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Upregulation of YY1/EZH2 and MLH1 as Therapeutic Targets for Adult T-Cell Leukemia/Lymphoma

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ABSTRACT

The clinical and genetic presentation of adult T-cell leukemia/lymphoma (ATLL) ranges from indolent to aggressive, making it difficult to identify common therapeutic targets. Inhibiting EZH1/2 suppresses ATLL through epigenetic modulation; however, the diverse genetic background of ATLL precludes its mode of action from being clearly elucidated. We conducted single-cell RNA sequencing (scRNA-seq) of primary ATLL cells and identified an epigenetic regulative axis. First, flow cytometry showed that the proliferative potential of CADM1⁺ HTLV-1-infected cells ranges from stable to treatment-required. Second, scRNA-seq identified a CCR4⁺CD48⁻ cluster, the population of which increased in treatment-required patients. In silico promoter analysis of this cluster identified a transcription factor YY1 as a candidate regulator. Intracellular flow cytometry confirmed that YY1 and EZH2 were upregulated in acute-type. By contrast, MLH1 but not MSH2 within CADM1⁺ cells was downregulated in remitted ATLL ($p < 0.05$), suggesting that MLH1 is associated with YY1/EZH2. Notably, lentiviral YY1 knockdown and the EZH1/2 inhibitor valemetostat downregulated MLH1 in ATLL cell lines and primary ATLL cells. Finally, knockdown of YY1 or MLH1 suppressed the proliferation of ATLL cells. Our findings suggest that YY1/EZH2 overexpression in the ATLL subpopulation defines aggressiveness and that MLH1 downregulation through YY1/EZH2 inhibition may be an effective treatment for aggressive ATLL.

1 | Introduction

ATLL is a unique peripheral malignancy derived from T cells infected with human T-lymphotropic virus type 1 (HTLV-1) [1, 2]. The disease is classified into four clinical subtypes: smoldering, chronic, lymphoma, and acute [3]. Smoldering type and chronic type without poor prognostic factors are regarded as indolent,

with 4-year survival rates of 52% (smoldering) and 36% (chronic), respectively. Acute, lymphoma, and unfavorable chronic types are regarded as aggressive, with survival rates of 16% (lymphoma) and 11% (acute) [4]. However, clinical presentation and prognosis are heterogeneous, making the clinical definition of these subtypes difficult [5], that is, lymphoma-type ATLL extranodal primary cutaneous variant [6], and acute type ATLL in spontaneous

Abbreviations: ATLL, adult T-cell leukemia/lymphoma (ATLL); ChIP, chromatin immunoprecipitation; DEGs, differentially expressed genes; EZH2, enhancer of zeste homolog 2; GO, gene ontology; H3K27me3, histone H3Lys27; HTLV-1, human T-lymphotropic virus type 1; iATL-PI, indolent ATLL prognostic index; MMR, mismatch repair; NF- κ B, NF- κ B nuclear factor-kappa B; PI, prognostic index; PRC2, polycomb repressive complex 2; scRNA-seq, single-cell RNA sequencing; sIL-2R, soluble interleukin 2-receptor; UMAP, uniform manifold approximation and projection for dimension reduction.

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remission without chemotherapy [7]. To overcome this dilemma and optimize therapeutic strategies for ATLL, a prognostic index (PI) for aggressive ATLL [8–10] and indolent ATLL (iATL-PI) was developed [11]; however, even in patients with low-risk iATL-PI, some cases of smoldering-type progress to aggressive ATLL within years. By contrast, some cases of smoldering-type can remain stable for over years. Because the pathology of ATLL is heterogeneous, there is an unmet need to predict and prevent its progression.

Whole genome/exome sequencing of ATLL has provided genetic insight into the key molecules that function in the T-cell receptor signaling and nuclear factor-kappa B (NF- κ B) signaling pathways [12]. sc-RNA-seq revealed that key driver mutations emerge prior to the onset of aggressive ATLL, enabling identification of high-risk groups [13–16]. To understand the diverse genetic abnormalities, studies have identified Tax [17], HBZ [18], and BATF3/IRF4 [19–21] as possible master regulators of ATLL tumorigenesis. In addition, histone trimethylation and DNA repair abnormalities are thought to exert dynamic control over ATLL-related genes. For example, overexpression of enhancer of zeste homolog 2 (EZH2) and polycomb repressive complex 2 (PRC2)-mediated trimethylation at histone H3Lys27 (H3K27me3) is common in ATLL [22, 23]. This led to the development of the EZH1/2 inhibitor valemestostat [24, 25]; however, the downstream therapeutic targets of the inhibitors are so diverse that mechanisms underlying resistance require further investigation [26]. In addition, the mechanisms that act as upstream regulators of EZH1/2 were not fully understood.

There is a suggested molecular link between EZH2 and the DNA repair pathway [27, 28]. Downexpression of mismatch repair (MMR) genes has been reported in ATLL [29, 30]. Previously, we identified HBZ-mediated DNA repair abnormalities as therapeutic targets [31–33]. These suggest that DNA repair defects lead to genetic instability in ATLL cells. However, others suggest that DNA repair dysfunction might increase the expression of neoantigens, which is associated positively with antitumor immunity and provides a rationale for anti-PD-1 therapy [34–37]. In general, downexpression of MMR genes may be an unfavorable prognostic factor (e.g., as in Lynch syndrome) [38], but the significance of MMR in ATLL remains unclear.

Therefore, we decided to use a scRNA-seq analysis to investigate the mechanisms involved in ATLL progression. Then, we used clinical specimens to examine the interaction/relationships between target proteins and disease phenotypes.

2 | Materials and Methods

2.1 | Measurement of Peripheral Biomarkers

HTLV-1 carriers and ATLL patients who visited Kyoto University Hospital were included. The percentage of abnormal lymphocytes, soluble interleukin 2-receptor (sIL-2R), and HTLV-1 proviral load was measured.

2.2 | Cell Lines

ATL43T and ED-40515(+), both of which are ATLL patient-derived cell lines, were cultured in RPMI-1640 medium (Nacalai

Tesque) supplemented with 10% FBS (Life Technologies), 1% penicillin–streptomycin–glutamine (Nacalai Tesque), and 70 U/mL recombinant human IL-2 (Shionogi).

2.3 | Preparation of Peripheral Blood Mononuclear Cells (PBMCs)

Peripheral blood was obtained from HTLV-1 carriers and ATLL patients, and whole PBMCs were isolated by density gradient centrifugation using Lympholyte-H (Cedarlane Laboratories). PBMCs were cultured in RPMI medium supplemented with 10% FBS and 1% PSG at 37°C/5% CO₂. Alternatively, cells were lysed in Cellbanker 1 (Takara Bio) and stored in a –80°C deep freezer for subsequent analyses.

2.4 | Detection of HTLV-1-Infected Cells Using HTLV-1 Analysis System (HAS) flow

PBMCs were suspended in Dulbecco PBS (Nacalai Tesque) supplemented with 1% FBS and incubated for 15 min at room temperature in the dark with antibodies. CD4+ T cells were classified as normal T cells (CADM1-CD7⁺), ATLL cells (CADM1⁺CD7[–]), or HTLV-1-infected cells (CADM1⁺) [39]. The following antibodies were used for HAS-flow: anti-CCR4-FITC (R&D Systems), anti-CD7-PE (BD Biosciences), anti-CD4-PerCPCy5.5 (BD Biosciences), anti-CD25-PECy7 (BioLegend), anti-CADM1-Alexa Fluor 647 (MBL), anti-CD3-APC-Cy7 (BD Biosciences), and anti-IgG2b- κ -FITC (BD Biosciences). To stain intracellular proteins, cells were fixed and permeabilized using an Intracellular Flow Cytometry Kit (Cell Signaling Technologies) and then stained with the following antibodies (Cell Signaling Technologies): anti-MSH2, anti-MLH1, anti-YY1, anti-EZH2, anti-rabbit IgG, anti-mouse IgG, anti-rabbit-AlexaFluor 488, anti-mouse-AlexaFluor 488, anti-rabbit IgG Alexa Fluor 647, and anti-mouse IgG Alexa Fluor 647. Surface antigens were stained using anti-CD7-PE (BD Biosciences), anti-CD4-PECy7 (BD Biosciences), and anti-CADM1-Alexa Fluor 647 (MBL). The data were acquired using a FACS Lyric (BD Biosciences) and were analyzed using FlowJo software (version 10.8.2).

2.5 | scRNA-Seq and Analysis Pipelines

Whole PBMCs were isolated from three ATLL patients. Then, CD4+ T cells were isolated using the EasySep Human CD4+ T Cell Isolation Kit (Stem Cell Technologies). Isolated cells were labeled and pooled using a 3' CellPlex Kit Set A 48 rxns PN:1000261 (10X Genomics) according to the Cell Multiplexing Oligo Labeling for Single Cell RNA Sequencing Protocol (CG000391 Rev. A). Pooled samples were stained with trypan blue, then the condition and number of cells were evaluated using Countess3 (Invitrogen) and microscopy. The cell suspension was loaded into a Chromium Controller (10x Genomics) in accordance with the manufacturer's protocols. The labeled and pooled cells were used for cDNA synthesis and for generation of libraries using the Chromium Next GEM Single Cell 3' Reagent Kit v3.1 16 rxns PN:1000123 (Dual Index) (CG000388 Rev A) in accordance with the Feature Barcode technology for Cell Multiplexing. The following kits were used: Chromium

Next GEM Single Cell 3' Kit v3.1 16 rxns PN:1000123; 3' Feature Barcode Kit 16 rxns PN:1000262; Chromium Next GEM Chip G Single Cell Kit 48 rxns PN:1000120; Dual Index Kit TT Set A 96 rxns PN:1000215; and Dual Index Kit NN Set A 96 rxns PN:1000243. The quality of cDNA and the library was evaluated by using TapeStation2200 (Agilent Technologies). The library was sequenced using DNBSEQ-G400RS (MGI Tech. seq mode: 28×100bp). Cell Ranger software (10X Genomics, version 6.1.2), Seurat (version 4.3.0), R (version 4.0.2), and ggplot2 (version 3.4.2) were used for subsequent analyses.

2.6 | Lentiviral shRNA-Knockdown of ATLL Cell Lines

Lentivirus transduction of ATLL cell lines (ATL43T, ED-50515(+) or ED+) was performed using the Spinfection protocol [40], with minor modifications. Briefly, lentiviral particles bearing shYY1 and shMLH1 knockdown vectors were purchased from VectorBuilder Japan (vectorID: YY1, VB900067-2496kyp; MLH1, VB900051-5797upn; scramble, VB010000-0009mxc). Polybrene was omitted due to cytotoxicity. 1 µg/mL puromycin (Selleck) was added 48 h after transduction, and the cells were selected by culturing for 4 days. Lentivirus-transduced cells were isolated using an SH800 cell sorter (SONY Biotechnologies) prior to use in subsequent experiments. All experiments were conducted in accordance with local regulations and with the approval of Kyoto University.

2.7 | MTS Assay

Cells (1–3×10⁴ per well) were seeded into a 96-well plate. Cell viability was measured at different times using the CellTiter 96® AQueous One Solution Cell Proliferation Assay Kit (Promega). Optical density was measured in the iMark™ Microplate Reader (Bio-Rad) at a wavelength of 490 nm for 1 s. Each experiment was performed in triplicate and repeated three times.

2.8 | Analysis of Public Datasets

Gene-expression profiling of ATLL patients was performed previously [39], and the data were deposited in the Gene Expression Omnibus database (GSE55851). For microarray analysis, differentially expressed genes (DEGs) were screened by GEO2R. Publicly available chromatin immunoprecipitation (ChIP)-sequencing datasets were searched by ChIP-Atlas [41].

2.9 | Statistical Analyses

Data are presented as the mean ± SD (error bars denote the standard deviation). Statistical analysis using either a paired Student's t-test or one-way/two-way ANOVA followed by Tukey's multiple comparisons test was performed by GraphPad Prism (version 9). **p* < 0.05, ***p* < 0.01, ****p* < 0.001 were considered significant; N.S., not significant. Correlations between two groups were analyzed by calculating Pearson's correlation coefficient (*R*).

3 | Results

3.1 | Proliferative Potentials of CADM1⁺ Cells Are Heterogeneous

Peripheral blood samples were collected from 20 HTLV-1 carriers and 35 ATLL patients (11 smoldering-type, 7 chronic-type, 3 lymphoma-type, 6 acute-type ATLL, and 8 ATLL patients in remission for over 6 months) (Table 1). After separating PBMCs, cells were analyzed by HAS-flow [39]. Percentages of CADM1⁺ cells among CD4⁺ T cells were positively correlated with disease aggressiveness (Figure 1A). The percentages of CADM1⁺ cells among the CD4⁺ T population from HTLV-1 carriers were low (9.3% ± 6.4%), but were high in the population from patients with acute-type ATLL (77.3% ± 31.9%). In cases of smoldering-type, chronic-type, and lymphoma-type ATLL, the percentages were intermediate: 35.0% ± 28.4%, 34.1% ± 24.5%, and 25.8% ± 24.8%, respectively. For ATLL in remission, the percentages were 17.6% ± 17.9% (Figure 1B). Notably, there were some discrepancies between subtypes and %CADM1⁺ cells. Even in patients in remission, the percentages were heterogeneous. In one patient, the %CADM1⁺ cells decreased (Figure 1A, Lymphoma CR#1), but it was almost 50% in another who had been in remission for more than 5 years after CHOP therapy, without increase in sIL-2R (Figure 1A, Lymphoma CR#2).

Overall, the percentage of CD4⁺CADM1⁺ cells weakly correlated with sIL-2R (*R* = 0.25, *p* < 0.001), but it did correlate moderately with the percentage of abnormal lymphocytes (*R* = 0.35, *p* < 0.001).

TABLE 1 | Characteristics of HTLV-1 carriers and ATLL patients.

Variable	Median (range) or <i>N</i> (%)	
	63	(32–90)
Age, years		
Sex		
Male	21	(38)
Female	31	(56)
Unknown	3	(6)
Disease subtype		
HTLV-1 carrier	20	(36)
Smoldering	11	(20)
Chronic	7	(13)
Lymphoma	3	(6)
Acute	6	(11)
Remission	8	(14)
sIL-2R (U/mL)	1128	(175–11,500)
WBC (/µL)	8031	(2800–71,000)
Lymphocytes (%)	27	(5–69)
Abnormal lymphocytes (%)	4.8	(0–76)
HTLV-1 proviral load (copies/10 ng DNA)	427	(0–3500)

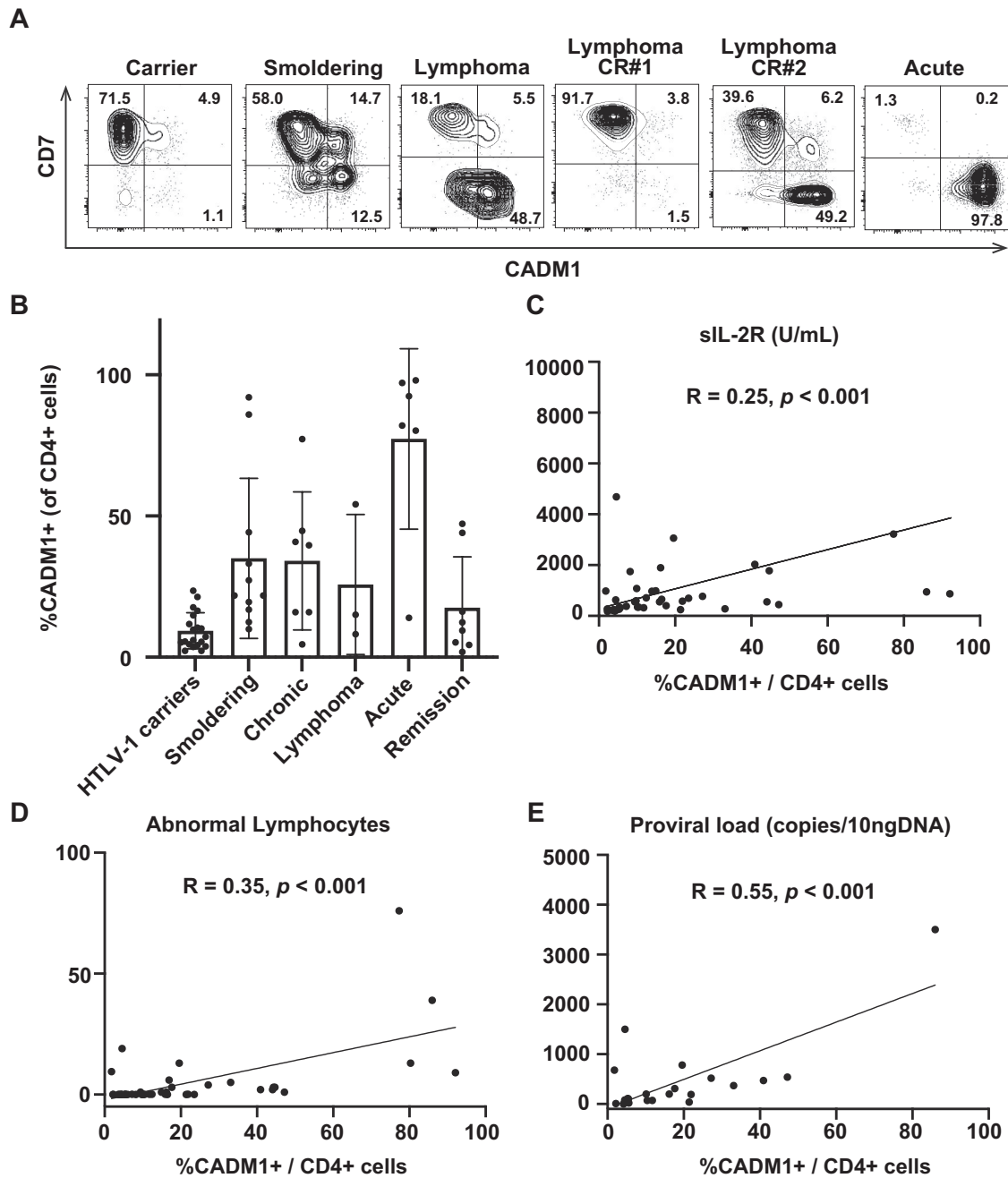


FIGURE 1 | Discrepancy between the frequency of CADM1⁺ cells and aggressiveness of ATLL. (A) Representative scattergrams for CADM1 versus CD7 gating on CD3⁺CD4⁺ cells (HAS-flow analysis) within each ATLL subtype. HTLV-1-infected cells are detected as CADM1⁺ cells. The numbers represent the percentage of cells in each quadrant. (B) Bar graphs showing the distribution of CADM1⁺ cells among CD4⁺ T cells across all the subtypes. X-axis = disease subtype; y-axis = percentage of CADM1⁺ cells among CD4⁺ T cells in each patient. Data are presented as the mean $\pm$ SD. (C–E) The percentage of CADM1⁺ cells among CD4⁺ T cells in HTLV-1 carriers and ATLL patients, and its correlation with clinical biomarkers, were analyzed by calculating Pearson's correlation coefficient (R). X-axis = percentage of CADM1⁺ cells among CD4⁺ T-cells; y-axis = soluble interleukin-2 receptor (sIL-2R) level (C), percentage of abnormal lymphocytes (D), and HTLV-1 proviral load (copies/10 ng DNA) (E) in peripheral blood. Abbreviation; CR, complete remission.

and the HTLV-1 proviral load ($R=0.55, p<0.001$) (Figure 1C–E). Taken together, these indicate that HTLV-1-infected cells belong exclusively to the CADM1⁺ fraction, as previously shown [39]. However, some CADM1⁺ cells were outliers, even in patients in remission (Figure 1B). Thus, the proliferative potential of CADM1⁺ cells is heterogeneous from stable to aggressive.

3.2 | Sc-RNA-Seq Identified a CD48-Deficient ATLL Cluster With High Expression of YY1/EZH2

We conducted sc-RNA-seq to highlight similarity and difference in gene expression profiles among stable (lymphoma-type ATLL in remission for over 5 years) and proliferative ATLL

(requiring treatments). The CADM1 versus CD7 plots of peripheral blood from analyzed patients are shown (Figure 2A). A stable patient (CM74) was diagnosed with lymphoma-type ATLL, kept in remission for over 5 years. CM75 and CM76 were classified as proliferative ATLL. CM75 was diagnosed with smoldering ATLL, but he required UVB therapy for active and progressive skin lesions on his extremities, that is, multiple erythema with some tumor-like lesions. CM76 was originally diagnosed with smoldering type with erythroderma and underwent UVB therapy. Ten years after the initial diagnosis, he developed systemic lymphadenopathy and progressed to acute type ATLL at the time of sample collection. He was successfully treated with mogamulizumab and CHOP therapy. The uniform manifold approximation and projection for dimension reduction (UMAP) of the scRNA-seq data using the CD4⁺ T cell-sorted three ATLL patients displayed 12 cell clusters (Figure 2B), and the cell clusters #0, 1, 3, 6, and 7 expressed *CCR4*, demonstrating ATLL cells. However, the *CCR4* expression varied in the ATLL populations and was the highest in cells of Cluster 7 (Figure S1). The further analysis of genes differentially expressed in Cluster #7 exhibited a significant reduction of *CD48* expression (Figure 2C), which is involved in NK cell activation and surveillance [42, 43]. Interestingly, the percentage of cells belonging to Cluster #7 in the case with remitted ATLL (CM74) was low (14.6%; Figure 2D, red bar); however, it was much higher in the case with smoldering ATLL with refractory skin lesions (CM75, 25.7%; Figure 2D, green bar) and in the case with acute ATLL (CM76, 59.7%; Figure 2D, blue bar). Also, *MALAT1* and *NEAT1* were expressed significantly higher in Cluster 7 (Table S1), showing a distinct expression pattern from other ATLL clusters (Figure S2). A gene ontology (GO) enrichment analysis showing that terms related to mRNA metabolic processes (GO: 0016071), chromatin organization (GO: 0006325), and activated T-cell responses (e.g., GO:0046649: lymphocyte activation, GO:0071345: cellular response to cytokine stimulus, GO:0043122: regulation of canonical NF- κ B signal transduction) were significantly enhanced in this cluster (Figure 2E), reflecting that ATLL cells in this cluster were prominently activated.

3.3 | Transcription Factor YY1 May Regulate the ATLL Cluster, Associated With Disease Progression

We address gene regulation of the Cluster #7 by motif analysis of promoter regions of Cluster 7-specific genes, and found that the consensus motif of a transcription repressor YY1-binding site was enriched on the promoter regions of Cluster 7-specific genes, implying that the property of the Cluster 7 cells was regulated by YY1 (Figure 2F). Although YY1 was expressed ubiquitously in ATLL Clusters 0, 1, 3, and 6, a subpopulation of cells in Cluster #7 showed high expression (Figure 2G), supporting YY1-involved gene regulation in Cluster 7. Expression of *EZH2* was also higher in Cluster #7 (Figure 2H) than in Clusters 0, 1, 3, and 6. These suggest that ATLL cells can be subclassified into YY1^{high} and YY1^{low} clusters. The top DEGs of Cluster 7 from each patient were plotted as a heatmap (Figure 2I). Interestingly, these three patients share common characteristics, that is, activation of *MALAT1*

and *NEAT1*, both of which are associated with *EZH2* in lymphomas [44]. Thus, scRNA-seq identified transcription factor YY1 as a regulator of a subcluster of cells that determines the aggressiveness of ATLL.

3.4 | Overexpression of YY1/EZH2 as a Potential Biomarker for Aggressive ATLL

Depending on a hypothesis that YY1 and *EZH2* in the cluster define tumor aggressiveness, the functional significance of YY1 and *EZH2* was verified, focusing on the expression of those proteins among CADM1⁺ cells of six cases by intracellular flow cytometry. The mean fluorescence intensity (MFI) values of the proteins were compared between ATLL cells (N fraction; CADM1⁺CD7⁻) and normal CD4⁺ T cells (P fraction; CADM1⁻CD7⁺). Interestingly, YY1 expression in ATLL cells was downregulated in patients in remission and in smoldering-type ATLL, but was upregulated in acute-type ATLL patients (Figure 3A).

In acute-type ATLL patients, YY1 (Figure 3B, middle row) and *EZH2* (Figure 3B, lower row) were upregulated in the N fraction (albeit with some fluctuation between cases). In acute-type #1, the YY1^{high}/*EZH2*^{high} ATLL cluster (Figure 3B, upper panel, red dots) and the YY1^{low}/*EZH2*^{low} ATLL cluster (Figure 3B, upper panel, blue dots) could be separated. In acute-type #2, *EZH2*^{high} ATLL cells were detected in the CADM1⁺ fraction (Figure 3B, upper panel, red dots). Interestingly, ATLL cells in acute-type #3 were YY1-positive but *EZH2*-dimly positive. These indicate that both YY1 and *EZH2* may be biomarkers of ATLL progression.

Due to difficulties in modulating YY1 expression in primary samples, we assessed the functional significance of YY1 using ATLL-derived cell lines. After confirming YY1 upregulation in ATL43T and ED+ (Figure 3C), they were transduced with a lentiviral shYY1 knockdown vector (Figure 3D). The proliferation of shYY1 knockdown cell lines was significantly lower than that of those transduced with a scramble vector (Figure 3E). Considering that primary samples showed bimodal expression of YY1 and *EZH2*, there may be a difference in the proliferative potential between YY1^{high} and YY1^{low} ATLL cells. These results suggest that YY1/*EZH2* overexpression is a potential biomarker of proliferation, making them an attractive therapeutic target in aggressive ATLL.

3.5 | Expression of YY1/EZH2 and *MLH1* Correlate With Disease Phenotype

YY1 and *EZH2* form the PRC2 complex, which is involved in gene regulation. Therefore, we hypothesized that MMR proteins might be downstream targets of YY1/*EZH2*. We used a microarray database (GSE55851) derived from ATLL patients to examine YY1, *EZH2*, and *MLH1* expression. ATLL cells (N fraction) from smoldering and acute ATLL were compared with normal CD4⁺ T cells (P fraction) from smoldering ATLL. In addition to YY1 ($p=0.10$), *EZH2* ($p=0.02$) and *MLH1* ($p=0.01$) were upregulated in acute ATLL (Figure 4A). These data raise potential correlations between them, but YY1 and *EZH2* expression were not always directly associated in

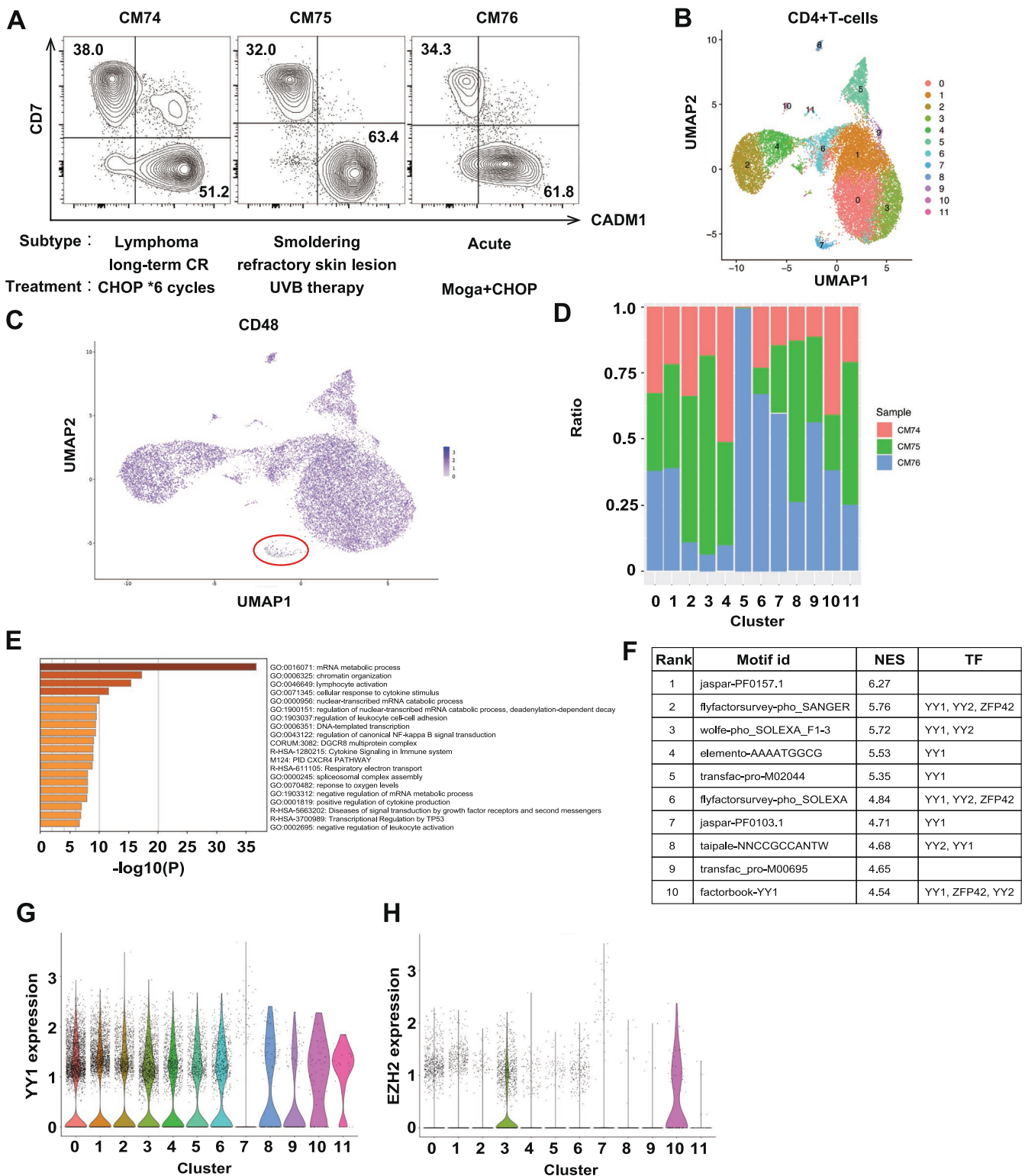


FIGURE 2 | Single-cell (sc) RNA-seq of ATLL cells identified a subcluster of cells showing downregulation of CD48 and overexpression of YY1/EZH2. Whole PBMCs and CD4⁺ T cells isolated by magnetic sorting were obtained from three ATLL patients and subjected to scRNA-seq. (A) CADM1 versus CD7 plots, gating on CD3⁺CD4⁺ T cells, the clinical subtype, and treatment regimens are also shown. (B) UMAP projection of single CD4⁺ cells from ATLL patients ($n=3$). Cells were classified into 12 clusters according to mRNA expression pattern. (C) CD48 was used to label clusters represented in the UMAP plot. Data are colored according to expression level, and the axes are labeled as a log scale. A CD48-downregulated cluster is gated (Cluster 7; red circle). (D) The percentage of cells in each of the 12 distinct clusters (X-axis) from the patients (Y-axis) is indicated in red (CM74), green (CM75), and blue (CM76). (E) Gene enrichment analysis of DEGs in Cluster 7. (F) In Cluster 7, the top 10 DEGs as well as in silico analysis of binding motifs for each gene are indicated. Violin plot showing expression of YY1 (G) and EZH2 (H). X-axis=cluster number; y-axis=expression level of each gene. (I) Heatmap showing expression of the top DEGs in Cluster 7 from each patient. The colored bars above the heatmap indicate patient ID. Y-axis=DEGs. CR, complete remission; Moga, mogamulizumab; NA, not available.

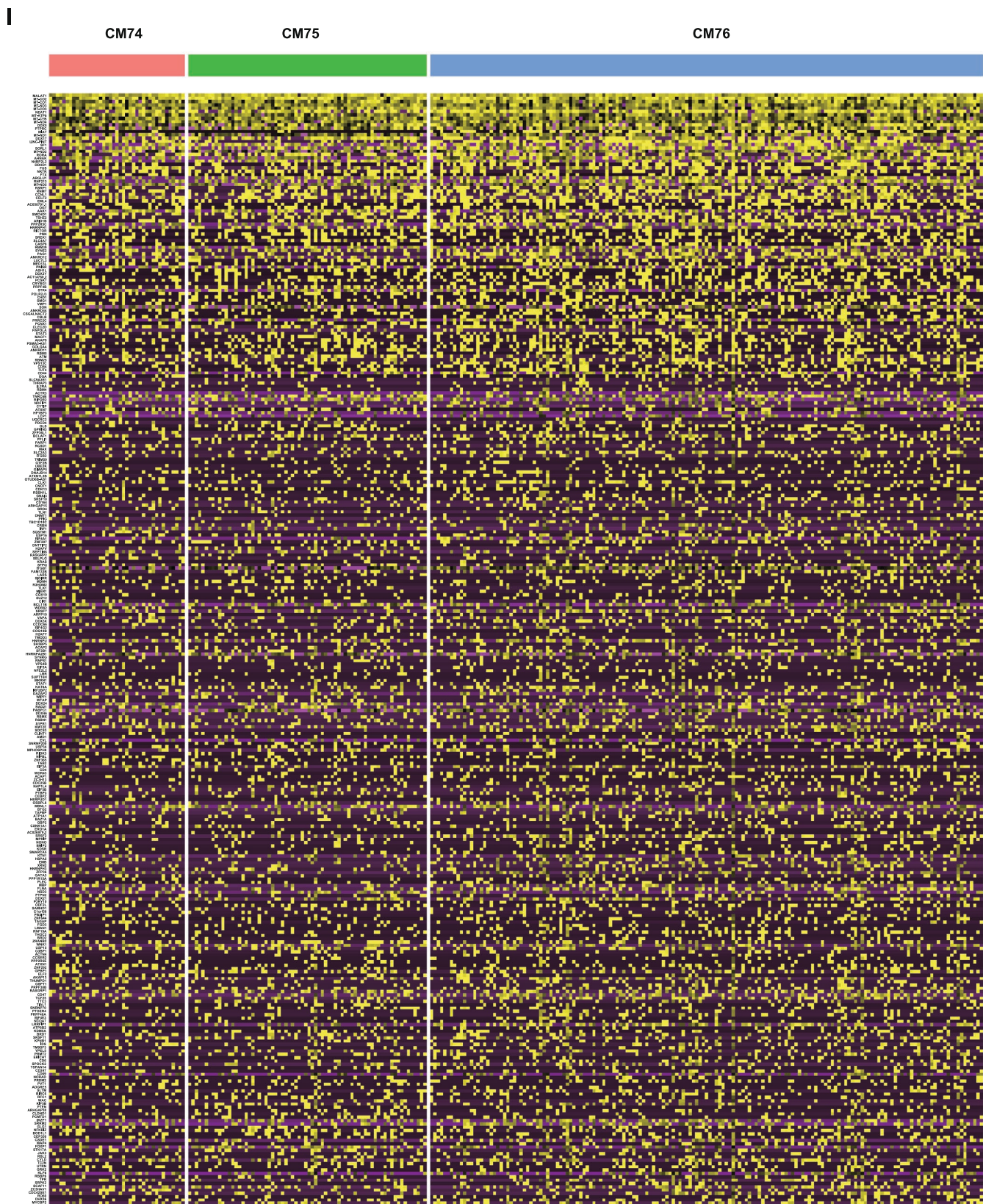


FIGURE 2 | (Continued)

individual cases (Figure 3B), suggesting complicated associations. On the other hand, there was no association between *MSH2* and disease phenotype ($p=0.44$). Next, we quantified

MLH1 expression in each cluster identified by scRNA-seq. Although *MLH1* was sparse, cells that expressed high levels of *MLH1* were present in Cluster 7 (Figure 4B).

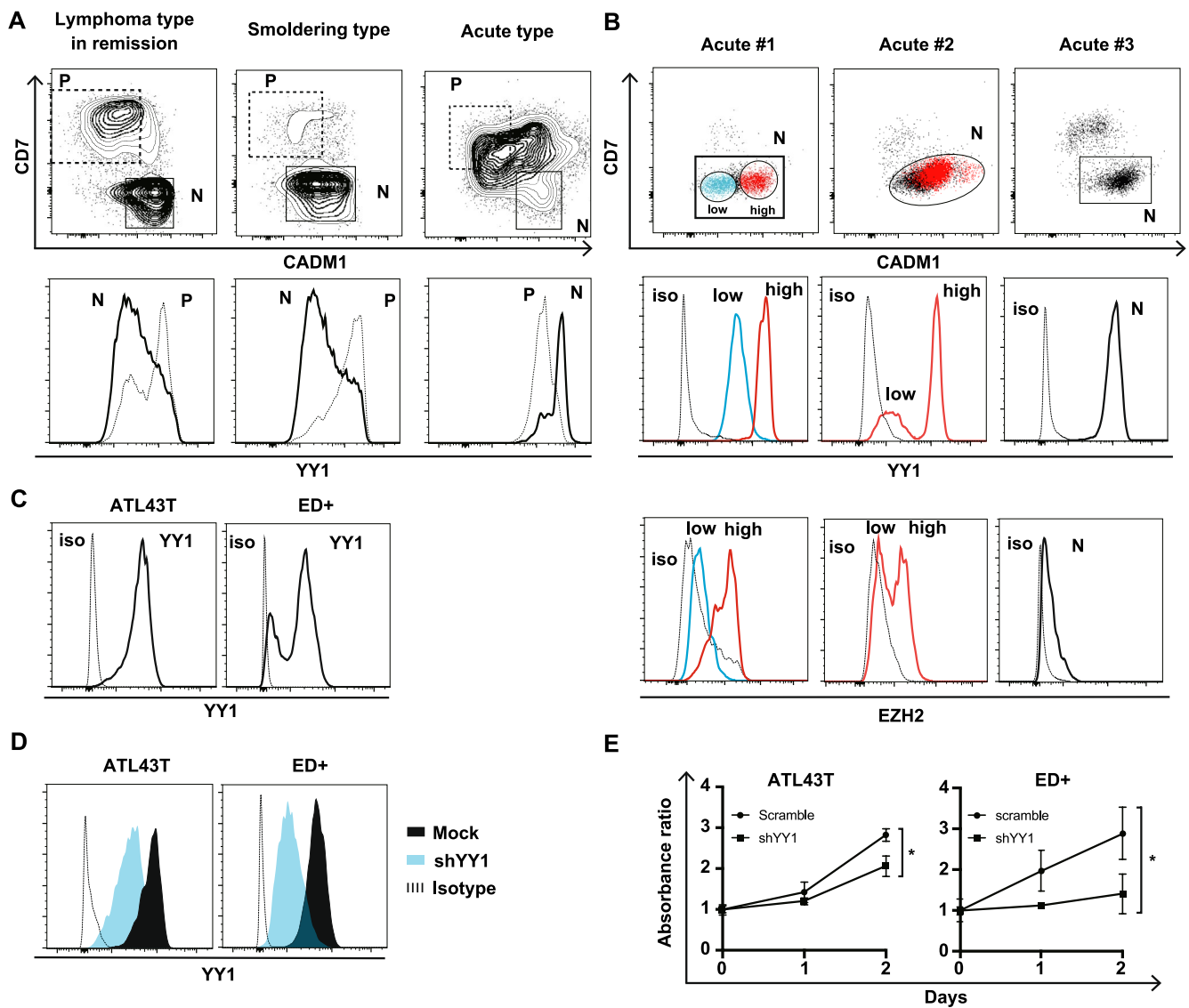


FIGURE 3 | Overexpression of YY1/EZH2 is a potential biomarker for aggressive ATLL. (A) Expression of intracellular YY1 protein in three ATLL patients was analyzed by flow cytometry. The mean fluorescence intensity (MFI) of YY1 expression in ATLL cells (N fraction; CADM1⁺CD7⁻ cells) was compared with that in normal CD4⁺ T cells (P fraction; CADM1⁻CD7⁺ cells). (B) Intracellular YY1 and EZH2 proteins in three patients with acute-type ATLL was analyzed by flow cytometry. Upper row: ATLL cells (N fraction) are gated with rectangles or an ellipse. The histogram shows expression of YY1 (middle row) and EZH2 (lower row) proteins in ATLL cells (N fraction). Expression of EZH2 was replotted on CADM1 versus CD7 plots. (C) Expression of YY1 in ATLL-derived cell lines (ATL43T, ED+) was analyzed by flow cytometry. YY1, bold line; isotype, dashed line. (D) A lentiviral shYY1 knockdown vector was transduced into ATLL-derived cell lines (ATL43T, ED+) and expression of YY1 protein (compared with that by a mock vector-infected cell line (black line), an shYY1-infected cell line (gray line), and an isotype control (dotted line)) was measured by flow cytometry. (E) MTS assay results following infection with the shYY1 vector. Absorbance ratio = absorbance on each day/absorbance at Day 0. The absorbance ratio was measured at triplicate and repeated three times with similar results. Results are expressed as the mean $\pm$ SD. Differences between groups were tested using two-way ANOVA. * $p < 0.05$.

To reveal the association between MMR proteins and disease subtype, we measured MLH1 and MSH2 in both normal CD4⁺ T cells (P fraction) and ATLL cells (N fraction) (Figure 4C). The MFI ratio for each patient was calculated as follows: (MFI of N fraction)/(MFI of P fraction). An MFI ratio > 1.0 indicates upregulation of the MMR protein in ATLL cells. The MFI ratio of MSH2 was not upregulated significantly in ATLL patients in remission when compared with other subtypes. With respect to MLH1, the ratio decreased in ATLL patients

in remission when compared with that in HTLV-1 carriers ($p = 0.04$) and acute-type ATLL ($p = 0.02$) (Figure 4D). This suggests that MLH1 downregulation is associated with remission. Interestingly, expression of MMR proteins fluctuated (Figure 4E). In this case, expression of MLH1 rather than that of MSH2 was downregulated in ATLL cells from patients in remission, but it was upregulated at relapse. These suggest that in addition to YY1/EZH2, MLH1 correlates with the proliferative capacity of HTLV-1-infected cells.

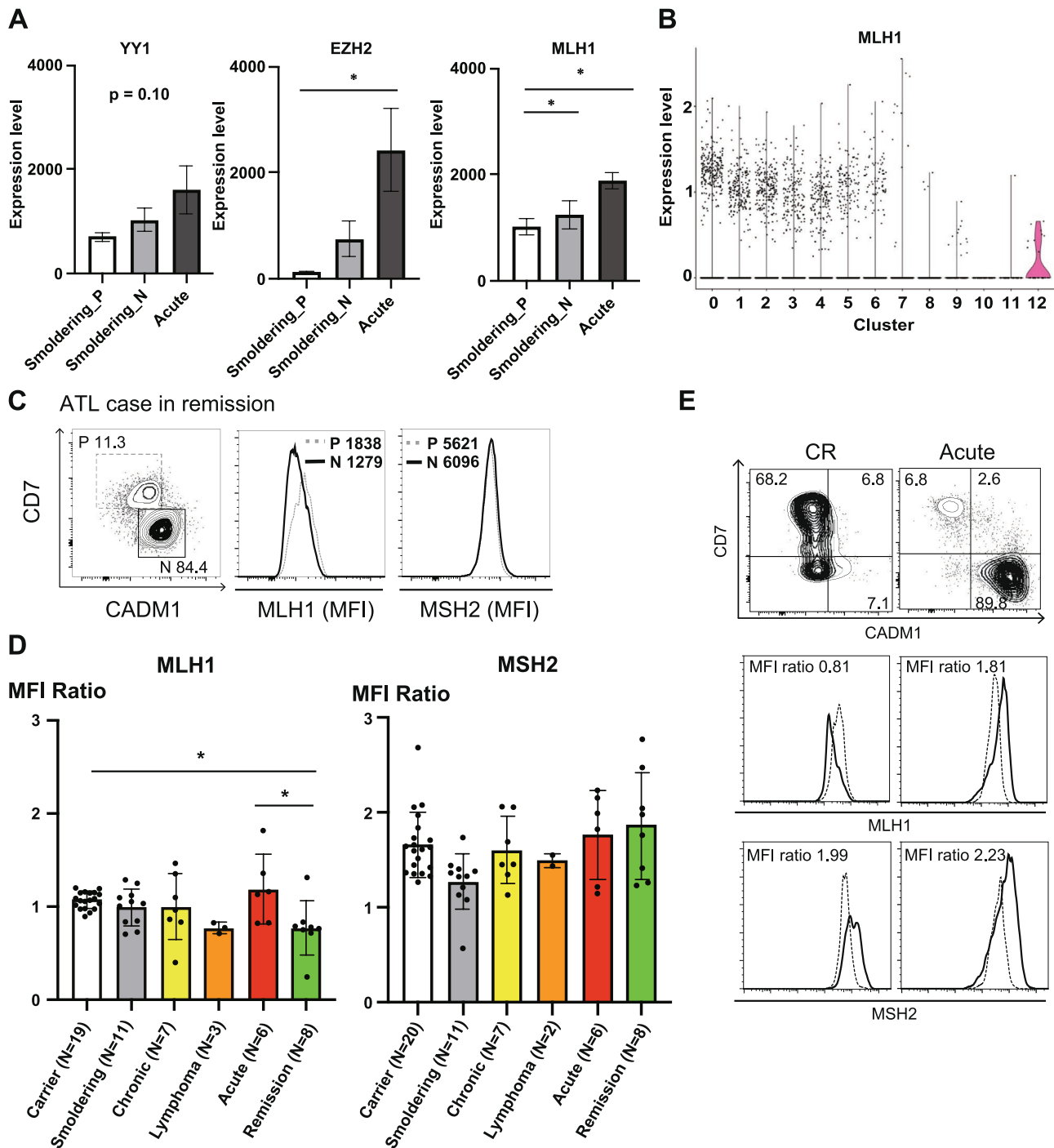


FIGURE 4 | Expression of YY1/EZH2 and MLH1 correlate with disease phenotype. (A) YY1/EZH2/MLH1 expression was extracted from microarray dataset (GSE 55851) and compared between the P fraction (CADM1⁻CD7⁺ cells) from patients with smoldering ATLL, N fraction (CADM1⁺CD7⁻ cells) from patients with smoldering ATLL and acute ATLL (one-way ANOVA). Data are presented as the mean $\pm$ SD. (B) Expression of MLH1 in each cluster, as determined by single cell-RNA-sequencing, is shown in violin plots. (C) Gating strategy used for analysis of mismatch repair protein (MLH1 and MSH2) expression by CD4⁺ T cells. CD4⁺ T cells were divided into a P fraction (CADM1⁻CD7⁺ cells, dotted line) and an N fraction (CADM1⁺CD7⁻ cells, bold line), and the MFIs of MLH1 and MSH2 were measured. (D) Expression of MLH1 and MSH2 by each subtype was compared. The MFI ratio for each patient was calculated as follows: MFI of the N fraction/MFI of the P fraction. X-axis = disease subtype; Y-axis = MFI ratio. Data are presented as the mean $\pm$ SD. (E) The MFI ratio for a representative ATLL patient was calculated at different time points (i.e., CR and progression of acute ATLL). N fraction (bold line) and P fraction (dotted line). CR, complete remission. N.S., not significant. * $p < 0.05$.

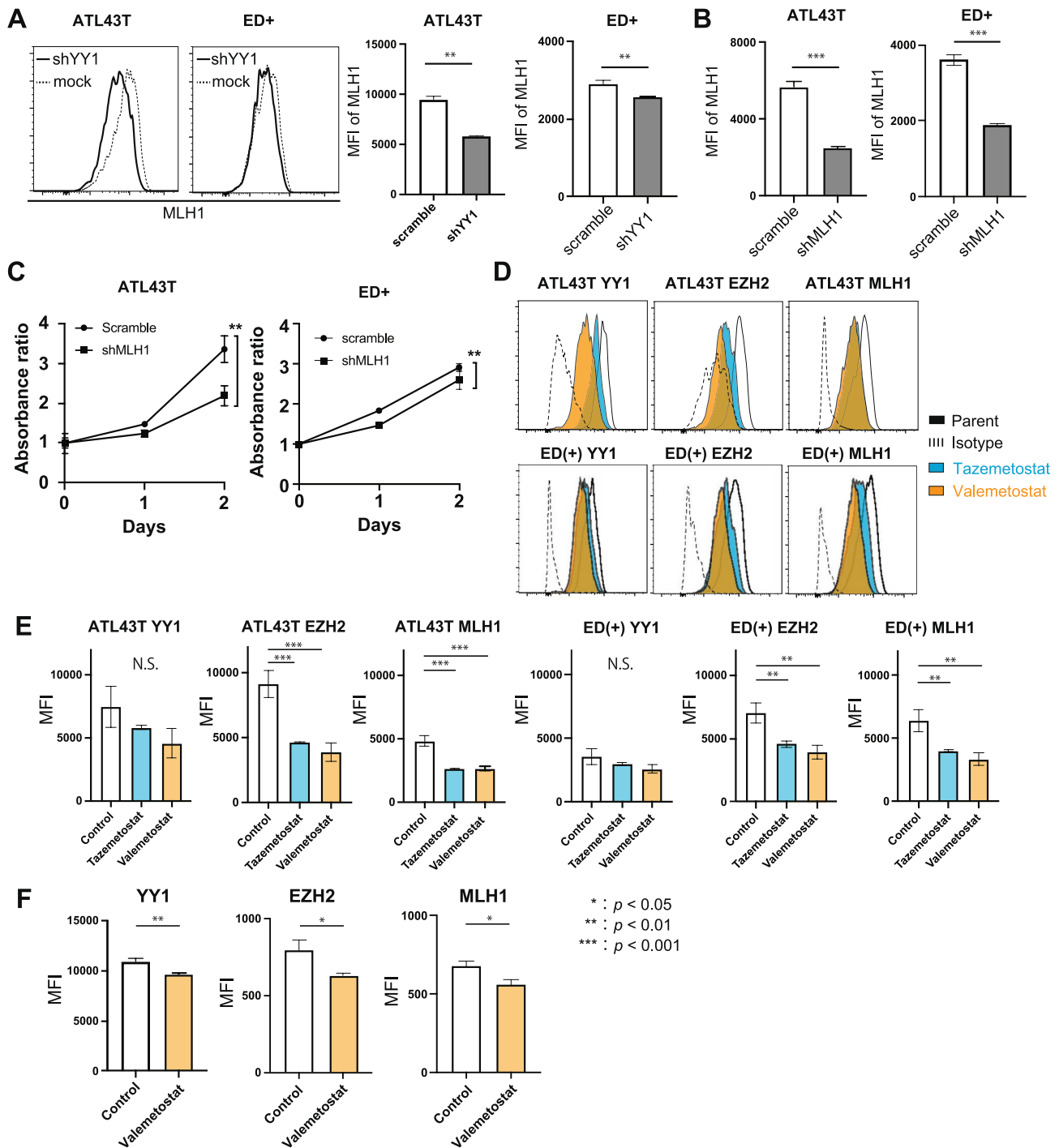


FIGURE 5 | MLH1 is a downstream target of YY1/EZH2 and a potential therapeutic target of valemetostat. (A) ATLL-derived cell lines (ATL43T, ED+) were transduced with either a lentiviral shYY1 knockdown vector (bold line) or a mock-vector (dotted line). MLH1 expression by shYY1-transduced cells was compared with that by mock-vector-transduced cells. Aggregate data are summarized in the right panel. (B) ATL43T and ED+ were transduced with either a lentiviral shMLH1 knockdown vector or a mock vector. MLH1 expression by both transduced cell lines was measured in triplicate by flow cytometry. Data were analyzed by one-way ANOVA. (C) An MTS assay was performed using either shMLH1-knockdown or mock-vector-transduced ATLL-derived cell lines (ATL43T, ED+). Absorbance at each time point was measured in triplicate and data were assessed using two-way ANOVA. (D) Intracellular expression of YY1, EZH2, and MLH1 in ATLL-derived cell lines (ATL43T, ED+) was measured by flow cytometry after treatment with an EZH2 inhibitor (tazemetostat) or an EZH1/2 inhibitor (valemetostat) for 48 h. Aggregate MFI data are shown (E). (F) Expression of YY1, EZH2, and MLH1 protein in the N (CADM1⁺CD7⁻) fraction of acute-type ATLL cells was measured after treatment with an EZH1/2 inhibitor (valemetostat) for 48 h. MFI was measured in triplicate. Results are plotted as the mean $\pm$ SD and analyzed by Student's *t*-test (A, B, D–F) or one-way ANOVA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. N.S., not significant.

3.6 | MLH1 Is a Downstream Target of YY1/EZH2 and a Potential Therapeutic Target of Valemetostat

To clarify the relationship between YY1/EZH2 and MLH1, we sought to determine whether YY1/EZH2 occupies the promoter region of MLH1. ChIP-seq datasets indicated that a YY1-binding site was present in the MLH1 promoter region in T-ALL, Jurkat, K562, and HeLa cells (Figure S3).

Therefore, we hypothesized that MLH1 is a downstream target of the YY1/EZH2 axis. To test this, we first confirmed that MLH1 expression was also downregulated in YY1 knockdown cell lines (ATL43T and ED+) (Figure 5A). ATLL-derived cell lines were transduced with a lentiviral MLH1 knockdown vector (Figure 5B), which suppressed proliferation (Figure 5C). These imply an association between YY1 and MLH1. Next, we assessed whether EZH2 inhibition downregulated MLH1 expression in ATLL cells. ATLL cells were cultured in vitro with either an EZH2 inhibitor (tazemetostat) or an EZH1/2 inhibitor (valemetostat). Expression of EZH2 and MLH1 in ATLL cells decreased in the presence of these inhibitors (Figure 5D,E). Finally, we found that not only tazemetostat but also valemetostat reduced the expression of EZH2 and MLH1 in acute-type ATLL (Figure 5F). Taken together, the data suggest that YY1/EZH2-mediated downregulation of MLH1 is a potential therapeutic target in ATLL patients.

4 | Discussion

In this study, we performed scRNA-seq and intracellular flow cytometry using primary ATLL samples and cell lines. First, we identified a cluster of ATLL cells showing downregulated expression of CD48 and overexpression of YY1/EZH2; this cluster was associated with a proliferative phenotype. Second, we found that YY1/EZH2 expression was hierarchical within ATLL clusters from acute-type ATLL patients. Finally, we showed that MLH1 expression is regulated by the YY1/EZH2 axis, making it a potential downstream target of EZH1/2 inhibitors. The clinical significance of this study lies in the finding that YY1^{high} cells within the CADM1⁺ fraction of ATLL cells are associated with higher proliferative capacity, and that YY1/EZH2-modulated downregulation of MLH1 may be associated with durable remission.

CD48 is an important immune checkpoint molecule involved in NK cell activation because it has marked effects on the survival of patients with solid cancers [42]. Decreased CD48 expression on tumor cells enables them to escape NK cell-mediated responses, which might be important for the progression of acute myeloid leukemia or peripheral T-cell lymphoma [43, 45]. ATLL is characterized by its heterogeneity; indeed, patients usually harbor several ATLL clusters. Although the population showing downregulation of CD48 was not large, it correlated clinically with disease aggressiveness. Expansion of the cluster may contribute to the progression and therapeutic resistance of ATLL. To identify it as a disease-defining clone, we need to conduct a prospective analysis of the YY1/EZH2^{high} cluster at multiple time points.

Notably, the ATLL cells showing downregulation of CD48 were similar to those identified by comprehensive CRISPR screening

[45], supporting the importance of this subcluster; however, we found that flow cytometry analysis of primary samples did not clearly identify the cluster showing downregulated expression of the CD48 protein. This may be attributed to a discrepancy between the expression of CD48 mRNA and the protein product. Thus, the functional significance of CD48 is still unclear. Differentially expressed genes in the subcluster included MALAT1 and NEAT1, both of which are associated with EZH2 upregulation [44]. We found that this subcluster has a survival advantage with respect to tumor proliferation; therefore, this distinct minor tumor subcluster may drive ATLL pathogenesis and define clinical outcome.

YY1 is expressed ubiquitously in various cells and is associated with early embryonic development, stemness potential during organogenesis, lineage development of tissues, and immune checkpoint regulation in T cells [46, 47]. In solid cancer cells, YY1 plays a key role in cancer stemness [47]. Here, in silico promoter analysis identified YY1 as a candidate master regulator of the aggressive ATLL cluster. YY1 and EZH2 form the PRC2 complex. PRC2 induces epigenetic alterations through H3K27me3. In particular, EZH2 and H3K27me3-mediated gene repression is the therapeutic target of aggressive ATLL [22–24]. However, our data indicate that EZH2 expression in ATLL cells is heterogeneous and that EZH2^{high} ATLL cells confer proliferative potential. We assume that YY1^{high} ATLL cells possess a stemness. This fraction might be present within the CD45RA⁺ T memory stem cell population [48], although this needs to be verified. The presence of this fraction may be important for predicting prognosis and responses to EZH1/2 inhibitors.

We also identified MLH1 as a downstream target of the YY1/EZH2 axis in ATLL. Moreover, MLH1 downregulation was marked in ATLL cases in remission. In solid cancers, MMR deficiency increases the burden of neoantigens, which increases sensitivity to immune surveillance [49]. By contrast, MLH1 is downregulated in ATLL cell lines [29, 30]. Decreased MLH1 expression in ATLL patients may increase genetic instability, which might lead to higher immunogenicity. Subsequent studies should clarify whether MLH1 downexpression is associated with increased neoantigens.

In summary, we identified that the YY1/EZH2/MLH1 axis is an important biomarker associated with ATLL aggressiveness and suggest that it is a common therapeutic target in ATLL patients. Furthermore, further study of the high YY1/EZH2-expressing fraction in ATLL may be useful for stratification of therapeutic responses.

Author Contributions

Takuya Shimizu: data curation, formal analysis, investigation, methodology, writing – original draft. **Takero Shindo:** conceptualization, data curation, funding acquisition, investigation, methodology, supervision, validation, writing – review and editing. **Hanako Ogawa:** data curation, formal analysis, methodology. **Kaori Teranaka:** data curation, formal analysis, investigation, methodology. **Akira Watanabe:** data curation, formal analysis, investigation, methodology, writing – review and editing. **Akifumi Takaori-Kondo:** supervision, writing – review and editing.

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Disclosure

Animal Studies: The authors have nothing to report.

Ethics Statement

Approval of the research protocol by an Institutional Review Board: The study was approved by the institutional review board of Kyoto University Hospital (accession ID: G0697-16, https://kyoto.bvits.com/rinri/publish_document.aspx?ID=1898).

Consent

All patients provided written informed consent to participate in accordance with the World Medical Association's Declaration of Helsinki.

Conflicts of Interest

The authors declare no conflicts of interest.

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Supporting Information

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