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Research Article

A Comprehensive Study of the Immunophenotype and its Clinicopathologic Significance in Adult T-Cell Leukemia/Lymphoma

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ABSTRACT

Adult T-cell leukemia/lymphoma (ATLL) is a mature T-cell tumor caused by human T-lymphotropic virus type 1 (HTLV-1). The typical ATLL immunophenotypes are described in the 2017 World Health Organization Classification of Tumours of Haematopoietic and Lymphoid Tissues (positive: CD2, CD3, CD5, CD4, and CD25; negative: CD7, CD8, and cytotoxic markers; and partially positive: CD30, CCR4, and FOXP3). However, limited studies are available on the expression of these markers, and their mutual relationship remains unknown. Furthermore, the expression status of novel markers associated with T-cell lymphomas, including Th1 markers (T-bet and CXCR3), Th2 markers (GATA3 and CCR4), T follicular helper markers (BCL6, PD1, and ICOS), and T-cell receptor (TCR) markers, and their clinicopathologic significance is unclear. In this study, we performed >20 immunohistochemical stains in 117 ATLL cases to determine the comprehensive immunophenotypic profile of ATLL, which were compared on the basis of clinicopathologic factors, including morphologic variants (pleomorphic vs anaplastic), biopsy locations, treatments, Shimoyama classification-based clinical subtype, and overall survival. CD3+/CD4+/CD25+/CCR4+ was considered a typical immunophenotype of ATLL, but approximately 20% of cases did not conform to this pattern. Simultaneously, the following new findings were obtained: (1) most cases were negative for TCR-β and TCR-δ (104 cases, 88.9%), indicating the usefulness of negative conversion of TCR expression to provide differentiation from other T-cell tumors; (2) the positivity of CD30 and CD15 and the negativity of FOXP3 and CD3 were significantly associated with anaplastic morphology; and (3) atypical cases, such as T follicular helper marker-positive (12 cases, 10.3%) and cytotoxic molecule-positive cases (3 cases, 2.6%), were

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identified. No single markers could predict the overall survival among patients with acute/lymphoma subtypes of ATLL. The results of this study illustrate the diversity of ATLL phenotypes. In T-cell tumors occurring in HTLV-1 carriers, the possibility of ATLL should not be eliminated even when the tumor exhibits an atypical phenotype, and the confirmation of HTLV-1 in the tissue is recommended.

Introduction

Adult T-cell leukemia/lymphoma (ATLL) is a mature T-cell tumor caused by human T-lymphotropic virus type 1 (HTLV-1) and composed of lymphocytes with severe nuclear pleomorphism. The 2017 World Health Organization (WHO) Classification of Tumours of Haematopoietic and Lymphoid Tissues describes its immunophenotype as having an expression of T-cell markers (CD2, CD3, and CD5), but CD7 expression is usually lost.[1] Most cases are CD4+/CD8-, but there are a small number of CD4-/CD8+ or CD4+/CD8+ cases. CD25 is positive in almost all cases, and CD30 is positive in large-transformed cells. Cytotoxic markers are negative, although CCR4 and FOXP3, the markers associated with regulatory T cells (Treg), are sometimes partially positive in tumor cells.

The expression status for several markers in ATLL has only been analyzed in a few studies[2-4]; however, the accurate positive rates in tumor cells for these markers and their mutual relationships remain unknown. Concurrently, several novel markers have recently been reported as useful in confirming a diagnosis and predicting prognosis for T-cell lymphomas. For peripheral T-cell lymphoma not otherwise specified (PTCL-NOS), prognosis can be predicted on the basis of expression status of Th1 (T-bet and CXCR3) and Th2 markers (GATA3 and CCR4).[5] The expression of T follicular helper (TFH) markers (such as BCL6, PD1, and ICOS) can differentiate nodal PTCL from TFH phenotype.[6] Primary cutaneous gamma delta T-cell lymphoma and others can be differentiated by the expression of T-cell receptor (TCR)-β or TCR-γ.[1] However, the expression status of these novel markers and their clinicopathologic significance in ATLL have not been studied.

Our study seeks to determine the comprehensive immunophenotypic profile of ATLL and explore its clinicopathological significance by performing 21 immunohistochemical (IHC) stains in 117 ATLL cases (23 stains for 68 cases and 22 stains for 12 cases).

Materials and Methods

Tissue Samples

We selected 131 of the 143 cases diagnosed with ATLL in the Okinawa Prefecture between January 2016 and December 2020 (University of the Ryukyus Hospital [82 cases], Nakagami Hospital [34 cases], and Okinawa Prefectural Nanbu Medical Center and Children's Medical Center [27 cases]), excluding those for which specimen preparation was difficult due to small sample volume (12 cases). We further excluded 14 cases in which the identification and the evaluation of tumor cells in all 21 IHC-stained specimens seemed difficult as the distribution of solid sheets of tumor cell were not identified. The final number of cases for this study was 117. We retrieved multiple samples in 31 cases; in 11 cases, we examined 1 sample each before and after treatment, comparing them for morphology and immunophenotype (Supplementary Table S1), and in the remaining 20 cases, 1 representative specimen with higher tumor content and less artifact was selected.

Medical records were reviewed for age, sex, sampling site, chemotherapy/immunotherapy (chemotherapy and/or anti-CCR4 antibody therapy: mogamulizumab), Shimoyama classification-based clinical subtype,[7] and overall survival (Table 1). Samples were collected from the lymph nodes (LN) in 47 cases, skin in 47 cases, bone marrow (BM) in 11 cases, and other sites in 12 cases. All cases were divided into the following 2 morphologic variants: (1) pleomorphic type (medium and large cell) and (2) anaplastic large cell type, on the basis of the morphology of the tumor in the hematoxylin and eosin stained specimen.[8] We included no rare morphologic variants, such as Hodgkin-like or T-prolymphocytic leukemia-like.[9,10]

Table 1
Patient characteristics of the 117 adult T-cell leukemia/lymphoma cases

Factor	Total (N = 117)
Age	
Median (range)	69 (32-91)
Sex	
Male/female	54/63
Sampling site	
Lymph node	47
Skin	47
Bone marrow	11
Others	12
Subcutaneous	2
Stomach	2
Pleural effusion	2
Pharynx	2
Bone	1
Breast	1
Nasal cavity	1
Paranasal cavity	1
Chemotherapy/immunotherapy[a]	
-/+	97/20
No chemotherapy/immunotherapy	97
After immunochemotherapy	10
After chemotherapy	9
After Mogamulizumab	1
Morphology[b]	
Pleomorphic/anaplastic	102/15
Shimoyama classification[7]	
Smoldering	12
Chronic	38
Lymphoma	9
Acute	58
Overall survival (mo)	
Median (range)	13 (0.5-240)

[a] Chemotherapy/immunotherapy: chemotherapy and/or mogamulizumab.
[b] Pleomorphic: pleomorphic (medium and large cell) type; anaplastic: anaplastic large cell type.

Of the 117 cases, 97 had not undergone chemotherapy/immunotherapy at the time of sampling, and 20 were biopsied after chemotherapy/immunotherapy. Of these 20 patients, 10 had received chemotherapy and mogamulizumab, 9 had received only chemotherapy, and 1 had received only mogamulizumab. Detailed information about genetic alterations was obtained in 10 cases according to a previous report in which their corresponding fresh frozen tissues or peripheral blood mononuclear cells were analyzed.[11]

Immunohistochemical Stains

We performed IHC stains using an automated staining system (Ventana Benchmark ULTRA, Roche Diagnostics) for the following 21 different stains: (1) CD2, (2) CD3, (3) CD4, (4) CD5, (5) CD7, (6) CD8, (7) CD15, (8) CD25, (9) CD30, (10) CCR4, (11) FOXP3, (12) TIA1, (13) BCL6, (14) PD1, (15) ICOS, (16) T-bet, (17) CXCR3, (18) GATA3, (19) TCR-β, (20) TCR-δ, and (21) TCL1 (Supplementary Table S2). Antigen retrieval was performed with CC1 (pH 8.5) for all staining. We also used the archived IHC slides at each institution. CD10 and CXCL13 were also examined in 12 cases and were stained using BOND-MAX (Leica Biosystems) as follows: (1) CD10 (56C6, Nichirei Biosciences, × 2, ER2, pH 9.0) and (2) CXCL13 (polyclonal Goat IgG, R&D systems, × 100, ER1, pH 6.0). p53 and MUM1 were additionally examined in 68 cases (mainly composed of acute and lymphoma subtypes) and were stained using BOND-MAX as follows: (1) p53 using the DO7 antibody (Agilent Technologies, × 200, ER1, pH 6.0) and (2) MUM1 using the M-17 antibody (Santa Cruz Biotechnology, × 1000, ER1, pH 6.0).

The percentage of positive cells in tumor cells (positive rate) was examined for each IHC stain. Referencing previous studies, the cutoff value was set at 30%, with 30% or more labeled "positive" and less than 30% labeled "negative."[4,12] The percentages of strong and weak positives were also examined for CD7, CCR4, p53, and MUM1 (Supplementary Fig. S1).

Flow Cytometry

Flow cytometry (FCM) was performed to evaluate the surface expression of CD2, CD3, CD5, CD4, CD8, CD7, CD25, and CD30 simultaneously with the IHC stains. Fresh single-cell suspensions were isolated by FCM on a FACSCanto II instrument (BD Biosciences) using fluorescein isothiocyanate-conjugated CD2, CD3, CD4, and CD30 antibodies and phycoerythrin-conjugated CD5, CD7, CD8, and CD25 antibodies, all of which were purchased from Beckman Coulter, except anti-CD25 (BD Biosciences) and CD30 (Agilent Technologies).

Ultrasensitive RNA in Situ Hybridization Targeting Human T-cell Leukemia Virus Type 1 bZIP factor

Ultrasensitive RNA in situ hybridization targeting HTLV-1 bZIP factor (HBZ-ISH) was performed according to the experimental procedure previously described.[13]

Southern Blot Hybridization

Southern blot hybridization (SBH) was performed using DNA extracted from raw tissue samples matching the formalin-fixed, paraffin-embedded specimens using an experimental procedure previously described.[14]

Statistical Analysis

Statistical analysis was performed using EZR with a P value $<.05$.[15] Fischer exact test was performed to analyze correlation between the IHC stains and clinicopathologic findings, including age, sex, morphology, presence/absence of chemotherapy/immunotherapy, sampling site, clinical subtype, or genetic alteration. The Kaplan–Meier overall survival curves for the IHC stains and clinical subtype were compared using the log-rank test.

Results

General Findings

Figure 1 shows the positive rate for the 21 IHC stains; T-bet and TCL1 were completely negative (0%) in all cases, whereas positive rates for other stains varied from case to case. Figure 2 represents a case with a typical immunophenotype. Table 2 lists the ratios of all positive and negative cases.

Of the 117 cases, T-cell marker expression was positive for CD2 in 108 (92.3%), CD3 in 114 (97.4%), and CD5 in 95 (81.2%) cases. Although there was 1 case (0.9%) in which expression of CD2, CD3, and CD5, all pan T-cell markers, were not detected, this case was evaluated as ATLL with anaplastic large cell type, positive for CD4, CD25, CCR4, CD30, and HBZ-ISH (Supplementary Fig. S2). Cases exhibiting CD3+/CD4+/CD8-/CD7-/CD25+ were 61/117 (52.1%), CD3+/CD4+/CD8-/CD7-/CD25+/CCR4+ were 61/117 (52.1%), CD3+/CD4+/CD8-/CD25+/CCR4+ were 77/117 (65.8%), and CD3+/CD4+/CD25+/CCR4+ were 96/117 (82.1%).

CD7 was evaluated as "negative" in 81 cases (69.2%, positive rate <30%), and it was entirely negative in 47 cases (40.2%, positive rate of 0%). Among the 36 CD7-positive cases (positive rate of ≥30%), none was confirmed with 100% strong positivity (Supplementary Fig. S3). In the present study, the attenuation of CD7 in comparison with CD3 was confirmed in 2 cases in which 80% of the tumor cells were strongly positive; this was the highest positive rate with strong intensity (Fig. 3). CD25 expression was mostly positive in 111/117 (94.9%) cases; CD30 was positive in 39 (33.3%) cases, whereas CD15 was positive in only 2 (1.7%) cases. The CD15-positive cases were also CD30-positive. TIA1, a cytotoxic marker, was negative in most cases (114 [97.4%]). The expression of Treg markers was positive for CCR4 and FOXP3 in 109 (93.2%) and 23 (19.7%) cases, respectively.

Most cases in the present study were CD4+/CD8− (91 [77.8%] cases), although CD4+/CD8+ cases also constituted a relatively large portion (20 [17.1%] cases) (Supplementary Table S3). There were 3 (2.6%) CD4-/CD8+ (2.6%) and 3 (2.6%) CD4-/CD8− cases. Looking at TCR expression, most cases were negative for both TCR-β and TCR-δ (104 [88.9%] cases); however, 12 (10.3%) cases were TCR-β positive/TCR-δ negative. One (0.9%) patient showed positive results for both TCR-β and TCR-δ. None was confirmed to be TCR-δ positive/TCR-β negative. The correlations of each IHC stain were examined for CD4 vs other antibodies, CD8 vs other antibodies, TCR-β vs TCR-δ, and CD30 vs FOXP3. As for the correlations between CD7 and CD8, 14/23 (60.9%) CD8-positive cases were also CD7 positive, whereas 72/94 (76.6%) CD8-negative cases were CD7 negative, indicating significant correlations in each expression (P <.01). No other combinations exhibited any correlation.

In the 4 cases in which IHC and FCM were performed simultaneously on LN samples, results were comparatively investigated for CD2, CD3, CD5, CD4, CD8, CD7, CD25, and CD30, all of which were consistent except for CD3. No markers were positive in FCM and negative in IHC. CD3 results were completely different in 2

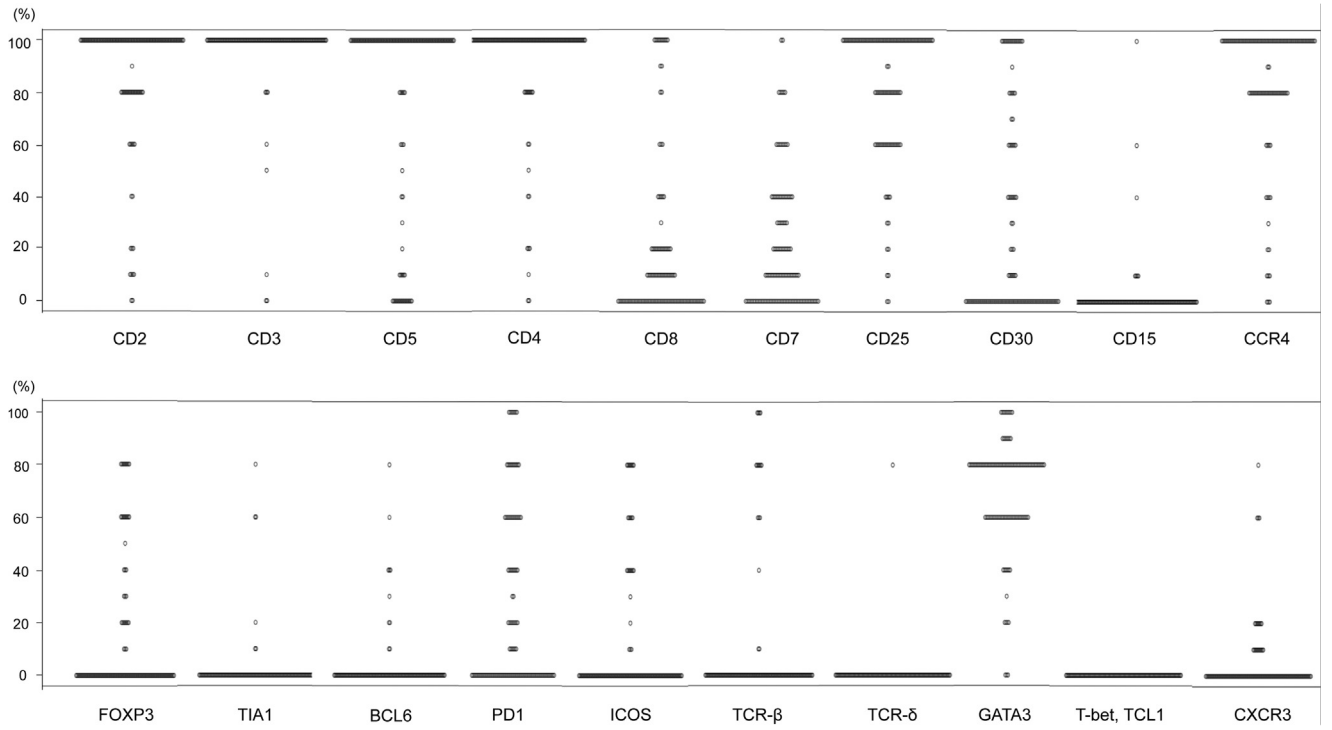

Figure 1.
Percentages of positive cells in tumors (positive rate) were estimated for 21 different immunohistochemical stains. Positive rates are shown without distinguishing staining intensity. Each dot represents 1 case.

cases showed 100% positivity in IHC and negativity in FCM, showing a discrepancy between the 2 methods.

Association of Each Phenotype With Morphology

When categorized morphologically into the pleomorphic and anaplastic large cell types, 102 (87.2%) and 15 (12.8%) cases were identified, respectively. No correlation was confirmed between the morphology and the presence/absence of chemotherapy/immunotherapy or sampling sites (data not shown). The expression of each IHC stain (positive vs negative) in each morphologic variant (pleomorphic vs anaplastic large cell type) was examined. The proportion of positive cases was significantly lower in the anaplastic large cell type for CD3 (102/102 [100%] cases vs 12/15 [80%] cases; $P < .01$), CD5 (86/102 [84.3%] cases vs 9/15 [60%] cases; $P < .05$), and FOXP3 (23/102 [22.5%] cases vs 0/15 [0%] cases; $P < .05$). This finding was in contrast with CD15 (0/102 [0%] cases vs 2/15 [13.3%] cases; $P < .05$) and CD30 (26/102 [22.2%] cases vs 13/15 [86.7%] cases; $P < .01$) (Supplementary Table S3).

Among the 15 cases of anaplastic large cell type in this study, CD30 was positive in 13 (86.7%) cases, 12 of which showed 100% positivity, whereas the 2 (13.3%) CD30 cases were completely negative. Among the 15 cases, 12 (80.0%) were CD2 positive, 12 (80.0%) were CD3 positive, and all were CD4 positive. One case with anaplastic large cell type was completely negative for all T-cell markers (CD2, CD3, and CD5) (Supplementary Fig. S2).

Relevance to Clinical and Genetic Findings

The expression of each IHC stain (positive vs negative) was compared in 2 groups according to clinical findings using age as follows: (1) 50 years as the cutoff value (<50 years, 7 cases; ≥50 years, 110 cases); (2) 65 years as the cutoff (<65 years, 33 cases; ≥65 years, 84 cases) and the presence or absence of therapy; (3) presence/absence of chemotherapy/immunotherapy (with therapy, 20 cases; without therapy, 97 cases); and (4) the presence/absence of immunotherapy (with therapy, 11 cases; without therapy, 106 cases). No difference in expression was observed in IHC stains when comparing age in (1) and (2) (data not shown). When comparing the effects of therapy, the number of cases with CCR4 positivity was significantly smaller in the cases with chemotherapy/immunotherapy ($P < .05$) (3), but CCR4 expression was not significantly correlated with immunotherapy alone (4) (Supplementary Table S3). In the multiple-sample collections, Supplementary Table S1 shows that no marker exhibited a significant difference in expression when comparing the morphology and immunophenotype of pre- and postchemotherapy/immunotherapies. There were no pre- and posttreatment changes in CCR4 in the 4 patients who underwent mogamulizumab administration.

The result of each IHC stain (positive vs negative) was compared on the basis of sample collection sites in the BM, LN, skin, and others. The proportion of CD2-negative cases (BM cases, 4/11 [36.4%]; LN cases, 2/47 [4.3%]; skin cases, 3/47 [6.4%]; and other cases, 0/12 [0%]) was significantly higher in BM ($P < .05$) (Supplementary Table S3). The other IHC stains showed no significant differences according to the sample collection site. The 11 BM cases were reviewed and did not exhibit significant differences in the frequency of positive cases, either in biopsy samples that underwent decalcification (4 cases) or those with coagulation clots, without decalcification (7 cases), suggesting that the influence of decalcification was highly unlikely to be the cause of the low CD2 expression in tumor cells in the BM (Supplementary Table S4).

On the basis of Shimoyama classification, 12 (10.3%) cases in the present study were identified as smoldering type, 38 (32.5%)

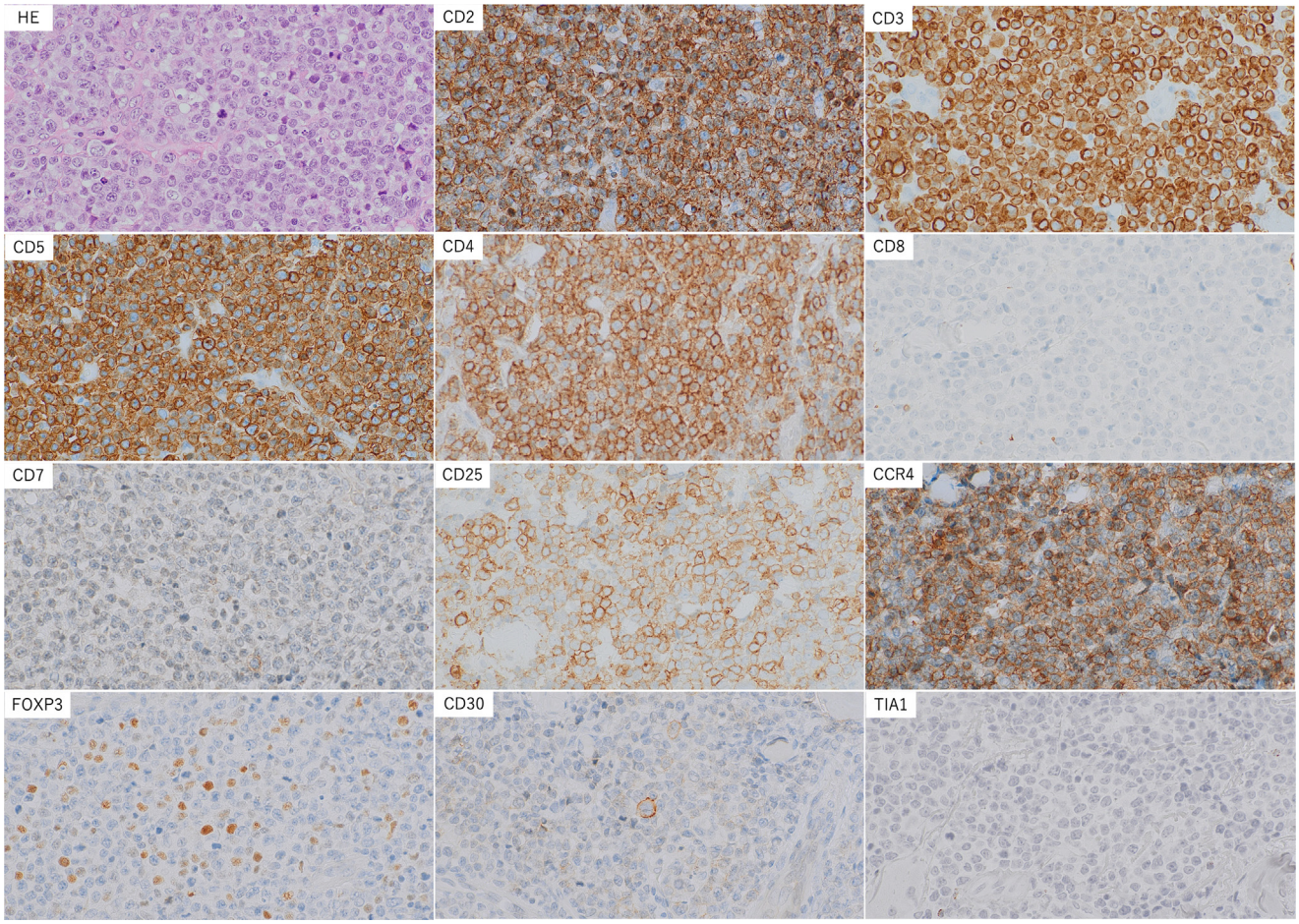

Figure 2.
A case with the typical adult T-cell leukemia/lymphoma immunophenotype (original magnification; objective × 40). HE staining showed the proliferation of atypical lymphocytes with severe nuclear pleomorphism. Tumor cells were positive for CD2, CD3, CD5, CD4, CD25, and CCR4; negative for CD7, CD8, and TIA1; partially positive for FOXP3; and positive for CD30 in large-transformed cells. *HE*, Hematoxylin and eosin.

Table 2
Results of each immunohistochemical staining

	Positive, n (%)	Negative, n (%)
CD2	108 (92.3)	9 (7.7)
CD3	114 (97.4)	3 (2.6)
CD5	95 (81.2)	22 (18.8)
CD4	111 (94.9)	6 (5.1)
CD8	23 (19.7)	94 (80.3)
CD7	36 (30.8)	81 (69.2)
CD25	111 (94.9)	6 (5.1)
CD30	39 (33.3)	78 (66.7)
CD15	2 (1.7)	115 (98.3)
CCR4	109 (93.2)	8 (6.8)
FOXP3	23 (19.7)	94 (80.3)
TIA1	3 (2.6)	114 (97.4)
BCL6	6 (5.1)	111 (94.9)
PD1	40 (34.2)	77 (65.8)
ICOS	20 (17.1)	97 (82.9)
TCR-β	13 (11.1)	104 (88.9)
TCR-δ	1 (0.9)	116 (99.2)
GATA3	110 (94.0)	7 (6.0)
T-bet	0 (0)	117 (100)
CXCR3	4 (3.4)	113 (96.6)
TCL1	0 (0)	117 (100)

as chronic type, 9 (7.7%) as lymphoma type, and 58 (49.6%) as acute type. Overall survival (OS), which was calculated from the day of diagnosis to the last follow-up day or death by any cause, ranged from 0.5 to 240 months (median, 13 months). There was a significant correlation between the clinical subtype and OS ($P < .01$) (Supplementary Fig. S4).

The correlation between the clinical subtype and the results of the 21 types of IHC staining tests (positive/negative) was examined. The number of CD8-positive cases was 6/58 (10.3%) for acute type, 3/9 (33.3%) for lymphoma type, 12/38 (31.6%) for chronic type, and 2/12 (16.7%) for smoldering type, which showed that lymphoma type and chronic type was significantly high ($P < .05$). The number of CD25-negative cases was 4/58 (6.9%) for acute type, 2/9 (22.2%) for lymphoma type, 0/38 (0%) for chronic type, and 0/12 (0%) for smoldering type, indicating a significantly low frequency of CD25 negative results ($P < .05$) for the chronic and smoldering subtypes. No significant correlation was found between the other markers and clinical subtypes. The association between OS and the results of IHC staining tests (including those for p53 and MUM1, whose genetic alterations were reported to correlate with poor prognosis) was analyzed in 67 cases,[16-19] including the acute and lymphoma subtypes. No significant difference in OS was observed between positive and negative cases for any of the 23 IHCs.

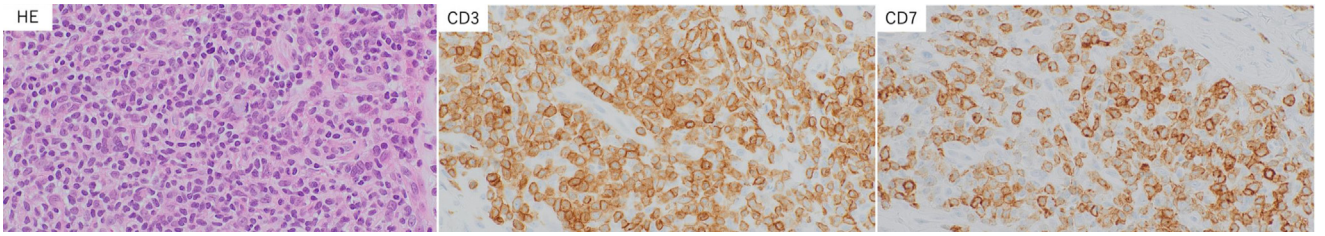

Figure 3.
A CD7-positive case in which 80% of tumor cells were strongly positive. Although this case showed the highest expression intensity and positivity rate for CD7 in the present study, CD7 was downregulated compared with CD3.

Regarding genetic findings, no significant correlation was found by the Fisher exact test between *TP53* mutation and p53 expression, *IRF4* mutation/amplification and MUM1 expression, or *CCR4* mutation and CCR4 expression in a review of 10 cases for which genetic analysis was conducted in a previous study.[11] However, 2 patients had 100% strong positivity for p53 had *TP53* mutations (Supplementary Table S5).

Adult T-Cell Leukemia/Lymphoma With an Atypical Phenotype

A recent study has categorized PTCL-NOS into T-bet and GATA3 subtypes using several IHC stains.[5] A total of 117 ATLL cases in this study were divided into 11 (9.4%) cases belonging to the T-bet subtype, 103 (88.0%) cases belonging to the GATA3 subtype, and 3 (2.6%) cases to the unclassified type. Among the 23 FOXP3-positive cases, 20 belonged to the GATA3 subtype. These findings suggest a correlation between ATLL and Th2 or Treg.[20]

Regarding CD4 and the 3 TFH markers (BCL6, PD1, and ICOS),[6] 12/117 (10.3%) ATLL cases had at least 2 positive TFH markers (TFH-positive cases). Of these, 10/12 (83.3%) were positive for PD1 and ICOS only; 1/12 (8.3%) was positive for BCL6 and PD1 only; 1/12 (8.3%) was positive for PD1, ICOS, and BCL6; and none (0%) were confirmed positive for ICOS and BCL6. Although no correlation was confirmed between BCL6 and PD1 or ICOS and BCL6, 14/40 (35%) PD1-positive cases were ICOS positive, and 71/77 (92.2%) PD1-negative cases were ICOS negative, exhibiting significant coexpression between both markers ($P < .01$) (Supplementary Table S3). In addition, the characteristics of CD10 and CXCL13 were examined in the 12 TFH-positive cases. CD10 was negative in all cases, and CXCL13 was negative in 11/12 (91.7%) cases. On re-examination, 3/12 cases exhibited a proliferation of clear cells and arborizing vessels, morphologically indicative of a differential diagnosis of angioimmunoblastic T-cell lymphoma (AITL), whereas tumor cells propagated in a sheet-like manner with no noticeable mixture of eosinophils in each case, less concordant with the diagnosis of AITL (Fig. 4). In this study, there were also 3 (2.6%) cases that were positive for TIA1, a cytotoxic marker; they were re-examined morphologically, and the angiocentric and angiodestructive growth patterns characteristic of extranodal NK/T-cell lymphoma, nasal type, were not evident. These 6 atypical cases, including 3 TFH-positive cases with the proliferation of clear cells and arborizing vessels and 3 TIA1-positive cases, had HTLV-1 in the tumor cells via HBZ-ISH or SBH. Among the 11 cases determined to be T-bet subtypes, none was TIA1-positive, whereas all the 3 TIA1-positive cases were GATA3 subtypes showing GATA3 positivity.

Discussion

This was an unprecedented study in which the ATLL immunophenotype was comprehensively analyzed for many cases. This

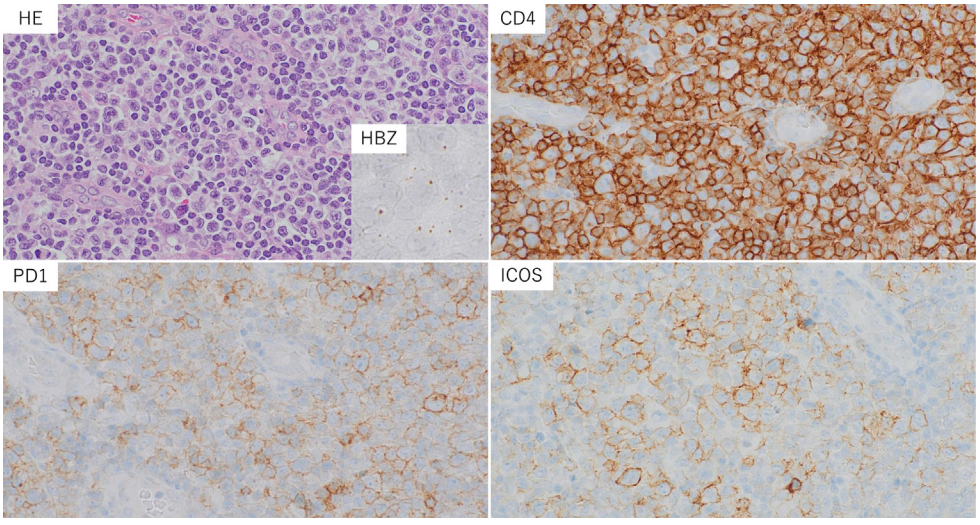

Figure 4.
A case requiring differentiation from angioimmunoblastic T-cell lymphoma. Atypical large lymphoid cells with clear cytoplasm in HE staining were positive for CD4 and 2 TFH markers (PD1 and ICOS), accompanied by the proliferation of arborizing blood vessels. Although angioimmunoblastic T-cell lymphoma was raised as a differential diagnosis, the diagnosis of adult T-cell leukemia/lymphoma was confirmed by the presence of human T-lymphotropic virus type 1 demonstrated by in situ hybridization for HBZ (inset). *HBZ*, Human T-cell Leukemia Virus Type 1 bZIP factor; *HE*, Hematoxylin and eosin.

study confirmed the typical ATLL immunophenotypes described in the 2017 WHO classification (positive: CD2, CD3, CD5, CD4, and CD25; negative: CD7, CD8, and cytotoxic markers; partially positive: CD30, CCR4, and FOXP3).[1] Furthermore, the following new findings were obtained: (1) the usefulness of negative conversion of TCR expression to provide differentiation from other T-cell tumors; (2) correlations of several immunophenotypes, including CD30 and FOXP3, with morphologic characteristics; and (3) identification of atypical cases that were positive for TFH markers or cytotoxic molecules.

CD7, a T-cell marker, has decreased expression in ATLL.[1] In the peripheral blood, CD7 decreases progressively according to the progression of the disease from the smoldering to acute subtypes.[21] Approximately 70% of the cases in this study were CD7 negative, confirming that the decrease in CD7 expression is useful for ATLL diagnosis. At the same time, approximately 30% of the cases were CD7 positive, although the rate exceeded the cutoff of 30%, the decrease in CD7 expression was confirmed at various rates (Fig. 1). Even among CD7-positive cases, those exhibiting strong positivity at a high rate were rare. Therefore, the evaluation of CD7 is still one of the most sensitive methods for diagnosing ATLL.

Most ATLL cases are CD4+/CD8-, although there is also a small occurrence of CD4+/CD8+ or CD4-/CD8+ cells.[1] The results of a previous study of 107 cases with ATLL using FCM indicated that the proportions of CD4+/CD8-, CD4+/CD8+, CD4-/CD8+, and CD4-/CD8− cases were 81%, 7%, 4%, and 7%, respectively.[2] A previous study that used IHC stains to analyze frozen samples from 70 cases found similar proportions at 73%, 14%, 6%, and 7%, respectively, for the same aforementioned ATLL phenotypes.[3] The present study was an IHC examination of 117 cases using formalin-fixed, paraffin-embedded samples and exhibited 91 (78%), 20 (17%), 3 (3%), and 3 (3%) cases, respectively, indicating similar results to those of the previous studies.

Defining a typical ATLL phenotype is challenging, and only half of the cases in the present study were CD3+/CD4+/CD8-/CD7-/CD25+. On the other hand, approximately 80% of the cases were CD3+/CD4+CD25+CCR4+, suggesting that it is a more typical phenotype of ATLL than other candidates. CCR4, in particular, has significance as a therapeutic target of mogamulizumab, and cases suspected of ATLL should first be analyzed for a combination of CD3, CD4, CD25, and CCR4 in routine diagnosis. The degree of CD7 expression could be additionally analyzed.

More than 90% of normal T cells circulating in the blood express TCR-α/β chains, and approximately 10% express TCR-γ/δ chains; the proportion of the latter is high in the intestinal tract and skin. T-cell lymphoma is classified into those expressing TCR-α/β chains and TCR-γ/δ chains. The latter is quite rare and occurs primarily as a special subtype of hepatosplenic T-cell lymphoma or primary cutaneous T-cell lymphoma, which are known to have poor prognoses.[22] According to the 2017 WHO classification, mycosis fungoides usually exhibits CD8-/TCR-β+/TCR-γ-,[1] and cases with cytotoxic phenotypes of CD8+/TCR-β+/TCR-γ+ have been reported as rare cases.[23] In addition, PTCL-NOS is usually considered TCR-β positive.[1] In this study, TCR-β and TCR-δ were both negative in most of the 104 cases with ATLL (88.9%), suggesting that TCR expression might be useful in differentiating ATLL from other T-cell lymphomas.

The anaplastic large cell type of ATLL is a distinct morphologic subtype showing uniform proliferation of cells with nuclei that are larger than the pleomorphic type, distinct nucleoli, and abundant cytoplasm; this type is sometimes confirmed by the presence of giant multinuclear cells, similar to Reed−Sternberg cells.[8] These tumor cells are considered CD30-positive and exhibit a peripheral T-cell phenotype. In this study, CD30 expression was not limited to the anaplastic large cell type; this suggests the necessity of CD30 immunostaining, regardless of morphology, to identify cases that can be treated using anti-CD30 monoclonal antibody agents. A previous study emphasized the negative relationship between CD30 and FOXP3 expression.[24] However, the results of our study suggest a stronger correlation between FOXP3 expression and tumor morphology than CD30 expression (Supplementary Table S3).

Previous studies have confirmed that the phenotype of tumor cells is often affected by treatment. A study reported that the CD20-positive rate decreased after anti-CD20 antibody treatment (rituximab) in diffuse large B-cell lymphoma and follicular lymphoma,[25] and another study reported that CD19-specific chimeric antigen receptor-modified T-cell therapy induced a lineage switch including the deletion of CD19.[26] In the present study, only CCR4 expression was significantly correlated with the presence/absence of chemotherapy/immunotherapy, but the CCR4 expression was not significantly correlated with the presence/absence of immunotherapy. Moreover, the relevance of the change in CCR4 expression was unclear in patients who underwent anti-CCR4 antibody treatment (mogamulizumab) (Supplementary Table S1), indicating the correlation between treatment and CCR4 expression was not attributed to the direct effect of mogamulizumab. Regarding the sample collection sites, the expression of CD2 was significantly reduced in BM compared with other organs in the present study. Differences in microenvironments between the BM and other organs are suspected to be the cause of reduced expression; this requires further investigation in the future.

According to the 2017 WHO classification,[1] CD10, CXCL13, BCL6, PD1, ICOS, CXCR5, SAP, MAF, and CD200 are representative TFH markers, but there have been no studies investigating TFH marker expression in ATLL. Regarding cytotoxic molecules, tumor cells of ATLL are considered negative in the 2017 WHO classification.[1] In the present study, 12/117 (10.3%) cases were positive for CD4, and at least 2 of the 3 TFH markers (BCL6, PD1, and ICOS) showed “AITL-like” morphology accompanied by a proliferation of clear cells and growth of arborizing vessels. In addition, the expression of the cytotoxic molecule TIA1 was confirmed in 3 of the 117 cases. Although these 6 cases described above were very atypical for ATLL, the presence of HTLV-1 was confirmed in all 6 by HBZ-ISH or SBH. These findings indicate the immunophenotypic diversity of ATLL. Although the results of the present study suggest the possibility of the presence of a few TFH-derived ATLLs, most cases were negative for CD10 and CXCL13, even for more specific TFH markers that were additionally analyzed. Further accumulation of relevant case data are necessary to establish the concept of TFH-derived ATLL.

A limitation of the present study was that we only analyzed organ tissues from BM, LNs, skin, and other sites; no peripheral blood samples were included. Next, the results of the present study mainly consisted of IHC stains, and very few cases were analyzed with FCM. A study reported that FCM and IHC stains in T-cell/NK-cell lymphomas were comparatively investigated and the results indicated complete matches for CD4, CD7, CD10, CD25, CD30, and CD56, although differences were observed for CD3, CD5, and CD8.[27] In the present study, almost the same results were observed in LN samples from 4 cases that underwent simultaneous IHC stains and FCM, but there were 2 cases in which the results of both analyses were completely mismatched, indicating that CD3 was 100% positive in IHC stains and negative in

FCM. In these cases, the cell surface expression of CD3 seemed to have disappeared, although cytoplasmic expression was retained. Although no significant associations were observed between genetic alterations and IHC staining for *TP53*, *IRF4,* and *CCR4,* the small number of cases indicates the necessity for further analyses with a larger sample size, especially for determining if there is a significant association between 100% strong positivity of p53 and *TP53* mutation.

In conclusion, the results of the present study illustrate the diversity of the ATLL phenotypes and the existence of markers, such as CD30 and FOXP3, which are linked to morphology. Although CD3+/CD4+/CD25+/CCR4+ was considered a typical immunophenotype of ATLL, approximately 20% of cases did not conform to this pattern, indicating that atypical phenotypes do not rule out the possibility of ATLL. The diagnosis of T-cell tumors occurring in HTLV-1 carriers should be confirmed by detecting HTLV-1 in the tumor tissues, especially when the tumors exhibit atypical phenotypes. As a next step, further detailed analyses should examine the phenotypic profile in the peripheral blood, significance of TFH marker expression, and correlation between phenotype and genotype.

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Author Contributions

T.T., K.K., and N.W. designed the research, reviewed the cases, analyzed the results, constructed the tables and figures, and wrote the manuscript. S.S. and T.F. provided the genetic data and discussed relevant analyses. The other authors discussed the content of the paper, and provided materials, clinical information, or pathologic information. All authors read and approved the final version of the manuscript.

Data Availability

The data sets used and/or analyzed during the current study are available from the corresponding authors upon reasonable request.

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Declaration of Competing Interest

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Ethics Approval and Consent to Participate

This study was approved by the Research Ethics Committees of Ryukyu University Hospital, Nakagami Hospital, Okinawa Prefectural Nanbu Medical Center and Children's Medical Center and performed in accordance with the Declaration of Helsinki.

Supplementary Material

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