








## ORIGINAL ARTICLE

# Anti-HTLV-1 immunity combined with proviral load as predictive biomarkers for adult T-cell leukemia-lymphoma

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## Funding information

Japan Agency for Medical Research and Development, Grant/Award Number: 21cm0106306h0006 and 21fk0108088h0003; Japan Science and Technology Corporation, Grant/Award Number: JPMJMI18G1; Japan Society for the Promotion of Science, Grant/Award Number: 19H03689, 20H03514 and Core-to-Core Program A, Advanced Research Networks; Core-to-Core Program A, Advanced Research Networks; Ministry of Education, Culture, Sports, Science and Technology, Grant/Award Number: 16H06277

## Abstract

Human T-cell leukemia virus type 1 (HTLV-1) establishes chronic infection in humans and induces a T-cell malignancy called adult T-cell leukemia-lymphoma (ATL) and several inflammatory diseases such as HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). Persistent HTLV-1 infection is established under the pressure of host immunity, and therefore the immune response against HTLV-1 is thought to reflect the status of the disease it causes. Indeed, it is known that cellular immunity against viral antigens is suppressed in ATL patients compared to HAM/TSP patients. In this study, we show that profiling the humoral immunity to several HTLV-1 antigens, such as Gag, Env, and Tax, and measuring proviral load are useful tools for classifying disease status and predicting disease development. Using targeted sequencing, we found that several carriers whom this profiling method predicted to be at high risk for developing ATL indeed harbored driver mutations of ATL. The clonality of HTLV-1-infected cells in those carriers was still polyclonal; it is consistent with an early stage of leukemogenesis. Furthermore, this study revealed significance of anti-Gag proteins

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to predict high risk group in HTLV-1 carriers. Consistent with this finding, anti-Gag cytotoxic T lymphocytes (CTLs) were increased in patients who received hematopoietic stem cell transplantation and achieved remission state, indicating the significance of anti-Gag CTLs for disease control. Our findings suggest that our strategy that combines anti-HTLV-1 antibodies and proviral load may be useful for prediction of the development of HTLV-1-associated diseases.

#### KEYWORDS

ATL, gag, HAM/TSP, HTLV-1, tax

## 1 | INTRODUCTION

Viruses that cause chronic infections possess strategies to propagate in vivo despite the pressure of host immunity. An imbalance between these viral persistence strategies and the host immune response can lead to the onset of various diseases. Human T-cell leukemia virus type 1 (HTLV-1) was the first human pathogenic retrovirus to be discovered.<sup>1</sup> It causes adult T-cell leukemia-lymphoma (ATL) and an inflammatory disease of the spinal cord, HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP).<sup>2,3</sup> HTLV-1 mainly infects CD4<sup>+</sup> T cells in vivo and propagates by two modes in vivo: de novo infection and clonal expansion.<sup>4</sup> The former mechanism involves cell-to-cell transmission of the viral particles, while the latter increases the number of cells of each infected clone. After infection with HTLV-1, the clonal proliferation of infected cells is limited by host immunity. However, HTLV-1 converts the immunophenotype of infected cells to regulatory T cell (Treg) like, which helps HTLV-1 infected cells escape host immune surveillance through expression of immunosuppressive molecules.<sup>5-7</sup> For infected people in the carrier state, the conflict between the virus and host immune system shapes the diversity of infected cells and the immune reactions of each individual. In a small subset of carriers, the balance is disrupted at some point, and HTLV-1-associated diseases develop; 2.6%–6% of carriers develop ATL, and approximately 0.25% of carriers in the Japanese population and approximately 1.9% of carriers in the Caribbean population develop HAM/TSP.<sup>8,9</sup>

The host immune response to HTLV-1 differs among the associated diseases. HTLV-1 encodes structural genes, such as *gag*, *pol*, and *env*, in addition to regulatory genes (*tax* and *rex*) and accessory genes (*p12*, *p13*, *p30*, and *HBZ*), which have various functions in infected cells.<sup>10</sup> Among them, Tax is recognized as a major target of cytotoxic T lymphocytes (CTLs) because of its high immunogenicity.<sup>11,12</sup> Tax is a potent transactivator and promotes viral replication by activation of the viral promoter, the 5' long terminal repeat (5'-LTR).<sup>13,14</sup> However, Tax expression is faint or inactivated by genetic/epigenetic mechanisms in ATL cells,<sup>15-19</sup> suggesting that escape from host immunity is closely associated with the development of ATL. In contrast, HAM/TSP cases show such strong immune responses to HTLV-1 that infiltration of infected cells into the central nervous system (CNS) causes chronic inflammation.<sup>20</sup> In addition to cellular immunity, humoral

immunity against HTLV-1 also differs between patients with different HTLV-1-associated diseases. While HAM/TSP patients have high levels of antibodies against HTLV-1 antigens in their blood and CSF,<sup>21,22</sup> ATL patients have low levels of antibody to Tax.<sup>23</sup> Having lower levels of antibody to Tax was suggested to be a risk factor for ATL.<sup>24</sup> These observations suggest that immunological parameters reflect the disease status induced by HTLV-1 infection. However, no clinically useful method to pinpoint disease status using antibody levels to each of the HTLV-1 antigens has been established until this one.

Previous studies proposed that high proviral load (PVL)<sup>25</sup> and clonality of infected cells<sup>26</sup> are possible risk factors for ATL; however, these criteria are insufficient to reliably identify groups at high risk for ATL or HAM/TSP. In this study, we employed the luciferase immunoprecipitation system (LIPS) assay, an efficient method to quantify the antibodies to HTLV-1 antigens, and evaluated the usefulness of antibody levels for predicting the development of ATL and HAM/TSP. Multivariate analysis using the titers of several antiviral antibodies and PVL was able to efficiently divide ATL and HAM/TSP cases into different groups, and this strategy also enabled us to identify carriers at high risk for ATL. In addition, Gag-specific CTLs were also observed in HTLV-1-infected individuals, indicating that Gag protein is immunogenic. These findings suggest that the evaluation of anti-Gag immunity is useful for the prediction of HTLV-1-associated diseases.

## 2 | MATERIALS AND METHODS

Details of clinical samples, protocols of LIPS assay, PVL quantification, next-generation sequencing, ELISPOT, experimental procedures for detection of Gag in ATL cells, and multivariate analysis are described in Appendix S1 (SI Text).

## 3 | RESULTS

### 3.1 | Antibody responses to human T-cell leukemia virus type 1 proteins

The initial purpose of this study was to find a way to identify HTLV-1 carriers at high risk for the development of associated diseases using simple biomarkers. It is thought that the immune responses to viral

antigens are suppressed in ATL patients but activated in HAM/TSP patients. Therefore, some immunological biomarkers may be useful to evaluate the risk of those diseases. Previously, we and another group reported that the LIPS assay was a sensitive and easy method to detect antibodies to HTLV-1 antigens.<sup>27,28</sup> In this study, we modified the assay and screened patients for antibodies against each of several viral proteins to identify parameters suitable for predicting disease progression. First, the presence of antibodies to three Gag proteins (p15 nucleocapsid, p19 matrix, and p24 capsid), envelope (Env), Tax, Rex, p12, p13, p30, and HBZ was evaluated using a relatively small number of samples from HTLV-1 carriers and non-infected subjects (Figure S1). We found that antibody responses to Gag proteins (p15, p19, p24), Env, and Tax were higher in HTLV-1-infected individuals than uninfected controls, whereas no increase was detected in the luminometer units (LU) for any of the other antigens in infected subjects compared with uninfected ones, probably due to low titers of antibody to these antigens. Thus, we focused our remaining studies on antibodies to Gag proteins, Env, and Tax, and determined the antibody titers to them in 263 carriers, 56 HAM/TSP patients, and 25 ATL patients (Table S1).

Different patterns of antibody responses were observed in each clinical subgroup. HAM/TSP patients had significantly higher antibody levels to all antigens except for Env than carriers (Figure 1A). In contrast, ATL patients had significantly lower antibodies to Env than carriers. Interestingly, antibodies to p19 and p24 were significantly higher in ATL patients than those in carriers, indicating that low antibody responses to Tax and Env are not simply due to the immunodeficiency induced by ATL. To track the changes in antibody responses over time in each individual, serial blood samples from seven carrier cases were analyzed by LIPS. Although the samples were collected at different times depending on the cases, the levels of antibodies to each antigen were stable and almost unchanged in all cases (Table 1).

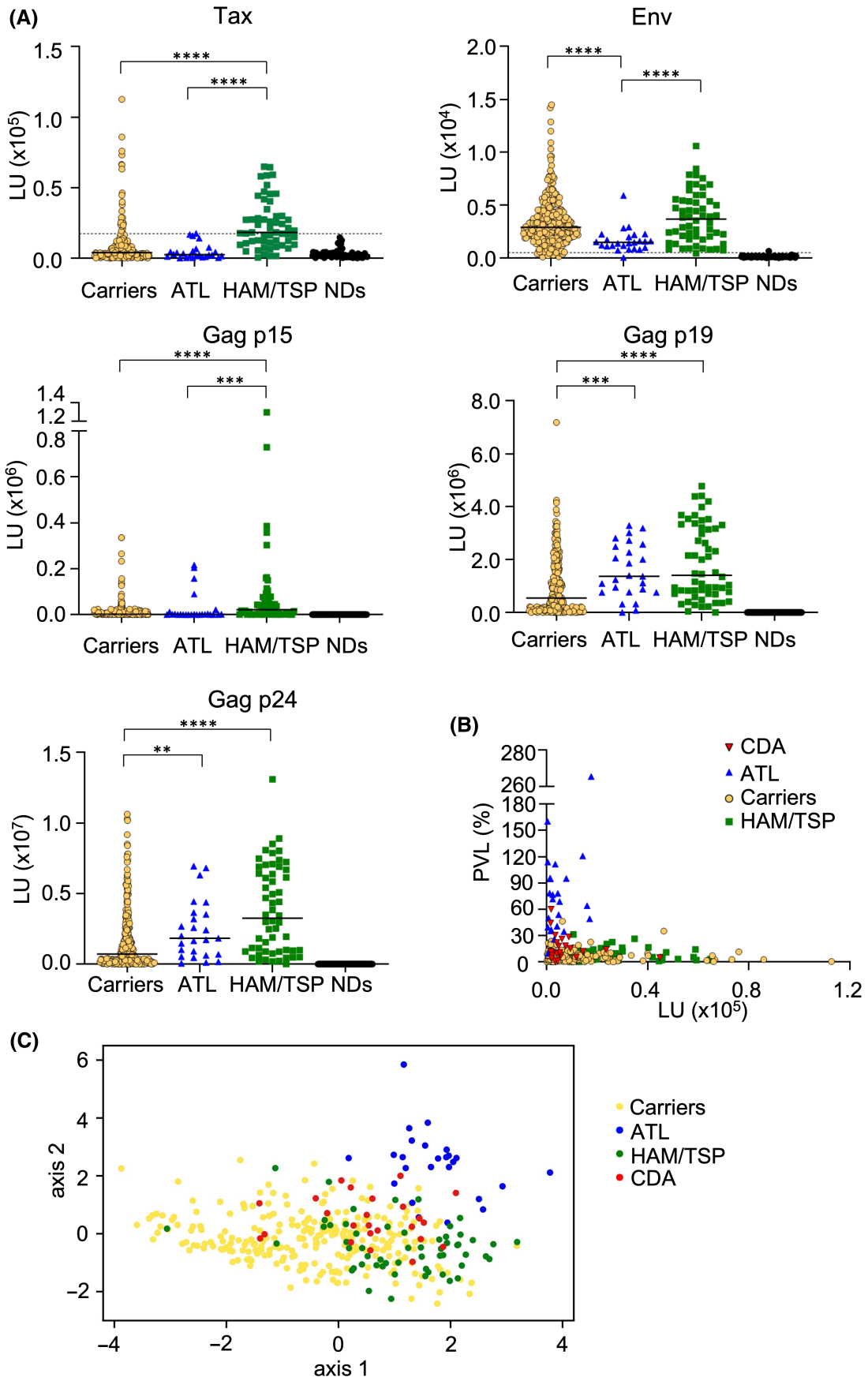
Next, we evaluated the correlation between those antibodies and PVL (Figure S2A-C, Table S2). In carriers, there were significant positive correlations between PVL and all antibodies (Figure S2A). In contrast, this correlation was not observed in ATL patients or HAM/TSP patients (Figure S2B,C), although it is possible that the sample sizes of these groups are too small to reveal a slight correlation. When the data from all subjects were combined, we saw that ATL and HAM/TSP were roughly divided into different populations, whereas carriers were widely distributed (Figure 1B; Figure S2D), indicating that carriers were heterogeneous. Some carriers had similar signatures to ATL or HAM/TSP. Samples from 25 carriers who later developed ATL (CDA) were additionally analyzed (Table S3). Antibody responses to viral antigens were also varied (red triangles in Figure 1B; Figure S2D).

### 3.2 | Multivariate analysis for identifying high-risk carriers for ATL

To develop more sensitive methods to identify high-risk carriers, we analyzed these data by combining all the parameters. Partial least square (PLS) analysis was performed to determine how well

the measured antibodies and PVL reflected the diagnosis. PLS is a variant of principle component analysis (PCA) used in medicine. PCA is a popular dimension reduction method that extracts a few useful interpretations from the whole array of measurements (antibody titers against Tax, Env, and Gag proteins and PVL) by minimizing the loss of information. PLS extends PCA by integrating diagnostic information such as HAM/TSP and ATL, enabling consideration of the association between the measurements and diagnostic information. Each diagnosis category (carrier, ATL, HAM/TSP, or CDA) formed a cluster on the plot (Figure 1C). Among several dimension reduction methods, we found that PLS performed better in cluster samples than other popular methods (as described in the Materials and Methods section). The following considerations may explain why only PLS, and not other popular methods, can achieve good clustering. First, a conventional PCA method can often face multicollinearity: highly correlated explanatory variables make it difficult to identify the explicit contribution of explanatory variables which are highly correlated. Multicollinearity is likely to occur in a clinical setting since selected biomarkers such as Gag-p19 and Gag-p24 often overlap biological characteristics. In several applications, PLS has been demonstrated to avoid the multicollinearity problem for analyzing metabolome datasets to which multicollinearity is inherent.<sup>29,30</sup> Second, PLS can utilize diagnostic information as a supervised machine learning method. This could be the reason why PLS outperforms nonlinear dimensionality reduction methods such as tSNE and uMAP. Using PLS analysis, HAM/TSP and ATL samples were well clustered in a two-dimensional plane, respectively (Figure 1C). As expected, carrier samples overlap with HAM/TSP and ATL clusters, and CDA samples were located around the boundary between ATL and carriers.

We assumed carriers whose samples were located close to the ATL cluster on the two-dimensional plane to be carriers at high risk for ATL. More specifically, the ranking of high-risk carriers was calculated based on the distance from the centroid of CDA samples (Table S4). Based on this ranking, carriers showing antibody titers and PVLs similar to CDA were estimated to be at high risk. To verify whether these carriers were indeed at elevated risk of developing ATL, we tried to detect genetic mutations of driver genes in these individuals. Samples from eight asymptomatic carriers were analyzed; seven cases were close to the CDA cluster, and one was located in the HAM/TSP cluster in the PLS plot (Figure 2A). HTLV-1-infected cells (CD4<sup>+</sup> CADM1<sup>+</sup>) were purified by cell sorting, and targeted sequencing was carried out. Five of the seven cases had somatic mutations associated with hematologic malignancies, including previously known driver mutations for ATL, such as *TP73*, *CCR4*, *GPR183*, and *ARID2* (Figure 2A, Table 2; Table S5).<sup>31</sup> In contrast, no mutations were detected in two cases with lower rankings for ATL, even though one of them, KC-30, had the highest PVL among the carriers analyzed. The median variant allele frequency of all analyzed cases was as low as 1.9%, and the clonality of infected cells in all cases was polyclonal (Figure 2A; Figure S3), suggesting that our prediction strategy has the potential to uncover carriers who are in a preleukemic stage.



**FIGURE 1** Antibody responses analyzed by luciferase immunoprecipitation system (LIPS) in human T-cell leukemia virus type 1 (HTLV-1) associated diseases. (A) The horizontal bar represents the median. The dotted lines are cutoff values calculated from the mean value of seronegative donors plus five standard deviations (SDs), as reported previously.<sup>28</sup> SDs of seronegative donors (NDs) were 2887, 75.63, 88.88, 455.6, and 1071 for Tax, Env, p15, p19, and p24, respectively.  $**P < 0.01$ ,  $***P < 0.005$ ,  $****P < 0.0001$ . (B) Tax antibody and proviral load (PVL) in carriers who developed ATL (CDA), ATL patients, HTLV-1 carriers, and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) patients. (C) PLS analysis using PVL and antibody responses to Tax, Env, and Gag. The scale of the PLS plot is a non-dimensional quantity since horizontal and vertical axes represent integrated characteristics regarding symptoms.

Name and date	Tax (LU)	Env (LU)	Gag p15 (LU)	Gag p19 (LU)	Gag p24 (LU)	PVL (%)
Carrier1-21	6447	2692	439	1,034,116	1,298,869	15.1
Carrier1-9	6137	2413	537	1,120,098	1,210,868	12.4
Carrier1-0	6347	2857	569	1,131,881	1,246,688	9.0
Carrier2-25	35,716	8497	706	2,565,449	5,518,157	22.5
Carrier2-13	33,634	7137	548	2,462,570	5,595,651	13.1
Carrier2-0	32,831	7784	810	2,267,793	5,167,180	9.5
Carrier3-22	18,805	1912	883	1,927,998	2,481,554	8.1
Carrier3-0	18,001	2030	771	2,255,472	2,298,831	4.1
Carrier4-11	53,202	7659	85,483	2,552,629	7,234,075	35.1
Carrier4-6	53,263	7718	95,916	2,608,591	7,623,197	58.0
Carrier4-0	53,338	7455	97,878	2,623,954	7,362,403	66.5
Carrier5-12	1833	3984	6315	273,786	521,893	15.0
Carrier5-0	1803	3869	6059	272,932	473,080	9.1
Carrier6-4	2766	769	321	326,841	4,209,567	9.5
Carrier6-0	3016	856	245	236,647	4,110,562	6.9
Carrier7-6	502	473	191	291,397	540,532	10.4
Carrier7-2	464	594	385	240,556	441,688	6.3
Carrier7-0	511	678	465	227,542	532,624	10.7
Carrier8-29	501	1147	58	575,385	2,842,144	15.2
Carrier8-2	463	1159	77	584,600	2,615,260	8.4
ATL8-0	491	994	63	596,678	2,405,165	6.9

**TABLE 1** Long-term stability of antibody responses in carriers.

Note: Date: 0 represents the date when the last sample was taken, and other numbers represent the number of months before the last sample was taken.

Case 8 developed ATL at time 0 after 29-month observation.

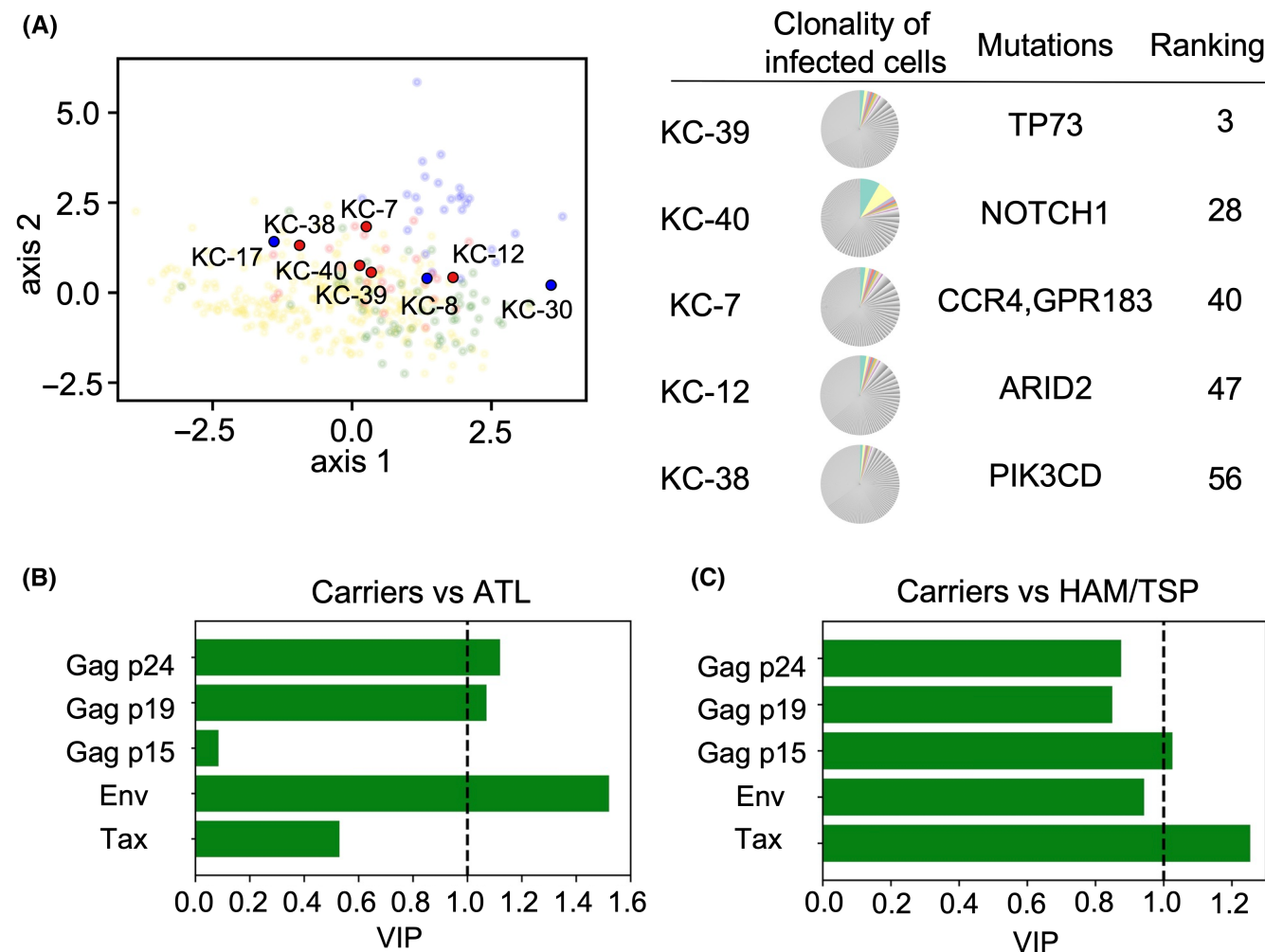
### 3.3 | Significance of elevated anti-Gag p19 and p24 antibodies in ATL cases

As expected, the titers of anti-Tax and anti-Env antibodies were lower in ATL cases compared with those of HAM/TSP patients and carriers. Unexpectedly, the titers of anti-Gag p19 and p24 antibodies of ATL cases were higher than those of carriers, although a finding that there was no significant difference in anti-Gag antibodies between ATL and HAM/TSP was consistent with previous reports.<sup>32</sup> To confirm which antibodies are important for discriminating ATL cases from carriers, we employed the variable importance in projection (VIP) scores derived from the PLS analysis. VIP scores identify antibody types that significantly contribute to the discrimination between the disease types (i.e., carrier, ATL, HAM/TSP, or CDA). A VIP score greater than one indicates an antibody type that makes an important contribution to the discrimination between disease types. (A more

detailed explanation of VIP scores is given in Appendix S1: Materials and Methods.) Using VIP scores as convenient indicators for ranking antibody types, we found that high antibody titers against Gag p19, p24, and Env were important markers of ATL (Figure 2B). In contrast, elevated antibody titers to Tax and p15 appeared to be markers for HAM/TSP (Figure 2C). These findings suggest that antibody titers to Gag antigens are useful in predicting HTLV-1-related diseases.

### 3.4 | Expression of Gag antigen in ATL cells

It is intriguing that the antibodies to Gag antigens have such an impact on the prediction of HTLV-1-associated diseases. In particular, we wondered why ATL patients had higher antibody responses against Gag p19 and p24 than carriers, even though responses to Tax and Env were lower in ATL cases (Figure 1). From those



**FIGURE 2** Identification of predictive markers for the development of ATL or human T-cell leukemia virus type 1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). (A) Eight carriers were analyzed for somatic mutations of driver genes and clonality of infected cells. Red dots represent cases with at least one detected mutation, and blue dots represent cases with no detected mutation. Colored portions of pie charts represent the top 10 clones of each case. (B,C) VIP scores for prediction of ATL (B) and HAM/TSP (C). The VIP score for each variable represents the mathematical degree of contribution of that variable to the disease type (ATL or HAM/TSP). To determine whether a variable (PVL/antibody titer) is important or not based on the VIP score, a conventional criterion (threshold equal to 1) was employed (See Appendix S1 for details). Note that the VIP score represents the relative importance of an antibody's contribution to the assignment of a sample to a cluster.

observations, we hypothesized that Gag antigens are expressed in ATL cells, even if Tax is not expressed. To detect *gag* transcripts, we synthesized cDNA using a *gag*-specific primer and carried out quantitative RT-PCR. As expected, *gag* mRNA was detected in all ATL cell lines, including Tax-negative cell lines, although its expression level was quite low (Figure 3A). Since ED and ATL-55 T cells have genetic mutations in the *tax* gene and TL-Om1 has lost Tax expression due to DNA methylation of the 5'LTR, Tax is not expressed in these cell lines. Therefore, the *gag* mRNA we found in these cell lines is transcribed in a Tax-independent manner. The *gag* gene transcript was also detected in fresh ATL cells (Figure 3B). There are no deletions in the provirus or mutations in the *tax* gene in these cases (Table S6). DNA methylation was observed in ATL-1 and ATL-3, consistent with the result that *tax* expression was repressed in those cases (Figure 3B). We also visualized *gag*

mRNA using RNA fluorescence in situ hybridization (RNA-FISH) in *tax*-defective cells (Figure 3C,D). Expression of Gag protein in ED was confirmed by proximity ligation assay (Figure 3E,F). It is reported that the titer of antibody against the well-known cancer-testis antigen NY-ESO-1 is correlated with the expression level of the antigen's mRNA.<sup>33</sup> Thus, transcription of *gag* in leukemic cells regardless of Tax status is a possible reason for the presence of anti-Gag antibodies in ATL cases.

### 3.5 | Cellular immunity against Gag in human T-cell leukemia virus type 1-infected individuals

Since HTLV-1 propagates mainly by causing the proliferation of infected cells, cellular immunity is more critical than humoral

TABLE 2 Somatic mutations in carriers.

Sample	Ranking	Gene	Type of mutation	VAF	PVL (%)	Tax	Env	Gag p15	Gag p19	Gag p24
KC-39	3	TP73	Nonsynonymous SNV	0.017	9.5	2969	4041	551	403,674	3,397,985
KC-40	28	NOTCH1	Nonsynonymous SNV	0.127	10.4	25,400	2001	1072	193,880	60,452
KC-8	36	ND			19.9	1649	7409	4386	2,312,792	4,837,946
KC-7	40	CCR4	Stopgain	0.02	15.1	2484	1102	516	569,784	332,024
		GPR183	Frameshift deletion	0.012						
KC-12	47	ARID2	Stopgain	0.032	22.5	17,410	5097	1540	2,868,154	3,644,769
KC-38	56	PIK3CD	Nonsynonymous SNV	0.01	8.9	248	2966	760	123,526	238,358
KC-17	98	ND			7.7	498	2313	419	104,451	25,882
KC-30	139	ND			66.5	52,423	6094	153,713	2,523,416	6,783,579

Abbreviation: ND, no mutation detected; PVL, proviral load; SNV, single nucleotide variant; VAF, variant allele frequency.

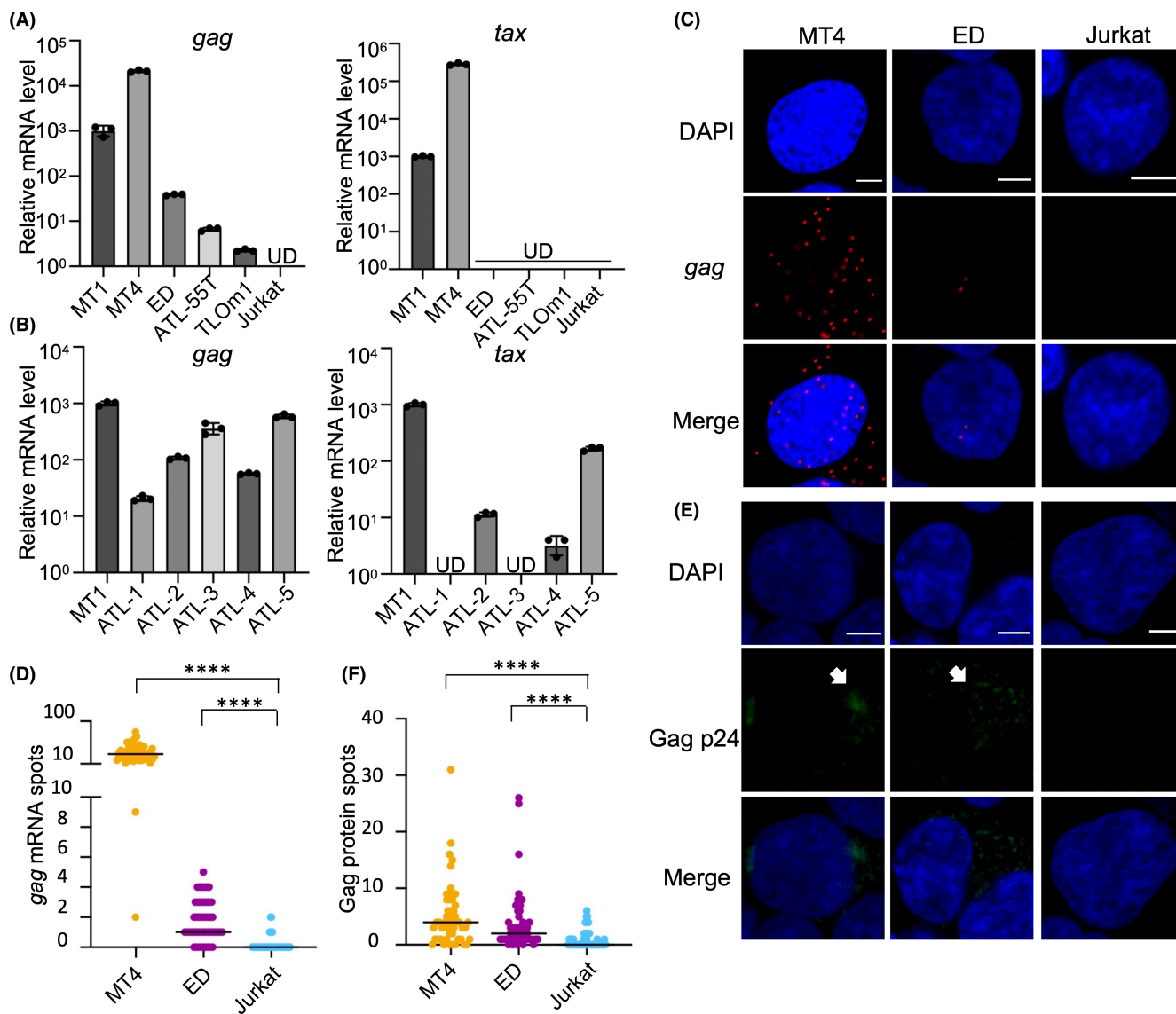
immunity in regulating the number of infected cells. Based on the existence of anti-Gag antibodies in ATL cases, we speculated that Gag-specific CTLs may have a significant impact on the development of ATL. To evaluate the number and activity of CTLs to Gag antigens, we conducted ELISPOT assays using samples from patients with various clinical statuses including ATL patients who received hematopoietic stem cell transplantation (HSCT; Table S7). As shown in Figure 4, IFN- $\gamma$ -producing cells were observed after stimulation with peptides from Gag antigens, indicating the presence of functional CTLs to those antigens. While the number of IFN- $\gamma$ -producing cells was generally low in ATL patients and carriers, IFN- $\gamma$ -producing cells were more common in some cases of HAM/TSP and, intriguingly, in several ATL patients who received HSCT. The two HSCT patients who had low numbers of IFN- $\gamma$ -producing cells (HSCT-3 and HSCT-5) suffered clinical relapse after transplantation. These results suggest that when HSCT is successful in treating ATL, HSCT is associated with the rise of functional CTLs against Gag antigens.

## 4 | DISCUSSION

ATL shows a dismal prognosis regardless of intensive chemotherapy. Therefore, the identification of a high-risk group among carriers is critical for successful treatment. ATL cells contain genetic changes in various pathways that are important for leukemogenesis.<sup>31</sup> Among these mutations, some of them are detected in the early stage of leukemogenesis.<sup>34,35</sup> However, in clinical practice, a method to identify high-risk carriers is required without analyses of genetic changes. Here, we show that disease status can be classified by the multivariate analysis using PVL and antibodies to multiple viral antigens, such as Tax, Env, and Gag proteins. Our strategy is useful for identifying HTLV-1 carriers at high risk for ATL at a very early stage of the disease since several driver mutations were found in the samples from individuals who are still in a polyclonal state. A previous study reported that all carriers who developed ATL had detectable

mutations, while most carriers with high PVL but no detectable mutations did not develop ATL during a median of 10 years follow-up,<sup>34</sup> suggesting that it is difficult to identify high-risk cases by PVL alone. A more recent study showed that in evaluating the abundance of each HTLV-1-infected clone, the so-called oligoclonality index is useful to identify a subset of HTLV-1 carriers with high PVL at increased risk of developing ATL.<sup>36</sup> In the carrier state, HTLV-1-infected clones survive for years, and a clone that acquires malignant characteristics preferentially expands, resulting in the development of ATL.<sup>37</sup> Possibly our prediction method will be able to identify carriers with oncogenic mutations before the expansion of pre-malignant clones. However, the number of the ATL cases analyzed is still small in this study. A larger number of cases and longer-term follow-up of the subjects will be needed to verify the usefulness of our strategy and to set the thresholds that define the risk of future disease development.

Among the antibodies to the viral antigens we analyzed, anti-Gag antibodies were shown to be useful in predicting the development of HTLV-1-associated diseases. Gag is initially translated as a polyprotein and processed into three mature proteins: p19 (matrix), p24 (capsid), and p15 (nucleocapsid). Previous studies using the LIPS assay demonstrated that the titer of the antibody to full-length Gag protein was not different between asymptomatic carriers, ATL, and HAM/TSP patients.<sup>32,38</sup> In this study, we intended to analyze the immune reaction against each of the mature Gag antigens and found that all antigens evoke both humoral and cellular immunities, which might be useful for the prediction of the disease statuses. It is noteworthy that the amount of anti-Gag p15 was found to be an important parameter for predicting the onset of HAM/TSP. Gag precursor polyprotein is processed into each component by the viral protease. For efficient viral replication, ribosomal frameshift in the sequence of the p15-encoding region is required to produce the Gag-Pol polyprotein encoding the Gag proteins and viral enzymes such as the protease, reverse transcriptase, RNaseH, and integrase.<sup>39</sup> In our LIPS assay, the mature form of p15, which is generated by ribosomal frameshift and processing with the viral protease, was utilized as an



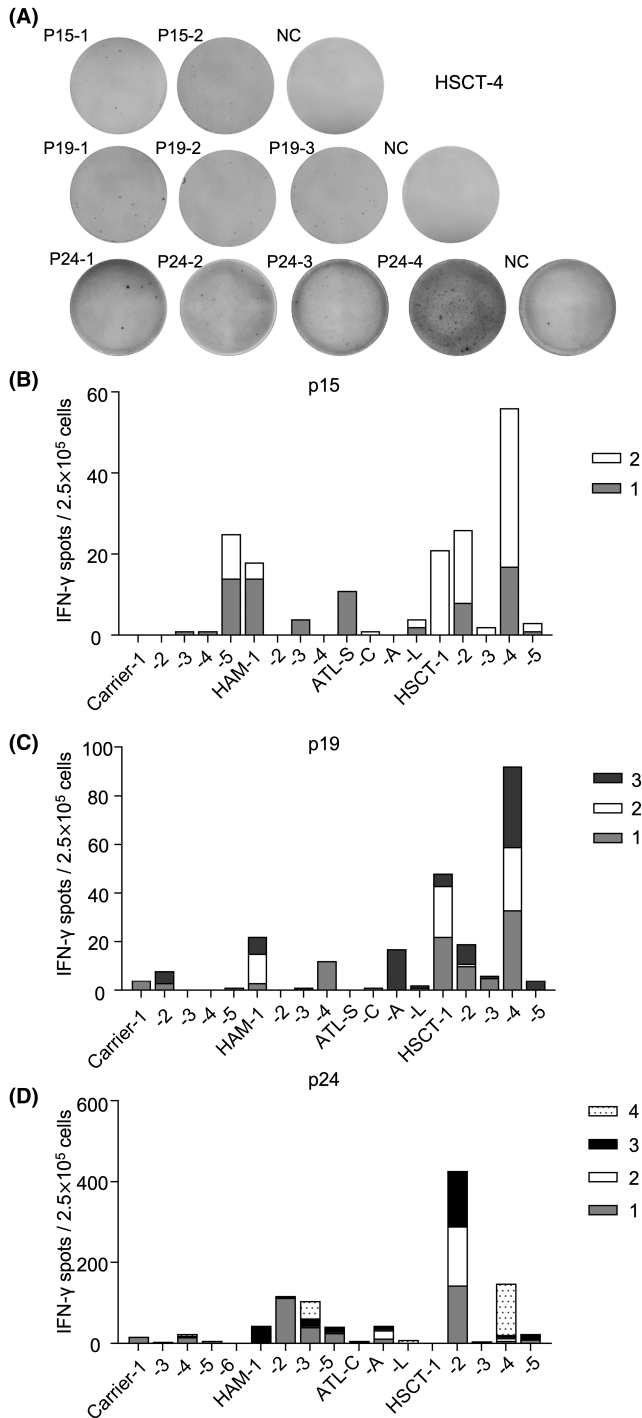
**FIGURE 3** Gag expression in ATL cells. (A) RT-qPCR of *tax* and *gag* in human T-cell leukemia virus type 1 (HTLV-1) infected cell lines and Jurkat cells. For quantification of *gag* mRNA, a no reverse transcriptase negative control was made, and *gag* mRNA was not detected. UD, undetected. (B) RT-qPCR of *tax* and *gag* in primary ATL cells. All samples were obtained from ATL patients in which more than 80% of PBMCs were CD4<sup>+</sup>CAD1<sup>+</sup>, and they were confirmed to be monoclonal by inverse PCR. The amount of 18S rRNA in each sample was quantified and used as a reference to calculate the relative expression levels of *tax* and *gag* with the ddCt method. (C) RNA-FISH of HTLV-1 infected cell lines and Jurkat cells (scale bar, 5  $\mu$ m). (D) *gag* mRNA spots per cell detected by RNA-FISH in HTLV-1-infected cell lines and Jurkat cells. \*\*\*\* $p$  < 0.0001. (E) Duolink PLA of HTLV-1 infected cell lines and Jurkat cells (scale bar, 5  $\mu$ m). Arrows indicate the signals of p24 protein. (F) Gag protein spots per cell detected by Duolink PLA in HTLV-1 infected cell lines and Jurkat cells. \*\*\*\* $p$  < 0.0001.

antigen. Therefore, a high amount of anti-p15 antibody in HAM/TSP patients might reflect the higher replication rate of the virus in those patients than in subjects with other disease statuses such as asymptomatic carriers or ATL. In sum, antibodies to these antigens appear to be promising markers for the prediction of both ATL and HAM/TSP, although more samples and different cohorts are still needed to verify this finding.

Human T-cell leukemia virus type 1 replication is generally suppressed *in vivo*. Since Tax is critical for viral expression,<sup>40,41</sup> it has been thought that all viral transcripts encoded in the plus strand of the provirus are also suppressed in the absence of Tax.

Tax is transiently induced in ATL cells by cellular stresses, and it functions to suppress apoptosis and promote viral replication.<sup>42</sup> In this study, through the profiling of antibodies against multiple viral antigens, we unexpectedly identified the expression of Gag in ATL cells. Gag proteins, which are translated from unspliced viral RNA, were observed even in Tax-deficient ATL cells, indicating that the gene can be transcribed in a Tax-independent manner. A previous study showed that, after *ex vivo* culture of ATL cells, induction of unspliced viral RNA was delayed but longer lasting than *tax/rex* expression,<sup>43</sup> suggesting that there are alternate mechanisms to regulate the expression of *gag* in addition to activation





**FIGURE 4** IFN- $\gamma$  ELISPOT assay for Gag. (A) Interferon-gamma (IFN- $\gamma$ ) spots of patient HSCT-4 after stimulation with Gag antigen peptide pools. (B–D) The proportion of IFN- $\gamma$  spots in patients with various human T-cell leukemia virus type 1 (HTLV-1)-associated diseases. Cells were stimulated with (B) p15 pooled peptides (1, 2), (C) p19 pooled peptides (1–3), and (D) p24 pooled peptides (1–4). The number of spots reported represents raw data minus the negative control (NC), which was stimulated without peptides. ATL-A, acute type; ATL-C, ATL chronic type; ATL-L, lymphoma type; ATL-S, smoldering type.

by Tax. Host transcription factors might be involved in this basal transcription. A recent study revealed that Yin Yang 1 (YY1) binds to viral RNA, but not DNA, and activates HTLV-1 transcription.<sup>44</sup>

The mechanism that triggers the initiation of viral transcription in a Tax-independent manner is not yet understood. It is also important to consider the virological significance of Tax-independent viral gene expression in ATL cells. It has been reported that the cis-acting regulatory sequence (CRS) in unspliced viral RNA is pivotal in its transportation to the cytoplasm and stabilization by Rex.<sup>45</sup> The presence of low levels of unspliced transcripts might be an idling mechanism that allows viral replication to accelerate quickly in response to some stimulation such as cytotoxic stress.

Gag might also be a novel target for immunotherapy. Since the persistence of HTLV-1 in vivo depends on the proliferation of infected cells rather than the replication of viral particles, cellular immunity plays an important role in regulating the abundance of the virus. Tax is recognized as the main target of CTLs, and a Tax-targeting immunotherapy has been developed.<sup>46</sup> In this study, the ELISPOT assay showed that functional Gag-specific CTLs are present in patients who respond well to HSCT. Cellular immunity against viral antigens is generally suppressed in ATL patients, and the graft versus ATL effect is thought to be important for long-term remission after HSCT,<sup>47</sup> suggesting that anti-Gag CTLs, in addition to anti-Tax CTLs, have suppressive effects on the development and recurrence of ATL.

This study demonstrates the potential of anti-Gag immunities as the predictive markers for HTLV-1-associated diseases. Evaluating the humoral immunity to Gag appears to be useful for predicting the onset of ATL and HAM/TSP, and Gag proteins have unforeseen, unexplored potential as novel targets for immunotherapy. It is hoped that further validation will lead to clinical application.

#### AUTHOR CONTRIBUTIONS

**Asami Yamada:** Conceptualization; data curation; formal analysis; methodology; writing – original draft. **Jun-ichirou Yasunaga:** Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; resources; supervision; writing – original draft. **Lihan Liang:** Data curation; formal analysis. **Wenyi Zhang:** Data curation; formal analysis. **Junya Sunagawa:** Data curation; formal analysis; methodology; writing – original draft. **Shinji Nakaoka:** Data curation; formal analysis; methodology; supervision; writing – original draft. **Shingo Iwami:** Methodology; supervision. **Yasunori Kogure:** Data curation; formal analysis; methodology; writing – original draft. **Yuta Ito:** Data curation; formal analysis. **Keisuke Kataoka:** Methodology; supervision. **Masanori Nakagawa:** Resources. **Masako Iwanaga:** Resources. **Atae Utsunomiya:** Resources. **Ki-Ryang Koh:** Resources. **Toshiki Watanabe:** Resources. **Kisato Nosaka:** Conceptualization; resources; supervision. **Masao Matsuoka:** Conceptualization; funding acquisition; investigation; supervision; writing – review and editing.

#### ACKNOWLEDGMENTS

The authors would like to thank Dr. Kaoru Uchimaruru for providing samples, Drs. Kosuke Toyoda, Takafumi Shichijo, and Mikiko Izaki for helpful discussion, Mrs. Miho Matsumoto for helping with the experiments, Dr. Rika A Furuta and Dr. Naoya Shinohara for technical advice, and Dr. Linda Kingsbury for proofreading.

## FUNDING INFORMATION

This work was supported by the Project for Cancer Research and Therapeutic Evolution (P-CREATE) (21cm0106306h0006 to J.Y. and M.M.) and the Research Program on Emerging and Re-emerging Infectious Diseases (21fk0108088h0003 to J.Y. and M.M.) from the Japan Agency for Medical Research and Development (AMED), KAKENHI (20H03514 to J.Y.; 19H03689 to M.M.) from the JSPS, the Japan Science and Technology Agency-Mirai Program (JPMJMI18G1 to J.Y.), and the Platform of Supporting Cohort Study and Biospecimen Analysis (CoBIA) (16H06277 to T.W.) from the MEXT. This study was also supported in part by the JSPS Core-to-Core Program A, Advanced Research Networks.

## CONFLICT OF INTEREST STATEMENT

Keisuke Kataoka and Masao Matsuoka are editorial board members, and the other authors have no conflicts of interest.

## ETHICS STATEMENT

Approval of the research protocol by an Institutional Reviewer Board: Our experiments were approved by the Institutional Ethics Committee of Kumamoto University (accession numbers: G489, G499, and E2214).

Informed Consent: All clinical samples were collected after written informed consent was obtained in accordance with the Declaration of Helsinki.

Registry and the Registration No. of the study/trial: N/A.

Animal Studies: N/A.

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#### SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

**How to cite this article:** Yamada A, Yasunaga J-i, Liang L, et al. Anti-HTLV-1 immunity combined with proviral load as predictive biomarkers for adult T-cell leukemia-lymphoma. *Cancer Sci*. 2024;115:310-320. doi:[10.1111/cas.15997](https://doi.org/10.1111/cas.15997)