Clonality analysis of HTLV-1-infected cells enhances prognostic precision in smoldering adult T-cell leukemia/lymphoma

Smoldering adult T-cell leukemia/lymphoma (ATL) is a clinically heterogeneous condition, with patients exhibiting varying risks for progression to aggressive ATL. Current diagnostic criteria, based solely on the presence of >5% abnormal lymphocytes, fail to capture this heterogeneity, creating a critical gap in risk assessment and clinical management. This study addresses this gap by introducing the clonality analysis of human T-cell leukemia virus type 1 (HTLV-1)-infected cells (Cv: clonality value) as a novel diagnostic and prognostic marker. Using the highly sensitive and cost-effective Rapid Amplification of Integration Sites without Interference by Genomic DNA (RAISING)-CLOVA (a clonality quantification software) method, we quantified the clonality of HTLV-1-infected cells and evaluated its prognostic impact alongside serum soluble interleukin-2 receptor (sIL-2R) levels. Our findings demonstrate that patients with smoldering ATL with the monoclonal or oligoclonal expansion of HTLV-1-infected cells are at high risk for progression to aggressive ATL, whereas those with polyclonal proliferation exhibit a more favorable prognosis. The combination of Cv and sIL-2R levels enables precise risk stratification, providing a framework for personalized, risk-adapted clinical management. Importantly, these results suggest that incorporating clonality analysis into the diagnostic criteria for smoldering ATL could refine prognostic accuracy, reduce unnecessary psychological burdens for low-risk patients, and improve therapeutic decision-making. This represents a paradigm shift in the diagnosis and management of smoldering ATL, with significant implications for patient outcomes and future treatment strategies.

HTLV-1 infects at least 5-10 million people worldwide and causes two distinct diseases: ATL and HTLV-1-associated-myelopathy/tropical spastic paraparesis (HAM/TSP).[1] ATL is highly aggressive, with a four-year survival rate of only 11% for the acute subtype, underscoring the urgent need for its early detection and risk stratification for effective treatment.[2]

Adult T-cell leukemia/lymphoma is classified into four clinical subtypes according to Shimoyama's criteria: smoldering, chronic, acute, and lymphoma types.[3-5] Patients with smoldering ATL are generally asymptomatic and are diagnosed based on the presence of >5% abnormal lymphocytes in peripheral blood.[3-5] However, prognosis varies widely. Although more than 60% progress to aggressive subtypes,[6] others remain stable or even transition between the smoldering ATL and asymptomatic carrier (AC) states, highlighting the heterogeneity of disease pathology and clinical progression. Thus, reliable methods for predicting progression risk are urgently needed to enable risk-stratified treatment strategies.

Assessing the risk of progression from smoldering to aggressive ATL is an actively evolving field. Serum sIL-2R levels ≥1,000 U/mL are associated with a higher risk of progression and increased mortality,[7] and this is the prognostic marker currently utilized in clinical practice.[8] Additionally, the peripheral blood HTLV-1 proviral load (PVL) is an established predictor of ATL onset,[9] but its utility in predicting the progression from smoldering to aggressive ATL remains unverified. Recent studies suggest that the degree of clonal expansion of HTLV-1-infected cells, including ATL cells, is a crucial risk factor for ATL progression.[10-14] However, clinical implementation has been limited because of a lack of large-scale longitudinal studies and technical challenges. Southern blot hybridization, historically used to assess the clonality of HTLV-1-infected cells in Japan, suffers from low sensitivity and qualitative limitations, restricting its clinical utility.[12,15]

To overcome these challenges, we developed RAISING-CLOVA, a highly sensitive and cost-effective method for quantifying virus-infected cell clonality (Cv).[14] Our previous longitudinal study in AC and HAM/TSP patients using RAISING-CLOVA demonstrated that a Cv ≥0.5 was associated with a higher risk of developing ATL.[14] In this study, we conducted a new longitudinal analysis to evaluate Cv as a predictive biomarker for progression risk in patients with smoldering ATL.

The present study was approved by the research ethics committees of Nagasaki University (16072504), St. Marianna University School of Medicine (1646, 2044, and 5807), and the National Institute of Infectious Diseases (1382). Cv, sIL-2R levels, and PVL were successfully obtained for all peripheral blood samples analyzed in this study, with no samples excluded because of technical failure.

First, we compared the distributions of Cv, sIL-2R levels, and PVL in AC (N=165), HAM/TSP (N=205), and smoldering ATL (N=125) (*Online Supplementary Table S1*). All three metrics were significantly higher in patients with smoldering ATL compared with AC or those with HAM/TSP (Figure 1A-C). Notably, some patients with smoldering ATL exhibited Cv comparable with those observed in AC and patients with HAM/TSP, suggesting underlying heterogeneity (Figure 1C). Next, we classified patients with smoldering ATL into three risk groups using the Cv cut-off values previously

established for ATL risk in AC and patients with HAM/TSP: low-risk ≤0.24; intermediate-risk 0.25–0.49; and high-risk ≥0.50.[14] Most patients with smoldering ATL (65.6%) were classified as high-risk, whereas the remainder were distributed between the intermediate-risk (19.2%) and low-risk (15.2%) groups (Figure 1D). This distribution closely mirrored previous reports based on Southern blot hybridization, in which patients were classified using a different set of three groups: those with monoclonal (57.4%), oligoclonal (21.4%), or polyclonal bands (21.2%) bands.[15] Indeed, both metrics clearly demonstrated there is substantial heterogeneity among patients with smoldering ATL, who were all diagnosed using the same criterion of >5% abnormal lymphocytes.[3-5]

We then evaluated the utility of Cv, sIL-2R, and PVL as predictors of progression from smoldering to aggressive ATL. Progressors (N=23), who progressed to aggressive ATL during follow-up (overall period 0-167 months; average period 56.4 months) exhibited significantly higher Cv, sIL-2R, and PVL than non-progressors (N=61) (Figure 2A). Receiver operating characteristic analysis revealed that Cv (area under the curve [AUC] = 0.780) and sIL-2R (AUC = 0.777) were more effective than PVL (AUC = 0.717) at distinguishing progressors from non-progressors (Figure 2B). To further evaluate the accuracy of Cv (≥0.50) and sIL-2R (≥1,000 U/mL) cut-off values, we analyzed their distributions across different ATL subtypes, including chronic ATL (total N=48; favorable N=24; unfavorable N=24), acute ATL (N=101), and smoldering ATL (total N=84; non-progressor N=61; progressor N=23), as well as non-ATL (total N=370; AC N=165; HAM/TSP N=205) (Figure 3A). The distribution based on four regions (R1-R4) demonstrated that non-ATL patients were predominantly located in R1, whereas chronic and acute ATL patients were primarily found in R4. In contrast, smoldering ATL patients were heterogeneously distributed across R1, R2, and R4, further highlighting the marked variability within this population. These findings support the potential utility of this method for the risk assessment of disease progression in patients with smoldering ATL.

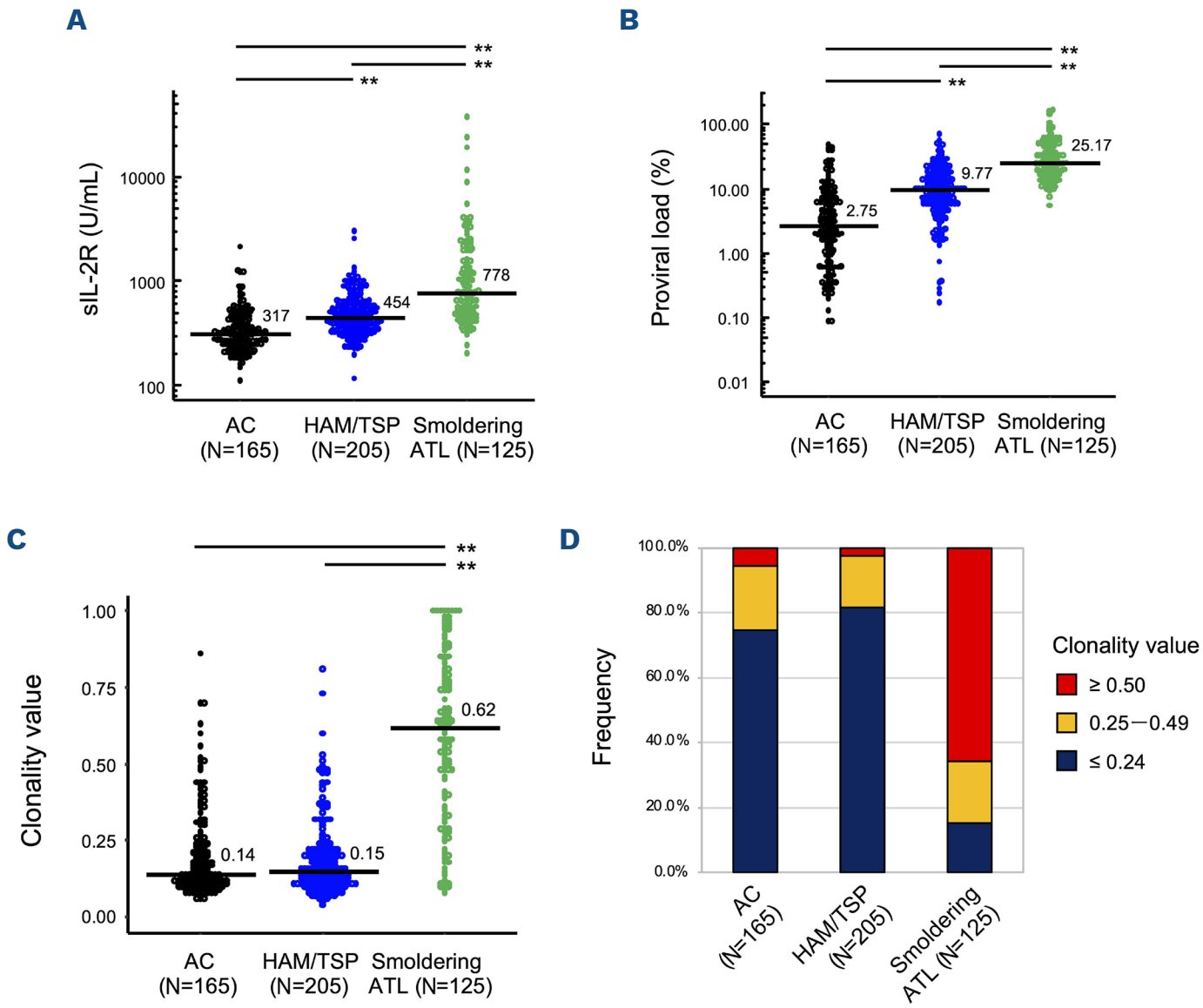

Figure 1. Characteristics of asymptomatic carriers, patients with HTLV-1-associated myelopathy/tropical spastic paraparesis, and patients with smoldering adult T-cell leukemia/lymphoma. (A) Serum soluble interleukin 2 receptor (sIL-2R) concentrations in asymptomatic carriers (AC) (N=165), and patients with HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (N=205) or smoldering adult T-cell leukemia/lymphoma (smoldering ATL) (N=125). Proviral loads (B) and clonality values (C) were measured in the same samples as in (A). (D) AC, HAM/TSP, and smoldering ATL were categorized into three groups based on the indicated clonality values, and the frequencies are displayed. Median values are shown by horizontal lines. *P* values were calculated using Dunn multiple comparisons tests; ***P*<0.01.

Based on this classification, we assigned patients with smoldering ATL to these four regions (*Online Supplementary Table S2*) and stratified them further into three progression risk groups: low-risk (R1), intermediate-risk (R2 and R3), and high-risk (R4). Although the number of patients with smoldering ATL in R3 was relatively small and no progressors were identified in this category, previous reports indicating a poor prognosis in patients with smoldering ATL with sIL-2R levels of ≥1,000 U/mL led us to categorize R3 as part of the intermediate-risk group.

Finally, we assessed the cumulative incidence rates of progression from smoldering to aggressive ATL, analyzing the impact of Cv and sIL-2R cut-off values independently and in combination (Figure 3B-D). Only patients with a Cv ≥0.5 progressed to the aggressive form (mean time to progression [MTTP]: 99.0 months), confirming Cv as a highly reliable predictor of progression risk for smoldering ATL (Figure 3B). Consistent with previous studies, sIL-2R alone was also a significant prognostic marker, with patients exhibiting sIL-2R levels ≥1,000 U/mL progressing significantly earlier to the aggressive form (MTTP: 44.0 months (Figure 3C).[7,8] Notably, when the Cv and sIL-2R cut-off values were combined, patients were effectively stratified into three risk groups (R1, R2 and R3, and R4), which correlated with no, slow, and rapid progression to aggressive ATL, respectively (MTTP: low-risk, not reached; intermediate-risk, 156.0 months; high-risk, 25.0 months) (Figure 3D). This combined approach provides a more refined tool for assessing progression risk, clearly differentiating the high-risk group (R4), which may require therapeutic intervention, from the low-risk group (R1), where intervention is unnecessary.

Despite these promising findings, two important limitations must be addressed in future investigations. First, our study excluded patients with smoldering ATL with skin or lung lesions, necessitating further research to determine the progression risk of these patient subgroups. Second, the small number of patients in the R3 category limited the strength of conclusions regarding their classification as intermediate-risk, warranting additional studies to validate this approach.

In conclusion, the combination of Cv and sIL-2R as prognostic markers effectively captured the heterogeneity of smoldering ATL patients and was a highly effective predictor of progression to aggressive ATL. Moreover, our findings provide compelling evidence that integrating Cv

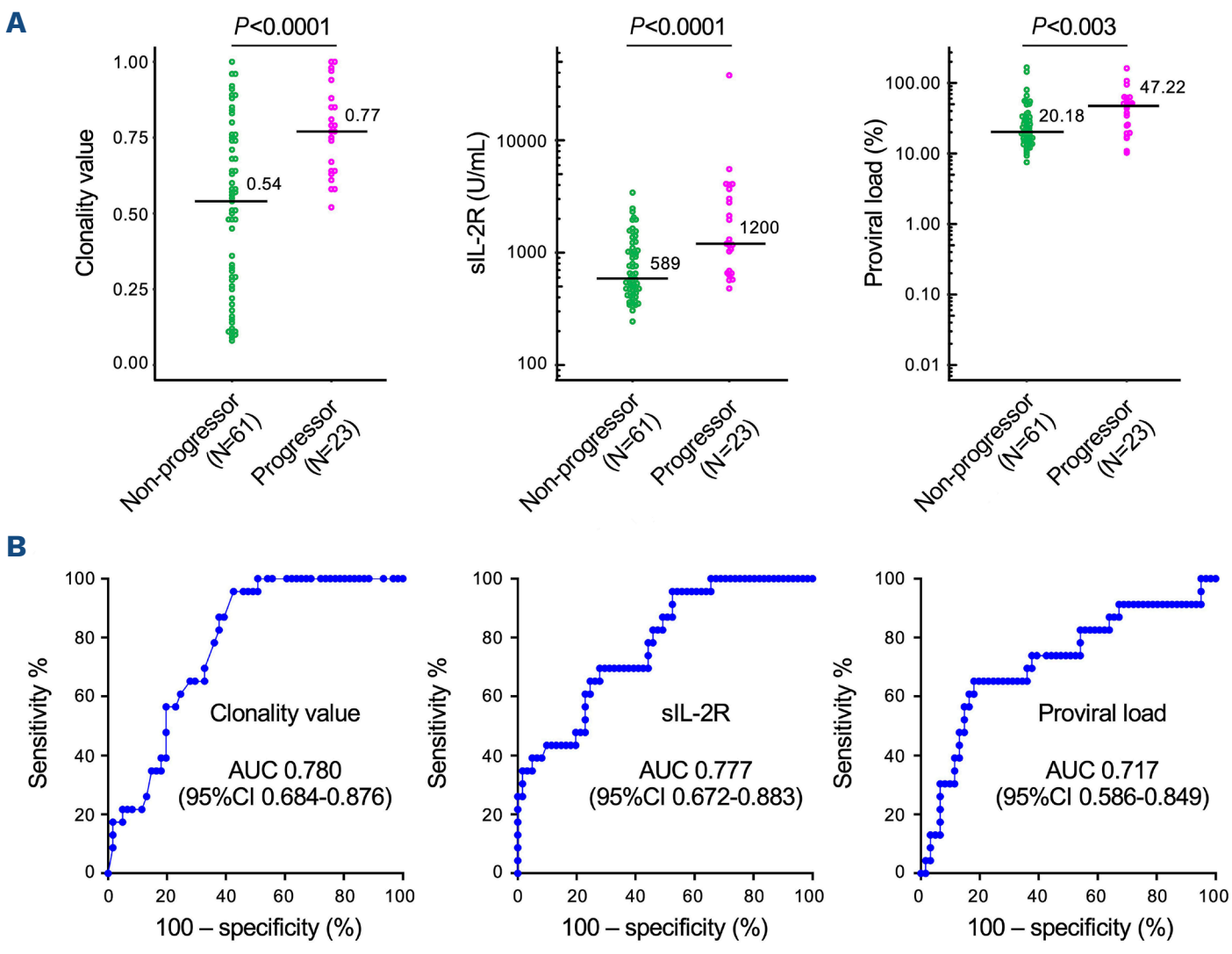

Figure 2. Characteristics of non-progressors and progressors among patients with smoldering adult T-cell leukemia/lymphoma. (A) Clonality values, soluble interleukin 2 receptor (sIL-2R) concentrations, and proviral loads in non-progressors (N=61) and progressors (N=23) among patients with adult T-cell leukemia/lymphoma (smoldering ATL) patients. Median values are represented by horizontal lines. *P* values were calculated using the Mann-Whitney *U* test. (B) Receiver operating characteristics analysis was performed using data from the same samples in (A). The effectiveness of clonality value, sIL-2R concentration, and proviral load to discriminate between progressors and non-progressors is shown. AUC: area under the curve; CI: confidence interval.

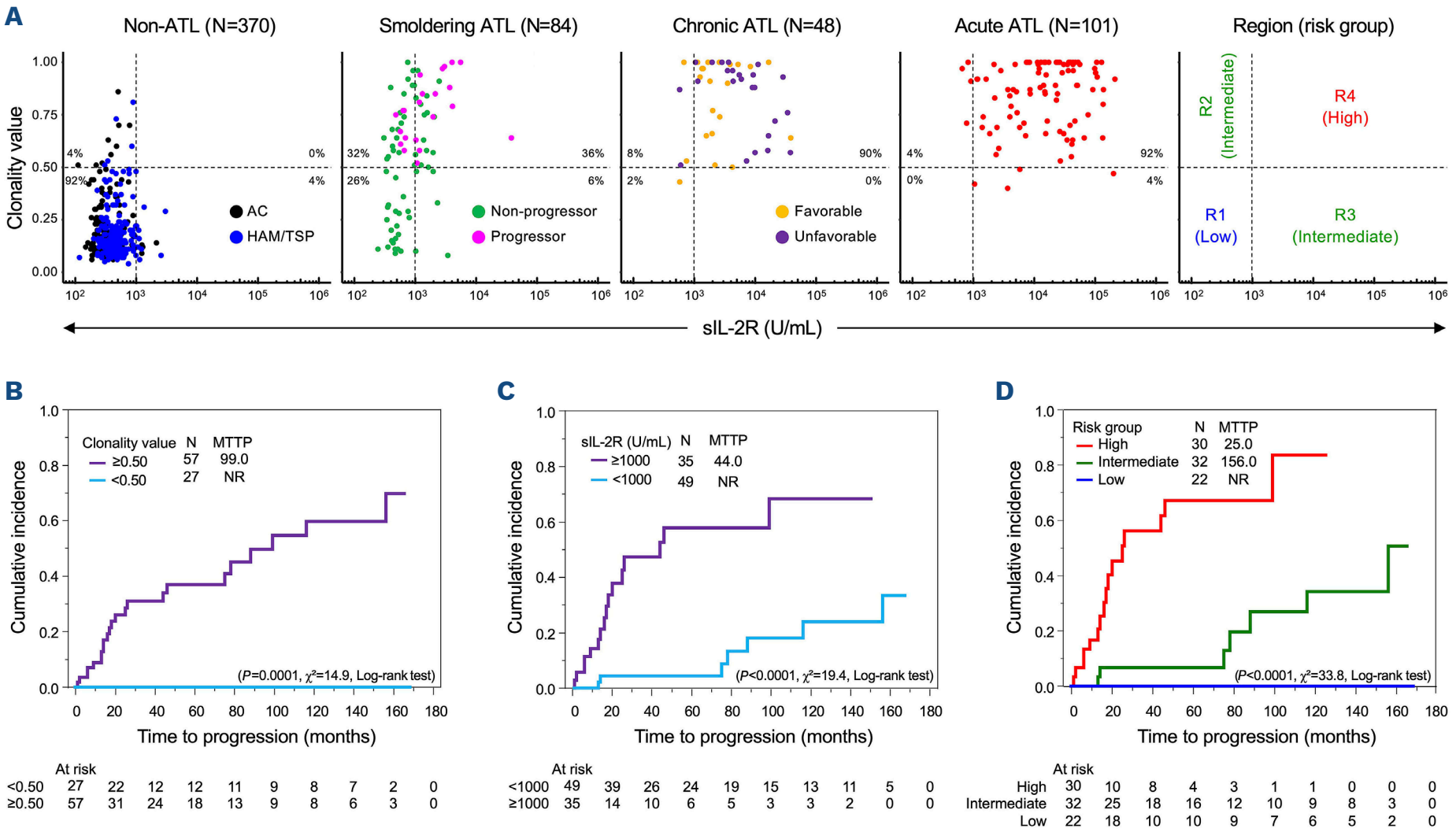

Figure 3. Stratification of progression risk from smoldering to aggressive adult T-cell leukemia/lymphoma. (A) Bivariate analysis of clonality values and soluble interleukin 2 receptor (sIL-2R) levels for non-adult T-cell leukemia/lymphoma (ATL) (total, N=370; asymptomatic carrier [AC], N=165; patients with HTLV-1-associated myelopathy/tropical spastic paraparesis [HAM/TSP], N=205), smoldering ATL (total, N=84; non-progressor, N=61; progressor, N=23), chronic ATL (N=48), and acute ATL (N=101). The dotted lines indicate the cut-off for the clonality value (0.50) and sIL-2R concentrations (1,000 U/mL). The frequencies of samples in each population are shown across four regions (R1, R2, R3, and R4). These regions were further categorized into three groups to assess the risk of progression from smoldering to aggressive ATL (low-, intermediate-, and high-risk groups). (B-D) Cumulative incidences of progression from smoldering to aggressive ATL using the clonality value alone (B), sIL-2R concentration alone (C), and a combination of both (D). Risk groups are defined as indicated in (A). N: number of samples; MTTP: median time to progression; NR: not reached.

cut-off values into diagnostic criteria enhances prognostic precision. If validated in larger cohorts and confirmed by comparing the other clonality analysis methods, such as flow cytometry and Southern blot hybridization, in the same cohort,[12,13] this approach could fundamentally shift the clinical management of smoldering ATL, enabling more precise risk stratification and tailored therapeutic strategies.

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https://doi.org/10.3324/haematol.2025.287521

Received: February 4, 2025.
Accepted: April 18, 2025.
Early view: April 30, 2025.

Disclosures
No conflicts of interest to disclose.

Contributions
MS, HY-F and NN performed the research. MS, TS, KT, HY-F, EH, SY, MN, NA, NY, KT, NN, DS, TW and YY performed the data analysis. TS, HH, EH, SY, MN, NY, DS, YS, KU, AU, KK-R, TK, HI, YM, KO, AA, TW and YY provided the patients' clinical information and samples. MS, TS, HH, AC-R, KT, TK, HI and YY wrote the manuscript. MS, TS, HH, TW and YY supervised the study.

Acknowledgments
We thank the asymptomatic carriers and patients who participated in this study and the Joint Study on Predisposing Factors of ATL Development (JSPFAD) research group for providing the clinical information and specimens. We thank J. Ludovic Croxford, PhD, from Edanz (https://jp.edanz.com/ac) for editing a draft of this manuscript.

Funding
This work was supported in part by grants from the Japan Society for the Promotion of Science (JSPS) KAKENHI (N. JP22H02972, JP22H02987, JP22H04923 [CoBiA]), the Japan Agency for Medical Research and Development (AMED) (N. JP23ek0109529, JP24ek0109735, JP23ek0109529, JP23ek0109548, 24ek0109617, 24ek0109735, 24ek0109759, JP23fk0108672, JP23wm0325056), and Rare and Intractable Diseases from the Ministry of Health, Labour and Welfare of Japan (N. JPMH22FC1013, 24FC2002).

Data-sharing statement
Data are available on request from the corresponding authors.

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