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MEF2D-BCL9 Fusion Gene Is Associated With High-Risk Acute B-Cell Precursor Lymphoblastic Leukemia in Adolescents

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ABSTRACT

Purpose

Acute lymphoblastic leukemia (ALL) makes up a significant proportion of all pediatric cancers, and relapsed ALL is a leading cause of cancer-associated deaths in children. Identification of risk factors and druggable molecular targets in ALL can lead to a better stratification of treatments and subsequent improvement in prognosis.

Patients and Methods

We enrolled 59 children with relapsed or primary refractory ALL who were treated in our institutions. We primarily performed RNA sequencing (RNA-seq) using patients' leukemic cells to comprehensively detect gene fusions and analyze gene expression profiles. On the basis of results obtained by RNA-seq, we performed genetic validation, functional analysis, and in vitro drug sensitivity testing using patients' samples and an exogenous expression model.

Results

We identified a total of 26 gene fusions in 22 patients by RNA-seq. Among these, 19 were nonrandom gene fusions already described in ALL, and four of the remaining seven involved identical combination of *MEF2D* and *BCL9*. All *MEF2D-BCL9*–positive patients had B-cell precursor immunophenotype and were characterized as being older in age, being resistant to chemotherapy, having very early relapse, and having leukemic blasts that mimic morphologically mature B-cell leukemia with markedly high expression of *HDAC9*. Exogenous expression of *MEF2D-BCL9* in a B-cell precursor ALL cell line promoted cell growth, increased *HDAC9* expression, and induced resistance to dexamethasone. Using a primary culture of leukemic blasts from a patient, we identified several molecular targeted drugs that conferred inhibitory effects in vitro.

Conclusion

A novel *MEF2D-BCL9* fusion we identified characterizes a novel subset of pediatric ALL, predicts poor prognosis, and may be a candidate for novel molecular targeting.

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INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most common hematologic malignancy in childhood. ALL was originally classified into several subtypes on the basis of morphologic findings and surface protein markers reflecting cell lineage and differentiation. In addition, its pathognomonic molecular mechanisms include chromosomal copy number aberration, chromosomal rearrangements, and somatic point mutations, each of which provides better understanding of leukemia progression and directs the therapeutic approach.[1] Historical and recent comprehensive genetic studies have unraveled a list of druggable gene rearrangements that activate kinase signaling, including *BCR-ABL1*.[2,3]

A significant improvement in treatment outcome in pediatric ALL was achieved by the incorporation of precise risk stratification, combined chemotherapy approaches, allogeneic hematopoietic stem cell transplantation (HSCT), and, in *BCR-ABL1*–positive ALL, the clinical use of tyrosine kinase inhibitors.[2,4] The 5-year overall survival rate of childhood ALL has reached

approximately 90% in developed countries.[1,4] However, a considerable number of patients still experience relapse, for which the prognosis is often poor. To tackle current issues in ALL, we applied comprehensive transcriptome analysis to patients with relapsed/refractory ALL to identify genetic alterations that may contribute to the improvement of treatment outcome.

In this study, we identified a novel gene fusion of *MEF2D* and *BCL9* recurrently in patients with B-cell precursor ALL (B-ALL). To our knowledge, this is the first report that describes *MEF2D-BCL9* fusion and its functional analysis in leukemic cells. We further provide encouraging data indicating its potential for molecular targeted therapies obtained by in vitro drug sensitivity testing.

PATIENTS AND METHODS

Patients

Fifty-nine children with relapsed (56 patients) or primary refractory (three patients, all with Philadelphia chromosome [Ph1]) ALL who were treated in Nagoya University Hospital or Japanese Red Cross Nagoya First Hospital were included in this study. Among them, three patients were infants, three had mature B-cell, and four had T-cell immunophenotype. Ph1 was identified in six patients. Written informed consent was obtained from the patients or the patients' parents. The institutional review board of Nagoya University Graduate School of Medicine approved this study. All patients were primarily treated according to a Japanese protocol for pediatric ALL.[5,6]

Samples

We used cryopreserved mononuclear cells obtained from patients' bone marrow or peripheral blood containing leukemic blasts. In 56 patients with relapse, the samples were obtained at the time of the first relapse, except for three patients for whom they were not available. For these three patients, we used the samples obtained at the time of the second relapse. RNA and genomic DNA were extracted using RNeasy Mini Kits (QIAGEN, Hilden, Germany) and QIAamp DNA Blood Mini Kits (QIAGEN), respectively.

Polymerase Chain Reaction

Amplification of target regions containing break point of chromosomal structural variations from template genomic DNA was performed using PrimeSTAR GXL DNA Polymerase (TaKaRa Bio, Ohtsu, Japan). Primer sequences are listed in the Data Supplement.

RNA Sequencing

The quality of extracted RNA was assessed using an RNA ScreenTape and a TapeStation 2200 system (Agilent, Santa Clara, CA). Sequencing libraries were prepared using a NEBNext Ultra RNA Prep Kit for Illumina (New England Biolabs, Ipswich, MA) according to the manufacturer's instructions. Prepared libraries were run on a HiSeq 2500 next-generation sequencing (NGS) platform (Illumina, San Diego, CA). Obtained reads were analyzed using Tophat-fusion (for gene fusions),[7] Cufflinks (for expression analysis),[8] DESeq2 (for differential expression),[9] and VarScan2 (for nucleotide variations).[10] Candidate gene fusions were validated by reverse transcription–polymerase chain reaction (RT-PCR) using a ThermoScript Reverse transcription system (Life Technologies, Carlsbad, CA) and PrimeSTAR GXL DNA Polymerase.

Clustering Analysis

We used Cluster 3.0 to group samples by hierarchical clustering using the average linkage method.[11] Fragments per kilobase of transcript per million mapped reads values for each gene in each sample were log transformed, and the values were adjusted to center the genes relative to

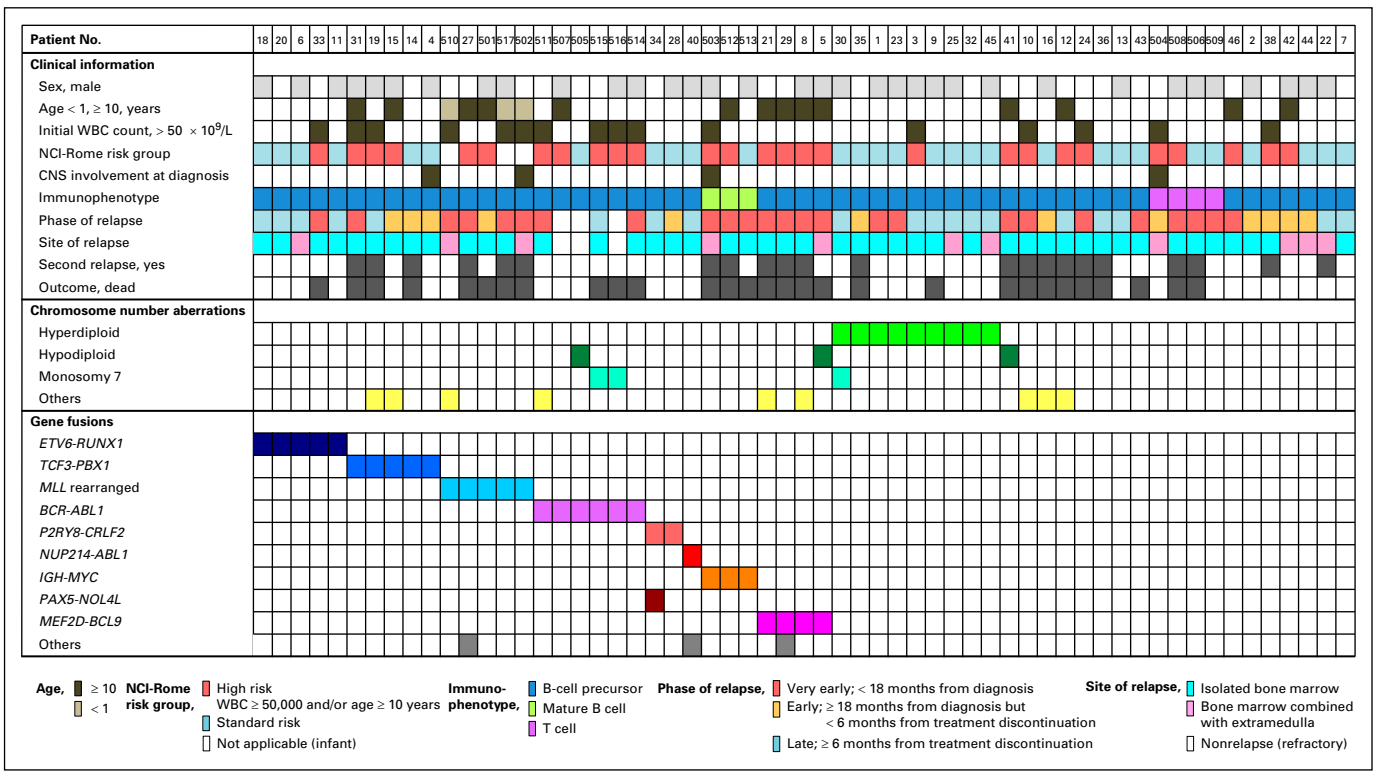

Fig 1. Clinical features and fusion genes identified in each patient with acute leukemia analyzed by RNA sequencing. Each column indicates an individual patient. NCI, National Cancer Institute.

medians. We used both Spearman and Pearson's correlation methods. Clustering results were visualized with Java TreeView.[12]

Real-Time Quantitative PCR

Real-time quantitative PCR was performed using Mastermix, probes (cat no. 4331182) and machine ABI PRISM 7000 sequence system (all obtained from Life Technologies). The obtained data were analyzed by ABI Prism 7000 SDS Software. Data were normalized to the expression level in NALM-6 cells (*MEF2D-BCL9*–negative B-ALL cell line).

Whole-Exome Sequencing

Sequencing libraries were prepared using a SureSelect XT target enrichment system and SureSelect Human All Exon v5 bait (Agilent) according to the manufacturer's instructions. The prepared libraries were run on a HiSeq 2500 (Illumina). Sequences were aligned to the hg19 reference genome using Burrows-Wheeler aligner (http://bio-bwa.sourceforge.net/) with a-mem option. PCR duplicates were removed using Picard tools (http://broadinstitute.github.io/picard/). Sequence variations were detected using VarScan2.

For germline variations, common single-nucleotide polymorphisms with $>$ 1% allele frequency defined by ExAC (http://exac.broadinstitute.org/), 1000 genomes (http://www.1000genomes.org/), and ESP6500 (http://evs.gs.washington.edu/EVS/) were removed. Of the remaining variants, those with inactivating effects (nonsense, frameshift, or splice site) or pathogenicity reported in the literature were searched using the Human Genome Mutation Database (http://www.hgmd.cf.ac.uk/) and extensive search of existing literature. Genetic diagnoses were considered on the basis of the mode of inheritance in each disease.

For somatic mutations, candidates with $P < .01$ were validated by PCR-based deep sequencing of samples at diagnosis, complete remission, and relapse. The sequences of primers are available on request. For copy number analysis, sequence reads normalized for the mean coverage of each sample were compared between paired tumor-normal samples. An estimated copy number was simply calculated as 2 $\times$ (normalized read number in tumor sample)/(normalized read number in normal sample).

Immunofluorescent Analysis

Cells (5×10^4) were suspended in 500 μL of 1% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO) –supplemented phosphate-buffered saline (PBS; Life Technologies) and attached to a slide glass using Cytospin4 (Thermo Fisher Scientific, Waltham, MA) at 1,000 rpm for 5 minutes. The cells were then fixed using 4% paraformaldehyde dissolved in PBS for 15 minutes at room temperature, washed with PBS three times, and blocked in 1% FBS and 0.3% Triton X-100 for 60 minutes at room temperature. The cells were then incubated with an anti-PTPRZ1 antibody (HPA015103, Sigma-Aldrich; 1:100 dilution) overnight at 4°C, followed by staining with Alexa Fluor 488-conjugated goat anti-rabbit IgG antibody (Thermo Fisher Scientific) for 120 minutes at room temperature. Cells were also stained with 4,6-diamidino-2-phenylindole. Microscopic images were obtained using an FSX100 (Olympus, Tokyo, Japan).

Establishment of Primary Cultured Cells

Patients' peripheral blood mononuclear cells containing leukemic blasts were cultured in RPMI1640 medium (Life Technologies) supplemented with 10% FBS. Half of the medium was replaced every 4 days over 6 weeks. After establishment of stably expanding cells, cells were maintained at a concentration of 0.2 to 1 $\times$ 10^6 cells/mL.

Establishment of MEF2D-BCL9–Transfected Cell Line

The *MEF2D-BCL9* complete coding sequence was cloned into CSIV-CMV-MCS-IRES2-Venus, a self-inactivating lentiviral vector construct (which was a generous gift from Dr. Hiroki Miyoshi, Riken BioResource Center). HEK293T cells were transfected with the above-mentioned vector

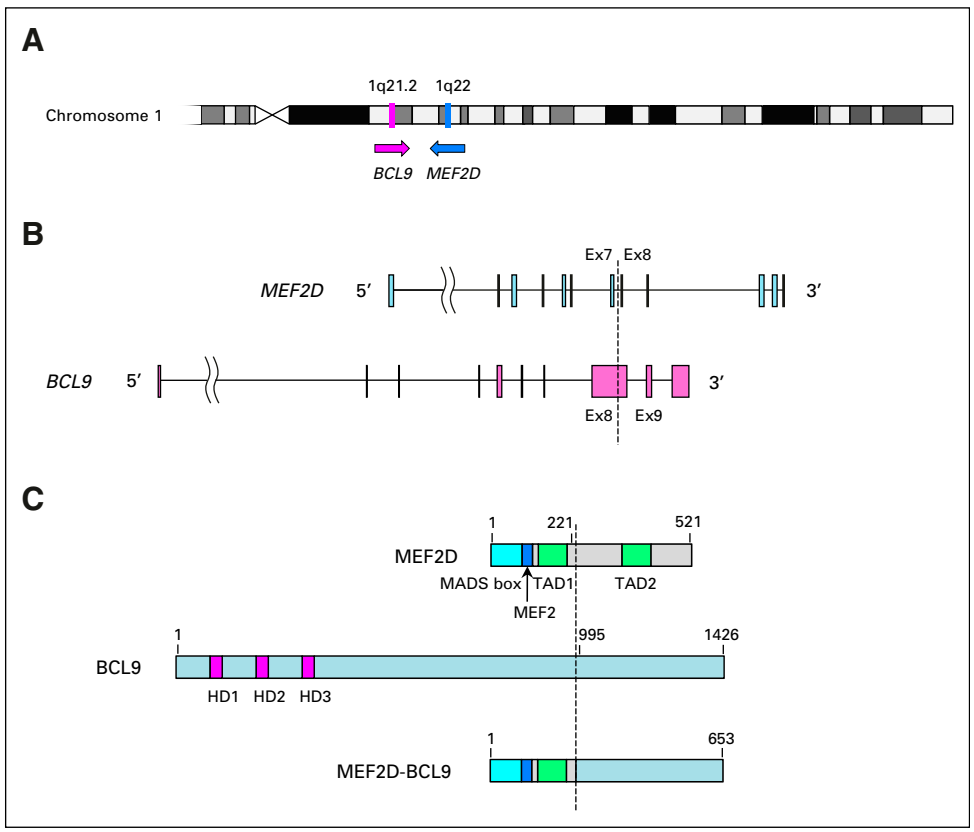

Fig 2. Genetic architecture of *MEF2D-BCL9* fusion. (A) Location of *MEF2D* and *BCL9* in chromosome 1. (B) Schema of *MEF2D-BCL9* fusion in patient 29. Dashed line indicates genomic break points. (C) Predicted domain structure of an MEF2D-BCL9 protein. Dashed line indicates break points. HD, homology domain responsible for interaction with Pygopus (HD1, HD3) and β-catenin (HD2); MADS, DNA binding and protein dimerization domain; MEF2, cofactor interactions domain; TAD, transcriptional activation domain.

plasmid, the packaging plasmid (pCAG-HIVgp), and the VSV-G– and Rev-expressing plasmid (pCMV-VSV-G-RSV-Rev) using ScreenFect A (Wako Pure Chemical Industries, Osaka, Japan). The medium was removed 16 hours later and the cells were incubated with complete RPMI1640 medium containing 10 μM forskolin for 48 hours. The supernatant was filtered through a 0.45-μm filter, and 1×10^6 NALM-6 cells were incubated with 2 mL of the supernatant. Venus-positive cells were selected 5 days later using a FACSAria2 (BD Biosciences, Franklin Lakes, NJ).

In Vitro Drug Sensitivity Testing

For the testing of cell lines and leukemic blasts, cells were cultured in RPMI1640 medium supplemented with 20% FBS at a cell density of 1.25×10^5 cells/mL. Dexamethasone sodium phosphate (Wako Pure Chemical Industries), vorinostat (Cayman Chemical, Ann Arbor, MI), quisinostat (Selleck Chemicals, Houston, TX), and bortezomib (Selleck Chemicals) dissolved in dimethylsulfoxide were added to the culture at the indicated concentrations. Cells were harvested 48 hours later and stained with fluorescein isothiocyanate or allophycocyanin-conjugated antiannexin-V antibody (MBL, Nagoya, Japan) and 7-amino-actinomycin D (Beckman Coulter, Brea, CA) and measured using a FACSCalibur flow cytometer (BD Biosciences).

Minimal Residual Disease Detection

We measured minimal residual disease using an NGS-based method. Briefly, PCR amplification of the *IGH* locus complementarity-determining region 3 (CDR3) was performed essentially as described.[13,14] A total of 3,200 ng of genomic DNA was mixed in a 100:1 ratio with spike-in reference DNA containing a known CDR3 sequence. The prepared DNA was then amplified using multiplexed primers and PrimeSTAR GXL DNA Polymerase (Takara Bio). All sequence data obtained by NGS were evaluated using IMGT V-QUEST software.[15,16] The sequences of primers are available on request.

Statistical Analysis

The probability of overall survival (OS) and event-free survival (EFS) was calculated from first relapse (for EFS, second relapse, secondary malignant neoplasm, and death with any cause were censored as events) using the Kaplan-Meier method. Differences between the two groups were calculated using log-rank tests at 5 years from first relapse. All *P* values reported are two sided, and *P* values $< .05$ were considered significant. All statistical analyses were performed using EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria).[17]

RESULTS

We performed RNA-sequencing (RNA-seq) in 59 children with relapsed or primary refractory ALL, including three infant patients, three with mature B-cell leukemia, and four with T-cell immunophenotype ALL (Fig 1; Data Supplement). We identified a total of 26 gene fusions in 22 patients. Among these gene fusions, 19 were nonrandom gene fusions already described in ALL, including *ETV6-RUNX1*, *TCF3-PBX1*, *BCR-ABL1*, *MLL-AFF1*, *MLL-MLLT3*, *P2RY8-CRLF2*, *NUP214-ABL1*, and *PAX5-NOL4L*. Interestingly, four of the remaining seven in-frame fusions involved identical combination of *MEF2D* and *BCL9* (Fig 1; Data Supplement).

MEF2D and *BCL9* are located on chromosome regions 1q22 and 1q21.2, respectively. The distance between these two genes is approximately 9 Mb, which is too small for chromosomal aberrations to be detected by G-banding (Fig 2A; Data Supplement). We identified break points on chromosome 1 by direct sequencing based on genomic DNA and confirmed that inversion in this chromosomal region resulted in this fusion (Fig 2B). The product generated by in-frame fusion of *MEF2D* and *BCL9* had a length of 573 to 653 amino acids, which contained *N*-terminal domains derived from MEF2D and C-terminal peptides without reported functional domains derived from BCL9 (Fig 2C). The expression of *MEF2D-BCL9* was detectable both at the time of diagnosis and at relapse (Data Supplement). *BCL9-MEF2D* was undetectable in these four patients (data not shown).

All four patients with *MEF2D-BCL9* fusion had similar clinical characteristics, including age 10 years or older (range, 10 to 13 years), a diagnosis of B-ALL on the basis of a $CD19^+$ $CD20^-$ $HLA\text{-}DR^+$ immunophenotype, very early relapse, and uniform morphologic findings characterized by large, densely basophilic, and heavily vacuolated leukemic blasts, mimicking those of mature B-cell leukemia (Fig 3; Data Supplement). Minimal residual disease was detectable in three of four patients either at day 15 or the end of induction therapy (Data Supplement). They died despite several courses of intensified chemotherapy and allogeneic HSCT (case reports in Data Supplement). Although the number of patients with this fusion gene was limited, OS and EFS after relapse were significantly inferior in four patients with *MEF2D-BCL9* fusion than in other patients (5-year OS, 0%; 95% CI, 0 to 0%; *v* 5-year OS, 54.5%; 95% CI, 36.9 to 69.0%; $P < .001$; and 5-year EFS, 0%; 95% CI, 0 to 0%; *v* 5-year EFS, 49.3%; 95% CI, 32.7 to 64.0%; $P < .001$; Data Supplement). We screened this fusion gene among the other 115 patients without relapse (85 B-ALL, 16 T-cell ALL, 12 acute myeloid leukemia, two mixed lineage leukemia) by RT-PCR and genomic PCR; however, we did not detect *MEF2D-BCL9* in this nonrelapse cohort.

We performed whole-exome sequencing of paired tumor-normal samples within the four patients with *MEF2D-BCL9* fusion (Fig 4; Data Supplement). We did not detect any germline variations that were diagnostic of inherited blood disorders or predisposition to cancer. We detected a total of 41 somatic

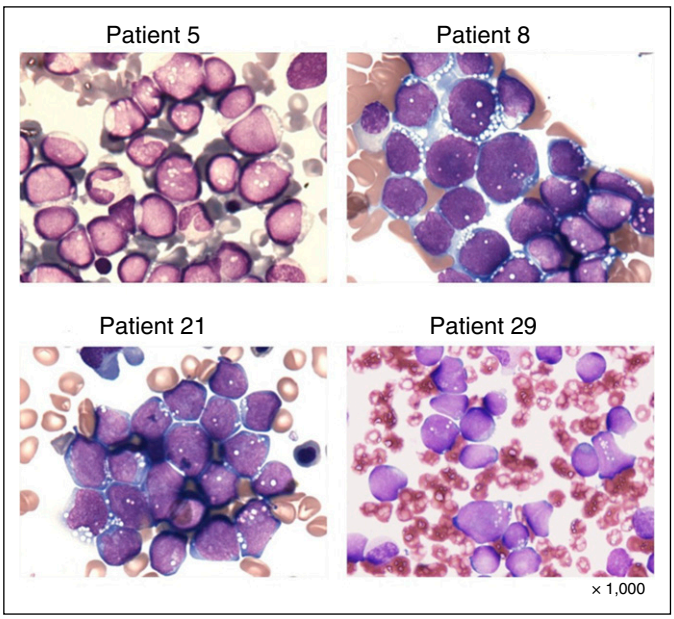

Fig 3. Morphology of leukemic blasts in patients with *MEF2D-BCL9*. May-Grunwald-Giemsa stain of bone marrow smears at relapse in each *MEF2D-BCL9*–positive patient (patients 5, 8, 21, and 29).

mutations, of which 31 were specific to relapse phase. There was no obvious driver mutation within mutations detectable at diagnosis, whereas relapse-specific mutations contained driver somatic alterations, including *TP53*, *KMT2D*, *ASXL1*, and *IKZF1* mutations. Every patient carried at least one relapse-specific somatic mutation that suggested clonal evolution during therapy.

Expression profiles of leukemic blasts with *MEF2D-BCL9* were assessed by clustering analysis (Data Supplement). Blasts with *MEF2D-BCL9* grouped into a compact cluster distinguishable from those with *ETV6-RUNX1*, *TCF3-PBX1*, or *BCR-ABL1* (Data Supplement). The top three differentially expressed genes were *HDAC9*, *PTPRZ1*, and *DPYSL4* (Data Supplement). *HDAC9* is a class IIA histone deacetylase (HDAC), and its high expression has been reported to predict poor prognosis in several cancers, including ALL.[18,19] We confirmed increased expression of *HDAC9* in blasts with *MEF2D-BCL9* compared with those without *MEF2D-BCL9* by quantitative RT-PCR (Data Supplement). *PTPRZ1* encodes a membrane-bound protein tyrosine phosphatase. Its expression is restricted to the CNS, although aberrantly high expression has been reported in several cancers.[20,21] We confirmed the protein expression of PTPRZ1 by immunostaining (Data Supplement). *DPYSL3*, a member of *DPYSL4*, is strongly upregulated by *HLF*, a transcription factor involved in *TCF3-HLF* fusion and associated with poor prognosis.[22] Markedly low *CDKN2A* expression levels were observed, resulting from copy number aberrations affecting this gene (Data Supplement).

NALM-6 cells exogenously overexpressing *MEF2D-BCL9* showed increased growth rate by 17% at 48 hours compared with mock control in vitro (Fig 5A). This cell line showed resistance to dexamethasone, one of the key drugs in the treatment of ALL (Fig 5B). High expression of *HDAC9* in *MEF2D-BCL9*–transduced cells was confirmed by quantitative RT-PCR (Fig 5C). Several other genes that showed strong differential expression in *MEF2D-BCL9*–positive patient samples were also upregulated in *MEF2D-BCL9*–transduced NALM-6 cells (Data Supplement). These findings suggest that this fusion gene is a driver gene and that some proportion of phenotypes observed in *MEF2D-BCL9*–positive patients were a result of this gene fusion.

We were able to establish a primary culture of leukemic blasts harboring *MEF2D-BCL9*. Therefore, we performed in vitro assays using these cells to test sensitivity to several anticancer drugs. Although dexamethasone had no inhibitory effect on cell growth, two HDAC inhibitors, vorinostat and quisinostat, showed inhibitory activities to cultured leukemic cells at therapeutic concentration (Figs 6A–6C).[23,24] Bortezomib, a proteasome inhibitor expected to be potent against refractory B-ALL, also showed inhibitory activity in vitro (Fig 6D).[25]

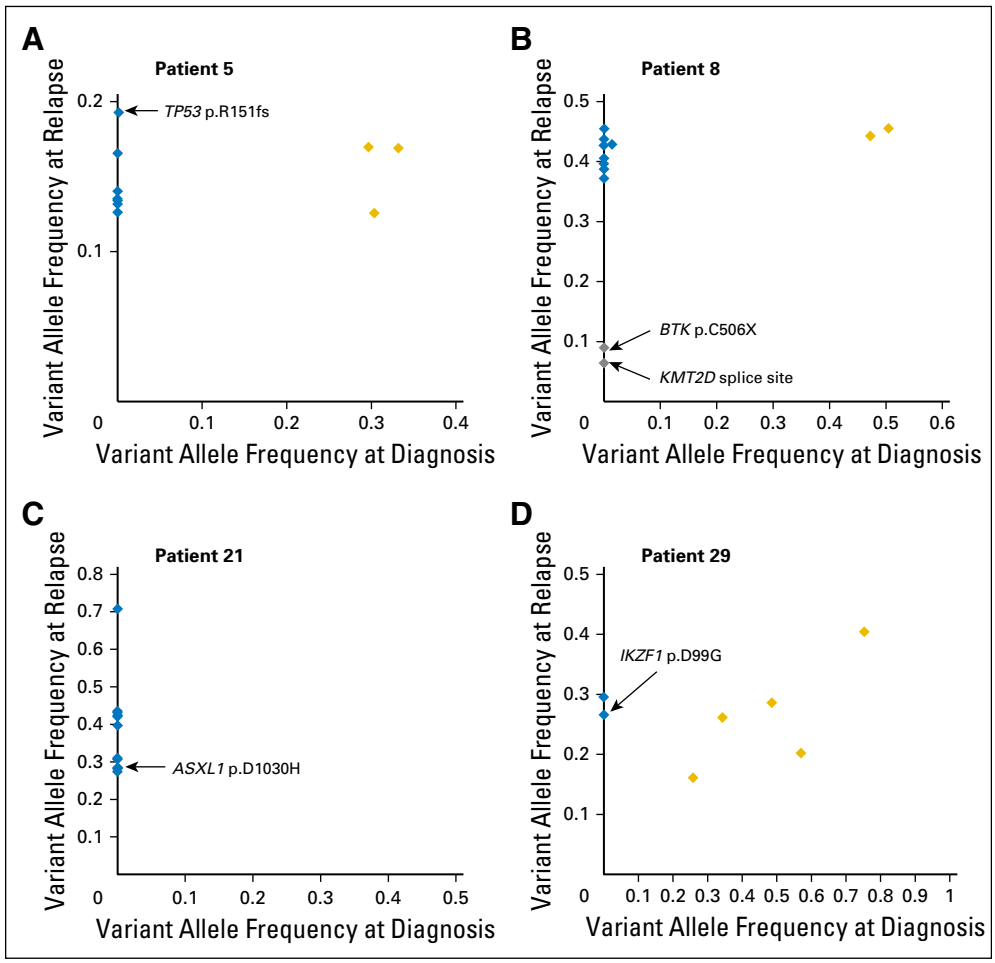

Fig 4. Whole-exome sequencing analysis. (A) Patient 5, (B) patient 8, (C) patient 21, (D) patient 29. Blue, gold, and gray dots indicate relapse-specific, shared, and subclone somatic mutations, respectively.

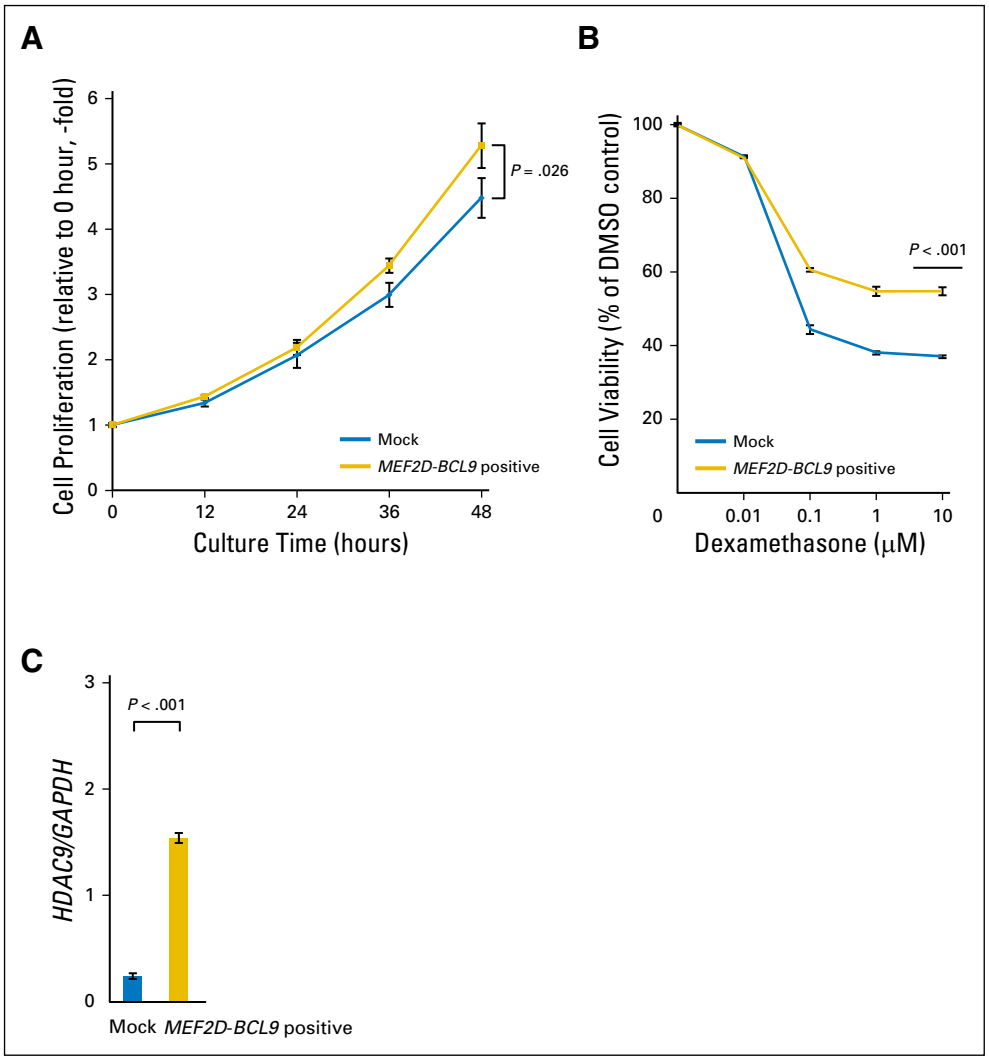

Fig 5. Exogenous expression of *MEF2D-BCL9* in NALM-6 cells. (A) Growth of *MEF2D-BCL9*–transduced NALM-6 cells. The proliferation rate was compared with mock-transduced NALM-6. (B) In vitro sensitivity to dexamethasone. Cells were cultured with the indicated concentrations of dexamethasone and viability was measured 48 hours later. Data are presented as percentages of dimethylsulfoxide (DMSO) control and depict the mean ± standard deviation. (C) *HDAC9* expression in NALM-6 cells with or without *MEF2D-BCL9*.

DISCUSSION

A current focus in ALL research is the discovery of genetic lesions that provide prognostic implications and novel therapeutic approaches. One of the most important discoveries in recent years has been the identification of Ph1-like ALL and druggable kinase lesions,[26] including *NUP214-ABL1* fusion detected in one of our patients. In this study, we also identified a novel gene fusion of *MEF2D* and *BCL9* recurrently in four patients with B-ALL, which indicated dismal prognosis. Patients with *MEF2D-BCL9* formed a subset in our B-ALL cohort on the basis of unique characteristics, which included older age, a leukemic blast morphology mimicking mature B-cell leukemia, and distinct expression profiles.

Neither *MEF2D* nor *BCL9* is well documented in the context of ALL. *MEF2D* encodes a ubiquitously expressed transcription factor originally identified as a major transcriptional activator for muscle differentiation.[27] However, the regulatory role of MEF2 proteins has also been associated with other types of cells, including hematopoietic differentiation.[28] A homo- or heterodimer form of MEF2 binds to specific DNA-binding sequence through the MADS and MEF2 domains and, with the activation signals, facilitates target gene transcription. Retroviral insertional analysis in a murine model identified *MEF2D* as a candidate gene involved in leukemogenesis.[29] *BCL9* is also a transcription factor and a nuclear component of the Wnt signaling cascade, first identified through cloning the (1;14)(q21;q32) translocation from a patient with B-ALL.[30] This gene was associated with Wnt/β-catenin signaling and has an HD2 domain required for interaction with β-catenin in its N terminus. However, this domain was truncated in the *MEF2D-BCL9* fusion we identified. *BCL9* is overexpressed in several types of human cancer and can promote tumor progression,[31,32] although its significance in ALL is not studied well.

We hypothesize that *MEF2D-BCL9* is a primary genetic event that establishes a leukemic cell, because this fusion product was detectable at the time of diagnosis, whereas other founder mutations or recurrent genetic events were absent. The small number of somatic mutations in whole-exome sequencing also supports this. As previously reported in *MEF2D-DAZAP1* fusion in the TS-2 B-ALL cell line, retained MADS and MEF2 domains might play a key role in leukemogenesis by enhancing transcriptional activity of MEF2D.[33-35] However, to confirm this hypothesis, further accumulation and genetic study of patient samples are required.

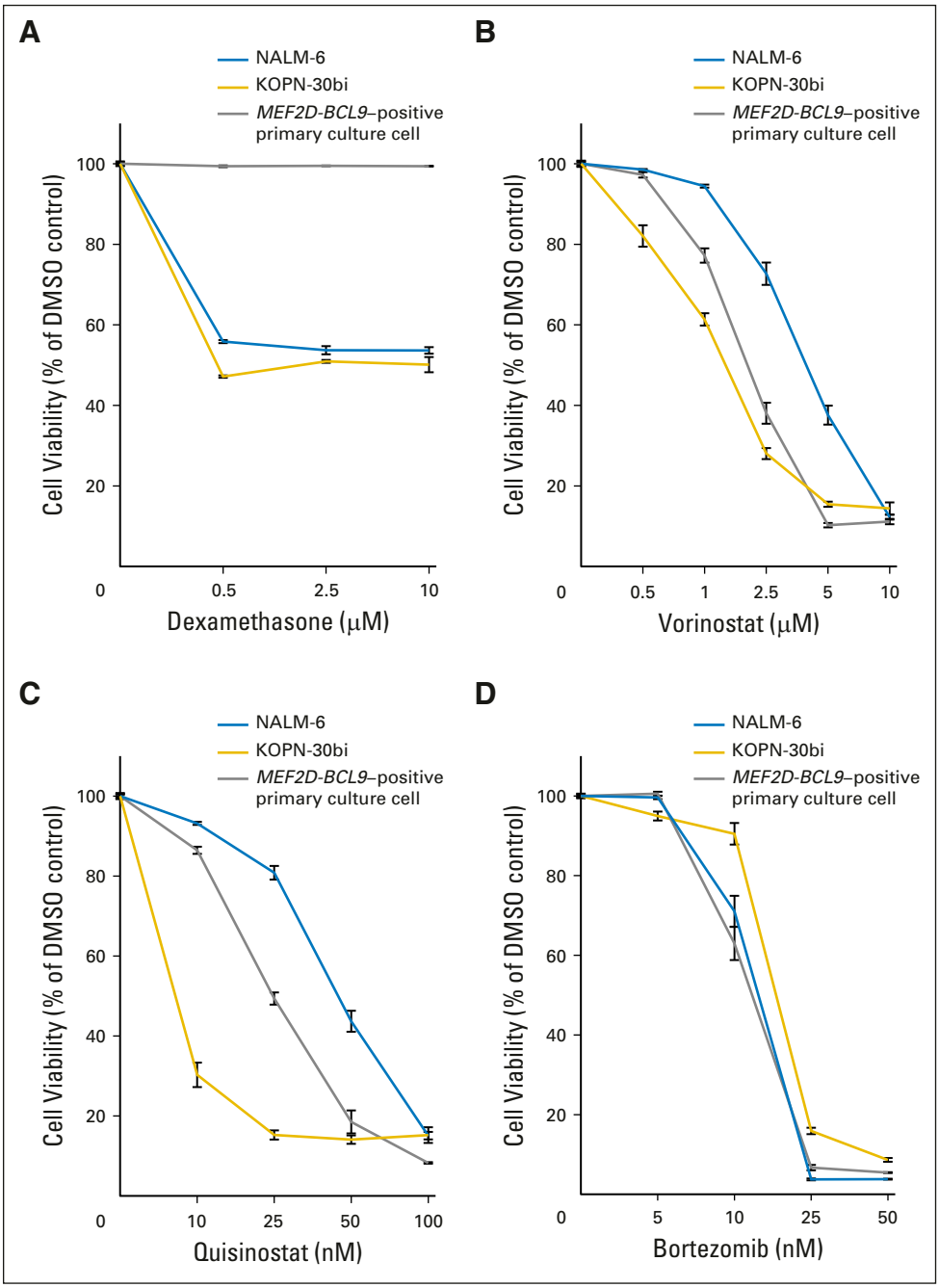

Fig 6. In vitro drug sensitivity. (A) Dexamethasone, (B) vorinostat, (C) quisinostat, (D) bortezomib. Acute lymphoblastic leukemia cell lines (NALM-6, KOPN-30bi) and primary cultured cells established from patient 21 were cultured with the indicated concentrations of anticancer drugs, and viability was measured at 48 hours. Data are presented as percentages of dimethylsulfoxide (DMSO) control and depict the mean ± standard deviation.

Although four patients with *MEF2D-BCL9* were categorized as high risk by the National Cancer Institute–Rome risk classification, they were not indicative of HSCT during their first complete remission. Our study identified *MEF2D-BCL9* as a marker of dismal prognosis, possibly calling for more intensified and earlier treatment. In vitro assays suggest that the leukemic cells with *MEF2D-BCL9* showed sensitivity to several molecular targeted drugs, such as vorinostat and bortezomib, possibly providing a treatment option in these patients.[23,25] Clinical trials for the detection of this fusion gene along with subsequent intensified therapy or incorporation of targeted therapies are required to confirm our findings. Mature B-cell leukemia-like morphology would help the detection of this fusion gene and facilitate subsequent clinical trials.

In conclusion, to our knowledge, we have identified a previously unknown genetic fusion event, which characterizes a novel subset of pediatric B-ALL. *MEF2D-BCL9* explained a considerable proportion of patients with relapse and conferred dismal prognosis. Identification of this gene fusion at diagnosis might provide a diagnostic marker indicating a high risk of treatment failure. Prospective clinical trials investigating combinatorial approaches of molecular targeted therapies with current chemotherapies are warranted for the treatment of this intractable disease.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Disclosures provided by the authors are available with this article at www.jco.org.

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Manuscript writing: All authors

Final approval of manuscript: All authors

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AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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