apparently hematopoietic lineage–specific (Figure 1A) and present in approximately 80% of the Japanese population (data not shown), had not yet been determined. Using these CTL clones, a panel of B-LCLs expressing the restriction HLA (HLA-B44 for CTL-2A12 and HLA-A24 for CTL-1B9) endogenously or retrovirally transduced, were subjected to "immunophenotyping" for the presence or absence of the minor H antigen by ELISA and, if necessary, by standard chromium release assay (CRA). Based on the assay results, for CTL-2A12 we initially collected 44 cytotoxicity-positive (CTX^+) and 44 cytotoxicity-negative (CTX^-) B-LCLs after screening 132 B-LCLs, while 57 CTX^+ and 38 CTX^- B-LCLs were obtained from 121 B-LCLs for CTL-1B9. From these sets of B-LCL panels, pools of DNA were generated and subjected to analysis on Affymetrix GeneChip 100 K and 500 K microarrays in duplicate.[19,20]

Detection of association between minor H phenotypes and marker SNPs

Genetic mapping of the minor H locus was performed by identifying marker SNPs that showed statistically significant deviations in allele-frequencies between CTX^+ and CTX^- pools based on the observed allele-specific signals in the microarray experiments. For this purpose, we evaluated the deviations of observed allele ratios between CTX^+ and CTX^- pools for each SNP on a given array (Document S1). An SNP was considered as positive for association if its test statistic exceeded an empirically determined threshold that provided a "genome-wide" P value of .05 in duplicate experiments (Document S1, Figures S1,S2, and Table S1). Threshold values for different pool sizes are also provided in Table S2 for further experiments. The positive SNPs eventually obtained for both CTLs are summarized in Table 1, where the 10 SNPs showing the highest test statistics are listed for individual experiments.

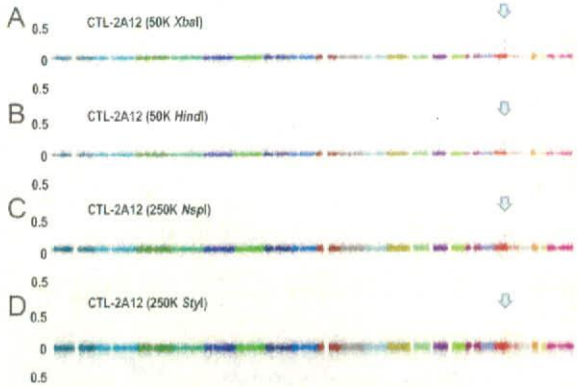

Figure 2. Whole genome association scans performed with pooled DNA generated based on immunophenotyping with CTL-2A12. Pooled DNAs generated from 44 CTX^+ and 44 CTX^- B-LCLs were analyzed with 50 K *Xba*I (A), 50 K *Hind*III (B), 250 K *Nsp*I (C), and 250 K *Sty*I (D) arrays. Test statistics were calculated for all SNPs and plotted in the chromosomal order. In all SNP array types, a common association peak is observed at 18q21, to which the minor H antigen for CTL-2A12, encoded by the *HMSD* gene, had been mapped based on expression cloning[9] (arrows).

Mapping of the minor H loci by WGASs

All the SNPs significantly associated with susceptibility to CTL-2A12 were correctly mapped within a single 115 kb LD block at chromosome 18q21 containing the *HMSD* gene (Figures 2 and 3A), which had been previously shown to encode the ACC-6 minor H antigen recognized by CTL-2A12.[9] According to the above criteria, no false-positive SNPs were reported in any array types (Table 1). Confirmation genotyping of individual B-LCLs from both panels revealed none of the 44 that had been immunophenotyped as CTX^- were misjudged, while 8 of the 44 CTX^+ B-LCLs were found to actually carry no minor H-positive allele for ACC-6, which was likely due to the inclusion of individual B-LCLs showing borderline cytotoxicity (data not shown).

On the other hand, positive association of the target minor H antigen with CTL-1B9 was detected in 2 independent loci: SNP rs1879894 at 15q25.1 in 250 K *Nsp*I (Table 1, Figure 4A-B, and Figure S5) and SNP rs1842353 at 8q12.3 in 50 K *Hind*III (Table 1 and Figure S3A). We eventually focused on rs1879894, as it showed a much more significant genome-wide P value than SNP rs1842353 (Table 1). In contrast to the CTL-2A12 case, where many mutually correlated SNPs around the most significant one created a broad peak in the statistic plots (Figure 2 arrows and Figure S3), the adjacent SNPs (rs6495463 and rs2562756; Figure 3B solid arrows) around rs187894 (Figure 3B open arrow) did not show large test statistic values, reflecting the fact that no marker SNPs on 100 K and 500 K arrays exist in high LD (Figure 3B dashed red lines encompassing 26 kb) with this SNP according to the HapMap data. To further confirm the association, we generated additional B-LCL pools consisting of 75 CTX^+ and 34 CTX^-

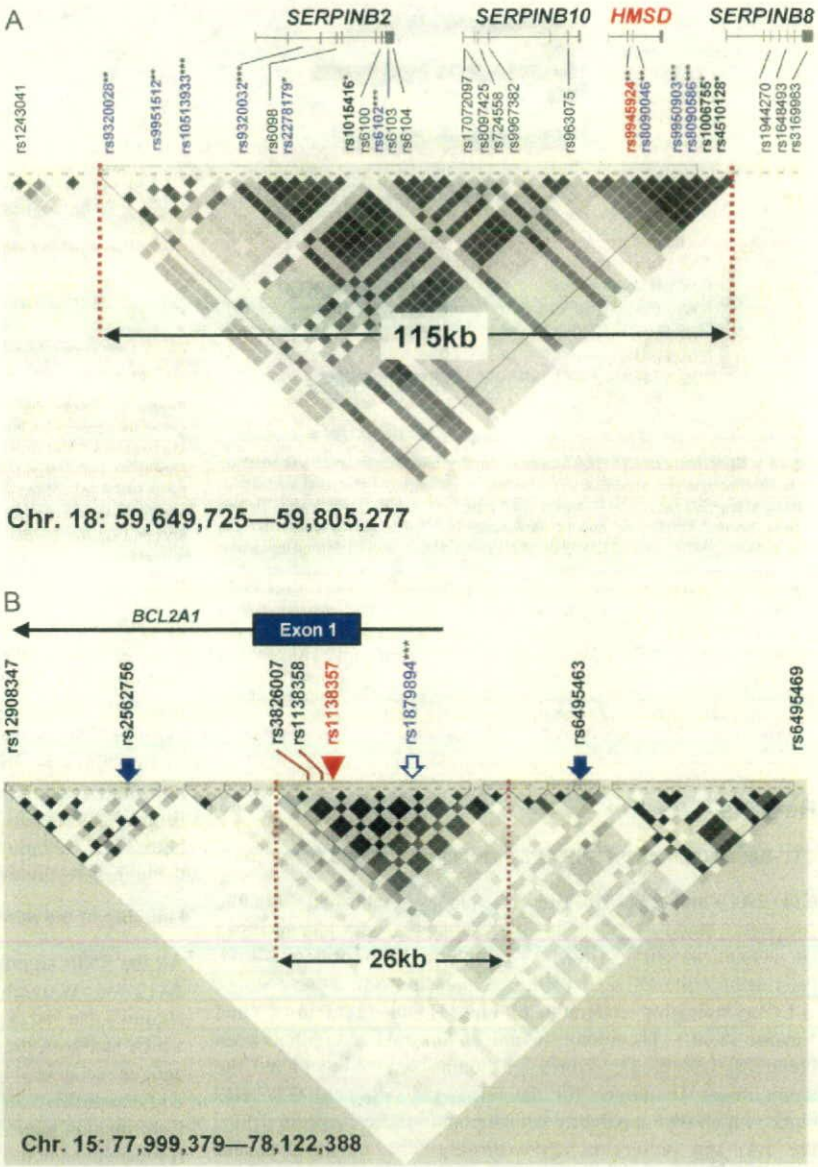

Figure 3. Linkage disequilibrium (LD) block mapped by CTL-2A12 and CTL-1B9. (A) An LD block map identified by pairwise r^2 plot from HapMap CEU data are overlayed with SNPs available from Affymetrix GeneChip SNP-genotyping microarrays (arrows) and 4 genes in the 115 kb block. SNPs that emerged repeatedly in the 2 independent experiments are indicated in blue. The genomewide *P* values for positive SNPs are shown as follows: $*P < .05$; $**P < .01$; $***P < .001$. The intronic SNP (rs9945924) controlling the alternative splicing of *HMSD* transcripts and expression of encoded ACC-6 minor H antigen is indicated in red. (B) LD blocks identified by pairwise r^2 plot from HapMap JPT data are overlayed with SNPs available from Affymetrix GeneChip SNP-genotyping microarrays (arrows) and exon 1 of the *BCL2A1* gene. The only SNP showing a high association with CTL-1B9 immunophenotypes (rs1879894) is shown as an open arrow. The nonsynonymous SNP (rs1138357) controlling the expression of the minor H antigen recognized by CTL-1B9 is indicated by a red arrowhead. ***SNP with genomewide $P < .001$. The 2 SNPs adjacent to the 26 kb LD block (rs2562756 and rs6495463) never gave a significant genomewide *P* value.

B-LCLs from another set of 128 B-LCLs, and performed a WGAS. As expected, the WGAS of the second pools also identified the identical SNP with the highest test statistic value in duplicate experiments, unequivocally indicating that this SNP is truly associated with the minor H locus of interest (Figure 4C,D and Table S3). The association was also detected when the references in the first and second pools were swapped (data not shown).

Identification of the minor H epitope recognized by CTL-1B9

The LD block containing SNP rs1879894 that was singled out from more than 500 000 SNP markers with 2 sets of DNA pools only encodes exon 1 of *BCL2A1* (Figure 3B). To our surprise, this was the region to which we had previously mapped an HLA-A24–restricted minor H antigen, ACC-1^{Y}.[7] We first confirmed that full-length *BCL2A1* cDNA cloned only from the recipient but not his donor could stimulate interferon-γ secretion from CTL-1B9 when transduced into donor B-LCL (Figure 5A), indicating that *BCL2A1* is a bona fide gene encoding minor H antigen recognized by CTL-1B9. We next genotyped 3 nonsynonymous SNPs in the *BCL2A1* exon 1 sequence (Figure 3B) and comparison was made between the genotypes and the susceptibility to CTL-1B9 of 9 HLA-A*2402^{+} B-LCLs, including ones generated from the recipient (from whom CTL-1B9 was established) and his donor. Susceptibility to CTL-1B9 correlated completely with the presence of guanine at SNP rs1138357 (nucleotide position 238, according to the mRNA sequence for NM_004049.2) and thymine at SNP rs1138358 (nucleotide position 299) (Table 2), suggesting that the expression of the minor H epitope recognized by CTL-1B9 is controlled by either of these SNPs. We searched for nonameric amino acid sequences spanning the 2 SNPs using BIMAS software,[22] since most reported HLA-A*2402 binding peptides contain 9 amino acid residues.[23] Among these, a nonameric peptide, DYLQ*C*VLQI (the polymorphic residue being underlined), has a predicted binding score of 75 and was considered as a candidate minor H epitope. As shown in Figure 5B, the DYLQ*C*VLQI was strongly recognized by CTL-1B9, whereas its allelic counterpart,

Table 1. Positive SNPs from pooled DNA analysis

CTL-2A12, Exp 1				CTL-2A12, Exp 2				CTL-1B9, Exp 1				CTL-1B9, Exp 2			
rsID	Chr	Position	$\Delta R_{A\Delta R_B}$	rsID	Chr	Position	$\Delta R_{A\Delta R_B}$	rsID	Chr	Position	$\Delta R_{A\Delta R_B}$	rsID	Chr	Position	$\Delta R_{A\Delta R_B}$
50K X bal															
rs10513933	18	59699669	0.366*	rs10513933	18	59699669	0.511†	rs1363258	5	103297593	0.239	rs10499174	6	131209689	0.352*
rs9320028	18	59668150	0.255‡	rs9320028	18	59668150	0.360*	rs726083	3	67093729	0.203	rs30058	5	122325602	0.240
rs6102	18	59721450	0.221	rs10485873	7	3503743	0.157	rs639243	5	31392931	0.198	rs150724	16	61960443	0.213
rs724533	23	116440574	0.137	rs219323	14	59510440	0.150	rs1936461	10	56519024	0.186	rs1993129	8	63618836	0.208
rs1341112	6	104919391	0.136	rs10506892	12	82478539	0.147	rs763876	12	94922502	0.186	rs356946	13	69066751	0.201
rs470490	18	61182216	0.136	rs10492269	12	97786333	0.144	rs958404	7	133054441	0.179	rs2869268	4	86421898	0.184
rs2826718	21	21471423	0.134	rs10483466	14	35986827	0.139	rs10486727	7	41672315	0.178	rs287002	12	40312537	0.183
rs10506697	12	73241741	0.128	rs5910124	23	116408616	0.137	rs2833488	21	32010112	0.176	rs1146808	13	67688608	0.182
rs10506891	12	82393029	0.127	rs10512545	17	66337079	0.134	rs379212	5	60977687	0.172	rs10501287	11	42446011	0.180
rs308995	14	59657919	0.125	rs295678	5	58186928	0.131	rs1954004	14	58627872	0.170	rs564993	5	31393476	0.177
50K *Hind*III															
rs9320032	18	59712191	0.486†	rs9320032	18	59712191	0.506†	rs1842353	8	63617543	0.244*	rs9300692	13	101216476	0.225‡
rs8090046	18	59773066	0.207‡	rs8090046	18	59773066	0.245*	rs10521202	17	12755289	0.201‡	rs1842353	8	63617543	0.210‡
rs1474220	2	108525317	0.193‡	rs10498752	6	41876488	0.210‡	rs7899961	10	59696431	0.198‡	rs10520983	5	31314700	0.195‡
rs10498752	6	41876488	0.178	rs1941538	18	37994337	0.176	rs9320974	6	124421441	0.197‡	rs1334375	13	80897038	0.173
rs2298578	21	21632551	0.167	rs7682770	4	152748018	0.174	rs10520983	5	31314700	0.179	rs10519164	15	75412758	0.163
rs7516032	1	91618962	0.165	rs1445862	5	3675257	0.169	rs1862446	5	147460749	0.170	rs9322063	6	146852196	0.152
rs5030938	10	70645922	0.164	rs4696976	4	21058616	0.167	rs1358778	20	13266796	0.169	rs8067384	17	37926265	0.150
rs1883041	21	44921845	0.158	rs5030938	10	70645922	0.165	rs1873790	4	83422480	0.166	rs10521202	17	12755289	0.147
rs3902916	4	189045176	0.155	rs3902916	4	189045176	0.165	rs1220724	4	70888705	0.162	rs7914904	10	62749969	0.141
rs1000551	20	58709208	0.154	rs1883041	21	44921845	0.164	rs9300692	13	101216476	0.157	rs1220724	4	70888705	0.141
250K *Nsp*I															
rs9950903	18	59781783	0.534†	rs9950903	18	59781783	1.036†	rs1879894	15	78055874	0.846†	rs1879894	15	78055874	1.072†
rs1463835	3	23539615	0.532†	rs8090586	18	59781864	0.518†	rs9646294	16	6110019	0.484†	rs6771859	3	190642054	0.387†
rs16975459	18	37802275	0.383*	rs6473170	8	80664840	0.338*	rs17734332	5	134945240	0.365†	rs10512261	9	98804394	0.299*
rs8090586	18	59781864	0.367*	rs4510128	18	59782312	0.310‡	rs566619	7	41381538	0.345*	rs12122772	1	60384564	0.287*
rs16872621	4	22081055	0.312‡	rs1006755	18	59782026	0.300‡	rs17737566	6	50345280	0.310*	rs2153155	4	26034162	0.248‡
rs870582	6	125097114	0.301‡	rs7039378	9	118735938	0.258	rs3849955	9	28350374	0.285*	rs17126896	14	53320494	0.246‡
rs1015416	18	59720363	0.270‡	rs1860563	16	6418899	0.258	rs4616156	13	86581518	0.273*	rs1328652	13	35607527	0.240
rs2155907	11	97599883	0.227	rs4699126	4	105709109	0.212	rs2484698	1	217474460	0.263*	rs7021551	9	27446645	0.237
rs2112948	5	50994294	0.222	rs10275055	7	156212079	0.204	rs17139603	11	79638632	0.262*	rs252817	5	106752487	0.237
rs2919747	2	129681506	0.217	rs1526411	7	124658309	0.201	rs2156737	4	100642529	0.246‡	rs10772587	12	12681356	0.235
250K *Sty*I															
rs6102	18	59721450	0.597†	rs6102	18	59721450	0.495†	rs9383925	6	151975774	0.819†	rs201204	6	104842863	0.688†
rs9951512	18	59690885	0.374*	rs9945924	18	59771746	0.407*	rs6497397	16	19646258	0.311‡	rs12556155	23	108836419	0.442†
rs6496897	15	90493249	0.320‡	rs9951512	18	59690885	0.317‡	rs917252	7	22219990	0.289‡	rs4791422	17	10605304	0.435†
rs9945924	18	59771746	0.315‡	rs1983205	3	157782892	0.314‡	rs1019403	3	7823997	0.260‡	rs7749012	6	106459559	0.336*
rs12707805	8	107404746	0.303‡	rs950865	5	2720684	0.307‡	rs17053134	5	155373544	0.259‡	rs509951	5	31385483	0.308‡
rs10971778	9	33893184	0.296‡	rs2278179	18	59715512	0.292‡	rs11710880	3	72214965	0.246	rs16879024	8	32225711	0.256‡
rs6565076	16	81487818	0.294‡	rs10427722	22	36417752	0.289‡	rs17167866	7	13919264	0.237	rs2100054	15	75293482	0.252
rs2278179	18	59715512	0.291‡	rs17156659	7	82046820	0.271	rs10867062	9	137935241	0.237	rs11811023	1	143805934	0.240
rs7806238	7	29906442	0.290‡	rs4502324	18	4811261	0.262	rs5925800	23	23278707	0.235	rs17382798	15	75256074	0.231
rs965888	18	38062658	0.283‡	rs1348428	2	225927288	0.260	rs2255831	4	146614313	0.234	rs2030302	17	12526591	0.231

Significant SNPs that appeared on both experiments are underlined.
*Genomewide $P < .01$.
†Genomewide $P < .001$.
‡Genomewide $P < .05$.

DYLQ*Y*VLQI, was not. Decameric peptide, QDYLQ*C*VLQI, on the other hand, appeared to be weakly recognized; however, it is likely that the nonameric form was actually being presented after N-terminal glutamine cleavage by aminopeptidase in the culture medium. Because it was possible that the cystine might be cysteinylated, recognition of synthetic peptides DYLQCVLQI and cysteinylated DYLQC*VLQI were assayed using CTL-1B9. Half-maximal lysis for the former was obtained at a concentration of 200 pM, whereas recognition of the latter was several-fold weaker (Figure 5C). Thus, we concluded that DYLQCVLQI defines the cognate HLA-A*2402–restricted CTL-1B9 epitope, now designated ACC-1^C. This incidentally provides a second example of products from both dichotomous SNP alleles being recognized as HLA-A*2402–restricted minor H antigens, the first example being the HB-1 minor H antigen.[24] Finally, real-time quantitative PCR revealed that T cells carrying the complementarity-determining region 3 sequence identical to CTL-1B9 became detectable in the patient's blood at the frequencies of 0.22%, 0.91%, 1.07% and 0.01% among $TCR\alpha\beta^+$ T cells at days 30, 102, 196, and 395 after transplantation, respectively, suggesting that ACC-1^C minor H antigen is indeed immunogenic (Figure 5D).

Discussion

Recent reports have unequivocally demonstrated that WGASs can be successfully used to identify common variants involved in a wide variety of human diseases.[25-27] Our report represents a novel

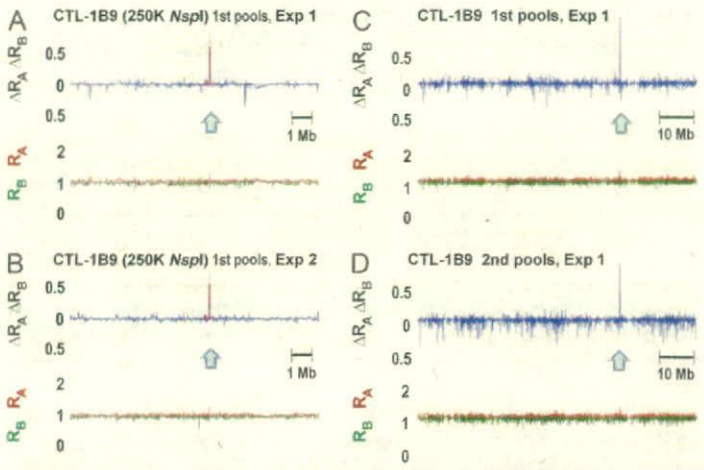

Figure 4. Reproducible detection of association with the immunophenotypes determined by CTL-1B9 at the *BCL2A1* locus. The maximum test statistic value was observed at a single SNP (rs1879894) within 15q25.1 in duplicate experiments for the first pools consisting of 57 CTX$^+$ and 38 CTX$^-$ B-LCLs (A-C). The peak association at the same SNP was reproduced in the experiments with the second pools consisting of 75 CTX$^+$ and 34 CTX$^-$ LCLs (D). Test statistic values ($\Delta R_A \Delta R_B$) are plotted by blue lines together with their R_A (red) and R_B (green) values. The expected $\Delta R_A \Delta R_B$ values multiplied by r^2 correlation coefficients for the adjacent SNPs within 500 kb from the SNP rs1879894 are overlaid by red lines (A,B).

application of WGASs to transplantation immunology, which provides a simple but robust method to fine-map the genetic loci of minor H antigens whose expression is readily determined by standard immunophenotyping with CTL clones established from patients who have undergone transplantation.

The current WGA/CTL method has several desirable features that should contribute to the acceleration of minor H locus mapping. In comparing the method to those of linkage analysis and other nongenetic approaches, including direct peptide sequencing of chemically purified minor H antigens[5,6,10,13] and conventional

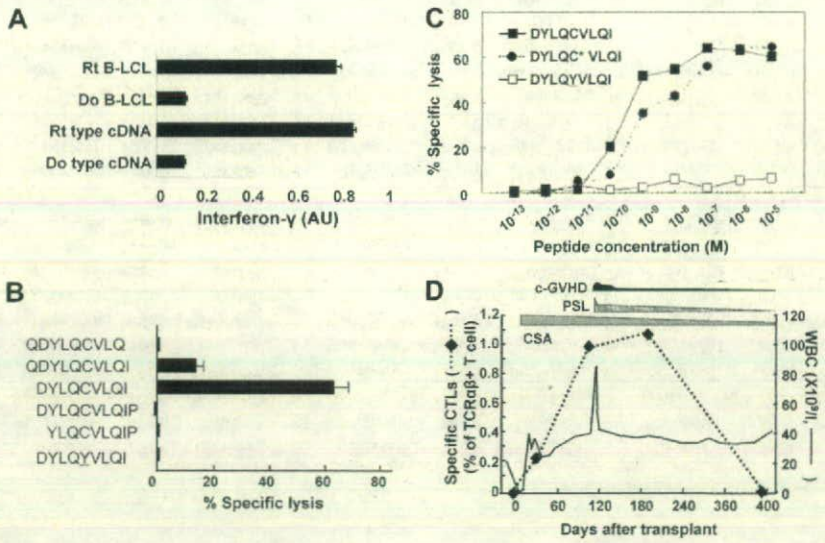

Figure 5. Identification of the CTL-1B9 minimal minor H epitope. (A) Interferon-γ production from CTL-1B9 against HLA-A*2402–transduced 293T cells transfected with plasmid encoding full-length *BCL2A1* cDNA cloned from either the recipient (Rt) from whom CTL-1B9 was isolated or his donor (Do). Rt B-LCL and Do B-LCL were used as positive and negative controls, respectively. Secreted interferon-γ was measured by ELISA and is expressed in arbitrary units (AUs) corresponding to optical density at 630 nm. Results are typical of 2 experiments and data are the mean plus or minus SD of triplicates. (B) A peptide reconstitution assay was conducted to determine the minimal epitope for CTL-1B9. Nonameric peptide (DYLQCVLQI), 2 nonameric peptides shifted by one amino acid to N- or C-terminus, N- and C-terminal extended decameric peptides, and its allelic counterpart (DYLQ**Y**VLQI) were synthesized and tested by adding to antigen-negative donor B-LCL at 10 nM in a standard ^{51}Cr release assay. Results are typical of 2 experiments and data are the mean plus or minus SD of triplicates. (C) Titration of the candidate minor H peptide by epitope reconstitution assay. Chromium-labeled donor B-LCLs were distributed to wells of 96-well round-bottomed plates, pulsed with serial dilutions of the indicated peptides for 30 minutes at room temperature, and then used as targets for CTL-1B9 in a standard ^{51}Cr release assay. A cysteinylated peptide (indicated by an asterisk) was included as an alternative form of the potential epitope. Results are typical of 2 experiments. (D) Tracking of ACC-1^C–specific T cells in the recipient's peripheral blood. In order to longitudinally analyze the kinetics of the ACC-1^C–specific CTLs in peripheral blood from the patient from whom CTL-1B9 was established, a real-time quantitative PCR was conducted. Complementary DNAs of peripheral blood mononuclear cells from the donor and patient before and after HSCT were prepared from the patient. Real-time PCR analysis was performed using a TaqMan assay as described previously.[9] The primers and fluorogenic probe sequences spanning the CTL-1B9 complementarity-determining region 3 (CDR3) were used to detect T cells carrying the CDR3 sequences identical to that of CTL-1B9. The primers and fluorogenic probe sequences spanning constant region of TCR beta chain (TCRBC) mRNA were used as internal control. Samples were quantified with the comparative CT method. The delta CT value was determined by subtracting the average CT value for TCRBC from the average CTL-1B9 CDR3 CT value. The standard curve for the proportion of CTL-1B9 among TCR$\alpha\beta^+$ T cells was composed by plotting mean delta CT values for each ratio, and the percentages of T cells carrying the CDR3 sequence identical to CTL-1B9 were calculated by using this standard curve. During this period, quiescent chronic GVHD, which required steroid treatment, developed; however, involvement of immune reaction to ACC-1^C minor H antigen was unlikely since its frequency increased even after resolution of most chronic GVHD symptoms. c-GVHD, chronic GVHD; CSA, cyclosporine A; PSL, prednisolone; WBC, white blood cell count.