Original Research

Inhibition of BCL2A1 by STAT5 inactivation overcomes resistance to targeted therapies of FLT3-ITD/D835 mutant AML

Kotoko Yamatani a, Tomohiko Ai a, Kaori Saito a, Koya Suzuki a, Atsushi Hori a,b, Sonoko Kinjo c, Kazuo Iko e, Vivian Ruvolo i, Weiguo Zhang d, Po Yee Mak d, Bogumil Kaczkowski c, Hironori Harada a, Kazuhiro Katayama a, Yoshihazu Sugimoto h, Jered Myslinski j, Takashi Hato i, Takashi Miida a, Marina Konopleva l, Yoshihide Hayashizaki k, Bing Z. Carter d, Yoko Tabe a,d,i,* , Michael Andreeff d,i,*

a Department of Clinical Laboratory Medicine, Juntendo University Graduate School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan
b Center for Genomic and Regenerative Medicine, Juntendo University Graduate School of Medicine, Tokyo, Japan
c Center for Information Biology, National Institute of Genetics, Shizuka, Japan
d Department of Leukemia, Section of Molecular Hematology and Therapy, The University of Texas MD Anderson Cancer Center, Houston, TX, United States
e Department of Hematology, Juntendo University Graduate School of Medicine, Tokyo, Japan
f Laboratory of Molecular Targeted Therapeutics, School of Pharmacy, Nihon University, Chiba, Japan
g Division of Chemotherapy, Faculty of Pharmacy, Keio University, Tokyo, Japan
h Department of Medicine, Indiana University School of Medicine, Marion, IN, United States
i Department of Leukemia, Section of Leukemia Biology Research, The University of Texas MD Anderson Cancer Center, Houston, TX, United States
j Kabushiki Kaisya Dnaform, Yokohama, Japan
l Department of Next Generation Hematology Laboratory Medicine, Juntendo University Graduate School of Medicine, Tokyo, Japan

ARTICLE INFO

Keywords:
BCL2A1
AML
FLT3
CAGE
Venetoclax

ABSTRACT

Tyrosine kinase inhibitors (TKIs) are established drugs in the therapy of FLT3-ITD mutated acute myeloid leukemia (AML). However, acquired mutations, such as D835 in the tyrosine kinase domain (FLT3-ITD/D835), can induce resistance to TKIs. A cap analysis gene expression (CAGE) technology revealed that the gene expression of BCL2A1 transcription start sites was increased in primary AML cells bearing FLT3-ITD/D835 compared to FLT3-ITD. Overexpression of BCL2A1 attenuated the sensitivity to quinazolinib, a type II TKI, and venetoclax, a selective BCL2 inhibitor, in AML cell lines. However, a type I TKI, gilteritinib, inhibited the expression of BCL2A1 through inactivation of STAT5 and alleviated TKI resistance of FLT3-ITD/D835. The combination of gilteritinib and venetoclax showed synergistic effects in the FLT3-ITD/D835 positive AML cells. The promtor region of BCL2A1 contains a BRD4 binding site. Thus, the blockade of BRD4 with a BET inhibitor (CP-T0610) downregulated BCL2A1 in FLT3-mutated AML cells and extended profound suppression of FLT3-ITD/D835 mutant cells. Therefore, we propose that BCL2A1 has the potential to be a novel therapeutic target in treating FLT3-ITD/D835 mutated AML.

Introduction

Acute myeloid leukemia (AML) is associated with chromosomal disorders, germline, and somatic driver mutations [1]. Specifically, internal tandem duplications in the juxtamembrane domain of the FMS-like tyrosine kinase 3 gene (FLT3-ITD) and missense mutations in the tyrosine kinase domain (TKD) of the FLT3 gene play critical roles in the pathophysiology of AML. In fact, FLT3-ITD and FLT3-TKD mutations were detected in approximately 15–35% and 5–10% of AML patients, respectively [2], and approximately 1–3% of the patients carry both mutants.

* Corresponding authors at: Department of Leukemia, Section of Molecular Hematology and Therapy, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Boulevard, Unit 448, Houston, TX 77030, United States.

E-mail addresses: tabe@juntendo.ac.jp (Y. Tabe), mandreef@mdanderson.org (M. Andreeff).

Received 19 November 2021; Received in revised form 7 January 2022; Accepted 22 January 2022

https://doi.org/10.1016/j.tranon.2022.101354

© 2022 Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
Cap analysis gene expression (CAGE) technology revealed that -ITD AML cells decreases the sensitivity to quizartinib. Based on FLT3 compared to FLT3 by transcriptome analysis and compared with those with proliferation of refractory clones after TKI treatments. Targeted TKIs have shown favorable effects in refractory AML with sorafenib, selectively bind to the inactive state of American Type Culture Collection (Manassas, VA), and Molm13 cells.

Preparation of cell lines and primary AML cells

Materials and methods

Preparation of cell lines and primary AML cells

Human MV4;11 cells (RRID: CVCL_0064) were purchased from the American Type Culture Collection (Manassas, VA), and Molm13 cells (RRID: CVCL_2119) were purchased from DSMZ (Braunschweig, Germany). These cells were cultured in IMDM or RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. MV4;11 cells bearing FLT3-ITD/D835 mutations (MV4;11-ITD/D835) were established from parental MV4;11 bearing FLT3-ITD mutation (MV4;11-ITD) by exposure to quizartinib at incremental concentrations of from 0.3 to 1.5 nM for six months, followed by clone cultures. The FLT3-ITD/D835 mutations were confirmed by direct sequencing. The established FLT3-ITD/D835 clone had a 50% inhibitory concentration (IC50) of 6.7 nM for quizartinib, which was 34-times higher than that of MV4;11-ITD. MV4;11 cells bearing FLT3-ITD/D835 mutations were maintained in IMDM containing quizartinib (1.5 nM). Quizartinib was removed three to four days before experiments.

To stably overexpress BCL2A1 in AML cell lines, a lentiviral vector was used for gene delivery. A lentiviral transfection vector with the BCL2A1 open reading frame (RefSeq NM_004049.4) under regulation of the human EF1A promoter was purchased from Vector Builder (Chicago, IL). As a negative control, a lentiviral transfection vector was used with a 300 bp non-coding stuffer sequence under the same promoter from the same vendor. Lentivirus was prepared by transient co-transfection of HEK-293T cells (ATCC, RRID: CVCL_0063) with an equimolar mix of transfer vector and packaging plasmids (psPAX2 and pMD2.G, RRID: Addgene_12.260 and RRID: Addgene_12.259, respectively, from Addgene) using JetPrime transfection reagent as directed by the manufacturer (Polyplus, Illkirch, France). Lentiviral supernatants were harvested 48-h post transfection and passed through 0.45-micron, surfactant-free-cellulose acetate membranes. AML cell lines were incubated with undiluted viral supernatant overnight at 37 °C under 5% CO2; infected cells were then washed and selected with puromycin (Invivogen, San Diego, CA) at 0.5 μg/mL. Increased expression of each transgene was verified by immunoblot analysis.

Primary peripheral blood and bone marrow (BM) samples were obtained from newly diagnosed or relapsed AML patients (n = 26) after written informed consent was obtained in accordance with the University of Texas MD Anderson Cancer Center Institutional Review Board regulations under the Declaration of Helsinki principles. The protocol was approved by the respective Institutional Ethics Committees. The patients carrying nucleophosmin (NPM1) mutations were excluded since NPM1 mutations are associated with favorable prognosis in negative or low allelic ratio FLT3-ITD AML. Their age varied from 24 to 87 years. Ficoll-Hypaque density gradient centrifugation was used to separate mononuclear cells (Sigma-Aldrich, St Louis, MO). Venetoclax (ABT-199/GDC-0199) and quizartinib (AC220) were purchased from Selleck-chem (Houston, TX). Gilteritinib and CPI-0610 were purchased from Funakoshi (Tokyo, Japan) and Abcam (Cambridge, UK), respectively.

Analyses of cell viability and apoptosis

Cell proliferation was assessed by Cell Counting Kit-8 (WST-8, Dojindo, Kumamoto, Japan). Effects of the reagents on cellular proliferation were evaluated as percent-decrease of cell viability compared to the cell viability in the culture medium containing 0.01% dimethyl sulfoxide. The half-maximal inhibitory concentration (IC50) and combination index (CI) were calculated by the Chou-Talalay method based on the median-effect principle (23), using CalcuSyn 2.0 software (Biosoft, Cambridge, UK). The dosage of each reagent was determined based on the previously reported therapeutic concentrations in humans: 270 nM for quizartinib (24), 310 nM for gilteritinib (25), 2.5 μM for venetoclax (26), and 6 μM for CPI-0610 (27).

Immunoblot analysis

Immunoblot analysis was performed as previously described (28). For immunohublotting, the following antibodies were used: β-actin (Sigma-Aldrich, St. Louis, MO, Cat# A5316, RRID:AB_476,743); MCL-1 (BD
Translational Oncology 18 (2022) 101354

Biosciences, San Diego CA, Cat# 559,027, RRID:AB_397,176); BCL2 (Cell Signaling Technology, Danvers, MA Cat# 2872, RRID:AB_10,693, 462), BCL2A1 (Cell Signaling Technology, Cat# 14,093, RRID:AB_2, 798,390), BCL-XL (Cell Signaling Technology, Cat# 2762, RRID:AB_10, 694,844), STAT5 (Cell Signaling Technology, Cat# 9363, RRID:AB_2, 196,923), phosphorylated (p-) STAT5 (Cell Signaling Technology, Cat# 9351, RRID:AB_2,315,225), horseradish peroxidase–linked anti-mouse (Cell Signaling Technology, Cat# 7076, RRID:AB_330,924) and anti-rabbit IgG (Cell Signaling Technology, Cat# 7074, RRID:AB_2,099, 233).

Mutational analyses

Mutation screening was performed using paired-end sequencing on the MiSeq sequencer (Illumina, San Diego CA) using DNA from fresh BM samples or peripheral blood. NGS sequencing libraries were created by polymerase chain reaction (PCR) amplification of target regions using TrueSeq chemistry (Illumina) or Haloplex probe capture followed by PCR amplification of target regions using Haloplex chemistry (Agilent, Santa Clara, CA). The panel interrogated either selected hotspot or entire coding regions of 25 genes (ASXL1, BRAF, CEBPA, DNMT3, EGF, EZH2, GATA1, GATA2, IDH1, IDH2, IKZF2, JAK2, KIT, MDM2, MLL, MPL, MYD88, NOTCH1, PDGFRA, PTEN, RAS, RUNX1, TET2, TP53, WT1) using DNA from fresh BM samples or peripheral blood. NGS sequencing libraries were created by TrueSeq Amplicon Cancer Panel kit (Illumina) as described previously [29]. Human genome build 19 (hg19) was used as the reference for sequence alignment. Reporter and Agilent SureCall were used for variant calling for Trueseq and Haloplex workflows, respectively. FLT3-ITD and FLT3-TKD mutation testing was performed using PCR followed by capillary electrophoresis on Genetic Analyzer (Applied Biosystems, Foster City, CA), as described previously [30].

mRNA quantification

Total RNA was extracted from cells with the RNeasy Mini Kit (Qiagen, Hilden, Germany). First-strand cDNA was synthesized with oligo (dT) as primer (Superscript II System; Invitrogen). Real-time reverse transcription-PCR (RT-PCR) was performed by the Model 7500 Real-time PCR System (Applied Biosystems). Expression of the mRNAs encoding BCL2, BCL2A1, and GAPDH was detected by TaqMan Gene Expression Assays (BCL2: Hs00608023_m1; BCL2A1: Hs00187845_m1; GAPDH: Hs99999905_s1; Applied Biosystems). The expression of each gene transcript relative to that of GAPDH was calculated as follows: relative expression = 2^(- ΔCt), where ΔCt is the mean Ct of the transcript of interest minus the mean Ct of the transcript for GAPDH. The ΔCt data from duplicate PCRs were averaged for calculation of relative expression.

CAGE

CAGE libraries from the RNA samples were prepared as described previously [31]. CAGE peaks that represent transcription start sites were defined by the decomposition-based peak identification method and annotated to genes [32]. Peaks were given a name in the form pN@GENE, where GENE indicates gene name and N indicates the rank in the ranked list of promoter activities for that gene. For example, p1@BCL2 represent the highest expression among alternative promoters among the peaks associated with BCL2 gene, according to the FANTOM5 CAGE profiles [32]. The relative log expression method was utilized to calculate normalization factors for the expression of promoters. Normalized data were subjected to the R Bioconductor package “edgeR” for differential expression (Bioconductor, RRID:SCR_006442). Then, gene ontology (GO) analysis of differentially expressed genes was performed with DAVID Bioinformatics Resources (DAVID, RRID: SCR_001881) [33].

RNA-Seq analysis

Sequencing libraries were generated using NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA) following the manufacturer’s recommendations. The library fragments were purified with QIAQuick PCR kits (QIAGEN). The clustering of the index-coded samples was performed on a cBot cluster generation system using HiSeq PE Cluster Kit v4-cBot-HS (Illumina) according to the manufacturer’s instructions. After cluster generation, the libraries were sequenced on an Illumina platform and 150 bp paired-end reads were generated.

The reads were aligned to the GRCh38/hg38 genome reference and indexed using STAR aligner (STAR, RRID:SCR_004463) and Samtools (SAMTOOLS, RRID:SCR_002105) with default parameters. VarDict, an RNA-sequencing data compatible variant caller, was used with default settings to identify edited regions. Edits were searched for against hg38 genome FASTA (FASTA, RRID:SCR_011819) and RefSeq (RefSeq, RRID: SCR_003496) hg38 bed file references with a minimum allele frequency of 0.01. Only single nucleotide mutations were kept. The output was further filtered by keeping edits occurring in exons (biomart) with > 100 reads in each experiment, > 0.1 differences in allele frequency of high-quality bases between the experimental conditions (higher in MV4;11 with FLT3-ITD/D835 vs. FLT3-ITD). Sites corresponding to A- to-I edit sites were removed from the list using REDTools (REDTools, RRID:SCR_012133) [34]. Finally, the list of edit sites was fed into Ensembl Variant Effect Predictor (VEP) [35] to identify associated amino acid changes, accounting for the strandness of the reads.

Statistical analyses

Normalization for heatmap and the statistical tests were executed in R (version 3.4.2). Differences between groups were assessed by a two-tailed Student’s t-test. A p-value ≤ 0.05 was considered statistically significant. Where indicated, the results are expressed as the mean ± SD of three or more samples.

Synergism, additive effects, or antagonism were assessed by the Chou-Talalay method, utilizing CalcuSyn software (Biosoft). The average CI value for the experimental combination was calculated from the 50, 75, and 90% dose-effect levels of cell growth inhibition. By this method, CI values indicate the following: 0.3 - 0.7, strong synergism; 0.7 - 0.85, moderate synergism; 0.85 - 0.9, slight synergism; 0.9 - 1.1, nearly additive; 1.1 - 1.2, slight antagonism; 1.2 - 1.45, moderate antagonism; 1.45 - 3.3, antagonism; 3.3 - 10, strong antagonism [23].

Data availability

The CAGE and RNA-seq data generated during this study have been deposited to GEO under the access codes GSE149962 and GSE151249.

Results

Primary AML cells bearing FLT3-ITD/D835 mutations show a higher frequency of co-mutations and higher levels of BCL2A1 gene transcripts

To investigate differences in transcriptome and co-mutation profiles in primary AML patient cells bearing FLT3-ITD and FLT3-ITD/D835 mutations, we obtained cells from 26 patients (14 bearing FLT3-ITD and 12 bearing FLT3-ITD/D835 mutations). Table 1 summarizes clinical characteristics. Gene panel sequencing showed that co-mutations were identified more frequently in primary AML cells bearing FLT3-ITD/D835 mutations than cells bearing FLT3-ITD alone (Table 1). In particular, mutations in RAS, ASXL1, and TET2 frequently co-existed in AML cells with FLT3-ITD/D835 mutations.

To investigate transcriptome profiles, CAGE transcriptome analyses were performed. CAGE peaks indicate transcription start sites, which allows the investigation of differentially expressed transcripts and
promoter activities across the whole genome [32]. Out of 18,032 promoters, CAGE identified 310 upregulated and 22 downregulated promoters with a false discovery rate (FDR) of < 0.05 in AML cells bearing FLT3-ITD/D835 compared to cells bearing FLT3-ITD mutation. We then extracted 60 upregulated and 13 downregulated promoters that were functionally annotated by mammalian genomes, FANTOM 5 (Table 2) [36]. The expression levels of promoters for each sample are shown in a MA-plot diagram (Supplementary Panel) (\(y\)) and \(x\). The expression levels of promoters for each sample are shown in a MA-plot diagram (Supplementary Panel) (\(x\)). The expression levels of promoters for each sample are shown in a MA-plot diagram (Supplementary Panel) (\(y\)) and \(x\).

**Table 1**: Clinical characteristics and mutation status of primary AML samples.

| Patient number | Diagnosis | Sex/Age | Prior therapy | Mutation status | Other genes |
|---------------|-----------|---------|---------------|-----------------|-------------|
|               |           |         |               | FLT3-ITD/VAF (%) | FLT3-D835/VAF (%) |
| 1             | AML       | M/39    | AML (3 + 7; DHAD + VP-16; IAC; ARA-C + TOPO) | Positive/47.0 | Negative Not detected |
| 2             | AML       | F/86    | None          | Positive/47.5 | Negative Not detected |
| 3             | AML       | M/32    | AML (FLAG + Gemtuzumab ozogamicin) | Positive/24.3 | Negative Not detected |
| 4             | AMOL      | M/81    | None          | Positive/24.9 | Negative Not detected |
| 5             | AMOL      | F/40    | None          | Positive/15.4 | Negative Not detected |
| 6             | AMOL      | F/55    | None          | Positive/47.9 | Negative Not detected |
| 7             | AMOL      | M/78    | None          | Positive/48.2 | Negative Not detected |
| 8             | AMOL      | F/62    | None          | Positive/48.2 | Negative Not detected |
| 9             | APL       | F/36    | None          | Positive/0.01 | Negative Not detected |
| 10            | AML       | M/46    | None          | Positive/2.1  | Negative RAS |
| 11            | AML       | M/71    | None          | Positive/43.6 | Negative CEBPA |
| 12            | AML       | M/47    | None          | Positive/1.6  | Negative KIT |
| 13            | AMOL      | F/50    | None          | Positive/43.7 | Negative RAS |
| 14            | AMOL      | M/29    | None          | Positive/29.1 | Negative RAS |
| 15            | AML       | M/32    | AML (FLAG + Gemtuzumab ozogamicin) | Positive/24.3 | Positive/54.3 |
| 16            | AMOL      | F/51    | AML (FLAG + IDA/ASA; FLAG + IDA/BMT; Sorafenib; Clofa + AraC) | Positive/46.0 | Positive/33.8 Not detected |
| 17            | AML       | F/87    | AML (Hydra + AraC; AraC + DXR; Sorafenib) | Positive/39.6 | Positive/13.3 TET2, RUNX1, RAS |
| 18            | AML       | M/66    | AML          | Positive/6.4  | Positive/3.4 TET2, RUNX1, IKZF2, EZH2, RAS |
| 19            | AML       | M/81    | None         | Positive/3.1  | Positive/34.9 CEBPA, DNMT3, IDH2, MPL, RUNX1 |
| 20            | AML       | M/76    | ET (hydrastreyside) + AML (DAC + RUX) | Positive/46.3 | Positive/3.9 ASXL1, RUNX1, TET2, WT1 |
| 21            | AML       | M/62    | AML (3 + 7)  | Positive/1.7  | Positive/13.1 IDH2, RAS |
| 22            | AML       | M/32    | None         | Positive/24.3 | Positive/12.8 CEBPA |
| 23            | AML       | M/24    | AML (3 + 7; VP-16 + Cit; Quizartinib) | Positive/54.3 | Positive/29.4 IDH1, RAS |
| 24            | AML       | M/58    | MDS (AZA); AML (3 + 7; Quizartinib) | Positive/87.3 | Positive/59.1 ASXL1, MLL, NOTCH1 |
| 25            | APL       | M/80    | Unknown      | Positive/6.9  | Positive/6.9 DNMT3, EGFR, MPL |
| 26            | APL       | F/51    | Unknown      | Positive/2.0  | Positive/27.7 EGFR, EZH2, MLL, MPL, NOTCH1, RUNX1, TET2, WT1 |

AML, acute myelogenous leukemia; APL, acute promyelocytic leukemia; AMOL, acute monocytic leukemia; AMML, acute myelomonocytic leukemia; MDS, myelodysplastic syndromes; VAF, variant allele frequency; ET, essential thrombocythemia; DHAD, mitoxantrone; VP-16, etoposide; DAC, decitabine; AraC, cytarabine; TOPO, topoisomerase; DXR, daunorubicin; RUX, ruxolitinib; Ctx, cyclophosphamide; AZA, azacitidine; FLAG, fludarabine, cytarabine and granulocyte colony stimulating factor; IDA, idarubicin; BMT, blood or marrow transplantation; Clofa, clofarabin.

All samples were wt-NPM. The mutational status of 25 genes was analyzed. *Data are not available.

**Upregulation of BCL2A1 and increased confounding gene mutations in MV4;11 cells bearing FLT3-ITD/D835**

We then examined the differences in mRNA and protein expression levels of BCL2A1 and BCL2 in isogenic MV4;11 cells bearing FLT3-ITD (MV4;11-ITD) and bearing FLT3-ITD/D835 mutations (MV4;11-ITD/D835). Fig. 2A shows that mRNA levels of BCL2A1 were higher in MV4;11-ITD/D835 compared to MV4;11-ITD (left panel, p < 0.01) while mRNA levels of BCL2 were comparable between the two groups (middle panel, t = 0.05). Concordant with these results, the western blot analyses showed that BCL2A1 expression was 1.6-fold higher in MV4;11-ITD/D835 compared to MV4;11-ITD cells (Fig. 2A right panel), while BCL2 protein expression was comparable between the two groups. In contrast, expressions of MCL-1 and BCL-XL levels were decreased in MV4;11-ITD/D835 compared to MV4;11-ITD. MV4;11-ITD/D835 cells have been established by the prolonged exposure to quizartinib, and our observation was consistent with our previous report that quizartinib downregulates the expression of MCL-1 and BCL-XL [37].

We further compared occurrence of co-mutations in the aforementioned cells. Compared to MV4;11-ITD, RNA-Seq analysis detected newly acquired mutations in MV4;11-ITD/D835 including CC2I c.538C>A; (p.H180N), PTPN11 c.218C>T; (p.T73I), ZNF508 c.1675C>T; (p.P559S), DXD5 c.427G>T; (p.G143W), and ARFGAP3 c.1421G>A; (p.S474N).
Upregulation of BCL2A1 correlates with decreased sensitivity to quizartinib

To investigate whether drug resistance of AML cells bearing FLT3-ITD/D835 is associated with BCL2A1, we utilized Molm13 cells bearing the FLT3-ITD mutation and transfected with BCL2A1 using lentiviral systems. Western blots confirmed that BCL2A1 was overexpressed in the transfected cells, but not in the cells with mock transfections (Fig. 2B left panel). These cells were then treated with various concentrations of quizartinib, and the apoptogenic effects were evaluated by Annexin V-FITC and compared to the cells with mock transfections. As shown in Fig. 2B (right panels), overexpression of BCL2A1 significantly decreased the fraction of quizartinib-induced apoptotic Molm13 cells compared to the cells with mock transfection.

A phase Ib/II clinical trial using quizartinib, a Type II FLT3 inhibitor, with venetoclax, a specific BCL2 inhibitor, targeting AML with FLT3-ITD is ongoing (NCT03735875) and is showing encouraging results [18]. However, the effect of quizartinib combined with venetoclax specifically

Table 2
CAGE-defined promoters differentially expressed between primary AML cells bearing FLT3-ITD and cells bearing FLT3-ITD/D835 (log2 FC > 2, FDR < 0.05).

| CAGE-defined promoter Log2 FC P value FDR | CAGE-defined promoter Log2 FC P value FDR |
|-------------------------------------------|-------------------------------------------|
| upregulated (FLT3-ITD/D835 vs. FLT3-ITD) | upregulated (FLT3-ITD/D835 vs. FLT3-ITD) |
| p1@TTL1 8.22 3.61E-07 3.92E-03 | p5@CD1E -6.38 1.49E-05 2.10E-02 |
| p5@HI1F0 6.09 6.00E-05 4.29E-02 | p1@CLIP3 -6.05 4.90E-05 3.46E-02 |
| p3@SGEB3A1 5.76 6.58E-05 4.46E-02 | p3@CYBSR3 -6.04 4.90E-05 3.46E-02 |
| p1@Coef126 5.39 9.38E-06 1.17E-02 | p1@CD1B -6.04 4.35E-05 3.58E-02 |
| p6@QMT1 5.25 9.68E-06 1.17E-02 | p2@KCN45 -5.83 7.01E-05 4.58E-02 |
| p5@ILF 5.11 7.50E-06 1.43E-02 | p1@CD1E -5.32 5.57E-05 4.20E-02 |
| p2@S100A8 5.80 1.63E-07 3.66E-03 | p1@PCDHGB5 -5.07 1.00E-05 1.63E-02 |
| p1@D4S234E 6.09 6.00E-05 4.29E-02 | p1@KLAP -5.07 1.00E-05 1.63E-02 |
| p6@QPCT 6.04 4.09E-05 3.47E-02 | p2@SLC4A3 -4.89 6.48E-05 4.46E-02 |
| p5@LTF 6.04 5.90E-05 4.26E-02 | p1@CD1B -5.63 7.01E-05 4.58E-02 |
| p2@S100A8 6.04 4.35E-05 3.58E-02 | p1@KLAP -5.63 7.01E-05 4.58E-02 |
| p5@LTF 6.04 4.35E-05 3.58E-02 | p1@CD1B -5.63 7.01E-05 4.58E-02 |
| p2@S100A8 6.04 4.35E-05 3.58E-02 | p1@KLAP -5.63 7.01E-05 4.58E-02 |

FC, Fold Change; FDR, False Discovery Rate.
Fig. 1. Reference in promoter expression in primary AML cells bearing FLT3-ITD or FLT3-ITD/D835 mutations. (A) Gene ontology (GO) enrichment analysis of the biological process of up-regulated promoters using network DAVID. The top 10 GO terms relevant to biological process were sorted according to promoter counts and \( p < 0.05 \). (B) The y-axis shows counts per million (CPM) of human BCL2A1 or BCL2 promoters, detected by CAGE. The x-axis shows patient number. The patients in the FLT3-ITD or FLT3-ITD/D835 groups were further divided into groups with or without co-mutations. Peaks were given a name in the form pN@GENE, where GENE indicates gene name and N indicates alternative promoters of the same gene. (C) Transcript expression levels of either BCL2A1 or BCL2 in AML patients harboring the FLT3-ITD or FLT3-ITD/D835 mutations were confirmed by q-RT-PCR. The relative expression of each mRNA was normalized to GAPDH.
in AML with FLT3-ITD/D835 has not been well investigated. We therefore examined the dose-dependent inhibitory effects of quizartinib and venetoclax on cell proliferation of MV4;11-ITD or MV4;11-ITD/D835 (Fig. 3A). The top-left panel of Fig. 3A shows that MV4;11-ITD/D835 were significantly less sensitive to quizartinib compared to MV4;11-ITD (IC\textsubscript{50}: 0.20 ± 0.02 nM vs. 7.69 ± 2.14 nM, p < 0.01, n = 5). The top-right panel of Fig. 3A shows that MV4;11-ITD/D835 were less sensitive than MV4;11-ITD to venetoclax (IC\textsubscript{50}: 1.90 ± 0.58 nM vs. 6.78 ± 1.60 nM, p < 0.01, n = 5). This data is consistent with previous reports demonstrating that BCL2A1 causes resistance to venetoclax [20, 21]. We confirmed that overexpression of BCL2A1 attenuated sensitivity of AML cells to venetoclax (Supplementary Fig. S3). The bottom panel of Fig. 3A shows effects of quizartinib in combination with venetoclax on proliferation of MV4;11-ITD (left panel) and MV4;11-ITD/D835 cells (right panel). While proliferation of MV4;11-ITD was almost completely suppressed by the quizartinib (0.25 nM) and venetoclax (2 nM) combination in a synergistic manner (combination index, CI = 0.41), no synergistic combinational effects were observed in MV4;11-ITD/D835 (CI = 1.61).

**Synergistic effects of gilteritinib and venetoclax on cell growth of MV4;11-ITD and MV4;11-ITD/D835**

It has been shown that gilteritinib, a Type I FLT3 inhibitor, effectively blocks cell growth of AML cells bearing FLT3-ITD/D835 [38], and that the combination of gilteritinib and venetoclax is synergistically effective in AML cells with FLT3-ITD [39]. We therefore investigated the effects of gilteritinib and venetoclax on MV4;11-ITD and MV4;11-ITD/D835 cells.

The top-left panel of Fig. 3B shows that gilteritinib inhibited cell growth of MV4;11-ITD and MV4;11-ITD/D835 in a dose-dependent manner. The top-middle and right panels of Fig. 3B shows that combined treatment of gilteritinib and venetoclax synergistically reduced cell proliferation of both MV4;11-ITD and MV4;11-ITD/D835, although to a lesser degree in the double-mutant cells. To investigate underlying mechanism(s) of the combinational effects, we assessed protein levels of STAT5, a transcriptional regulator of BCL2 family proteins [40, 41]. As shown in Fig. 3B (bottom panel), western blot analysis demonstrated that treatment with gilteritinib only diminished phosphorylation of STAT5 (p-STAT5) and decreased BCL2A1 levels in both MV4;11-ITD and MV4;11-ITD/D835 cells. The combination of gilteritinib and venetoclax further downregulated MCL-1 in MV4;11-ITD/D835. These results indicate that downregulation of p-STAT5 and its downstream BCL2 family proteins may be associated with the anti-leukemic effects of gilteritinib in AML cells bearing FLT3-ITD and FLT3-ITD/D835.
Inhibition of bromodomain and extra-terminal motif (BET) induced apoptosis in MV4;11-ITD/D835

We next examined the anti-leukemic effects of CPI-0610, a BET inhibitor, known to exert anti-tumor effects by also inhibiting BCL2A1 [21]. Currently, no specific inhibitors of BCL2A1 are available, but a clinical trial using CPI-0610 for treatment of AML has been reported [42]. Fig. 4A shows that CPI-0610 inhibited cell growth dramatically with a modest increase in apoptosis in MV4;11-ITD (IC₅₀ 256 ± 87.4 nM) and MV4;11-ITD/D835 (IC₅₀ 304 ± 119 nM) in a dose dependent manner. Western blot analysis demonstrated that CPI-0610 decreased BCL2A1 in both cells (Fig. 4B).

Discussion

In this study, we asked the question: what factors are involved in the well-established resistance of double-mutated FLT3-ITD/D835 AML cells compared to that with FLT3-ITD only? We conducted a systemic global analysis of transcription start sites using CAGE analysis or primary clinical samples. Comprehensive bioinformatics analysis revealed apoptosis resistance as a top pathway. Next, we demonstrated upregulation of BCL2A1 both at the transcriptional and protein levels in AML cells bearing FLT3-ITD/D835 mutations compared to ones with FLT3-ITD mutation only. The upregulation of BCL2A1 was found to account for resistance of AML cells bearing FLT3-ITD/D835 mutations to quizartinib as well as to venetoclax. BCL2A1 has been reported to play an important role in tumor expansion and/or metastasis of various solid cancers [43,44], hematopoietic malignancies [20,45], and resistance to venetoclax [20,21]. Indeed, the combination of quizartinib and venetoclax was not effective in AML cells bearing FLT3-ITD/D835 that highly express BCL2A1. However, the combination of gilteritinib and venetoclax showed synergistic effects in AML cells bearing FLT3-ITD/D835. We confirmed that gilteritinib reduced the expressions of p-STAT5 and BCL2A1 in MV4;11 cells bearing FLT3-ITD/D835. The expression of BCL2A1 is known to be regulated through JAK/STAT signaling [46,47] and here it is demonstrated that gilteritinib, at least in part, overcomes resistance of AML cells with FLT3-ITD/D835 to venetoclax by inhibition of JAK/STAT-BCL2A1 signaling.

While BCL2A1 may be a promising therapeutic target in AML with FLT3-ITD/TKD mutations, specific BCL2A1 inhibitors are currently unavailable. AML cells are vulnerable to BET family/BRD4 inhibitors, which transcriptionally suppress the expression of BCL2A1 and also downregulate the activity of important cell survival factors, such as MYC, BCL2, and CDK6 that are highly expressed in AML cells [21,48]. We observed that CPI-0610, a BET inhibitor [27], showed anti-tumor effects on AML cells bearing FLT3-ITD and FLT3-ITD/D835. Recently,
a combinatorial therapy of INCBO54329, a novel BET inhibitor, and venetoclax has been shown to successfully reduce cell viability of AML cells associated with reduced transcriptional activation of key oncogenes as well as with genes involved in cell cycle and metabolism [49]. The promoter regions of MYC and BCL2A1 contain BRD4 binding sites, suggesting that these genes are potentially sensitive to BRD4 inhibition [48]. BRD4 also activates NFXb via binding to the NFXb co-activator RELA [50], which is known to induce transcription of BCL2A1 [51]. These studies and our findings reported here may explain the anti-tumor effects of BET inhibitors on AML cells with upregulated BCL2A1.

In our data, co-mutations in the genes, RAS, ASXL1, and TET2, were frequently detected in primary AML cells bearing FLT3-ITD/D835 mutations. These findings are consistent with a previous study of the crenolanib-resistant cases exhibiting greater numbers of coexisting driver mutations in the TET2, IDH1, RAS, and ASXL1 genes compared to the responders [52]. Similarly, RAS mutations were frequently found in the relapsed cases after treatment with gilteritinib [14]. Recently, Zhang et al. reported that NRAS mutations induced resistance against venetoclax by upregulating of BCL2A1 through activation of NFXb signaling [53].

This study has significant limitations: (1) western blot analyses were only performed in cell lines because of inadequate amounts of primary patient samples in our repository; and (2) roles of other co-mutations detected in refractory AML samples could not be addressed though patient samples in our repository; and (2) roles of other co-mutations detected in refractory AML samples could not be addressed though patient samples in our repository.

In summary, we demonstrated that BCL2A1 was upregulated in AML cells with FLT3-ITD/D835 mutations, which is a novel underlying mechanism of drug resistance. A combination of gilteritinib and venetoclax that suppresses BCL2A1 has a potential to improve the prognosis of AML with FLT3-ITD/D835 mutations. In addition, BET inhibitors that downregulate BCL2A1 can be alternative reagents to treat AML bearing multiple mutations including FLT3-ITD/D835.

Financial support for this work

This work was supported in part by Grants-in Aid for Scientific Research (18J22436 to KY, 18K07424 to YT), International Joint Research Programs (19KK0221 to YT) from Japan Society for the Promotion of Science. This research was also partially supported by the MD Anderson MDS/AML moonshot program, the CPRIT MIRA grant and funds from the Endowed Haas Chair in Genetics (all to MA).

CRediT authorship contribution statement

Kotoko Yamatani: Methodology, Investigation, Data curation, Formal analysis, Visualization, Funding acquisition, Writing – original draft. Tomohiko Ai: Methodology, Writing – review & editing. Kaori Saito: Investigation. Koya Suzuki: Methodology. Atsushi Hori: Methodology. Sonoko Kinjo: Formal analysis. Kazuho Ikeo: Formal analysis. Vivian Ruvo1: Methodology, Investigation, Resources. Weiguo Zhang: Methodology, Resources. Po Yee Mak: Methodology, Investigation. Bogumil Kaczkowski: Formal analysis. Hironori Harada: Methodology. Kazuhiro Katayama: Resources, Yoshikazu Sugimoto: Resources. Jered Mylskina: Formal analysis. Takashi Hato: Formal analysis. Takashi Miida: Methodology. Marina Konopleva: Methodology. Yoshihide Hayashizaki: Formal analysis, Methodology. Bing Z. Carter: Methodology, Investigation, Resources. Yoko Tabe: Conceptualization, Methodology, Formal analysis, Supervision, Writing – review & editing, Funding acquisition. Michael Andreeff: Conceptualization, Supervision, Funding acquisition.

Declaration of Competing Interest

MA has received research support from Daiichi-Sankyo; all other authors have no competing financial interests to declare.

Acknowledgments

The authors wish to thank the Laboratory of Molecular and Biochemical Research and Research Support Center at Juntendo University Graduate School of Medicine for technical assistance. We are also grateful to Dr. Toshikai Takano and Dr. Hidetaka Eguchi from Intractable Disease Research Center at Juntendo University Graduate School of Medicine for technical assistance. The authors would also like to thank Dr. Numsen Hail for assistance in editing the manuscript, Dr. Kaoru Mogushi, Dr. Masaki Hosoya, and Dr. Shigeo Yamaguchi for useful discussions.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2022.101354.

References

[1] A.A. Petti, S.R. Williams, C.A. Miller, L.T. Fiddes, S.N. Srivatsan, D.Y. Chen, et al., A general approach for detecting expressed mutations in AML cells using single cell RNA-sequencing, Nat. Commun. 10 (1) (2019) 3660, https://doi.org/10.1038/s41467-019-11591-1.
[2] D.L. Stirewalt, J.P. Radich, The role of FLT3 in haematopoietic malignancies, Nat. Rev. Cancer 3 (9) (2003) 650–665, https://doi.org/10.1038/nrc1169.
[3] U. Bacher, C. Haferlach, W. Kern, T. Haferlach, S. Schnittger, Prognostic relevance of FLT3-TKD mutations in AML: the combination matters–an analysis of 3082 patients, Blood 111 (5) (2008) 2527–2537, https://doi.org/10.1182/blood-2007-09-092155.
[4] C. Thiede, C. Steudel, B. Mohr, M. Schaich, U. Schakel, U. Platzecker, et al., Analysis of FLT3-activating mutations in 979 patients with acute myelogenous leukemia: association with FAB subtypes and identification of subgroups with poor prognosis, Blood 99 (12) (2002) 4326–4335, https://doi.org/10.1182/blood-2002-10-4326.
[5] D. Staudt, H.C. Murray, T. McLachlan, F. Alvaro, A.K. Enjeti, N.M. Verrills, et al., Targeting oncogenic signaling in mutant FLT3 acute myeloid leukemia: the path to least resistance, Int. J. Mol. Sci. 19 (10) (2018), https://doi.org/10.3390/ijms19103198.
[6] W. Zhang, M. Konopleva, Y.X. Shi, T. McQueen, D. Harris, X. Ling, et al., Mutant FLT3: a direct target of sorafenib in acute myelogenous leukemia, J. Natl. Cancer Inst. 110 (3) (2008) 184–198, https://doi.org/10.1093/jnci/djm328.
[7] J.E. Cortes, S. Khaled, G. Martinelli, A.E. Perl, S. Gangoly, N. Russell, et al., Quizzartinib versus salvage chemotherapy in relapsed or refractory FLT3-ITD acute myeloid leukaemia (QuAANTUM-R): a multicentre, randomised, controlled, open-label, phase 3 trial, Lancet Oncol. 20 (7) (2019) 984–997, https://doi.org/10.1016/S1470-2045(19)30151-0.
[8] A.E. Perl, G. Martinelli, J.E. Cortes, A. Neubauer, E. Berman, S. Paolini, et al., Quizzartinib or chemotherapy for relapsed or refractory FLT3-ITD mutated AML, N. Engl. J. Med. 381 (18) (2019) 1728–1740, https://doi.org/10.1056/N EnglJMed.1902608.
[9] R.M. Stone, S.J. Mandrekar, B.L. Sandford, K. Laumann, S. Geyer, C.D. Bloomfield, et al., Midostaurin plus chemotherapy for acute myeloid leukemia with a FLT3 mutation, N. Engl. J. Med. 377 (5) (2017) 454–464, https://doi.org/10.1056/NEJMoa1614355.
[10] T. Yamashita, K. Uchikawa, S. Kida, C. Andoh, K. Ueda, M. Kitagawa, et al., Heterogeneous resistance to quizartinib in acute myeloid leukemia revealed by NGS analysis of 3082 patients, Cancer Discov. 9 (8) (2019) 1050–1063, https://doi.org/10.1158/2159-8290.CD-18-1453.
K. Yamatani et al.

11