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ORIGINAL ARTICLE

Inhibition of CDK4/6 and autophagy synergistically induces apoptosis in t(8;21) acute myeloid leukemia cells

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Abstract

The t(8;21) translocation is the most common cytogenetic abnormality in acute myeloid leukemia (AML). Although t(8;21) AML patients have a relatively favorable prognosis, relapse is a frequent occurrence, underscoring the need to develop novel therapeutic approaches. Here, we showed that t(8;21) AML is characterized by frequent mutation and overexpression of *CCND2*. Analysis of 19 AML cell lines showed that t(8;21) AML cells had lower IC50 values for the selective CDK4/6 inhibitors palbociclib and abemaciclib than non-t(8;21) AML cells. CDK4/6 inhibitors caused cell cycle arrest at G1 phase and impaired cell proliferation in t(8;21) AML cells. CDK4/6 inhibition decreased MAP-ERK and PI3K-AKT-mTOR signaling pathway activity, induced LC3B-I to LC3B-II conversion, and enhanced autophagosome formation, suggesting autophagy induction. Treatment of t(8;21) AML cells with the autophagy inhibitors chloroquine (CQ) or LY294002 in combination with the CDK4/6 inhibitor abemaciclib significantly increased the percentage of apoptotic (Annexin V positive) cells, whereas CQ or LY294002 single treatment had no significant effects. The effectiveness of co-inhibiting CDK4/6 and autophagy was confirmed in primary t(8;21) AML cells. The results suggest that the combination of CDK4/6 and autophagy inhibitors had a synergistic effect on inducing apoptosis, suggesting a novel therapeutic approach for the treatment of t(8;21) AML.

Keywords Acute myeloid leukemia · t(8;21) · Cyclin D2 · CDK4/6 · Autophagy

Introduction

Acute myeloid leukemia (AML) is a genetically and clinically heterogeneous disease [1]. t(8;21))(q22;q22)/*RUNX1-RUNX1T1* [also known as AML1-ETO, hereafter referred to as t(8;21)] is one of the most frequent cytogenetic abnormalities in AML. The incidence of t(8;21) is 12–14% in pediatric AML and approximately 7% in adult AML [2–6]. Although t(8;21) AML patients have a more favorable prognosis than other cytogenetic subgroups, nearly 40% of t(8;21) AML patients experience relapse [7–10]. Therefore, novel therapeutic approaches based on a better understanding of the biology of t(8;21) AML need to be developed.

The *RUNX1-RUNX1T1* fusion gene blocks myeloid differentiation, although the abnormality alone is not sufficient to induce leukemia [11, 12]. Additional abnormalities including driver mutations are required for leukemogenesis, and comprehensive analyses of genetic variations have been performed to identify these mutations. Several groups including ours recently identified novel *CCND2* mutations in t(8;21) AML [13–15]. *CCND2* encodes cyclin D2, which is one of three D-type cyclins (cyclin D1–3). D-type cyclins form complexes with CDK4/6 and induce cell cycle progression from G1 to S phase by phosphorylating the retinoblastoma protein [16, 17]. Most *CCND2* mutations are located on the PEST domain, which is present in short-lived proteins and plays a role in their degradation [18]. *CCND2* PEST domain mutations stabilize cyclin D2 and promote cell proliferation

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[13]. Deregulation of the cell cycle is a hallmark of cancer, and CDK4/6 inhibitors are currently in clinical trials for the treatment of solid cancers including breast cancer [19]. The efficacy of these inhibitors for improving survival rates has been reported [19, 20]. Because abemaciclib, a potent CDK4/6 inhibitor, is effective in cancer cells with deregulation of D-type cyclins [21], we hypothesized that CDK4/6 inhibitors may be effective for t(8;21) AML cells.

Here, we show that *CCND2* mutations/overexpression is common in t(8;21) AML, and we demonstrate the effect of CDK4/6 inhibitors on blocking cell cycle progression and inhibiting the proliferation of t(8;21) AML cells. CDK4/6 inhibition induced autophagy in t(8;21) AML cells. Autophagy is an intracellular degradation system that recycles cellular constituents by engulfing them into double-membrane vesicles called autophagosomes [22]. The conversion of light chain (LC)3B-I to LC3B-II is a marker for autophagy induction [23]. Autophagy is regulated by several kinases: activation of class I phosphoinositide 3-kinase (PI3K) suppresses autophagy via the PI3K-AKT-mTOR pathway. By contrast, activation of class II and III PI3Ks contributes to initiation and progression of autophagy [24, 25]. MAP-ERK signaling pathway also negatively regulates autophagy [26]. Autophagy is associated with resistance to chemotherapy in cancer, and autophagy inhibition is a promising strategy for overcoming resistance [27, 28]. Several studies of solid tumors report CDK4/6 inhibition-induced autophagy and show that targeting both CDK4/6 and autophagy is a promising therapeutic strategy [29–31]. Therefore, we examined the effect of combination treatment with a CDK4/6 inhibitor and an autophagy inhibitor, and found that the two inhibitors acted synergistically to induce apoptosis in t(8;21) cells. These data suggest a novel therapeutic option based on the mechanism underlying the biology of t(8;21) AML.

Methods

CCND2 mutations and expression

Published data from mutational analyses of t(8;21) AML were searched. For pediatric t(8;21) AML, mutational data from 149 patients were collected from St. Jude Children's Research Hospital tissue resource core facility and the Japanese Pediatric Leukemia/Lymphoma Study Group (JPLSG) AML-05 study [13, 15]. For adult t(8;21) AML, mutational data from 134 patients were collected from Cancer and Leukemia Group B (CALGB)/Alliance for Clinical Trials in Oncology (Alliance) trials and the University Hospital of Ulm [13, 14]. Lollipop plots for visualizing *CCND2* mutations were generated using ProteinPaint (https://pecan.stjude.org/proteinpaint/).

CCND2 expression data from 1707 AML patients in the GSE13159, GSE15434, GSE61804, GSE14468, and Cancer Genome Atlas (TCGA) deposited to BloodSpot (https://servers.binf.ku.dk/bloodspot/) were compared between t(8;21) AML and non-t(8;21) AML patients [32]. Data on *CCND2* mRNA expression in AML cell lines were also collected from the Cancer Cell Line Encyclopedia (CCLE) database (https://portals.broadinstitute.org/ccle) and compared between t(8;21) AML cell lines (Kasumi-1 and SKNO-1) and other AML cell lines [33].

Primary AML cells

Primary AML cells were obtained from the bone marrow of a pediatric t(8;21) AML patient at Kyoto University Hospital after provision of informed consent and institutional review board approval. The study was conducted in accordance with the Declaration of Helsinki.

AML cell lines

Kasumi-1, SKNO-1, ML-2, HL-60, HEL, MV4-11, NB-4, KG-1a, Kasumi-6, KG-1, KO52, MOLM-16, U937, Kasumi-3, UF-1, CMK-86, and MOLM-13 cell lines were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). THP-1 and NOMO-1 cells were purchased from the Japanese Collection of Research Bioresources Cell Bank (Ibaraki, Japan). These cell lines were cultured in Roswell Park Memorial Institute 1640 medium supplemented with 10% or 20% fetal bovine serum and 1% penicillin/streptomycin at 5% CO_2 and 37 °C.

Compounds

Palbociclib (PD0332991) and abemaciclib (LY2835219) were obtained from AdooQ BioScience (Irvine, CA, USA). Both compounds were dissolved in dimethyl sulfoxide (DMSO) and used at a concentration of 500 nM according to previously published data [15]. Chloroquine (CQ) (#C6628, Sigma-Aldrich, St. Louis, MO, USA) was dissolved in PBS and used at a concentration of 10 μM. LY294002 (#129-04,861, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) was dissolved in DMSO and used at a concentration of 1 μM.

Small hairpin RNA (shRNA) interference

shRNA targeting human cyclin D2 (shCCND2) (CTGCAA TATGGGAACAAAT) and the control shRNA against luciferase (shLuc) were designed and cloned into pENTR4-H1tetOx1 and CS-RfA-ETBsd purchased from RIKEN BRC. Lentivirus was produced in HEK293T cells with

psPAX2 and pMD2.G, and cells were transfected using polyethyleneimine (Sigma-Aldrich) for 48 h. The virus was collected, cells were infected, and infected cells were selected using Blasticidin S (Sigma-Aldrich).

Small interfering RNA (siRNA)

Experiments used siRNA targeting the human ATG7 transcript (siATG7) (sc-41447, Santa Cruz Biotechnology, Dallas, TX, USA) and control siRNA (siControl) (sc-37007, Santa Cruz Biotechnology, Dallas, TX, USA). Kasumi-1 cells (4×10^5 cells/mL) were cultured at 37 °C in RPMI 1640 medium containing 10% fetal bovine serum without 1% penicillin/streptomycin, and then 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. Three days after transfection, cells were collected for apoptosis analysis.

Cell fertility assay

Cells ($0.8–2\times10^5$/mL) were seeded according to the respective growing speed. SF reagent (Nacalai Tesque, Kyoto, Japan) was added after 48 h, and the absorbance was measured using the Tecan i-control infinite 200 (Tecan, Männedorf, Switzerland). The relative value was calculated based on the absorbance of the control.

Cell proliferation assay

Cells (2×10^5/mL) were cultured in DMSO in the presence of palbociclib or abemaciclib and counted daily for 4–5 days. Data are presented as the mean ± standard error (SE) of three independent experiments.

Cell cycle analysis

Cells (2×10^5/mL) were cultured in DMSO in the presence of palbociclib or abemaciclib for 72 h. Then, cells were stained with propidium iodide solution (PI) and analyzed using a FACS Canto II flow cytometer (BD Biosciences, San Jose, CA, USA).

Microarray and pathway analyses

Kasumi-1 and SKNO-1 cells were treated with DMSO or abemaciclib for 72 h. Cells were harvested and RNA was extracted using an RNase mini kit (QIAGEN). Microarrays were performed on GeneChip Arrays (Clariom™ S Assay, Human) (Filgen, Nagoya, Japan). The data were analyzed using the Microarray Data Analysis Tool Ver 3.2 (Filgen).

A total of 468 genes exhibiting a strong transcriptomic change (> 1.5-fold change in expression in DMSO-treated cells vs. abemaciclib-treated cells in both Kasumi-1 and SKNO-1 cell lines) were called. Pathway analysis was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

Immunoblot analysis

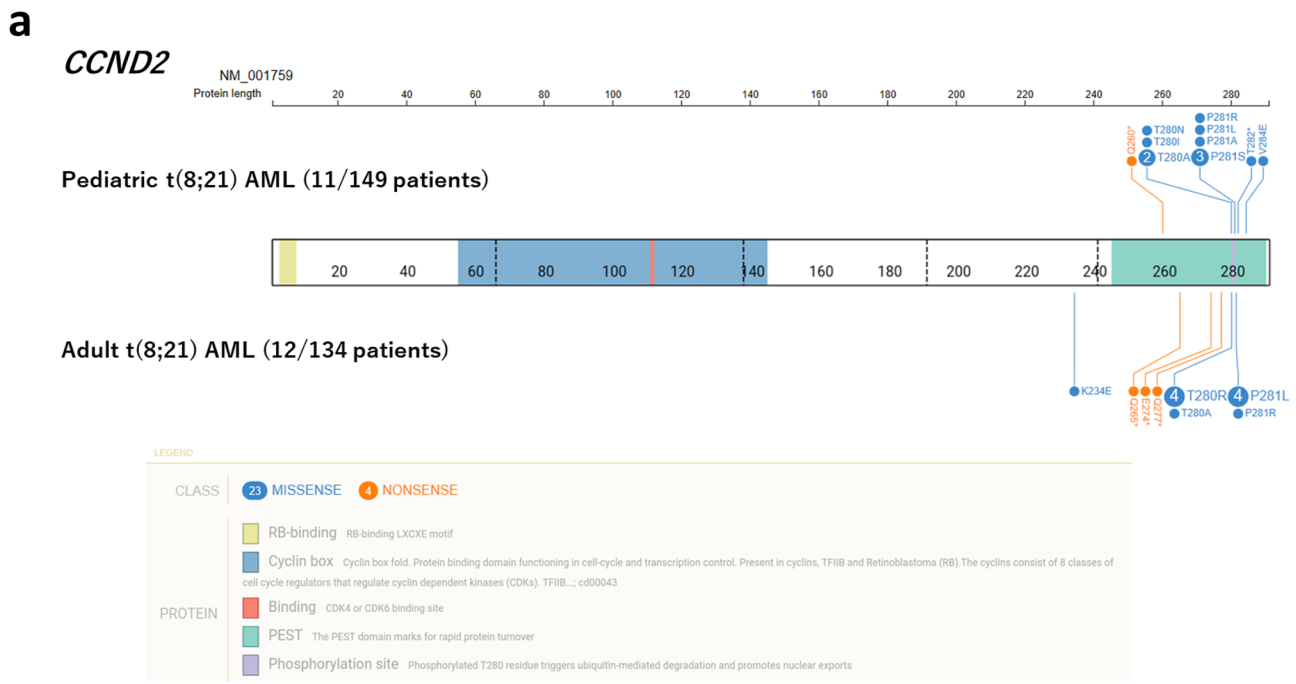
Cells were washed in PBS and lysed in RIPA buffer including a protease inhibitor cocktail (Nacalai Tesque). Whole-cell lysates were separated using 15% SDS polyacrylamide gels (SDS-PAGE) and transferred to Immobilon-P transfer membranes (Merck, Darmstadt, Germany). Membranes were blocked with Blocking One reagent (Nacalai Tesque) for 1 h at room temperature and incubated with the following primary antibodies overnight at 4 °C: anti-cyclin D2 (1/1000; #10,934-1-AP, Proteintech, Rosemont, IL, USA), anti-LC3B (1/1000; #L7543, Sigma-Aldrich), anti-cleaved caspase3 (1/500; #9661, CST, Danvers, MA, USA), or anti-GAPDH (1/3000; sc-47724, Santa Cruz Biotechnology, Dallas, TX, USA). Membranes were then incubated with HRP-conjugated anti-mouse IgG or anti-rabbit IgG (1/4000; #NA931; NA934, GE Healthcare Bio-Sciences, Little Chalfont, UK) for 1 h at room temperature. Protein bands were detected using an HRP Novex ECL Chemiluminescent Substrate Reagent Kit (Invitrogen, Carlsbad, CA, USA) and ChemiDoc™ XRS + Imager (Bio-Rad Laboratories, Hercules, CA, USA).

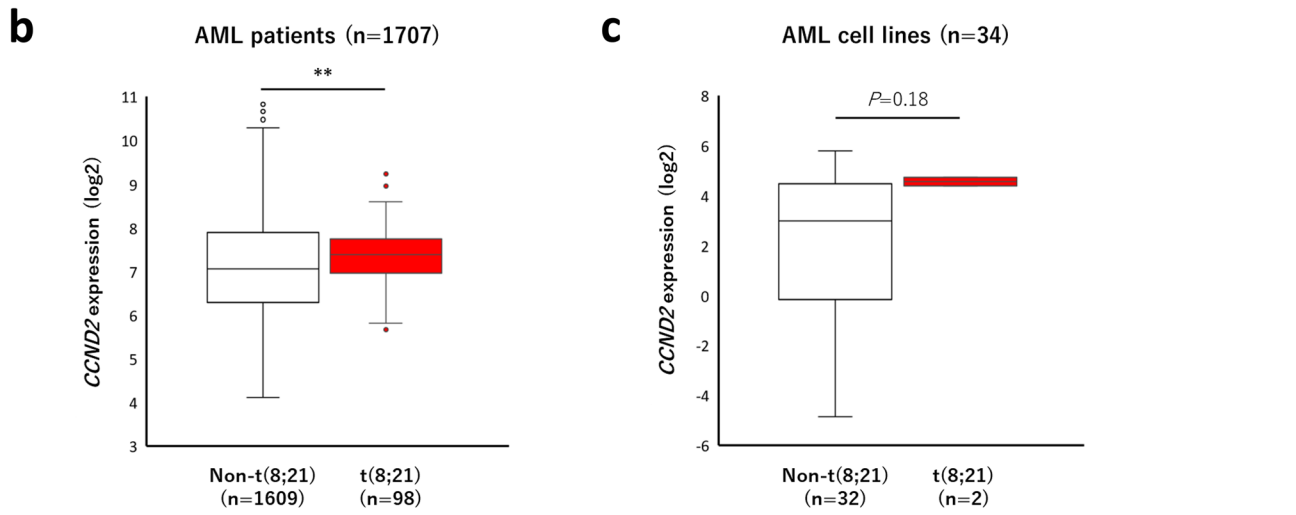
Transmission electron microscopy

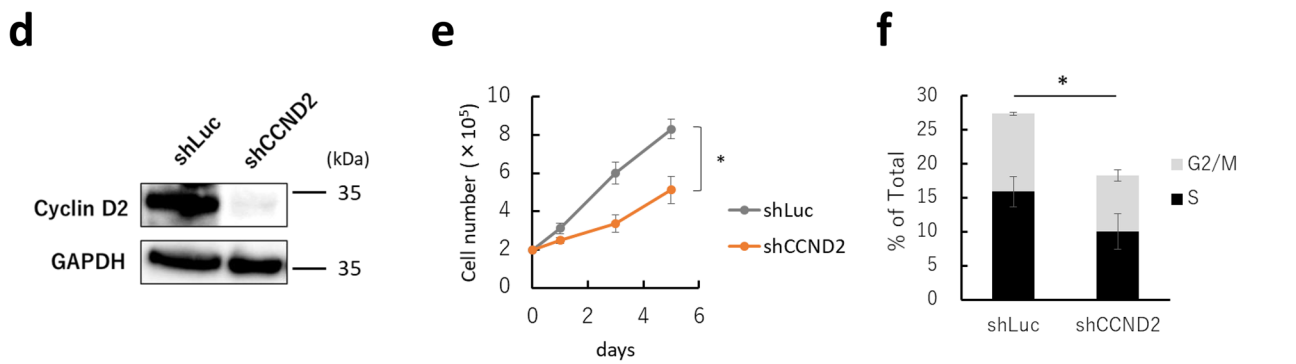
Cells (2.5×10^6) cultured in DMSO containing palbociclib or abemaciclib were pelleted by centrifugation, fixed in 4% paraformaldehyde/2% glutaraldehyde/0.1 M phosphate buffer at 4℃, washed in isotonic phosphate-buffered sucrose, re-fixed in phosphate-buffered 1% OsO_4, dehydrated through a graded series of ethanol solutions, and embedded in Luveak 812 (Nacalai Tesque). Thin sections (70–90 nm thick) were cut with a diamond knife on an EM UC6 ultramicrotome (Leica, Wetzlar, Germany), stained with uranyl acetate and lead citrate, and observed using a Hitachi H-7650 electron microscope (Hitachi, Tokyo, Japan).

Apoptosis assay

Cells were seeded at a density of 2×10^5 cells/mL and treated as indicated for 24 h. The collected cells were washed once with PBS- and suspended in 100 µL Annexin V binding buffer and 5 µL Annexin V using an apoptosis detection kit (BioLegend, San Diego, CA, USA). After addition of 10 µL PI and incubation at room temperature for 20 min, samples


a
CCND2
NM_001759
Protein length
Pediatric t(8;21) AML (11/149 patients)
Adult t(8;21) AML (12/134 patients)
T280N
T280I
T280A
P281R
P281L
P281A
P281S
K234E
T280R
P281L
T280A
P281R
LEGEND
CLASS
MISSENSE
NONSENSE
PROTEIN
RB-binding
RB-binding LXCXE motif
Cyclin box
Cyclin box fold. Protein binding domain functioning in cell-cycle and transcription control. Present in cyclins, TFIIB and Retinoblastoma (RB).The cyclins consist of 8 classes of cell cycle regulators that regulate cyclin dependent kinases (CDKs). TFIIB...; cd00043
Binding
CDK4 or CDK6 binding site
PEST
The PEST domain marks for rapid protein turnover
Phosphorylation site
Phosphorylated T280 residue triggers ubiquitin-mediated degradation and promotes nuclear exports


b
AML patients (n=1707)
**
CCND2 expression (log2)
Non-t(8;21) (n=1609)
t(8;21) (n=98)
c
AML cell lines (n=34)
P=0.18
CCND2 expression (log2)
Non-t(8;21) (n=32)
t(8;21) (n=2)


d
shLuc
shCCND2
(kDa)
Cyclin D2
35
GAPDH
35
e
Cell number (×10⁵)
days
shLuc
shCCND2
f
% of Total
G2/M
S
shLuc
shCCND2

◂**Fig. 1** Frequent *CCND2* mutations and high *CCND2* expression in t(8;21) AML. **a** Domain structure and location of *CCND2* mutations in pediatric and adult t(8;21) AML patients. *CCND2* mutations were detected in 11 of 149 pediatric t(8;21) AML patients and 12 of 134 adult t(8;21) AML patients. **b** *CCND2* mRNA expression data from 1707 patients [non-t(8;21) AML patients ($n=1609$); t(8;21) AML patients ($n=98$)] in the GSE13159, GSE15434, GSE61804, GSE14468, and Cancer Genome Atlas (TCGA) deposited to BloodSpot (https://servers.binf.ku.dk/bloodspot/) were compared. **c** *CCND2* mRNA expression in 34 AML cell lines [non-t(8;21) AML cell lines ($n=32$) and t(8;21) AML cell lines ($n=2$, Kasumi-1 and SKNO-1)] from the CCLE database was compared. **d** Kasumi-1 cells were transfected with shLuc or shCCND2. Immunoblot analysis showed significantly lower expression of cyclin D2 in shCCND2-transfected cells than in shLuc-transfected cells. **e** Kasumi-1 cells transfected with shCCND2 showed impaired cell proliferation. f. Cell cycle analysis showed a significantly lower percentage of cells in the S/G2/M phase in shCCND2-transfected Kasumi-1 cells compared with in shLuc-transfected cells. Data are presented as the mean ± SE of three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$

were prepared in 300 µL PBS and analyzed using the FACS Canto II flow cytometer (BD Biosciences).

Statistical analysis

Continuous variables were compared using the Student's *t*-test or Mann–Whitney *U*-test. A *P*-value of <0.05 was considered statistically significant. Statistical significance was defined as follows: $P<0.05$ (*), $P<0.01$ (**), or $P<0.001$ (***).

Results

Frequent *CCND2* mutations and high *CCND2* expression in t(8;21) AML

To examine the frequency of *CCND2* mutations in t(8;21) AML patients, we analyzed 149 pediatric patients from St. Jude Children's Research Hospital tissue resource core facility and the JPLSG AML-05 study, and 134 adult patients from CALGB/Alliance trials and the University Hospital of Ulm. In 149 pediatric t(8;21) AML patients, 13 mutations were detected in 11 patients (11/149, 7.4%) (Fig. 1a). In 134 adult t(8;21) AML patients, 14 mutations were detected in 12 patients (12/134, 9.0%). In both cohorts, *CCND2* mutations were located on the PEST domain, suggesting that the mutations stabilize the cyclin D2 protein.

Next, we compared *CCND2* expression in t(8;21) AML patients with that in other AML patients. RNA expression data from 1707 patients in the GSE13159, GSE15434, GSE61804, GSE14468, and TCGA deposited to BloodSpot (https://servers.binf.ku.dk/bloodspot/) were analyzed. The results showed that *CCND2* expression t(8;21) in AML patients ($n=98$) was significantly higher than that in other AML patients ($n=1609$) ($P=0.003$, Fig. 1b). In AML cell lines, those with t(8;21) ($n=2$: Kasumi-1 and SKNO-1; both *CCND2* wild-type) showed higher *CCND2* expression than those without ($n=32$); however, the difference was not statistically significant ($P=0.18$, Fig. 1c), probably due to the very small sample size.

To determine the role of high *CCND2* expression in t(8;21) AML, we performed shRNA knockdown analysis. Kasumi-1 cells transfected with shCCND2 showed a lower rate of cell proliferation than those transfected with shLuc (control) (Fig. 1d, e). Cell cycle analysis showed that the percentage of cells in S/G2/M phase was significantly lower in shCCND2-transfected Kasumi-1 cells than in the controls, suggesting that cells were arrested in G1 phase (Fig. 1f; Supplementary Fig. 1). These results suggest that the frequency of *CCND2* mutations and *CCND2* expression are increased in t(8;21) AML, and high *CCND2* expression plays an important role in t(8;21) AML cell proliferation.

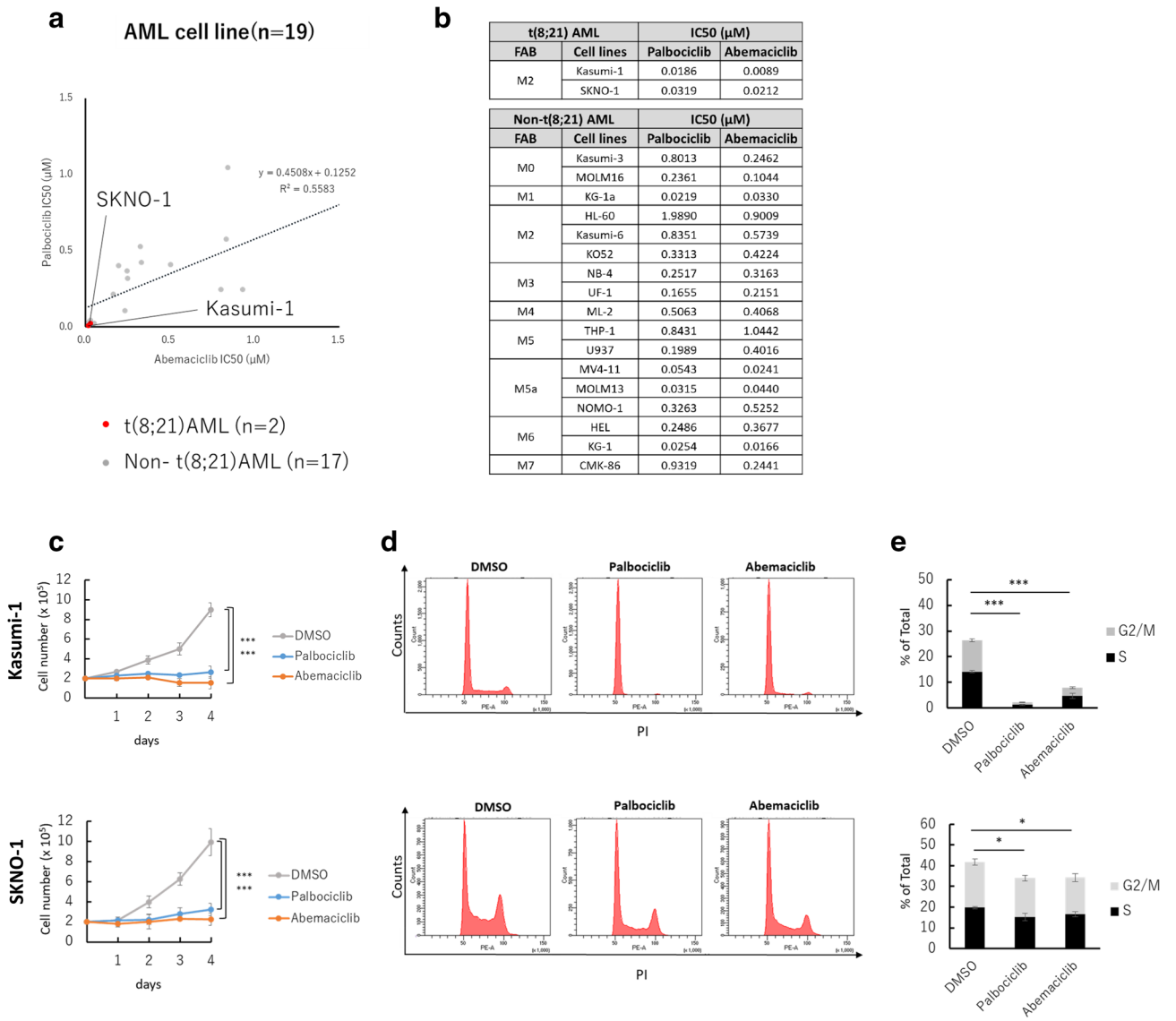
Efficacy of CDK4/6 inhibitors in t(8;21) AML cell lines

Because *CCND2* is not a druggable target, we examined the effect of CDK4/6 inhibitors (palbociclib and abemaciclib) on t(8;21) AML cells. The IC50 (50% inhibitory concentration) of CDK4/6 inhibitors was screened using 19 AML cell lines including two t(8;21) AML cell lines (Fig. 2a). There was a moderate correlation in IC50 values between palbociclib and abemaciclib ($R^2=0.56$). The IC50 values for both palbociclib and abemaciclib were significantly lower in the two t(8;21) AML cell lines than in the non-t(8;21) AML cell lines (Fig. 2a, b).

Investigation of the mechanism of action of CDK4/6 inhibitors in t(8;21) AML cell lines showed that the inhibitors impaired proliferation in Kasumi-1 and SKNO-1 cells (Fig. 2c). In addition, treatment with CDK4/6 inhibitors decreased the percentage of S/G2/M phase cells detected by flow cytometry, suggesting that CDK4/6 inhibition arrested cells in G1 phase (Fig. 2d, e). We also examined the effect of CDK4/6 inhibition on proliferation in shLuc-transfected and shCCND2-transfected Kasumi-1 cells. We found that shLuc + abemaciclib and shCCND2 + abemaciclib cells gave similar results in that both showed a significant decrease in proliferation compared with shLuc and shCCND2 cells (Supplementary Fig. 2). These results indicate that CDK4/6 inhibitors may be effective for the treatment of t(8;21) AML.

CDK4/6 inhibitors induce autophagy in t(8;21) AML cells

To identify potential therapeutic approaches in combination with CDK4/6 inhibitors in t(8;21) AML, we performed microarray analysis to examine the effects of CDK4/6 inhibition in addition to cell cycle arrest.



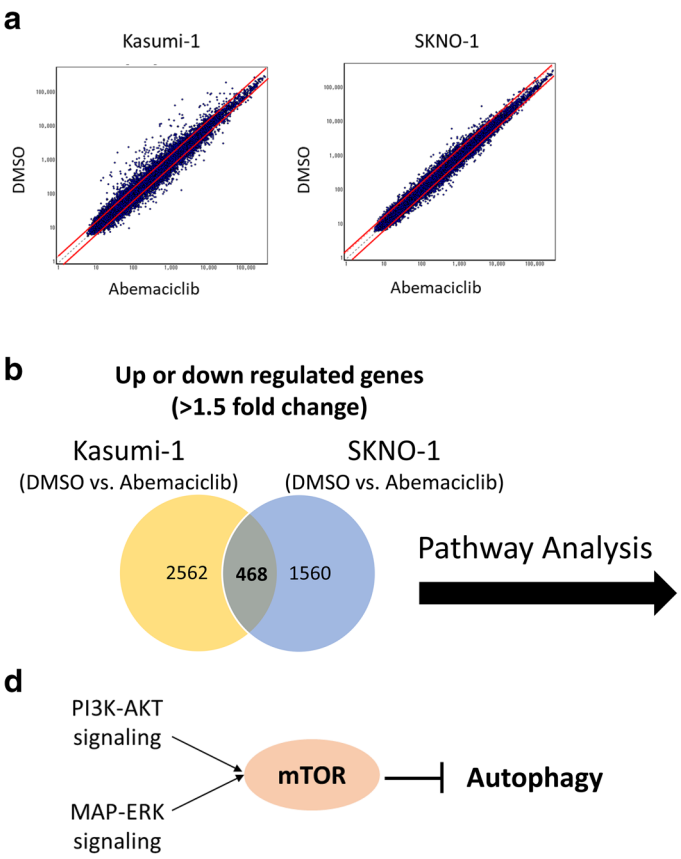
t(8;21) AML		IC50 (μM)	
FAB	Cell lines	Palbociclib	Abemaciclib
M2	Kasumi-1	0.0186	0.0089
	SKNO-1	0.0319	0.0212

Non-t(8;21) AML		IC50 (μM)	
FAB	Cell lines	Palbociclib	Abemaciclib
M0	Kasumi-3	0.8013	0.2462
	MOLM16	0.2361	0.1044
M1	KG-1a	0.0219	0.0330
M2	HL-60	1.9890	0.9009
	Kasumi-6	0.8351	0.5739
	KO52	0.3313	0.4224
M3	NB-4	0.2517	0.3163
	UF-1	0.1655	0.2151
M4	ML-2	0.5063	0.4068
M5	THP-1	0.8431	1.0442
	U937	0.1989	0.4016
M5a	MV4-11	0.0543	0.0241
	MOLM13	0.0315	0.0440
	NOMO-1	0.3263	0.5252
M6	HEL	0.2486	0.3677
	KG-1	0.0254	0.0166
M7	CMK-86	0.9319	0.2441

Fig. 2 CDK4/6 inhibitors induce cell cycle arrest in t(8;21) AML cell lines. **a** IC50 of abemaciclib and palbociclib in 19 AML cell lines including two t(8;21) and 17 non-t(8;21) AML cell lines. **b** Raw data from IC50 screening according to FAB subtypes of cell lines. **c** t(8;21) AML cells treated with palbociclib or abemaciclib showed impaired cell proliferation. **d**, **e** Treatment with CDK4/6 inhibitors decreased the percentage of S/G2/M phase cells in t(8;21) AML cells. Data are presented as the mean ± SE of three independent experiments. $*P < 0.05$, $***P < 0.001$

Kasumi-1 and SKNO-1 cell lines were incubated with DMSO or abemaciclib, and RNA expression levels were compared. The number of genes showing a significant change (> 1.5-fold up or down in DMSO vs. abemaciclib) was 2562 in Kasumi-1 cells, 1560 in SKNO-1 cells, and 468 in both cell lines (Fig. 3a, b). Pathway analysis of the 468 genes changed in both cell lines was performed using the KEGG database, and pathways with $P < 0.05$ were extracted. In addition to the pathways associated with the cell cycle (regulation of sister chromatid separation, retinoblastoma gene, and cell cycle), the MAP-ERK and (Class I) PI3K-AKT-mTOR signaling pathways were downregulated by CDK4/6 inhibition (Fig. 3c). Because these pathways are involved in autophagy regulation via mTOR (Fig. 3d), we focused on examining autophagy in subsequent experiments.

Assessment of the effect of CDK4/6 inhibition on autophagy in t(8;21) AML cells showed that inhibitor treatment induced LC3B-I to LC3B-II conversion in both Kasumi-1 and SKNO-1 cells (Fig. 4a, b). Transmission electron microscopic examination of autophagosome formation detected a large number of autophagosomes in the cytoplasm of Kasumi-1 and SKNO-1 cells treated with CDK4/6 inhibitors, whereas few autophagosomes were detected in control



c

Pathways (p<0.05)
Zinc_homeostasis
Regulation_of_sister_chromatid_separation
Copper_homeostasis
Retinoblastoma_Gene
Histone_Modifications
Cell_Cycle
Genotoxicity_pathway
FBXL10_enhancement_of_MAP-ERK_signaling
Photodynamic_therapy-induced_unfolded_protein_response
Gastric_Cancer_Network_1
The_effect_of_progerin_on_the_involved_genes
DNA_IR-damage_and_cellular_response_via_ATR
PI3K-AKT-mTOR_signaling_pathway_and_therapeutic_opportunities
Prion_disease_pathway
NAD_Biosynthesis_II_(from_tryptophan)
NAD+_biosynthetic_pathways
DNA_Mismatch_Repair
Glycosylation_and_related_congenital_defects
Degradation_pathway_of_sphingolipids,_including_diseases
Integrated_Cancer_Pathway
Nucleotide_GPCRs
GPCRs,_Class_A_Rhodopsin-like

Fig. 3 Microarray and pathway analysis of t(8;21) AML cell lines treated with abemaciclib. **a** Comparison of gene expression between DMSO-treated control cells and abemaciclib-treated cells in the Kasumi-1 and SKNO-1 cell lines. **b** The number of genes showing a significant change (> 1.5-fold up or down) was 2562 in Kasumi-1 cells, 1560 in SKNO-1 cells, and 468 in both cells. **c** Pathway analysis of the 468 genes changed in both cell lines was performed using the KEGG database, and pathways with $P < 0.05$ were extracted. The upregulated pathways are indicated in red characters and the downregulated pathways in blue. MAP-ERK and PI3K-AKT-mTOR signaling pathways are highlighted in bold. **d** Schematic figure showing the contribution of the MAP-ERK and PI3K-AKT-mTOR signaling pathways to autophagy regulation via mTOR

samples (Fig. 4c, d). These results suggest that autophagy is induced by CDK4/6 inhibition in t(8;21) AML cells.

CDK4/6 and autophagy inhibitors synergistically induce apoptosis in t(8;21) AML cells

Autophagy is involved in the resistance to chemotherapy in cancer cells [27]. Therefore, we hypothesized that autophagy inhibition may be a promising therapeutic approach. Two autophagy inhibitors with different mechanisms of action were selected: CQ neutralizes the acidic pH of intracellular vesicles and blocks lysosomal degradation, and LY294002 inhibits class III PI3Ks [34]. The percentage of Annexin V positive (apoptotic) cells was determined by flow cytometry in Kasumi-1 cells. The percentage of apoptotic cells did not differ significantly between cells treated with CQ or LY294002 and control cells (control vs. CQ: $P = 0.24$, control vs. LY294002: $P = 0.22$) (Fig. 5a, b). However, the percentage of apoptotic cells was significantly higher in cells treated with autophagy inhibitors in combination with abemaciclib than in cells treated with abemaciclib alone (abemaciclib vs. abemaciclib plus CQ: $P = 0.00001$, abemaciclib vs. abemaciclib plus LY294002: $P = 0.03$). Consistently, treatment with both abemaciclib and autophagy inhibitors upregulated the expression of cleaved caspase3, a marker of apoptosis (Fig. 5c). The combinatorial effect was confirmed by silencing the autophagy-related protein ATG7 using small interfering RNA in abemaciclib-treated t(8;21) AML cells (Supplementary Fig. 3).

To confirm the result using a t(8;21) AML cell line, we examined the efficacy of co-inhibiting CDK4/6 and autophagy in a primary bone marrow sample from a pediatric t(8;21) AML patient. Treatment of primary t(8;21) AML cells with CQ resulted in LC3B-I to LC3B-II conversion, suggesting induction of autophagy (Supplementary Fig. 4). The percentage of apoptotic cells did not differ significantly between control cells and cells treated with CQ ($P = 0.40$) (Fig. 5d, e). However, the percentage

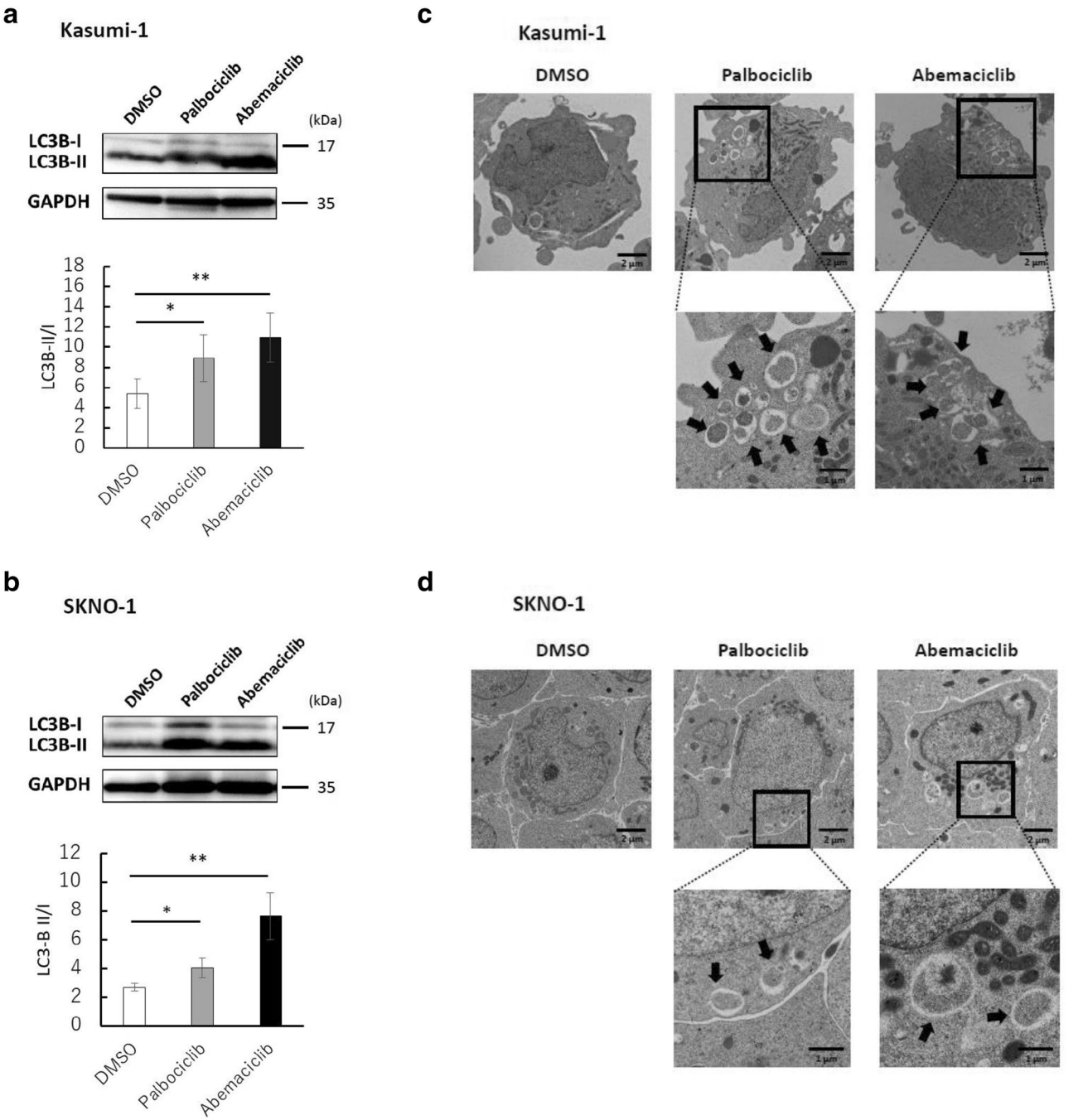


Fig. 4 Induction of autophagy in t(8;21) AML cells treated with CDK4/6 inhibitors. **a**, **b** Immunoblot analysis showing the effect of CDK4/6 inhibitors on inducing LC3B-I to LC3B-II conversion in t(8;21) AML cells. Data from immunoblots were quantified by densitometric analysis ($n=4$). **c**, **d** The formation of double-membrane structures (autophagosomes) in t(8;21) AML cells treated with CDK4/6 inhibitors was observed by TEM. The black arrows indicate autophagosomes. $^{*}P<0.05$, $^{**}P<0.01$

of apoptotic cells was significantly higher after treatment with autophagy inhibitors plus abemaciclib than after treatment with abemaciclib alone ($P=0.049$). These results suggest that CDK4/6 and autophagy inhibitors synergistically induce apoptosis in primary t(8;21) AML cells (Fig. 5f).

Discussion

In this study, we showed that *CCND2* mutations located on the PEST domain occur at a high rate in t(8;21) AML. *CCND2* mutations in t(8;21) AML were reported by three independent groups although the number of patients

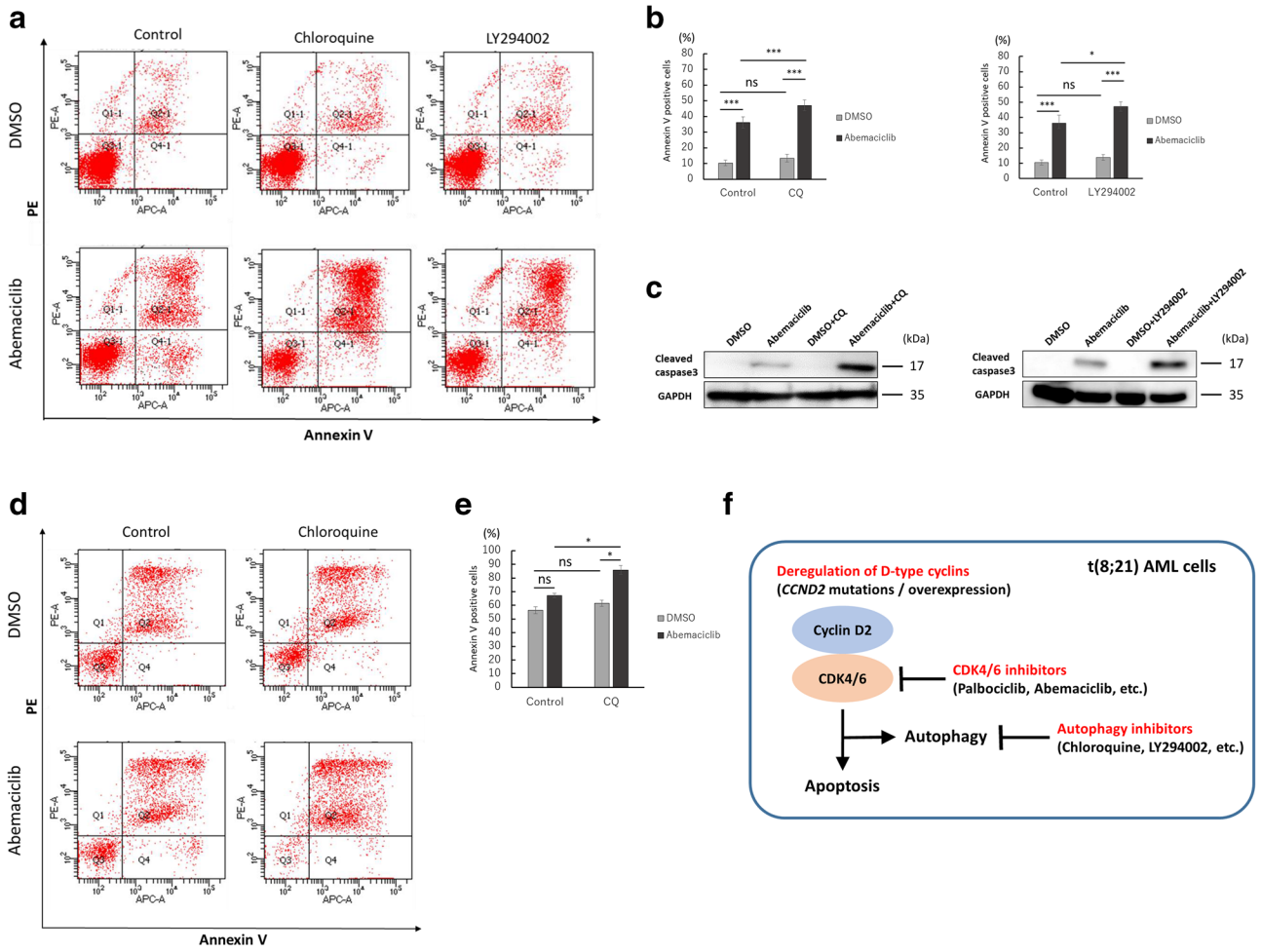


Fig. 5 CDK4/6 and autophagy inhibitors in combination synergistically induce apoptosis in t(8;21) AML cells. **a**, **b** The percentage of apoptotic (Annexin V positive) cells was examined by flow cytometry. The percentage of apoptotic cells did not differ significantly between chloroquine (CQ) or LY294002 treated Kasumi-1 cells and control cells. The percentage of apoptotic cells was significantly higher in Kasumi-1 cells treated with autophagy inhibitors in combination with abemaciclib than in cells treated with abemaciclib alone. **c** The expression of cleaved caspase 3 was examined by immunoblotting. Combination treatment with abemaciclib and autophagy inhibitors upregulated cleaved caspase 3 expression. **d**, **e** The percentage of apoptotic cells was examined using a primary t(8;21) AML sample. The number of apoptotic cells did not differ significantly between CQ-treated cells and control cells. The percentage of apoptotic cells was significantly higher in primary t(8;21) AML cells treated with CQ plus abemaciclib than in cells treated with abemaciclib alone. **f** Schematic figure showing that co-inhibition of CDK4/6 and autophagy enhances apoptosis in t(8;21) AML cells, which are characterized by frequent *CCND2* mutations or high *CCND2* expression. Data are presented as the mean ± SE of three independent experiments. $*P < 0.05$, $***P < 0.001$; *ns* not significant

analyzed in each study was small. We showed that the frequency of *CCND2* mutations was comparable between pediatric and adult t(8;21) AML patients (7.4% and 9.0%, respectively), suggesting that the genetic abnormality is a common feature in t(8;21) AML regardless of patient age. The frequency of *CCND2* mutations in patients with non-core binding factor (CBF)-AML is 0.4% (6/1426), supporting the high frequency of *CCND2* mutations in t(8;21) AML [14]. We also showed that *CCND2* expression in t(8;21) AML patients is higher than that in non-t(8;21) AML patients. This result may be explained by a recent study showing that *CCND2* is a crucial transcriptional target of RUNX1-ETO protein and that this fusion protein upregulates *CCND2* expression [35]. *CCND2* mutations located in the PEST domain stabilize cyclin D2 protein [13]. Therefore, the effect of *CCND2* mutations on *CCND2* expression would be reflected at the protein level. Comparison of cyclin D2 protein expression between patients with *CCND2* wild-type and those with the *CCND2* mutant would be an interesting topic for future study.

Among the 19 AML cell lines analyzed, t(8;21) AML cell lines (Kasumi-1 and SKNO-1) showed high sensitivity to CDK4/6 inhibition. In the IC50 screening, three non-t(8;21) AML cell lines with FAB-M2 (HL-60, Kasumi-6, and KO52) were included. Despite the fact that these cell

lines share the same FAB-subtype with Kasumi-1 and SKNO-1 cells, the IC50 values were > 10-fold higher than those of t(8;21) AML cell lines. This difference may be explained by the fact that deregulation of D-type cyclins increases the sensitivity of cancer cells to CDK4/6 inhibitors [21]. Several non-t(8;21) AML cell lines were also sensitive to CDK4/6 inhibitors, suggesting that these cell lines may harbor other D-cyclin activating features including *CCND3* amplification. Although CDK4/6 inhibitors are currently in clinical trials for the treatment of various solid tumors, there are few clinical trials assessing their efficacy in leukemia [19]. Clinical trials for AML, especially for t(8;21) AML, should be considered. We also showed that the proliferation curves of shLuc-transfected and shCCND2-transfected Kasumi-1 cells are similar when CDK4/6 is inhibited. This result is reasonable because *CCND2* and CDK4/6 share the same pathway: cyclin D2 forms complexes with CDK4/6 and induces cell cycle progression [16, 17].

CDK4/6 inhibition induced autophagy in t(8;21) AML cells. Despite the promising results of clinical trials of CDK4/6 inhibitors in solid tumors, various resistance mechanisms against CDK4/6 inhibition have been reported [36, 37]. In this study, combination treatment with CDK4/6 and autophagy inhibitors induced apoptosis, suggesting that autophagy is involved in the mechanism of resistance to CDK4/6 inhibition in t(8;21) AML. Targeting both CDK4/6 and autophagy showed promising results with respect to treatment of solid tumor cells [30, 31], supporting the notion that inhibiting autophagy may be a key strategy for overcoming the resistance to CDK4/6 inhibition. CQ, one of the autophagy inhibitors used in this study, is a well-tolerated antimalarial drug, and several clinical trials combining CQ with anticancer therapies are ongoing [27, 38]. Thus, CQ may be a promising agent in combination with CDK4/6 inhibitors.

In conclusion, the present results indicate that inhibition of CDK4/6 and autophagy may be a novel and promising biomarker-driven therapeutic strategy for the treatment of t(8;21) AML. The results of this study were obtained using in vitro analyses, and in vivo experiments are necessary to confirm the results in the future.

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Author contributions KN and HM analyzed sequencing data and performed the functional analysis. YH and MH helped with data acquisition. KN, HM, YH, MH, AK, MN, YN-A, YK, and SA contributed to the interpretation of data. KN and HM prepared the figures and wrote the manuscript. HM, YK, and SA supervised the project.

Compliance with ethical standards

Conflict of interest The authors declare no competing financial interests.

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