IDH2 and NPM1 Mutations Cooperate to Activate Hoxa9/Meis1 and Hypoxia Pathways in Acute Myeloid Leukemia

Yoko Ogawara1, Takuo Katsumoto1, Yukiko Aikawa1, Yutaka Shima1, Yuki Kagiyama1, Tomoyoshi Soga2, Hironori Matsunaga3, Takahiko Seki3, Kazushi Araki3, and Issay Kitabayashi1

Abstract

IDH1 and IDH2 mutations occur frequently in acute myeloid leukemia (AML) and other cancers. The mutant isocitrate dehydrogenase (IDH) enzymes convert α-ketoglutarate (α-KG) to the oncometabolite 2-hydroxyglutarate (2-HG), which dysregulates a set of α-KG–dependent dioxygenases. To determine whether mutant IDH enzymes are valid targets for cancer therapy, we created a mouse model of AML in which mice were transplanted with Npm1- deficient mice (TaconicArtemis GmbH) were maintained on the C57BL/6 genetic background. CreERT2 mice (Crea Japan). CreERT2 mice and Npm1-deficient mice (TaconicArtemis GmbH) were maintained on the C57BL/6 genetic background. Mouse experiments

Introduction

Mutations in genes encoding isocitrate dehydrogenase (IDH) 1 and 2 are frequently observed in acute myeloid leukemia (AML), brain tumors, and other cancers (1–7). Mutant IDHs catalyze the formation of the oncometabolite 2-hydroxyglutarate (2-HG; ref. 8), which dysregulates a set of α-ketoglutarate (α-KG)–dependent dioxygenases, including epigenetic regulators (TETs and histone demethylases), and others (EGLN and collagen prolyl 4-hydroxylases; refs. 9–14). The roles of mutant IDHs in tumorigenesis have been analyzed extensively both in vitro and in vivo. Mutant IDHs increased proliferation and repressed differentiation in vitro cultured cells (12, 15). Small molecules that potently and selectively inhibit tumor-associated mutant IDHs were developed (16, 17). These inhibitors induced differentiation of mutant IDH-expressing transformed cells in vitro. These in vitro experiments strongly indicate that mutant IDH is a druggable oncogene, and a mutant IDH-mediated leukemia and sarcoma mouse model was developed (18–21). These studies have shown that mutant IDH is actually an oncogene and can cause cancer in vivo. One report excitingly showed that induction of mutant IDH confers the addiction to mutant IDH itself in a Hoxa9/Meis1–mediated AML model (21). However, to clarify whether IDH mutants are valid targets in cancer therapy, it is necessary to show the effect of IDH mutant inhibition in a system close to the actual state of patients, such as an IDH mutant-mediated cancer mice model. IDH mutations in AML frequently occur simultaneously with other mutations such as NPMc (a cytoplasmic nucleophosmin mutation), DNMT3A, and FLT3/ITD (an internal tandem duplication; ref. 22) in AML. Indeed, the IDH1 mutation alone is not sufficient to induce AML in mice (23). These findings suggest that IDH mutation acts in multistep carcinogenesis, so the accumulation of additional mutations in conjunction with IDH mutation is necessary for development of AML. Here, we established new IDH2 mutant-mediated AML model mice through the combination of coexisting mutant genes in AML patients. By using this AML model, we report that AML harboring an IDH2 mutation can be blocked by conditional deletion of the mutant IDH2 gene, even after leukemia has developed. Our findings strongly suggest that inhibition of mutant IDHs represents an effective strategy for the treatment of AML harboring IDH mutations.

Materials and Methods

Mice

C57BL/6 mice were purchased from CREA Japan. CreERT2 mice and Npm1-deficient mice (TaconicArtemis GmbH) were maintained on the C57BL/6 genetic background. Mouse experiments
were performed in a specific pathogen-free environment at the National Cancer Center (Tokyo, Japan) animal facility according to the institutional guidelines, and with the approval of the Japan National Cancer Center Animal Ethics Committee.

Plasmids and retrovirus infection
- pMy-NPMc-ires-EGFP, pGCDN-IDH2/R140Q-ires-NGFR, pMSCV-DNMT3A/R882H, and pMSCV-FLT3-ITD constructs were generated by inserting cDNAs encoding each of the genes into the corresponding retroviral vectors: pMy-ires-EGFP (Cell Biolabs), pGCDN-ires-NGFR, pMSCV-neo, and pMSCV-puro (Clontech Laboratories). Ecotropic retrovirus was produced using Plat-E packaging cells (24). Plat-E cells were transfected using the GeneJuice reagent (Merck Millipore), and supernatants containing retrovirus were collected 48 hours after transfection. One third volume of PEG solution [30% PEG-8000, 0.4 mol/L NaCl, and 40 mmol/L HEPES (pH 7.4)] was added to the centrifuged supernatants and incubated overnight at 4°C. Samples were centrifuged at 1,500 rpm for 45 minutes at 4°C. Pellets were resuspended in StemPro medium (Invitrogen) and immediately stored at –70°C in single-use aliquots. c-Kit+ cells were selected from the bone marrow of 8-week-old mice using CD117-specific MicroBeads (Miltenyi Biotec). The cells were then incubated with retrovirus using RetroNectin (Takara Bio) in StemPro-34 medium (Invitrogen) containing cytokines (50 ng/mL of stem cell factor, 10 ng/mL of IL3, and 10 ng/mL of oncostatin M). For serial infections, each virus was incubated with the cells for 12 hours, and excess virus was washed away before the next infection.

RNA isolation and quantitative RT-PCR
- Total RNA was extracted from cells using the ISOGEN II reagent (Nippongene). Next, cDNA was generated using the GoScript Reverse Transcription (RT) System (Promega). Real-time PCR was conducted on an Applied Biosystems 7500 Fast real-time PCR system. TaqMan probes were used for mEbf1 (Mm00395519), mTBP was used as a control for normalization.

Colony formation assay
- Cells were infected with pMy-ires-EGFP or pMy-NPMc-ires-EGFP, cultured in StemPro-34 medium for 4 days, and EGFP+ cells were sorted on an LS cell sorter (Biotage). The sorted cells were plated in methylcellulose medium (M3234; STEMCELL Technologies) supplemented with mouse cytokines (10 ng/mL of IL3, 50 ng/mL of stem cell factor, and 10 ng/mL of GM-CSF). After that, cells were cultured and replated every 7 days.

Bone marrow transplantation assay and tamoxifen treatment
- Infected cells were transplanted into 8-week-old C57Bl/6 mice after irradiation (9.5 Gy) by intravenous injection. Secondary transplants were performed by intravenous injection of bone marrow cells from primary AML mice into C57Bl/6 mice after irradiation (6 Gy). When cells were infected with floxed IDH2/R140Q, a second bone marrow transplantation (BMT) was performed after irradiation with 3 Gy. Mice secondarily transplanted with floxed IDH2/R140Q received tamoxifen (80 mg/kg body weight) three times, every other day, by intraperitoneal injection. Subsequently, tamoxifen was administered once per week.

May–Giemsa staining
- Bone marrow cells were sprayed onto glass slides using a Cytospin (Thermo Scientific). The air-dried slides were stained with modified May–Grünwald’s eosin methylene blue solution (Merck), and then with Giemsa’s azure eosine methylene blue solution (Merck).

Flow cytometric analysis
- Bone marrow cells were preincubated with rat IgG (Sigma), and then incubated on ice with the following antibodies conjugated to staining reagents:
  - Transplanted cells: NGFR-APC
  - Myeloid lineage: Mac-1-PE-Cy7, Gr-1-PE
  - LSC (leukemia stem cell) markers: MCSFR-PE, biotin-conjugated lineage markers (Mac-1, CD3e, B220, Gr-1, and Ter119), Sca-1-Bio, c-Kit-APC, c-Kit-APC-eFluor780, Gr-1-PE, and CD33-Alexa647
- sAvi-PE was used to detect biotin-conjugated antibodies:
  - B lineage: CD19-PE
  - T lineage: CD3e-Bio (sAvi-PE)
- Erythroid lineage: CD71-PE and Ter119-Bio (sAvi-PE-Cy7)

2-HG analysis
- Peripheral blood was taken from mice, and 50 μL was centrifuged at 15,000 rpm for 5 minutes. The supernatant was collected as plasma. Fifty microliters of peripheral blood was lysed with red-cell lysis buffer [0.8% NH₄Cl and 17 mmol/L Tris (pH 7.65)], and the remaining white cells were collected. The volume of plasma and peripheral blood cells was adjusted to 100 μL prior to the addition of 400 μL of ethanol. Samples were incubated at –20°C for more than 1 hour, and then 300 μL of water was added. The samples were centrifuged and the supernatant was collected. The 2-HG level was analyzed in this purified sample using LS-MS/MS.

Genotyping
- Genomic DNA was isolated from peripheral blood cells using the KAPA Express Extract Kit (Kapa Biosystems). PCR was conducted to detect floxed and delta IDH2/R140Q using the following PCR primers: (TACGGGTCATCTCATCACCA), (CTTTATATGTTCTTGTTGTC), (GCCGACACCAGAC-TAAGAAC), and (GCCGACACCAGAC-TAAGAAC). The samples were centrifuged and the supernatant was collected.

Microarray analysis and data processing
- Microarray expression profiling was performed using Affymetrix GeneChip Mouse Gene 1.0 ST Arrays in the cells specified below:
  - TAM: Nf(I)DF-AML bone marrow cells untreated with tamoxifen.
  - +TAM: Nf(I)DF-AML bone marrow cells treated with tamoxifen.
  - Control: normal bone marrow cells.

NI: NPMc- and IDH2/R140Q-infected bone marrow cells.
NID: NPMc-, IDH2/R140Q-, and Dnmt3a/R882H-infected bone marrow cells.
NIF: NPMc-, IDH2/R140Q-, and FLT3-ITD-infected bone marrow cells.
Npm1

NPMc into leukemogenic function of NPMc. To test this idea, we transduced NPMc may inhibit wild-type (WT) NPM by localizing it to surviving cells formed increased numbers of colonies in methylcellulose medium. To determine whether expression of IDH2/R140Q is required for maintenance of AML, we inserted loxP sequences into the 5' and 3' regions of IDH2/R140Q and NPMc, respectively (Fig. 1B). Expression analysis revealed that Meis1 and Hoxa9 expression levels were elevated in cells expressing IDH2/R140Q and NPMc, respectively (Fig. 1A and B). Because the expression of Hoxa9 immortalsizes myeloid progenitor cells (27), we tested whether the NPMc-expressing cells could be maintained in vitro. The NPMc-expressing cells formed increased numbers of colonies in methylcellulose media, but could not be maintained for a long time (Fig. 1C). NPMc may inhibit wild-type (WT) NPM by localizing it to cytoplasm (28); if so, the levels of WT NPM may affect the leukemogenic function of NPMc. To test this idea, we transduced NPMc into Npm1−/− hematopoietic stem/progenitor cells to mimic the situation in an AML patient heterozygous for a mutation in the NPM1 gene. The Npm1+/− cells expressing NPMc exhibited serial colony-forming activity (Fig. 1C). Moreover, expression levels of Hoxa9 induced by NPMc were higher in the Npm1−/− cells than in the WT cells (Fig. 1D). Thus, we used Npm1+/− hematopoietic stem/progenitor cells for further analyses.

Results

IDH2/R140Q and NPMc upregulate expression of Meis1 and Hoxa9 in vitro, respectively

To investigate the roles of the aforementioned mutations in AML development, we infected mouse hematopoietic stem/progenitor cells with retroviruses, each encoding IDH2/R140Q, NPMc, DNMT3A/R882H, or FLT3/ITD. We used the most frequently used NPMc mutant containing a duplication of the TCTGCGCACACA, and reverse, (CTCGGAACAACCCCTGCTGTT). Follows:

12.5 software. Gene set enrichment analysis (GSEA) was performed using Illumina HiSeq2000. The acquired data were analyzed using Illumina HiSeqSeq software (Strand).

Enrichment of 5hmC-containing DNA, deep sequencing, and peak detection

Genomic DNA was purified from bone marrow cells using NucleoSpin Tissue (Takara). 5hmC-enriched DNA was obtained using Hydroxymethyl Collector (Active Motif). DNA libraries were generated by following the Illumina protocol for "Preparing Samples for ChIP Sequencing of DNA" (Part# 111257047 Rev. A). Genomic DNA was purified from bone marrow cells using Nucleospin Tissue (Takara). 5hmC-enriched DNA was obtained using Hydroxymethyl Collector (Active Motif). DNA libraries were generated by following the Illumina protocol for "Preparing Samples for ChIP Sequencing of DNA" (Part# 111257047 Rev. A). In total, 25 to 40 ng of input genomic DNA or 5hmC-enriched DNA was used. DNA fragments of approximately 150 to 300 bp were gel-purified after the adaptor-ligation step. PCR-amplified DNA libraries were quantified on an Agilent 2100 Bioanalyzer and diluted to a concentration of 6 to 8 pmol/L for cluster generation and sequencing. Thereafter, 100 cycle paired-end sequencing was performed using Illumina HiSeq2000. The acquired data were aligned to the mouse genome, and 5hmC peaks were detected using Avadis software (Strand).

The 5hmC mapping data have been deposited in the NCBI GEO database at http://www.ncbi.nlm.nih.gov/geo/ through accession number GSE63638.

qPCR validation of 5hmC-enriched loci

Input genomic DNA and 5hmC-enriched DNA were used for real-time PCR with Fast Start Universal SYBR Green Master (Roche) and forward and reverse primers. The primers were as follows: Ebf1: forward, (TGGCCTTTCCCGCTATT), and reverse, (CACCAGTGATTTAGGGCTCAGTT); Spib: forward, (GGATGCTCTGCAGCACA), and reverse, (CTCGAACAACCCCTGCTGTT).

Establishment of mouse AML model harboring IDH2/R140Q

To establish mouse AML models, we serially infected Npm1+/− hematopoietic stem/progenitor cells with retroviruses encoding NPMc-EGFP, IDH2/R140Q-ires-NGFR, DNMT3A/R882H, and FLT3/ITD, and transplanted the infectants into WT mice (Fig. 2A). About 60% of the recipient mice died of AML within 140 days after transplantation, and all the mice died within 275 days (Fig. 2B, NIDF). When only three out of the four mutant genes were transduced, the onset of leukemia was delayed in any combinations (Fig. 2B, IDF, NDF, NIF, and NID). These results clearly indicate that all four mutations are necessary for the efficient induction of AML. In our AML model, the expression of IDH2/R140Q and NPMc was monitored by measuring the expression of NGFR and EGFP, respectively, because these genes were encoded on the same vectors used to express IDH2/R140Q and NPMc (Fig. 2C). We analyzed bone marrow cells derived from AML mice transplanted with NDF cells, and found that most of the cells expressed both NPMc and IDH2/R140Q (Fig. 2D). We isolated RNA from NIDF-AML cells and confirmed the expression of DNMT3A/R882H and FLT3/ITD by performing PCR with TaqMan probes against human DNMT3A and FLT3 (data not shown). To examine expression of DNMT3A/R882H and FLT3/ITD at the single-cell level, we stained permeabilized AML cells with anti-DNMT3A and anti-FLT3 antibodies. Flow cytometric analysis showed that 30%–50% and 20%–40% of the AML cells expressed DNMT3A/R882H and FLT3/ITD, respectively (Supplementary Fig. S1). Morphologic analysis revealed that a large population of bone marrow cells derived from NDF-induced AML mice were blast cells (Fig. 2E). In our analysis of moribund mice transplanted with NID or NIF cells, the percentage of blast cells was around 10%, which did not meet the criteria for AML (Fig. 2E). Most bone marrow cells expressed myeloid markers such as Mac-1 and Gr-1 (Fig. 2F and Supplementary Fig. S2A). The percentages of cells expressing B-cell (CD19), T-cell (CD3ε), and Gr-1 (CD71 and Ter119) lineage markers were extremely low (Supplementary Fig. S2A). When the NIDF-induced AML bone marrow cells were transplanted into secondary recipient mice, all the mice developed AML (Fig. 2G). The secondary recipients of the AML cells die quicker than the primary recipients. This may be because the number of LSCs in primary recipients was lower than that in secondary recipients. Alternatively, it is also possible that the disease became more aggressive in secondary recipients.

IDH2/R140Q is necessary for the maintenance of AML

To determine whether expression of IDH2/R140Q is required for "maintenance" of AML, we inserted loxP sequences into the 5'- and 3'-regions of IDH2/R140Q within the viral vector (Fig. 3A). ERT2-Cre+ Npm1+/− hematopoietic progenitor cells were infected with the floxed IDH2/R140Q and the three other necessary genes (Nf1ΔDF), and then transplanted into mice to induce AML. Bone marrow cells from the AML mice were subsequently transplanted into secondary recipient mice (Fig. 3B). When the population of the EGFP+ leukemic cells within the total leukocyte population in peripheral blood of the secondary recipient mice reached 50% to 80% (7 weeks after transplantation), we commenced treatment.
with tamoxifen to delete IDH2/R140Q (Fig. 3C). The level of 2-HG, which is produced by IDH2/R140Q, was high in both the plasma and peripheral blood cells of AML mice, but decreased to the control levels within 7 days after initiation of tamoxifen treatment (Fig. 3D). This confirmed that the floxed IDH2/ R140Q system was functional. To test the effects of IDH2/ R140Q deletion on survival, we commenced treatment with tamoxifen when the population of EGFP+ leukemic cells reached 10% to 15% in peripheral blood. Although all 10 control AML mice died 45 to 94 days after transplantation, approximately 50%
of the AML mice treated with tamoxifen survived for at least 94 days (Fig. 3E). When we began tamoxifen treatment at an earlier stage (with 1%–2% EGFP+ cells in peripheral blood), 5 out of 9 mice survived for at least 130 days after secondary BMT (Fig. 3F). However, all 9 control mice died of AML within 87 days after transplantation (Fig. 3F). The number of EGFP+ leukemic cells did not increase in the peripheral blood of mice treated with tamoxifen for 4 weeks, whereas the number of EGFP+ cells in control AML mice increased dramatically over this period (Fig. 3G). To monitor the effects of IDH2/R140Q deletion on AML cells, we injected tamoxifen into mice once there was a high population (60%–70%) of EGFP+ leukemic cells in peripheral blood. The number of EGFP+ cells in peripheral blood was drastically reduced after 4 weeks of treatment (Fig. 3H). On the other hand, mice not treated with tamoxifen died within 4 weeks. Using the same set of mice as in Fig. 3H, we investigated expression of LSC markers in bone marrow cells of AML mice treated with tamoxifen for 2 weeks. Genotype analysis confirmed that flox-IDH2/R140Q was almost completely deleted after 2 weeks of tamoxifen treatment (Fig. 3I). Flow cytometric analysis revealed that IDH2/
IDH2/R140Q is necessary for the maintenance of AML. A, schematic of the IDH2/R140Q-flox system. Because loxP sequences were inserted in both 5' and 3' regions of IDH2/R140Q on the vector, IDH2/R140Q could be deleted by activating Cre recombinase. B, experimental scheme. c-Kit+ hematopoietic progenitor cells were isolated from ERT2-Cre+ Npm1+/-/C0 mice serially infected with pMy-NPMc-ires-EGFP, pGCDN-flox-IDH2/R140Q-ires-NGFR, pMSCV-DNMT3A/R882H-puro, and pMSCV-FLT3/ITD-neo [Nf(I)DF], and then transplanted into irradiated mice. The Nf(I)DF-induced AML cells were transplanted into secondary recipient mice. After 7 weeks (C and D), 3 weeks (E), and 2 weeks (F and G), the secondary recipient mice were injected with corn oil or tamoxifen (TAM). C, the genotyping of floxed IDH2/R140Q. Genomic DNA was prepared from peripheral blood (PB) cells 0, 1, 2, 4, and 7 days after TAM treatment, and genotyping of floxed and D'IDH2/R140Q was performed. D, 2-HG levels in blood plasma and peripheral blood cells. Plasma and peripheral blood cells were isolated from 3 mice described in C. E and F, survival of secondary recipient mice treated with corn oil or tamoxifen (TAM). C, the genotyping of floxed IDH2/R140Q. Genomic DNA was prepared from peripheral blood (PB) cells 0, 1, 2, 4, and 7 days after TAM treatment, and genotyping of floxed and A'IDH2/R140Q was performed. D, 2-HG levels in blood plasma and peripheral blood cells. Plasma and peripheral blood cells were isolated from 3 mice described in C. E and F, survival of secondary recipient mice treated with corn oil or tamoxifen. Tamoxifen treatment began 3 weeks (E) or 2 weeks (F) after transplantation. The inset panels show the percentage of EGFP+ leukemic cells in the peripheral blood at the start of tamoxifen treatment. E, P = 0.0029; F, P < 0.0001; log-rank test. G, deletion of IDH2/R140Q suppressed the expansion of EGFP+ cells in peripheral blood. Peripheral blood cells were isolated from the mice shown in F 4 weeks after tamoxifen treatment and analyzed for NPMc (EGFP) and IDH2/R140Q (NGFR) expression by flow cytometry. The number represents the mean percentage of EGFP+ cells in the peripheral blood of 10 mice. H, deletion of IDH2/R140Q decreased the population of EGFP+ cells in peripheral blood. Tamoxifen treatment began 7 weeks after transplantation. Peripheral blood cells were isolated from mice before and after tamoxifen treatment (1, 2, and 4 weeks), and were analyzed by flow cytometry. The graph represents the mean percentage of EGFP+ cells in the peripheral blood. I, deletion of IDH2/R140Q decreased the fraction of cells expressing LSC markers and exhausted EGFP+ cells in bone marrow. (Continued on the following page.)
R140Q-deleted EGFP+ AML cells were retained, but the populations of these cells that expressed LSC markers (MScFR, L-GMP, cKit+Gr1+, and CD34; refs. 29–31) were dramatically reduced in tamoxifen-treated mice (Fig. 3I and Supplementary Fig. S2B). We analyzed expression of B-cell (CD19), T-cell (CD3ε), and erythroid (CD71 and Ter119) lineage markers in IDH2/R140Q-deleted AML cells. The differentiated state of the cells was maintained, and most cells were of the myeloid lineage (Supplementary Fig. S2A). Consistent with decreased populations of cells expressing stem cell markers, the number of EGFP+ AML cells was markedly reduced after an additional 2 weeks of tamoxifen treatment (Fig. 3I). To confirm the decrease in the number of LSCs, we performed a third transplantation using bone marrow cells isolated from mice treated with or without tamoxifen for 2 weeks. When peripheral blood cells were analyzed 4 weeks later, the number of AML cells was increased in mice transplanted with untreated bone marrow cells (Fig. 3I). Conversely, AML cells were almost undetectable in mice transplanted with tamoxifen-treated bone marrow cells (Fig. 3I). All mice transplanted with tamoxifen-untreated bone marrow cells died of AML by 127 days after the third BMT, whereas none of the mice transplanted with tamoxifen-treated bone marrow cells died by 140 days (Fig. 3K). These results strongly indicate that IDH2/R140Q is necessary for the maintenance of the LSC population. To investigate the effect of the IDH2/R140Q deletion on mRNA expression profiles, we performed microarray analysis of bone marrow cells treated with or without tamoxifen for 2 weeks (Supplementary Table S1). The Meis1 expression level in tamoxifen-treated bone marrow cells was decreased to one third of that of tamoxifen-untreated bone marrow cells (Supplementary Table S1). This result is consistent with the data showing that IDH2/R140Q upregulated the Meis1 level in vitro (Fig. 1B). GSEA (25) using these microarray data showed that gene sets involved in the cell-cycle process (cell replication and nuclear replication) were downregulated in tamoxifen-treated cells compared with tamoxifen-untreated bone marrow cells (Fig. 3L and Supplementary Table S2). These results suggest that disruption of cell-cycle signaling contributes to the exhaustion of LSCs.

Roles of four mutant genes in AML development

Flow cytometric analysis of mice transplanted with NIDF-transduced cells revealed that most NPMc-expressing cells also expressed IDH2/R140Q and that the percentages of NPMc+ IDH2/R140Q- cells were extremely low in the bone marrow and peripheral blood (Fig. 4A). When Npm1+/− cells infected with NPMc alone were transplanted into mice, only a small population of NPMc+ cells was detected in the peripheral blood of recipients (Fig. 4B). On the other hand, when Npm1−/− cells were coinfected with NPMc and IDH2/R140Q (NI), a large population of NPMc+ IDH2/R140Q+ cells was retained in peripheral blood (Fig. 4B).

The infection efficiencies of the viruses were comparable (NPMc-ires-EGFP: ~40%, and IDH2/R140Q-ires-NGFR: ~50%). We analyzed mice 8 weeks after transplantation to determine the direct effects of each mutant gene. These data indicate that IDH2/R140Q was necessary and sufficient for the engraftment of or survival of NPMc+ cells in vivo. Six months after transplantation of Npm1−/− hematopoietic progenitor cells transduced with IDH2/R140Q alone, IDH2/R140Q− cells were predominant in the bone marrow (Fig. 4C). This indicates that IDH2/R140Q− cells could be maintained for a long period of time in vivo. We isolated RNA from these IDH2/R140Q-expressing cells and performed microarray analysis (Supplementary Table S3). GSEA using these microarray data showed that the expression of genes upregulated by hypoxia stimuli (32) was increased in IDH2/R140Q-expressing cells (Fig. 4D and Supplementary Table S4). These results suggest that the hypoxia pathway may facilitate the engraftment of NPMc+ cells in vivo. We analyzed bone marrow cells isolated from mice transplanted with NPMc and IDH2/R140Q transduced cells. Although NPMc−IDH2/R140Q− cells were predominant in the bone marrow of recipient mice (Fig. 4E), the proportion of blast cells in the bone marrow was less than 10% at 6 months after transplantation (Fig. 4F). These data suggest that these mice developed a myeloproliferative neoplasm (MPN)-like disease, not AML. Thus, in addition to IDH2/R140Q and NPMc, the expression of DNM3A/R882H and FLT3/ITD is also required for efficient development of AML. To investigate the roles of each of these mutants in leukemogenesis, we compared the in vivo mRNA expression profiles of control bone marrow cells, NPMc− IDH2/R140Q− (NI) cells, cells expressing all four mutant genes (NPMc, IDH2/R140Q, DNM3A/R882H, and FLT3/ITD; NIDF), and cells expressing three of the mutant genes (NID or NIF; Fig. 4G and Supplementary Table S5). Compared with vector control cells, the expression of Hoxa9 and Meis1 was upregulated in NI, NID, NIF, and NIDF cells (Fig. 4H). This is consistent with the finding that NPMc and IDH2/R140Q upregulate the expression of Hoxa9 and Meis1, respectively, in vitro (see Fig. 1B). Furthermore, the expression level of Meis1 was higher in NID and NIDF cells than in NI cells (Fig. 4I), suggesting that DNM3A/R882H promotes upregulation of Meis1. GSEA revealed that, compared with control bone marrow cells, the NI cells expressed significantly higher levels of a set of genes that are upregulated in NPMc− AML (26), hypoxia (32), and myeloid development (Fig. 4J and Supplementary Table S6; ref. 33). These results indicate that NPMc and IDH2/R140Q are sufficient to confer some of the properties of NPMc− AML cells on other cells. In addition, activation of the hypoxia pathway was consistent with the results obtained with cells expressing IDH2/R140Q only (see Fig. 4D). The myeloid differentiation signature is consistent with the MPN-like phenotype of the NI cells (see Fig. 4F). NID and NIDF cells expressed high levels of a set of genes that are
Figure 4.
Functional analysis of four genes involved in AML development. A, flow cytometric analysis of EGFP and NGFR in bone marrow (BM) and peripheral blood (PB) cells from mice transplanted with NIDF cells 3 months after transplantation. B, IDH2/R140Q is required for engraftment and survival of NPMc⁺ cells in vivo. c-Kit⁺ hematopoietic progenitor cells from Npm1⁺/− mice were infected with viruses as indicated and transplanted into irradiated mice. Peripheral blood cells were analyzed for expression of EGFP (NPMc) and NGFR (IDH2/R140Q) 8 weeks after transplantation. The number represents the mean percentage of EGFP⁺ cells in the peripheral blood of 4 mice. C, representative FACS plots of NGFR in bone marrow cells of mice at 6 months after transplantation of IDH2/R140Q-transduced cells. The bar graph shows the mean percentages of IDH2/R140Q⁺ cells for all 5 mice studied. D, results of GSEA. The microarray data of RNA isolated from IDH2/R140Q expressing cells in C and control bone marrow cells were compared. The IDs of the gene sets in the MSigDB (Molecular Signature Database) are indicated. E, representative FACS plots of EGFP and NGFR in bone marrow cells of mice at 6 month after transplantation of NI cells. The bar graph shows the mean percentages of NPMc⁺IDH2/R140Q⁺ cells for all 4 mice studied. F, representative morphology and blast population of NI cells. Bone marrow cells were prepared from the mice used in E and stained with May–Giemsa. The bar graph shows the percentage of blast cells. G, IDH2/R140Q⁺ and NPMc⁺ cells were enriched in bone marrow. c-Kit⁺ cells from Npm1⁺/− mice were infected with viruses as indicated (vector control, NI, NIF, NID, and NIDF) and transplanted into irradiated mice. Bone marrow cells were analyzed 8 weeks after transplantation. H, expression of HoxA9 and Meis1 in NI, NID, NIF, and NIDF cells. RNA was isolated from EGFP⁺ bone marrow cells prepared from the mice shown in G. In the case of vector control mice, whole bone marrow cells were used. Real-time PCR was performed to measure the expression levels of mHoxa9 and mMeis1. I, results of GSEA. The IDs of the gene sets in the MSigDB (Molecular Signature Database) are indicated. J, summary of GSEA. **, P < 0.05; ***, P < 0.001.
upregulated in hematopoietic early progenitors (compared with NI cells; ref. 34) and hematopoietic stem cells (compared with control bone marrow cells; Fig. 4I and J and Supplementary Table S6; ref. 35). These findings suggest that DNMT3A/R882H may confer stem/progenitor-cell properties on pre-AML cells, such as NI cells, to induce the development of AML.

IDH2/R140Q negatively regulates the 5hmC modification and expression of differentiation-associated genes

TETs, which convert 5mC into 5hmC, are α-KG–dependent enzymes; therefore, the level of 5hmC at TET2 target loci might be decreased in NIDF-AML cells. We isolated 5hmC-enriched DNA from normal bone marrow and NIDF-AML cells and performed high-throughput sequencing of these DNA fragments. We aligned these fragments with the mouse genome and identified gene loci with low levels of 5hmC in AML cells (top 974 genes had a score of >4). Comparison of loci that had a low level of 5hmC with microarray data revealed that one gene was upregulated (≥2-fold) and 35 genes were downregulated (<-2-fold) in AML cells compared with normal bone marrow cells (Fig. 5A). Among these 35 downregulated genes, eight genes were derepressed (≥2-fold) after deletion of IDH2/R140Q, according to microarray data (Fig. 5A and B). The level...
Quantitative PCR analysis indicated that 5hmC modification of these genes in tamoxifen-treated cells (Fig. 5C). Regulation of these genes in Nf(I)DF-AML cells and the derepression sequencing data. These data indicate that 5hmC modification and expression of Ebf1 and Spib are reversely downregulated by IDH2/R140Q.

Discussion

Taken together, our results show that IDH2/R140Q is necessary for the development and maintenance of AML. IDH1/2 mutations have been identified in AML, glioma, and many other cancers (22). Mutant IDHs dysregulate α-KG-dependent dioxygenases, such as TETs, EGLNs, collagen prolyl 4-hydroxylases, and histone demethylases (9–14). Because mutant IDH is an enzyme that acts via a mechanism that is completely different from those of other previously described oncogenes, it is expected to have potential as a therapeutic target. The IDH mutation occurs early in HSCs, and preleukemic cells engendered by the IDH mutation act as a reservoir for the evolution of AML (37, 38). IDH-mutated preleukemic cells display chemoresistance, thus making them potential initiators of relapsed disease. These reports point to the importance of eradicating IDH mutant-expressing cells for complete cure. Recent work demonstrated that selective inhibitors of mutant IDHs impair growth and induce differentiation of IDH mutant-expressing cells in vitro (16, 17). The results described herein clearly demonstrate that IDH mutations can play critical roles in oncogenesis in vivo. The deletion of mutant IDH2 from AML cells leads to the depletion of mutant IDH2-expressing AML stem cells.

To analyze the role of mutant IDH in oncogenesis, we established a mouse model of mutant IDH-mediated AML. As noted previously, IDH1/2 mutations in AML frequently occur simultaneously with mutations in other genes such as NPM (about 30% of IDH mutation-positive patients have the NPM mutation), DNMT3A (about 40%), and FLT3 (about 15%; ref. 39). In accordance with these observations, we found that IDH2/R140Q, NPMc, DNMT3A/R882H, and FLT3/ITD cooperatively induced AML in a mouse model (Fig. 2B–G). Our present data has shown that IDH2/R140Q is necessary for the engrafment or survival of NPMc+ cells in vivo. This is consistent with the previous reports showing that IDH1/2 mutations occur as preleukemic events, followed by NPM1 and FLT3 mutations as late proliferative events (37, 38).

NPMc and IDH2/R140Q cooperatively activated the Hoxa9/Meis1 pathway, and IDH2/R140Q activated the hypoxia pathway (Figs. 1B, 4D, and H–I). These two pathways are likely to be important for IDH2/R140Q-mediated engrafment/survival of NPMc+ cells in mice (Fig. 4A and B). In addition to IDH2/R140Q and NPMc, expression of DNMT3A/R882H and FLT3/ITD is also required for efficient induction of AML. DNMT3A/R882H further upregulated the expression levels of Meis1 (Fig. 4H). Furthermore, DNMT3A/R882H promoted the maintenance of cells in an undifferentiated state (Fig. 4I and J). Previous studies have shown that FLT3/ITD promotes cell growth and survival (40). Taken together, our results suggest that the activation of multiple signaling pathways is required for NIDF cells to induce AML (Fig. 5E).

By deleting floxed IDH2/R140Q from NIDF-induced AML mice, we found that AML cells could not expand in vivo (Fig. 3G). Furthermore, the number of AML cells expressing LSC markers in these mice decreased after deletion of IDH2/R140Q; however, the remaining AML cells continued to express myeloid lineage markers (Fig. 3I and Supplementary Fig. S2). After deletion of IDH2/R140Q from AML cells in our mouse model, the cells still expressed NPMc. NPMc has been found only in myeloid leukemia patients (26). These findings suggest that NPMc confers the myeloid phenotype on leukemia cells. It took time for the number of leukemia cells to decrease after deletion of IDH2/R140Q, suggesting that IDH2/R140Q is essential for the survival or inhibition of differentiation of LSCs, rather than for that of more differentiated cells (Fig. 3J). IDH2/R140Q-deleted AML cells showed defects in expansion in vivo and failed to induce AML after the third transplantation, indicating that LSCs were exhausted (Fig. 3I and K). In accordance with these results, deletion of IDH2/R140Q dramatically prolonged the survival of AML mice (Fig. 3E and F). IDH2/R140Q downregulated the 5hmC modification and expression of differentiation-inducing factors (Ebf1 and Spib). These data suggest that IDH2/R140Q-mediated repression of TET reduces 5hmC modification of Ebf1 and Spib. In T-cell development, 5hmC modification reportedly positively correlates with gene expression (41). It is possible that similar transcriptional control mechanisms operate in NIDF-AML cells. Importantly, when LSCs were depleted by IDH2/R140Q deletion, expression of Ebf1 and Spib was derepressed. It is highly probable that reactivation of these differentiation-inducing factors contributes to the differentiation of LSCs.

During the preparation of this article, four IDH mutant-mediated cancer model mice were reported (18–21). One previous report has shown that forced expression of Hoxa9 and the IDH1 mutant cooperatively induced MPN-like myeloid leukemia (18). This report is consistent with our results showing that NPMc increases the expression levels of Hoxa9 and cooperates with IDH2/R140Q to induce MPN-like disease. Another report has shown that introduction of an IDH2 mutant into FLT3-ITD knockin hematopoietic progenitor cells induced AML (19). Because FLT3-ITD knockin mice develop chronic myelomonocytic leukemia (42, 43), mutations have accumulated in FLT3-ITD knockin mice that effectively predispose them to develop AML by introducing the IDH2 mutations. Another report has shown that a xenograft model of IDH2-mutant cells was predisposed to develop sarcomas (20). The last report suggested that when the IDH2 mutant is expressed in a well-established surrogate AML model induced by Hoxa9 and Meis1 overexpression, the IDH2 mutant confers IDH2-mutant-addiction on the AML cells (21). This report suggests that the IDH mutant is still a good therapeutic target for cancer treatment, even if it is acquired during the late evolution of AML. In this study, we show very clearly that the function of IDH2/R140Q is critical for the maintenance of AML. This result strongly suggests that mutant IDHs are promising targets for anticancer therapy.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: Y. Ogawara, T. Katsumoto, H. Matsunaga, I. Kitabayashi
Development of methodology: Y. Ogawara, T. Soga, H. Matsunaga
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Ogawara, A. Iikawa, Y. Shima, Y. Kagiyaama, T. Soga, H. Matsunaga, T. Seki
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Ogawara, I. Kitabayashi
Writing, review, and/or revision of the manuscript: Y. Ogawara, I. Kitabayashi
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): I. Kitabayashi

Study supervision: K. Araki

References


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IDH2 and NPM1 Mutations Cooperate to Activate Hoxa9/Meis1 and Hypoxia Pathways in Acute Myeloid Leukemia

Yoko Ogawara, Takuo Katsumoto, Yukiko Aikawa, et al.

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