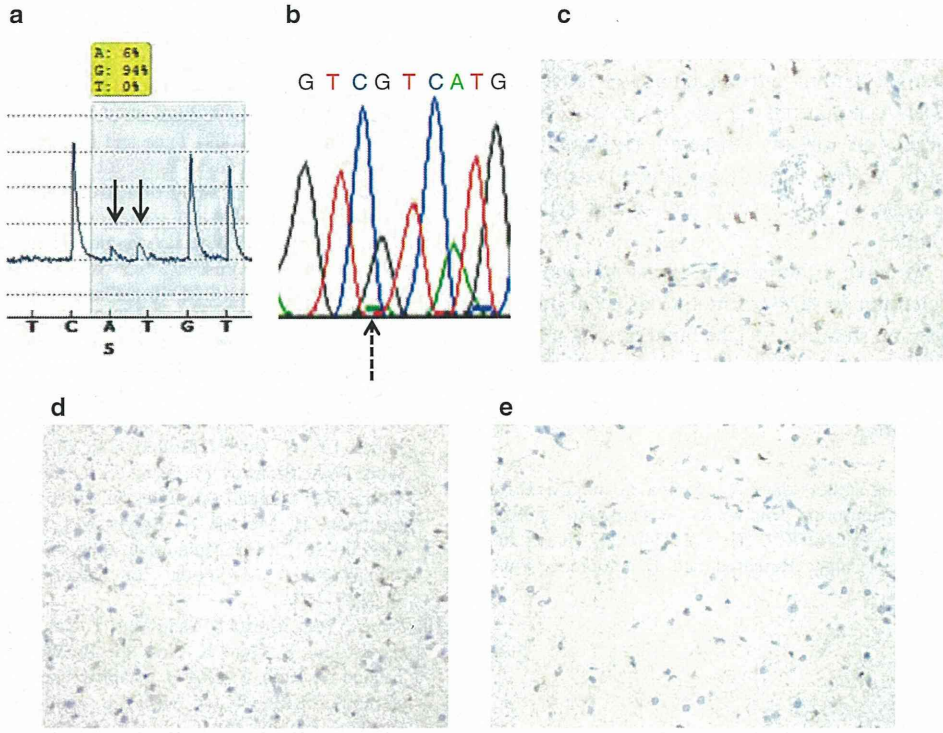

Fig. 4 The discordant case. A single case (DA068) showed discordance between pyrosequencing, Sanger sequencing and immunohistochemistry for *IDH1*. **a** The peaks indicating the R132H mutation (*arrows*) are shown in the pyrogram. The ratio of the mutant alleles was calculated to be 6.48 % by the AQ assay, suggesting the low density of tumor cells in this specimen. **b** A peak of adenine (*green*) at the hotspot (*broken arrow*) indicating the R132H mutation was obscure and hard to distinguish from non-specific peaks in the chromatogram. The cytoplasm of infiltrating tumor cells was positively stained in IHC using an antibody against the R132H mutation (original magnification ×400). The overall tumor cell content of this FFPE sample was low. Three separate areas with different tumor cell contents are shown (**c** the highest in this specimen, **d** intermediate, **e** low)

analysis depends on the design of the dispensation order, which determines the various types of mutation detectable by the assay. We successfully detected virtually all types of the known reported *IDH1/2* mutations using a single assay each for *IDH1* and *2*. Some of the previous studies applied more than one assay for this purpose [26]. Moreover, all mutations except one, *IDH2* R172K, are detected by the presence of mutation-specific peaks which are absent in the wild-type samples, making the assay highly sensitive. Not enough detailed information such as the dispensation order for example was provided in other previous studies [17]. With our novel assay, we set to achieve a fast and easy implementation aimed at the clinical setting.

We demonstrated the sensitivity and quantitativity of our assays by serial dilution analysis. It is a known fact that the sensitivity of conventional Sanger sequencing may be compromised by contamination of the samples with non-neoplastic tissues. Our results suggest that the conventional Sanger technique requires the presence of at least 20 % of the mutant alleles for the detection to be reliable [29]. By contrast, our assay detects as little as 5 % of the mutant alleles, and this sensitivity is comparable to previous studies [7, 26]. Glioma tissues often have a heterogeneous cell contents due to the invasive nature of the tumor into the surrounding brain tissue as well as its tendency to attract microglia infiltration [22]. The detection of *IDH1/2* mutations in samples containing a low percentage of tumor cells is challenging, but it is an absolute necessity for its clinical application. We showed that our pyrosequencing assays were applicable to FFPE samples, which are consistent with previous reports [6, 7, 26].

An optimal method for *IDH1/2* mutation testing remains under debate [28]. Some studies found that the results obtained by pyrosequencing were identical to those generated by Sanger sequencing [7, 26]. One study reported comparable frequencies of *IDH1/2* mutations detected between pyrosequencing, Sanger sequencing and IHC [17]. In the present study, the result of IHC was identical to that of pyrosequencing, while Sanger sequencing failed to detect mutations in three cases (4 % of *IDH1* mutated cases). The discordance between pyrosequencing and Sanger sequencing was attributable to the low tumor contents in each sample (see "Results"). Pyrosequencing thus provides higher sensitivity, robustness and throughput for

the analysis of *IDH1/2* mutation than Sanger sequencing [7]. Although the consistency between pyrosequencing and IHC needs further validation, pyrosequencing enables rapid screening for *IDH1/2* mutations (about 3–4 h) in a setting where DNA samples are already extracted. Ultimately, the optimal method for *IDH1/2* testing also depends on criteria such as the purpose of each study and/or the types of specimens available.

In summary, we have established a robust and sensitive assay for the detection of *IDH1/2* mutations. Our pyrosequencing assays are suitable for the analysis of a large number of samples, particularly if the samples are all simultaneously investigated such as in a large-scale retrospective clinical study.

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IDH2 and NPM1 mutations cooperate to activate Hoxa9/Meis1 and hypoxia pathways in acute myeloid leukemia

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IDH2 and NPM1 mutations cooperate to activate Hoxa9/Meis1 and hypoxia pathways in acute myeloid leukemia

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

YO designed the research, performed experiments, analyzed the data, and wrote the paper. TK and YS performed experiments. AY contributed to the construction of some plasmids. HM and TS measured 2-HG levels. TS performed metabolite analysis. IK designed the research, analyzed the data, and wrote the manuscript.

Abstract

IDH1 and IDH2 mutations occur frequently in acute myeloid leukemia (AML) and other cancers. The mutant 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 *nucleophosmin1* (*NPM1*)+/- hematopoietic stem/progenitor cells co-transduced with four mutant genes (*NPMc*, *IDH2/R140Q*, *DNMT3A/R882H*, and *FLT3/ITD*) which often occur simultaneously in human AML patients. Conditional deletion of *IDH2/R140Q* blocked 2-HG production and maintenance of leukemia stem cells, resulting in survival of the AML mice. IDH2/R140Q was necessary for the engraftment or survival of $NPMc^+$ cells *in vivo*. Gene expression analysis indicated that NPMc increased expression of Hoxa9. IDH2/R140Q also increased the level of Meis1 and activated the hypoxia pathway in AML cells. IDH2/R140Q decreased the 5hmC modification and expression of some differentiation-inducing genes (*Ebf1* and *Spib*). Taken together, our results indicated that IDH2 mutation is critical for the development and maintenance of AML stem-like cells, and they provided a preclinical justification for targeting mutant IDH enzymes as a strategy for anticancer therapy.

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) (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) (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 mice model was developed (18-21). These papers showed 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) (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.

Methods

Mice

C57BL/6 mice were purchased from CREA Japan (Tokyo, 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 animal facility according to 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, San Diego, CA, USA), 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 h after transfection. One-third volume of PEG solution (30% PEG-8000, 0.4 M NaCl, and 40 mM 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 BM (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 SCF, 10 ng/ml of IL-3, and 10 ng/ml of OSM). For serial infections, each virus was incubated with the cells for 12 h, 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), mHoxa9 (Mm00439364), mMeis1 (Mm00487664), mSpib (Mm03048233), and mTBP (Mm00446971). 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 a JSAN cell sorter (Bay Bioscience). The sorted cells were plated in methylcellulose medium (M3234, StemCell Technologies) supplemented with mouse cytokines (10 ng/ml of IL-3, 50 ng/ml of SCF, and 10 ng/ml of GM-CSF). After that, cells were cultured and re-plated every 7 days.

BM 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 BM cells from primary AML mice into C57BL/6 mice after irradiation (6 Gy). When cells were infected with floxed IDH2/R140Q, a second BM 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

BM 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

BM cells were pre-incubated 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, CD3ε, 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 bio-conjugated antibodies:

B lineage: CD19-PE

T lineage: CD3ε-Bio (sAvi-PE)

Erythroid lineage: CD71-PE and Ter119-Bio (sAvi-PE-Cy7)

Flow cytometry analysis was performed on a JSAN cell sorter (Bay bioscience), and the results were analyzed using FlowJo software (Tree Star).

2-HG analysis

Peripheral blood (PB) was taken from mice, and 50 μl was centrifuged at 15,000 rpm for 5 min. The supernatant was collected as plasma. Fifty microliters of PB was lysed with red-cell lysis buffer (0.83% NH_4Cl and 17 mM Tris [pH 7.65]), and the remaining white cells were collected. The volume of plasma and PB 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 PB 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), (CTTATACACGTGGCTTTTGGC) and (GCCGACACCAGACTAAGAAC).

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 BM cells untreated with TAM.

+TAM: Nf(I)DF-AML BM cells treated with TAM.

Control: normal BM cells.

IDH2/R140Q: IDH2/R140Q-infected BM cells.