

Recurrent *CCND3* mutations in *MLL*-rearranged acute myeloid leukemia

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Key Points

- Novel *CCND3* mutations were identified, and CDK4/6 inhibitors represent a promising therapeutic option for *MLL*-rearranged AML.
- Coexisting mutations had a negative prognostic impact on pediatric *MLL-MLLT3*-rearranged AML patients.

In acute myeloid leukemia (AML), *MLL* (*KMT2A*) rearrangements are among the most frequent chromosomal abnormalities; however, knowledge of the genetic landscape of *MLL*-rearranged AML is limited. In this study, we performed whole-exome sequencing (n = 9) and targeted sequencing (n = 56) of samples from pediatric *MLL*-rearranged AML patients enrolled in the Japanese Pediatric Leukemia/Lymphoma Study Group AML-05 study. Additionally, we analyzed 105 pediatric t(8;21) AML samples and 30 adult *MLL*-rearranged AML samples. RNA-sequencing data from 31 patients published in a previous study were also reanalyzed. As a result, we identified 115 mutations in pediatric *MLL*-rearranged AML patients (2.1 mutations/patient), with mutations in signaling pathway genes being the most frequently detected (60.7%). Mutations in genes associated with epigenetic regulation (21.4%), transcription factors (16.1%), and the cohesin complex (8.9%) were also commonly detected. Novel *CCND3* mutations were identified in 5 pediatric *MLL*-rearranged AML patients (8.9%) and 2 adult *MLL*-rearranged AML patients (3.3%). Recurrent mutations of *CCND1* (n = 3, 2.9%) and *CCND2* (n = 8, 7.6%) were found in pediatric t(8;21) AML patients, whereas no *CCND3* mutations were found, suggesting that D-type cyclins exhibit a subtype-specific mutation pattern in AML. Treatment of *MLL*-rearranged AML cell lines with CDK4/6 inhibitors (abemaciclib and palbociclib) blocked G1 to S phase cell-cycle progression and impaired proliferation. Pediatric *MLL-MLLT3*-rearranged AML patients with coexisting mutations (n = 16) had significantly reduced relapse-free survival and overall survival compared with those without coexisting mutations (n = 9) (*P* = .048 and .046, respectively). These data provide insights into the genetics of *MLL*-rearranged AML and suggest therapeutic strategies.

Introduction

Acute myeloid leukemia (AML) is a genetically and clinically heterogeneous disease characterized by expansion of undifferentiated myeloid precursor cells.¹⁻³ Recurrent chromosomal and genetic abnormalities, including t(8;21), inv(16), t(15;17), and *FLT3*-internal tandem duplication (*FLT3*-ITD), are well-established markers for risk-stratified therapy in AML^{4,5}; however, relapse remains common, and patients with relapsed AML have a poor prognosis.⁶ Recent genome-wide analyses revealed several recurrently mutated genes in AML, including *DNMT3A*, *IDH1*, *IDH2*, splicing factors, and cohesin complex genes⁷⁻⁹; however, few of these driver mutations have been developed as therapeutic targets to date. Moreover, because of the high level of genetic heterogeneity in AML, the clinical impact of driver mutations in each AML subtype remains unclear.

Rearrangements involving the *MLL* (mixed-lineage leukemia; official symbol, *KMT2A*) gene are among the most common chromosomal abnormalities in AML; these rearrangements are more common in pediatric AML (20%-24%), particularly infant AML (38%), than in adult AML (5%-10%),¹⁰⁻¹³ and >60 different *MLL* partner genes have been identified.¹⁴ Although *MLL* rearrangement has been regarded as a marker for poor prognosis in patients with AML, a recent study showed that the prognoses of patients with these changes vary considerably according to the *MLL* fusion partner. For example, pediatric AML patients with t(1;11)(q21;q23)/*MLL-MLLT11* have favorable prognoses, whereas those with t(6;11)(q27;q23)/*MLL-MLLT4(AF6)*, t(4;11)(q21;q23)/*MLL-AFF1*, t(10;11)(p12;q23)/*MLL-MLLT10(AF10)*, and t(10;11)(p11.2;q23)/*MLL-ABI1* have poor prognoses.¹⁵ The number of coexisting mutations is lower in *MLL*-rearranged AML than in other AML subtypes.^{2,13} RAS pathway mutations are well described; however, little is known about the prognostic significance of coexisting mutations in *MLL*-rearranged AML.¹⁶⁻¹⁸ A recent study of adult *MLL*-rearranged AML revealed novel somatic mutations in the transcription factor gene *SPI1*.¹⁸ Nevertheless, the genetic landscape of pediatric *MLL*-rearranged AML has yet to be fully elucidated, and further investigations are required to identify novel recurrent mutations and support improved therapeutic approaches in pediatric *MLL*-rearranged AML.

In this study, we identified recurrent mutations in *CCND3*, which encodes cyclin D3, in *MLL*-rearranged AML. Cyclins are cofactors that bind to cyclin-dependent kinases (CDKs), allowing cell-cycle progression. D-type cyclins form complexes with CDK4/6, which promote cell-cycle progression from G1 to S phase by phosphorylation of retinoblastoma protein (Rb).^{19,20} There are 3 D-type cyclins: cyclin D1 (encoded by *CCND1*), cyclin D2 (encoded by *CCND2*), and cyclin D3. Aberrant cell-cycle regulation mediated by deregulation of D-type cyclins is a major characteristic of cancer.²¹ In AML, recurrent mutations in *CCND1* and *CCND2* in t(8;21) AML were reported in previous studies^{22,23}; however, recurrent *CCND3* mutations have not been reported. A recent study showed that patients with deregulation of D-type cyclins may benefit from treatment with the CDK4/6 inhibitor²⁴; therefore, we also examined the effectiveness of CDK4/6 inhibitors in *MLL*-rearranged AML cells.

Methods

Patients and study protocol

The AML-05 study is a Japanese nationwide multi-institutional study of children (age <18 years) with de novo AML conducted by the

Japanese Pediatric Leukemia/Lymphoma Study Group (JPLSG) in which 485 patients were enrolled between 1 November 2006 and 31 December 2010. The trial was registered with the UMIN Clinical Trials Registry (#UMIN000000511; <http://www.umin.ac.jp/ctr/index.htm>), and details of the schedules and treatment regimens have been described previously.²⁵ AML was diagnosed via a central review system. The study was conducted in accordance with the principles set down in the Declaration of Helsinki and approved by the ethics committees of all participating institutions. All patients, or their parents/guardians, provided written informed consent.

Whole-exome sequencing

Whole-exome sequencing was performed as previously reported.²⁶ Whole-exome capture was accomplished by liquid phase hybridization of sonicated genomic DNA using a bait complementary RNA library (SureSelect Human ALL Exon V5 or V5 Inc RNA 5 kit; Agilent Technologies, Santa Clara, CA) following the manufacturer's protocol. Massively parallel sequencing of the captured targets was performed using a HiSeq 2000/2500 sequencing system (Illumina, San Diego, CA) with the paired-end 126- to 133-bp read option, following the manufacturer's instructions. Sequencing reads were aligned to hg19 using BWA-mem version 0.5.8 with default parameters. Details of the methods used to detect candidate somatic mutations using EBcell (Empirical Bayesian mutation Calling)²⁷ are presented in supplemental Methods.

Targeted sequencing

In total, 338 gene targets were examined for gene mutations in the 56 *MLL*-rearranged AML pediatric cases using targeted capture sequencing and selected if they were (1) known driver genes in myeloid malignancies or other neoplasms, (2) associated with myeloid malignancies, (3) mutated as detected by whole-exome sequencing in this study, and (4) therapeutically targetable.

Sample preparation, sequencing, and data analyses were performed using the same approach as for whole-exome sequencing. Target enrichment was performed using a SureSelect custom kit (Agilent Technologies), which is designed to capture all coding exons of the above 338 genes and 1216 single-nucleotide polymorphisms for copy-number analysis. Similarly, *CCND1*, *CCND2*, and *CCND3* were also captured and sequenced in samples from 105 pediatric cases with t(8;21)/*RUNX1-RUNX1T1* AML and 30 adult patients with *MLL*-rearranged AML. Detailed methods for detecting candidate somatic mutations are presented in supplemental Methods.

Detection of copy-number alterations

Copy-number analysis was performed as previously reported²⁸ using an in-house pipeline (Y. Shiozawa and S.O., manuscript in preparation) in which the total number of reads covering each bait region and the allele frequency of heterozygous single-nucleotide polymorphisms detected by targeted sequencing were used as input data.

Sanger sequencing and deep sequencing

First, polymerase chain reaction (PCR) was performed using a pair of primers targeting the PEST domain-encoding region of the *CCND3* gene (forward, 5'-TGAGAACTAAAGAGCGATTCC-TGG-3'; reverse, 5'-CTTTGTGAAGGGGGAACAGACG-3'). Reactions were performed in a volume of 20 μ L containing 2 μ L 10 $\times$ PCR buffer for KOD plus polymerase, 2 μ L 2'-deoxynucleoside

5'-triphosphate mix (2 mM), 1.2 μ L $MgSO_4$ (25 mM), 0.4 μ L KOD plus polymerase (1 U/ μ L) (Toyobo, Osaka, Japan), 0.2 μ L each primer (100 μ M; Invitrogen, San Diego, CA), and 20 ng template DNA. Reactions were carried out in a Veriti 96-Well Thermal Cycler (Applied Biosystems, Foster City, CA) using a touchdown PCR protocol (1 cycle of 96°C for 2 minutes; 3 cycles of 96°C for 10 seconds, 64°C for 10 seconds, and 70°C for 30 seconds; 3 cycles of 96°C for 10 seconds, 61°C for 10 seconds, and 70°C for 30 seconds; 3 cycles of 96°C for 10 seconds, 58°C for 10 seconds, and 70°C for 30 seconds; 35 cycles of 96°C for 10 seconds, 57°C for 10 seconds, and 70°C for 30 seconds; and 1 cycle of 70°C for 5 minutes). PCR products were analyzed by agarose gel electrophoresis and purified using a FastGene Gel/PCR Extraction Kit (NIPPON Genetics, Tokyo, Japan) according to the manufacturer's instructions. The sequences of purified PCR products were determined by direct sequencing using a forward primer (5'-CCAGACTTCCCCATGTGTTGG-3') and a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) on a 3130xl Genetic Analyzer (Applied Biosystems). For deep sequencing, PCR amplicons were sonicated and prepared using a NEBNext Ultra DNA Library Prep Kit for Illumina (New England Biolabs). Sequencing was performed using a MiSeq platform with the 77-bp paired-end read option.

Compounds

Palbociclib (PD0332991) and abemaciclib (LY2835219) were obtained from AdooQ BioScience (Irvine, CA). Both compounds were dissolved in dimethyl sulfoxide (DMSO).

Cell culture

ML-2, MV4-11, and MOLM-13 cell lines were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). THP-1 and NOMO-1 cell lines were obtained from the Japanese Collection of Research Bioresources Cell Bank (Ibaraki, Japan). All cell lines were cultured in RPMI 1640 medium containing 10% fetal bovine serum and 1% penicillin/streptomycin under 5% CO_2 and 95% air at 37°C.

Cell proliferation assay

Cells (2×10^5 /mL) were cultured in the presence of DMSO (control), palbociclib (500 nM), or abemaciclib (500 nM). Data are presented as the mean $\pm$ standard error of 3 independent experiments.

Cell-cycle analysis

Cells (2×10^5 /mL) were treated with DMSO (control), palbociclib (500 nM), or abemaciclib (500 nM) for 24 hours. Then, cells were stained with propidium iodide and analyzed using a FACS Canto II flow cytometer (BD Biosciences, San Jose, CA).

Immunoblot analysis

Cells were washed with PBS and then lysed in RIPA buffer containing a protease inhibitor cocktail (Nakalai, Kyoto, Japan). After centrifugation, the protein content in supernatants was measured using a DC Protein Assay (Bio-Rad Laboratories, Hercules, CA). Whole-cell lysates containing equal amounts of total protein were separated on 10% sodium dodecyl sulfate polyacrylamide gels and then transferred to Immobilon-P transfer membranes (Merck, Darmstadt, Germany). Membranes were blocked with Blocking One reagent (Nakalai) for 1 h, followed by incubation overnight at 4°C with an anti-cyclin D3 antibody (1/1000; K0013-3; MBL,

Nagoya, Japan) or an anti-GAPDH antibody (1/3000; sc-47724; Santa Cruz Biotechnology, Santa Cruz, CA). After washing thoroughly in Tris-buffered saline with Tween 20, membranes were incubated with horseradish peroxidase-conjugated whole anti-mouse immunoglobulin G (1/4000; NA931; GE Healthcare Bio-Sciences) for 1 hour at room temperature. Immunoreactive proteins were detected using a horseradish peroxidase Novex ECL Chemiluminescent Substrate Regent Kit (Invitrogen). Signals were captured, and the intensities of bands were quantified using a ChemiDoc XRS System (Bio-Rad Laboratories).

RNA interference

Small interfering RNA (siRNA) against human cyclin D3 transcript (sc-35136) and the nontargeting siRNA (sc-37007) were purchased from Santa Cruz Biotechnology. THP-1 cells (4×10^4 /mL) were cultured in RPMI 1640 medium containing 10% fetal bovine serum without 1% penicillin/streptomycin at 37°C. After 1 day, THP-1 cells were transfected with siRNA/Lipofectamine RNAiMAX (Invitrogen) complexes diluted in Opti-MEM Reduced Serum Medium (Gibco) (final siRNA concentration, 50 nM) according to the manufacturer's protocol. Two days after transfection, whole-cell lysates were prepared to determine knockdown efficiency by immunoblot analysis. Four days after transfection, THP-1 cells were collected for cell-cycle analysis by fluorescence-activated cell sorting.

Statistical analysis

Survival analyses were performed using the Kaplan-Meier method, and groups were compared using an unstratified log-rank test. Continuous variables were compared using the Student *t* test.

Results

Spectrum of mutations and copy-number alterations in pediatric *MLL*-rearranged AML

First, we analyzed paired AML tumor and germline samples from 9 pediatric *MLL*-rearranged AML patients enrolled in the JPLSG AML-05 study by whole-exome sequencing. Five of the patients were included in our previous study (supplemental Figure 1).²⁹ In total, 52 mutations (mean, 5.8 mutations/patient) were identified (supplemental Table 1; supplemental Figure 2), including known mutational targets in AML, such as genes associated with signaling activation (*FLT3* and *BRAF*), epigenetic regulation (*SETD2* and *BCORL1*), and encoding transcription factors (*WT1*). Moreover, *CCND3*, which is a well-known driver gene in lymphomas,³⁰ was mutated in 1 patient.

Next, we analyzed 56 samples from patients with pediatric *MLL*-rearranged AML enrolled in the JPLSG AML-05 study using targeted sequencing. Patient characteristics are presented in Table 1. The median age of patients was 3.0 years, which is consistent with data demonstrating that *MLL*-rearranged AML is frequent in infants.¹³ Over half of patients were classified in the M5a category (54%), followed by M4 (23%), M1 (7%), M5b (5%), M2 (2%), M4Eo (2%), and RAEB-T (2%). Among fusion types, *MLL-MLLT3* was the most frequent (45%), followed by *MLL-MLLT10* (20%), *MLL-ELL* (18%), *MLL-MLLT1* (9%), and *MLL-MLLT4* (*AF6*) (5%). We selected 338 genes, including *CCND3*, for targeted sequencing (supplemental Table 2). The driver or recurrent mutations detected by targeted sequencing are presented according to fusion type in Figure 1. Detailed results of targeted sequencing are provided in supplemental Table 3.

Table 1. Characteristics of pediatric patients with *MLL*-rearranged AML (n = 56)

Characteristic	Value
Age, y	
Median	3.0
Range	0.0-15.1
Sex, n (%)	
Male	24 (43)
Female	32 (57)
WBC, $\times 10^9/L$	
Median	27.4
Range	1.1-459.0
FAB subtype, n (%)	
M1	4 (7)
M2	1 (2)
M4	13 (23)
M4Eo	1 (2)
M5a	30 (54)
M5b	3 (5)
RAEB-T	1 (2)
ND	3 (5)
Fusion gene, n (%)	
<i>MLL-MLLT3</i>	25 (45)
<i>MLL-MLLT10</i>	11 (20)
<i>MLL-ELL</i>	10 (18)
<i>MLL-MLLT1</i>	5 (9)
<i>MLL-MLLT4</i>	3 (5)
Others	2 (4)

FAB, French-American-British; ND, not determined; WBC, white blood cell count.

We identified 115 mutations (mean, 2.1 mutations/patient), with the following mutations in signaling pathway genes being the most frequently detected (60.7%): *FLT3* (n = 13, 23.2%), *NRAS* (n = 11, 19.6%), *KRAS* (n = 9, 16.1%), *PTPN11* (n = 6, 10.7%), *CBL* (n = 5, 8.9%), and *BRAF* (n = 1, 1.8%). Mutations in genes associated with epigenetic regulation (*SETD2*, *ASXL1*, *ASXL2*, *BCOR*, *BCORL1*, *KDM6A*, *CREBBP*, and *EP300*) (21.4%), transcription factors (*SP1*, *WT1*, *MECOM*, *GATA2*, and *RUNX1*) (16.1%), and the cohesin complex (*STAG2*, *RAD21*, and *SMC3*) (8.9%) were also commonly detected. Interestingly, all *MLL-ELL* patients had at least 1 mutation of a signaling pathway gene, and all 3 *STAG2* mutations were restricted to *MLL-ELL* patients, suggesting that the distribution of coexisting mutations differs according to *MLL* fusion partner.

Copy-number alterations were also infrequent in *MLL*-rearranged AML, with trisomy 8 being the most frequently detected (identified in 6 cases; Figure 1; supplemental Figure 3), which is consistent with previous reports showing that trisomy 8 is among the most common cytogenetic aberrations in pediatric AML.^{31,32}

Recurrent *CCND3* mutations in pediatric and adult *MLL*-rearranged AML

We identified 8 mutations in *CCND3*, which encodes cyclin D3, in 5 pediatric *MLL*-rearranged AML patients (8.9%). Detailed

information regarding patients with *CCND3* mutations is presented in Table 2. All mutations were clustered in the PEST domain (Figure 2A), which is found in short-lived proteins and plays a role in their degradation.³³ Four of the 8 mutations were R271fs, which is a known hot-spot mutation in lymphoid malignancies.^{30,34-38}

To determine whether *CCND3* mutations were also present in adult *MLL*-rearranged AML, we analyzed 30 adult *MLL*-rearranged AML samples for *CCND3* mutations by targeted sequencing (patient information provided in supplemental Table 4). RNA-sequencing data from 31 patients published in a previous study were also reanalyzed.¹⁸ *CCND3* mutations affecting the PEST domain were detected in 2 out of 61 samples (3.3%) (Figure 2A). In total, 4 out of 7 patients (57.1%) with *CCND3* mutations were detected among those with *MLL-ELL*-positive AML. All *CCND3* mutations identified in our cohort were confirmed by Sanger sequencing or deep sequencing (supplemental Figure 4). Mutations of the PEST domain of *CCND3* elevate the stability of cyclin D3 protein and promote cell proliferation³⁰; therefore, these results suggest that the stabilization of cyclin D3 is important in both pediatric and adult *MLL*-rearranged AML.

Mutations of D-type cyclins exhibit a disease subtype-specific pattern in AML

Mutations of the other D-type cyclins (*CCND1* and *CCND2*) have been reported in t(8;21) AML^{22,23}; therefore, we also searched for mutations in *CCND1*, *CCND2*, and *CCND3* by targeted sequencing of samples from pediatric AML patients with t(8;21)/*RUNX1-RUNX1T1* (n = 105). *CCND1* mutations (n = 3; 2.9%) and *CCND2* mutations (n = 8; 7.6%) were detected in t(8;21) patients; however, no mutations of *CCND3* were found in this group (Figure 2B; supplemental Figure 5). By contrast, there were no mutations of *CCND1* or *CCND2* in *MLL*-rearranged AML (n = 56), suggesting that mutations of D-type cyclins exhibit a subtype-specific pattern in AML.

Furthermore, we compared the messenger RNA expression levels of *CCND1*, *CCND2*, and *CCND3* between t(8;21) AML (n = 60) and *MLL*-rearranged AML (n = 43) (in dataset GSE13159, generated as part of the MILE [Microarray Innovations in LEukemia] study program) using BloodSpot.³⁹ *CCND1* and *CCND2* expression was significantly higher in t(8;21) AML than *MLL*-rearranged AML (both $P < .001$), while *CCND3* expression was significantly higher in *MLL*-rearranged AML than t(8;21) AML ($P < .001$) (Figure 2C). These data support the hypothesis that deregulation of D-type cyclins exhibits a subtype-specific pattern in AML.

CDK4/6 inhibitors are a promising treatment of *MLL*-rearranged AML

A recent study demonstrated that cancers with *CCND3* amplification may benefit from treatment with the CDK4/6 inhibitor abemaciclib²⁴; therefore, we examined whether CDK4/6 inhibitors could be novel therapeutic agents for *MLL*-rearranged AML. The effects of major CDK4/6 inhibitors (abemaciclib and palbociclib) on the proliferation and cell-cycle progression of *MLL*-rearranged AML cell lines (ML-2 [*MLL-MLLT4* positive], MV4-11 [*MLL-AFF1* positive], MOLM-13 [*MLL-MLLT3* positive], THP-1 [*MLL-MLLT3* positive], and NOMO-1 [*MLL-MLLT3* positive]) were analyzed. The CDK4/6 inhibitor concentration employed was 500 nM, based on previously published data.⁴⁰ Among these cell lines, a *CCND3* mutation (R271fs) was detected

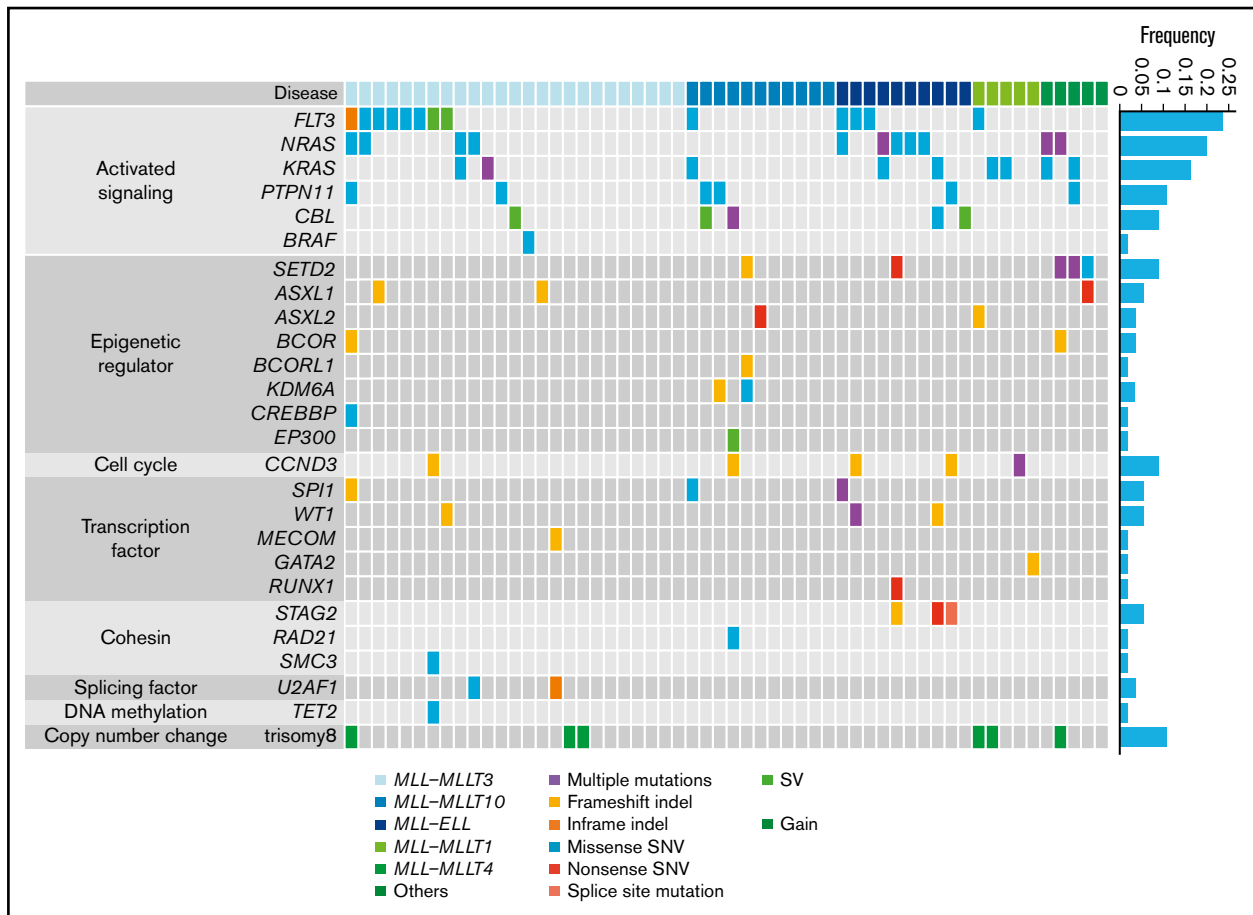


Figure 1. Mutational landscape of pediatric *MLL*-rearranged AML. Driver or recurrent mutations and copy-number alterations observed in pediatric *MLL*-rearranged AML (n = 56). The frequencies of mutated genes are shown on the right. SNV, single-nucleotide variant; SV, structural variant.

in ML-2 cells by Sanger sequencing (supplemental Figure 4). *CCND3*/cyclin D3 was highly expressed in *MLL*-rearranged compared with t(8;21) AML cell lines (Kasumi-1 and SKNO-1) (supplemental Figure 6). *MLL*-rearranged AML cell lines exhibited impaired proliferation after treatment with CDK4/6 inhibitors

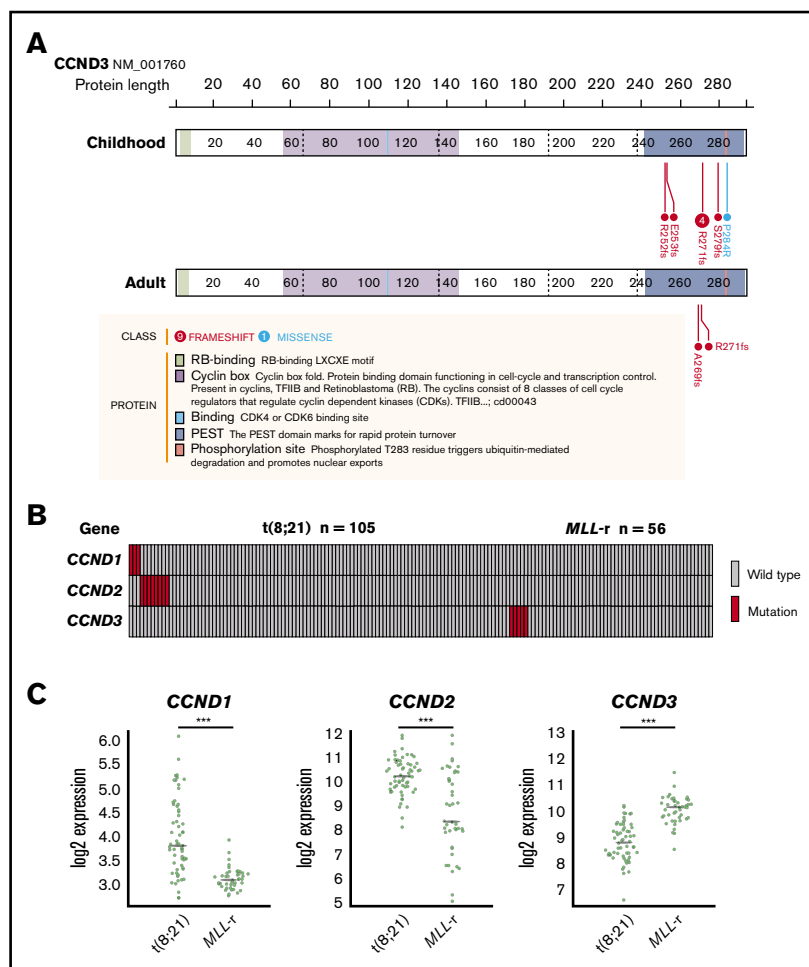
(Figure 3A). Furthermore, treatment of these cell lines with CDK4/6 inhibitors resulted in detection of lower frequencies of S/G2/M phase cells by flow cytometry, suggesting that cells were arrested in G1 phase via CDK4/6 inhibition (Figure 3B-C).

Table 2. Characteristics of patients with *MLL*-rearranged AML and *CCND3* mutation

Sample ID	Age, y	Sex	Karyotype	Exonic function	AA change	Mismatch rate	Relapse	Death
Childhood AML								
AML05-120	11	M	<i>MLL-ELL</i>	Frameshift insertion	exon5:c.811dupC p.R271fs	0.401	—	—
AML05-206	7	F	<i>MLL-MLLT3</i>	Frameshift insertion	exon5:c.811dupC p.R271fs	0.045	+	+
AML05-213	0	M	<i>MLL-MLLT10</i>	Frameshift insertion	exon5:c.836_837insAG p.S279fs	0.105	—	—
AML05-422	5	M	<i>MLL-ELL</i>	Frameshift insertion	exon5:c.811dupC p.R271fs	0.479	+	—
AML05-426	1	F	<i>MLL-MLLT1</i>	Frameshift deletion	exon5:c.754_755delAG p.R252fs	0.022	—	—
				Frameshift insertion	exon5:c.757dupG p.E253fs	0.063		
				Frameshift insertion	exon5:c.811dupC p.R271fs	0.040		
				Missense SNV	exon5:c.C851G p.P284R	0.286		
Adult AML								
OT-034	66	M	<i>MLL-ELL</i>	Frameshift insertion	exon5:c.811dupC p.R271fs	0.431	N/A	N/A
07H003*	25	F	<i>MLL-ELL</i>	Frameshift insertion	exon5:c.806dupC p.A269fs	0.282	N/A	N/A

AA, amino acid; F, female; M, male; N/A, not available.

*Data for 07H003 were obtained by reanalysis of RNA-sequencing data reported in a previous study.¹⁸



Next, we confirmed the role of cyclin D3 in *MLL*-rearranged AML cells. Among the 5 *MLL*-rearranged AML cell lines, THP-1 was selected because it could be knocked down efficiently by siRNA (Figure 3D). THP-1 cells transfected with siRNA against the human cyclin D3 transcript showed significant reductions in cell proliferation and cell cycling from G1 to S phase compared with THP-1 cells transfected with nontargeting siRNA (Figure 3E-G). Taken together, the results suggest that the cyclin D3-CDK4/6 complex plays an important role in cell-cycle progression in *MLL*-rearranged AML and that cyclin D3 and CDK4/6 are essential for the mechanism (Figure 3H).

Coexisting mutations are associated with poor prognosis in *MLL-MLLT3*-rearranged AML

Finally, we examined the prognostic significance of fusion partners and coexisting mutations in pediatric *MLL*-rearranged AML. Consistent with a previous report, patients with *MLL-MLLT4* exhibited poor outcomes (Figure 4A).¹⁵ The relapse-free survival (RFS) of patients with *MLL-MLLT4* was significantly shorter than that of those with *MLL-MLLT3* ($P = .004$), *MLL-MLLT10* ($P = .001$), or *MLL-ELL* ($P = .002$). Interestingly, patients with *MLL-MLLT1* also had poor outcomes, and the RFS of patients with this rearrangement was significantly shorter than that of patients with *MLL-ELL* ($P = .03$). P values for comparisons between patients with each fusion subtype are provided in supplemental Table 5.

We also evaluated the prognostic significance of coexisting mutations identified in *MLL-MLLT3*-rearranged AML ($n = 25$), because this rearrangement was the most common in the cohort. Patients with coexisting mutations listed in Figure 1 ($n = 16$) had significantly reduced RFS and overall survival (OS) compared with those without coexisting mutations ($n = 9$) ($P = .048$ and $.046$, respectively) (Figure 4B). Investigation of the prognostic impact of mutated pathways indicated that the RFS and OS of patients with activated signaling mutations (*FLT3*, *NRAS*, *KRAS*, *PTPN11*, *CBL*, and *BRAF*) tended to be shorter than those of patients without such mutations; however, the difference was not statistically significant (supplemental Figure 7). We also analyzed the prognostic significance of mutations of *FLT3*, *CCND3*, and RAS pathway (*NRAS*, *KRAS*, *PTPN11*, and *CBL*), epigenetic regulator (*SETD2*, *ASXL1*, *ASXL2*, *BCOR*, *BCORL1*, *KDM6A*, *CREBBP*, and *EP300*), transcription factor (*SPI1*, *WT1*, *MECOM*, *GATA2*, and *RUNX1*), and cohesin complex (*STAG2*, *RAD21*, and *SMC3*) genes (supplemental Figure 8). No significant differences in outcomes were associated with these mutation types. We also analyzed the prognostic significance of coexisting mutations in other subgroups (*MLL-MLLT10*, *MLL-ELL*, *MLL-MLLT1*, and *MLL-MLLT4*); however, there were no significant differences (data not shown). These analyses were limited by the small cohort size, therefore, further studies with larger cohorts should be conducted.

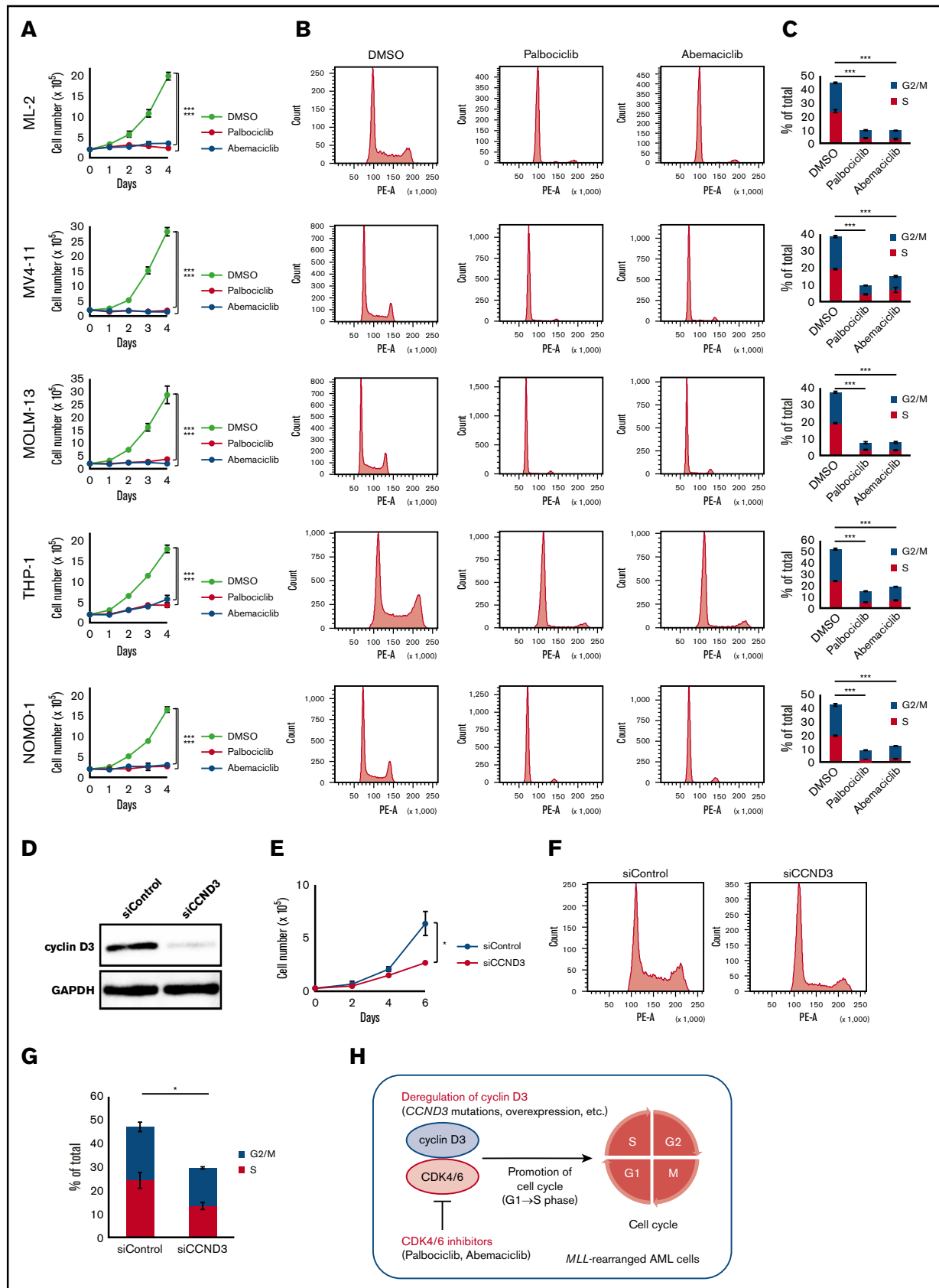


Figure 3.

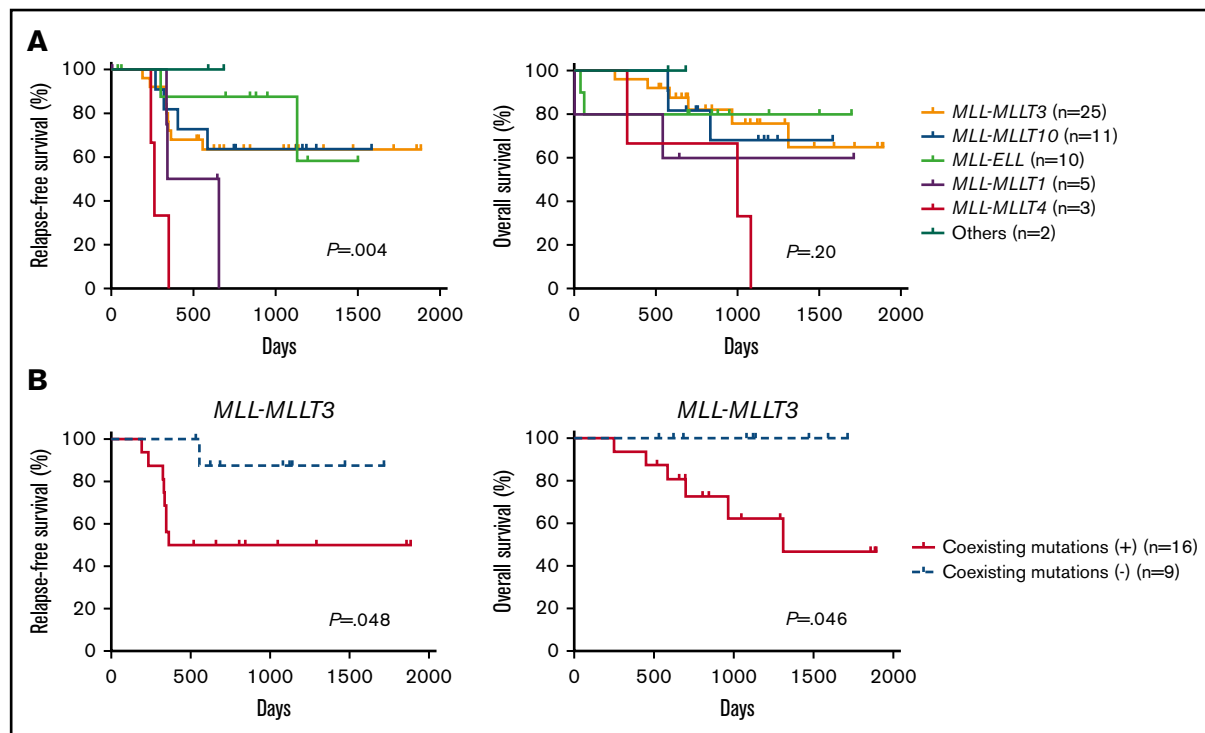


Figure 4. Prognostic significance of fusion partners in pediatric *MLL*-rearranged AML and coexisting mutations in pediatric *MLL-MLLT3*-rearranged AML.

(A) Comparison of patient RFS and OS according to *MLL* fusion partner. (B) Comparison of RFS and OS for patients with pediatric *MLL-MLLT3*-rearranged AML with and without coexisting mutations. Survival estimates were compared using the log-rank test.

Discussion

In this study, we performed whole-exome sequencing of samples from 9 pediatric patients with *MLL*-rearranged AML. The number of mutations was smaller (mean, 5.8 mutations/patient) compared with our previous findings from pediatric t(8;21) AML samples (mean, 7.8 mutations/patient) ($n = 5$)²⁹ and data from a cohort of pediatric core binding factor AML samples (mean, 7.4 mutations/sample) ($n = 87$).²² This suggests that *MLL* fusion proteins require fewer cooperating mutations than t(8;21) or inv(16).

We also performed targeted sequencing of 338 genes in 56 pediatric *MLL*-rearranged AML. Among the detected mutations, alterations of signaling pathway genes were the most frequently detected (34/56 patients; 60.7%), which is consistent with previous studies.^{16,17} A *BRAF* V600E mutation was detected in 1 patient. This mutation is found in some types of cancer, including hairy cell leukemia, and is a promising therapeutic target⁴¹; therefore, screening for the *BRAF* V600E mutation should be considered in *MLL*-rearranged AML. Patients with *MLL-MLLT3*-rearranged AML and coexisting mutations

had significantly worse RFS and OS. *MLL-MLLT3*-rearranged AML is categorized as an intermediate-risk group¹⁵; therefore, screening for coexisting mutations may enable improved risk assessment for these patients. Previous studies failed to determine the prognostic significance of coexisting mutations in *MLL*-rearranged AML, which may be because of the limited numbers of cases of each fusion partner included in the analyses.^{16,17} Further studies including larger numbers of patients are required.

We also observed recurrent mutations of epigenetic regulator genes (*SETD2*, *ASXL1*, *ASXL2*, *BCOR*, *BCORL1*, *KDM6A*, *CREBBP*, and *EP300*) in 12 patients (21.4%), suggesting that aberrant epigenetic regulation is strongly involved in *MLL*-rearranged AML pathogenesis. The result is not surprising, given its role as a histone methyltransferase. *CREBBP* and *EP300* encode highly related acetyltransferases that act as transcriptional coactivators in multiple signaling pathways.⁴² Recurrent mutations of *CREBBP* and *EP300* are reported in lymphomas and acute lymphoblastic leukemia (ALL); however, few studies have identified these mutations in AML, except for rare translocations involving *CREBBP* and *EP300*.^{35,43-46} Together

Figure 3. Effects of CDK4/6 inhibitors in *MLL*-rearranged AML cell lines. (A) Cell proliferation assay. ML-2, MV4-11, MOLM-13, THP-1, and NOMO-1 cells were cultured in the presence of DMSO, palbociclib (500 nM), or abemaciclib (500 nM). (B) Cell-cycle analysis. Flow cytometric analysis of cell lines treated with DMSO, palbociclib (500 nM), or abemaciclib (500 nM) for 24 hours and stained with propidium iodide. (C) Comparison of percentages of G2/M and S phase cells. (D) Immunoblot analysis revealed significantly lower expression of cyclin D3 proteins in THP-1 cells transfected with siRNA against human cyclin D3 transcript (left column: siCCND3) than in THP-1 cells transfected with non-targeting siRNA (right column: siControl). (E-G) THP-1 cells transfected with siCCND3 showed significant impairment in cell proliferation (E) and cell-cycle progression from G1 to S phase (F-G) compared with those transfected with nontargeting siRNA. (H) Schematic figure showing that the cyclin D3-CDK4/6 complex promotes cell-cycle progression and CDK4/6 inhibitors are promising therapeutic agents in *MLL*-rearranged AML cells. Data are presented as the mean $\pm$ standard error of 3 independent experiments. *** $P < .001$, * $P < .05$.

with the recurrent *CCND3* mutations detected in this study, these data indicate that *MLL*-rearranged AML may share some genetic features with lymphomas and ALL.

Mutations in cohesin complex genes (*STAG2*, *RAD21*, and *SMC3*) were detected in 5 patients (8.9%). Cohesin is a multimeric protein complex composed of 4 subunits: *STAG2*, *RAD21*, *SMC1A*, and *SMC3*.⁴⁷ The frequency of mutations in cohesin complex genes is very high in Down syndrome–related acute megakaryoblastic leukemia.⁴⁸ In de novo AML, the frequency of cohesin complex mutations is 10% to 12%,^{9,49} which is slightly higher than our findings for *MLL*-rearranged AML; however, *STAG2* mutations were detected in 3 patients with *MLL-ELL*, equating to a frequency of 30%. Furthermore, in a previous study of adult *MLL*-rearranged AML, both *STAG2* mutations identified were also restricted to patients with *MLL-ELL*.¹⁸ Hence, although the underlying mechanism is unclear, *STAG2* mutations may have an important role in the pathogenesis of *MLL-ELL*-rearranged AML.

We identified recurrent *CCND3* mutations in pediatric and adult *MLL*-rearranged AML. The frequency of *CCND3* mutations in adult patients (2/61; 3.3%) was lower than that in pediatric patients (5/56; 8.9%), which may be attributable to the different distribution of translocation partners between these groups (Table 1; supplemental Table 4). *CCND3* mutations are well described in lymphoid malignancies, including Burkitt lymphoma, diffuse large B-cell lymphoma, follicular lymphoma, hairy cell leukemia variant (HCL-V), splenic diffuse red pulp lymphoma, and T-lineage ALL.^{30,34-38} Nevertheless, there are few reports of *CCND3* mutations in AML other than 1 description of a case with normal karyotype AML and *CCND3* deletion.⁵⁰ All *CCND3* mutations detected in this study were clustered in the region encoding the PEST domain, similar to observations in lymphoid malignancies. *CCND3* mutations affecting the PEST domain cause stabilization of the cyclin D3 protein, resulting in cell-cycle deregulation³⁰; therefore, this mechanism may be associated with the pathogenesis of *MLL*-rearranged AML, as well as that of lymphoid malignancies. Mutation and overexpression of *CCND1*, *CCND2*, and *CCND3* exhibited an AML subtype-specific pattern. In a previous study, selective use of D-type cyclins was revealed in lymphoid malignancies by analysis of numerous cell lines⁵¹; however, little is known about this phenomenon in AML. Our data suggest that particular pairs of deregulated D-type cyclins and chromosomal abnormalities may be associated with AML pathogenesis.

MLL-rearranged AML cell lines showed blocked G1 to S phase cell-cycle progression and impaired proliferation after treatment with CDK4/6 inhibitors (palbociclib and abemaciclib) (Figure 3). These inhibitors are currently in clinical trials for treatment of multiple cancers, including breast cancer and mantle cell lymphoma, and some reports have already demonstrated that their use is linked to improved survival rates.⁵²⁻⁵⁴ *CCND3* amplification was recently identified as a promising marker for predicting responses to CDK4/6 inhibitors.^{24,55} In this study, CDK4/6 inhibitors were effective in inhibiting the growth of not only a *CCND3*-mutated cell line (ML-2)

but also that of cell lines lacking *CCND3* mutations (MV4-11, MOLM-13, THP-1, and NOMO-1). The *MLL*-rearrangement cell lines without *CCND3* mutations expressed abundant cyclin D3 protein (supplemental Figure 6); therefore, CDK4/6 inhibitors may be effective for the treatment of malignancies with these characteristics. According to previous studies on other D-type cyclins (*CCND1* and *CCND2*),^{24,56} increased *CCND3* expression in cell lines without *CCND3* mutations may be caused by mutations or posttranscriptional modifications in 3' untranslated regions of *CCND3*. According to the response profiles of abemaciclib across 560 reported cancer cell lines, ~20% of AML cells were sensitive; however, the subtype of AML exhibiting sensitivity was not discussed.²⁴ This study revealed recurrent *CCND3* mutations and high *CCND3* expression in *MLL*-rearranged AML patients; therefore, CDK4/6 inhibitors represent a promising therapeutic option, particularly for this patient subgroup. These data provide further insights into the genetic basis of, and potential therapeutic strategies for, *MLL*-rearranged AML.

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Authorship

Contribution: H. Matsuo analyzed clinical data and performed the functional analysis; K.Y. analyzed the sequencing data; H. Matsuo and K.Y. wrote the manuscript; Y. Shiozawa, Y. Shiraishi, K.C., H.T., A.O., and S. Miyano developed sequence data processing pipelines; H. Matsuo, K.Y., K.F., H.U., and H. Mano performed sequencing; Y. Noguchi helped with statistical analyses; K.N., S.T., and M.N. helped with functional analyses; H. Matsuo, Y. Nannya, J.T., N.S., G.Y., H.H., Y.O., N.H., T.I., K.U., K.I., S. Miyawaki, H.I., Y.M., M.K., H.Y., N.K., D.T., T.T., A.T., Y.H., and S.A. collected clinical samples; and H. Mano, Y.K., S.O., and S.A. supervised the project.

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