Supporting Information

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SI Methods

Sample Preparation and Gas Chromatograph–Time-of-Flight MS Analysis. Each 100-μL aliquot of serum sample was added into a 1.5-mL tube, spiked with two internal standards (10 μL L-2-chlorophenylalanine in water, 0.3 mg/mL; 10 μL heptadecanoic acid in methanol, 1 mg/mL), then followed by the addition of 300 μL organic mixture (methanol:chloroform = 3:1, vol/vol) for protein precipitation. The mixture was vortexed for 30 s. After storing at −20 °C for 10 min, the samples were centrifuged at 9,391 × *g* for 10 min. Next, 300 μL supernatant was transferred to a 500-μL glass tube and dried under vacuum conditions. The dried materials were dissolved in 80 μL of methoxylamine hydrochloride (15 mg/mL, dissolved in pyridine) for 90 min at 30 °C and then derivatized with 80 μL N,O-bis-trimethylsilyl-trifluoroacetamide (containing 1% Trimethylchlo-rosilane) for 1 h at 70 °C. After the sample was vortexed for 1 min and kept at room temperature for 1 h, a 1-μL aliquot of the solution was injected into an Agilent 6890N gas chromatography coupled with a Pegasus HT time-of-flight mass spectrometer (Leco Corporation) in the splitless mode. A DB-5MS capillary column coated with 5% (vol/vol) diphenyl cross-linked 95% (vol/vol) dimethylpolysiloxane (30 m × 250 μm i.d., 0.25-μm film thickness; Agilent J&W Scientific) was used for separation. Both the injection temperature and the interface temperature were set to 260 °C, and the ion source temperature was adjusted to 200 °C. Initial gas chromatography oven temperature was set at 80 °C for 2 min after injection, and was raised up to 285 °C with 5 °C/min and maintained at 285 °C for 7 min. Helium at a flow rate of 1 mL/min was used as the carrier gas. The measurements were carried out with electron impact ionization (70 eV) in the full-scan mode (*m/z* 30–550). Metabolic data files were converted into the NetCDF format via DataBridge (Perkin-Elmer) and a pretreatment was conducted as previously described (1).

Serum 2-hydroxyglutarate (2-HG) was identified by library searching and confirmed by D-2-HG standard (Toronto Research Chemicals). The spiked 2-HG standard solution was diluted to appropriate concentration ranges for establishment of a calibration curve. The 2-HG concentration of each sample was determined from the calibration curve.

Gene-Expression Microarrays and Data Analysis. Total cellular RNA was extracted and purified using the RNeasy micro kit (Qiagen) according to the manufacturer's protocol. RNA quality was assessed with the use of RNA 6000 Nano assay on the Agilent 2100 Bioanalyzer (Agilent Technologies). Total RNA were amplified, labeled and purified by using GeneChip 3′IVT Express Kit (Affymetrix) and the manufacturer's instructions were followed to obtain biotin-labeled cRNA. Labeled cDNA was hybridized to Affymetrix Human U133 Plus2.0 GeneChip. Array hybridization and wash were performed using GeneChip Hybridization, Wash and Stain Kit (Affymetrix) in Hybridization Oven 645 (Affymetrix) and Fluidics Station (Affymetrix) by following the manufacturer's instructions. Slides were scanned by GeneChip Scanner 3000 (Affymetrix) and Command Control Software 3.1 (Affymetrix) with default settings. The quality of samples and assays were high as indicated by measures of the background (40.63 ± 1.94), the percentage of genes presented (44.71 ± 1.66), and the ratio of GAPDH 3′ to 5′ (2.12 ± 0.91). Raw data were background corrected, normalized, probe-specific background-corrected, and summarized to expression values by a MAS 5.0 algorithm (2) performed on R 2.15.0 (www.r-project.org). The genes with *t* test $P < 0.05$ and fold-change >2 were considered differentially expressed between the high and normal 2-HG groups.

Quantitative Real Time-PCR. The differentially expressed genes identified by gene-expression microarrays were verified by quantitative-PCR, which was performed using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) on an Applied Biosystems ViiA7 Real-Time PCR machine. Primers were designed using Primer Premier 5.0, or obtained from PrimerBank (http://pga.mgh.harvard.edu/primerbank/) and qPrimerDepot (http://primerdepot.nci.nih.gov/). The PCR experiments were performed in triplicate.

Allele-Specific PCR. We designed the allele-specific PCR (AS-PCR) primers with primer 5.0. We positioned with the 3′-terminal end at the mutation base in the AS forward primers. For each AS primer, a mismatch should be added within five bases of the 3′-terminal end. The reverse primers, designed like routine primers, are adjusted so that the melting temperature is between 50 °C and 65 °C and with no more than 5 °C. The length of PCR product should be within 100–300 base pairs. The PCR amplification method was as in Shen et al. (3). Primer sequences used in the AS-PCR are as follows (all primers listed as 5′–3′-terminal end, the mismatch bases are marked with lowercase letters): *isocitrate dehydrogenase 1* (IDH1)R132-R: CCACCA CCTTCTTCAAAGTTATGT; *IDH1*R132CGT > CAT-F: GGTAAAACCTATCATCATAGGgCA; *IDH1* R132CGT > AGT-F: GGGTAAAACCTATCATCATcGGTA; *IDH1*R132CGT > GGT-F: GGGTAAAA CCTATCATCATcGGTG; *IDH1*R132CGT > TGT-F: GGGTAAAACCTATCATCATcGGTT; *IDH2* R140CGG > CAG-F: AAAAGTCCCAATGGAACTATaCA; *IDH2*R140-R: CTGAGATGGACTCGT CGGTGTTG; *IDH2*R172AGG > AAG-F: CAAGCCCATCACCATTGGgAA; *IDH2*R72-R: CTGAGA TGGACT CGTCGGTGTTG.

DNA Methylation Microarrays and Data Analysis. ***Genomic DNA extraction and fragmentation.*** Genomic DNA (gDNA) was extracted and purified using DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's protocol. gDNA was quantified and quality assessed by NanoDrop ND-1000. gDNA of each sample was sonicated to ∼200–1,000 bp with a Bioruptor sonicator (Diagenode) on "Low" mode for 10 cycles of 30 s "ON" and 30 s "OFF." The gDNA and each sheared DNA were analyzed on agarose gel.

Immunoprecipitation. One-microgram of sonicated gDNA was used for immunoprecipitation using a mouse monoclonal anti–5-methylcytosine antibody (Diagenode). For this process, DNA was heat-denatured at 94 °C for 10 min, rapidly cooled on ice, and immunoprecipitated with 1 μL primary antibody overnight at 4 °C with rocking agitation in 400 μL immunoprecipitation buffer (0.5% BSA in PBS). To recover the immunoprecipitated DNA fragments, 200 μL of anti-mouse IgG magnetic beads were added and incubated for an additional 2 h at 4 °C with agitation. After immunoprecipitation, a total of five immunoprecipitation washes were performed with ice-cold immunoprecipitation buffer. Washed beads were resuspended in TE buffer with 0.25% SDS and 0.25 mg/mL proteinase K for 2 h at 65 °C and then allowed to cool down to room temperature. MeDIP DNA was purified using Qiagen MinElute columns (Qiagen).

Whole-genome amplification. The MeDIP-enriched DNA was amplified using a whole-genome amplification kit from Sigma-Aldrich [GenomePlex Complete Whole Genome Amplification

(WGA2) kit]. The amplified DNA samples were then purified with QIAquick PCR purification kit (Qiagen).

DNA labeling and array hybridization. The purified DNA was quantified using a NanoDrop ND-1000. For DNA labeling, the NimbleGen Dual-Color DNA Labeling Kit was used according to the manufacturer's guidelines detailed in the NimbleGen MeDIP-chip protocol (NimbleGen Systems). One-microgram of DNA of each sample was incubated for 10 min at 98 °C with 1 OD of Cy5-9mer primer (IP sample) or Cy3-9mer primer (Input sample). Next, 100 pmol of deoxynucleoside triphosphates and 100 U of the Klenow fragment (New England Biolabs) were added and the mix incubated at 37 °C for 2 h. The reaction was stopped by adding 0.1 volume of 0.5 M EDTA, and the labeled DNA was purified by isopropanol/ethanol precipitation. Microarrays were hybridized at 42 °C for 16–20 h with Cy3/5 labeled DNA in NimbleGen hybridization buffer/hybridization component A in a hybridization chamber (Hybridization System; NimbleGen Systems). Following hybridization, washing was performed using the NimbleGen Wash Buffer kit (NimbleGen Systems). For array hybridization, Roche NimbleGen's HG19 Meth 2.1M CpG plus Promoter array was used, which is a single-array design containing 27,867 University of California at Santa Cruz-annotated CpG Islands and all RefSeq gene promoter regions (from approximately −8 kb to +3 kb of the transcription start site) totally covered by ∼2.1 M probes.

Data normalization and analysis. Raw data were extracted as pair files by NimbleScan software. We performed median-centering, quantile normalization, and linear smoothing by Bioconductor packages Ringo, limma, and MEDME (cran.r-project.org). After normalization, normalized $\log_2$-ratio data were created for each sample. From the normalized $\log_2$-ratio data, a sliding-window peak-finding algorithm provided by NimbleScan v2.5 (Roche-NimbleGen) was applied to find the enriched peaks with specified parameters (sliding-window width: 750 bp; mini probes per peak: 2; *P* value minimum cutoff: 2; maximum spacing between nearby probes within peak: 500 bp). The identified peaks were mapped to genomic features: transcripts, CpG Islands, and miRNAs. To avoid technical variability and evaluate methylation differences between samples, the $\log_2$-ratio from raw data value should be normalized. Correlation analysis of gene expression and DNA methylation was conducted by R gplots package (cran.r-project.org).

Statistical Methods. Prognosis-related factors examined for cytogenetically normal acute myeloid leukemia (CN-AML) in this study as covariates were age, white blood cells (WBC), percentage of bone marrow (BM) blasts, and gene mutations of *FLT3*-ITD, *NPM1*, *CEBPA*, *MLL* partial tandem duplication (*MLL*-PTD), *DNMT3A*, and *IDH1/2*. Chemotherapy consolidation by stem cell transplantation (BM transplantation) was not considered as one of the covariates. Because of small number of the patients ($n = 5$) who received BM transplantation, the follow-up time was censored at the time of BM transplantation for survival analysis.

The 2-HG distribution was not normal (Fig. 1*B*) in AML. The cutoff value of 2-HG was based on the mean and SD estimated from 405 healthy control individuals with appropriate age groups and sex ratio compared with AML patients, and then applied to the 367 AML cases for a comparison of clinical and laboratory features between the high and normal 2-HG groups, thus identifying potential prognostic confounders. Our attention was focused on CN-AML for survival analysis. Stratified analysis was performed to detect the confounding, and tests for interaction were also conducted between 2-HG and other factors. Multivariate logistic-regression models were constructed to analyze risk factors related to the probability of achieving complete remission (CR). Multivariable Cox proportional-hazard models were constructed to adjust for established clinical prognostic factors, including age, WBC, BM blasts, and treatment protocols, as well as presence or absence of *IDH1/2* mutations and five prognosis-related gene mutation markers (*FLT3*-ITD, *NPM1*, *CEBPA*, *MLL*-PTD, *DNMT3A*). For achievement of CR, 2-HG level was not significant in the multivariate model with adjustment of the above covariates. For the Cox models, the proportional-hazards assumption was checked for each variable individually.

Treatment Protocols for CN-AML. Three groups of CN-AML patients received their different treatment protocols as follows: 27 received either HAA (homoharringtonin, 2 mg·m^2·d for 7 d; cytarabine, 100 mg·m^2·d for 7 d; and aclarubicin, 20 mg·m^2·d for 5 d) or HDA (the same as HAA except for the replacement of aclarubicin with daunorubicin, 40 mg·m^2·d for 3 d) protocols; 146 patients received a standard DA (daunorubicin, 40 mg·m·/d for 3 d; cytarabine, 100 mg·m^2·d for 7 d) regimen; the remaining 61 senile patients were treated in an individual manner consisting of low-dose priming regimen of CAG (aclamycin, 12 mg/m^2 days 1–4; Ara-C 15 mg/m^2 once every 12 h, days 1–14; G-CSF, 200 μg/m^2 days 1–14) or a DA-like regimen. The chemotherapy consolidation was as described previously (3).

1. Mardis ER, et al. (2009) Recurring mutations found by sequencing an acute myeloid leukemia genome. *N Engl J Med* 361(11):1058–1066.
2. Gautier L, Cope L, Bolstad BM, Irizarry RA (2004) affy—Analysis of Affymetrix GeneChip data at the probe level. *Bioinformatics* 20(3):307–315.
3. Shen Y, et al. (2011) Gene mutation patterns and their prognostic impact in a cohort of 1185 patients with acute myeloid leukemia. *Blood* 118(20):5593–5603.

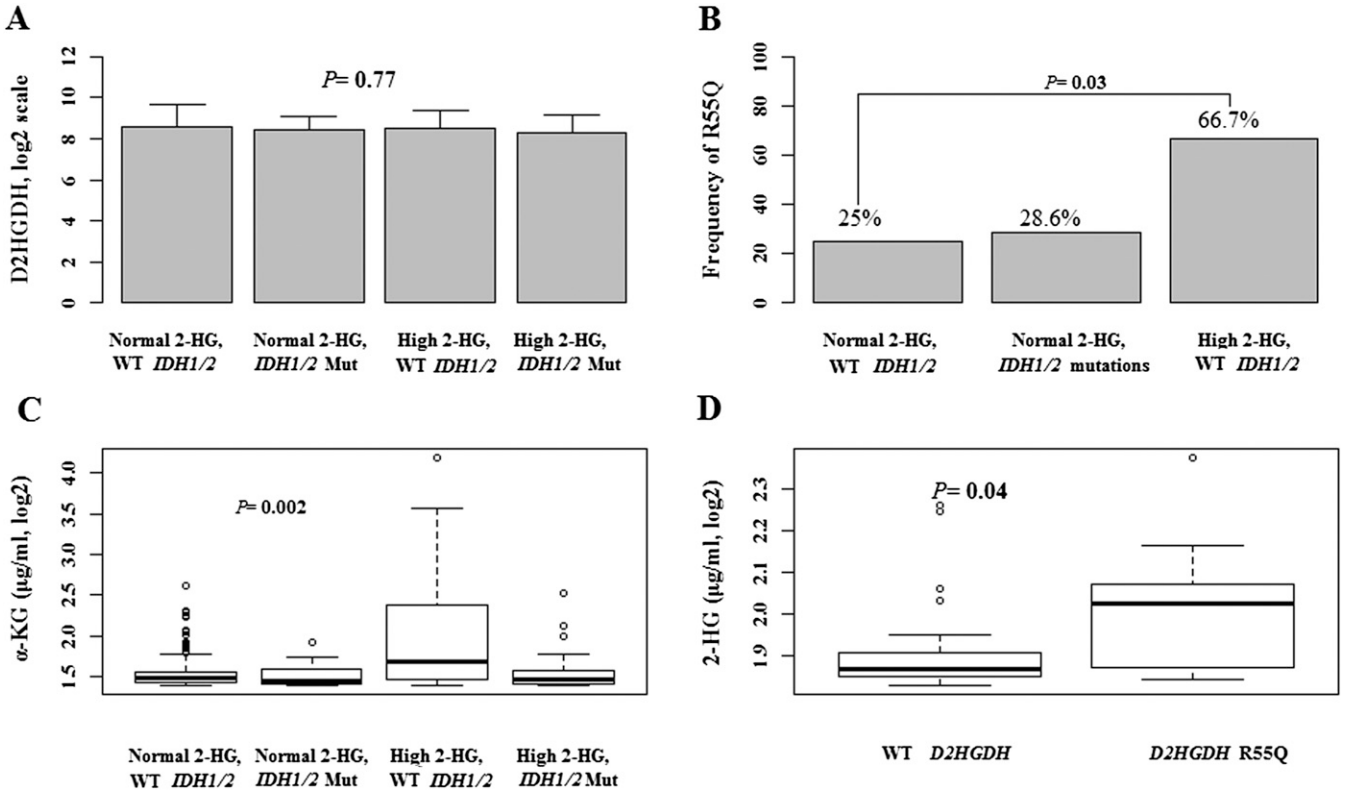

Fig. S1. The relationship between 2-HG levels and *IDH1/2* mutations or *D2HGDH* R55Q. No differences of serum D2HGDH were observed in patients with normal 2-HG and wild-type *IDH1/2*, with normal 2-HG and *IDH1/2* Mut (mutations), or with high 2-HG in the presence or absence of *IDH1/2* mutations status (*A*). The frequency of SNPs of 2-HG dehydrogenase (*D2HGDH*) was more common in patients with high 2-HG but wild-type *IDH1/2* (*B*). α-Ketoglutarate (α-KG) levels in patients with normal 2-HG and wild-type *IDH1/2*, with normal 2-HG and *IDH1/2* Mut (mutations), or with high 2-HG in the presence or absence of *IDH1/2* mutations status (*C*). The 2-HG level in AML with *D2HGDH* R55Q was significantly higher compared with cases with wild-type *D2HGDH* (*D*).

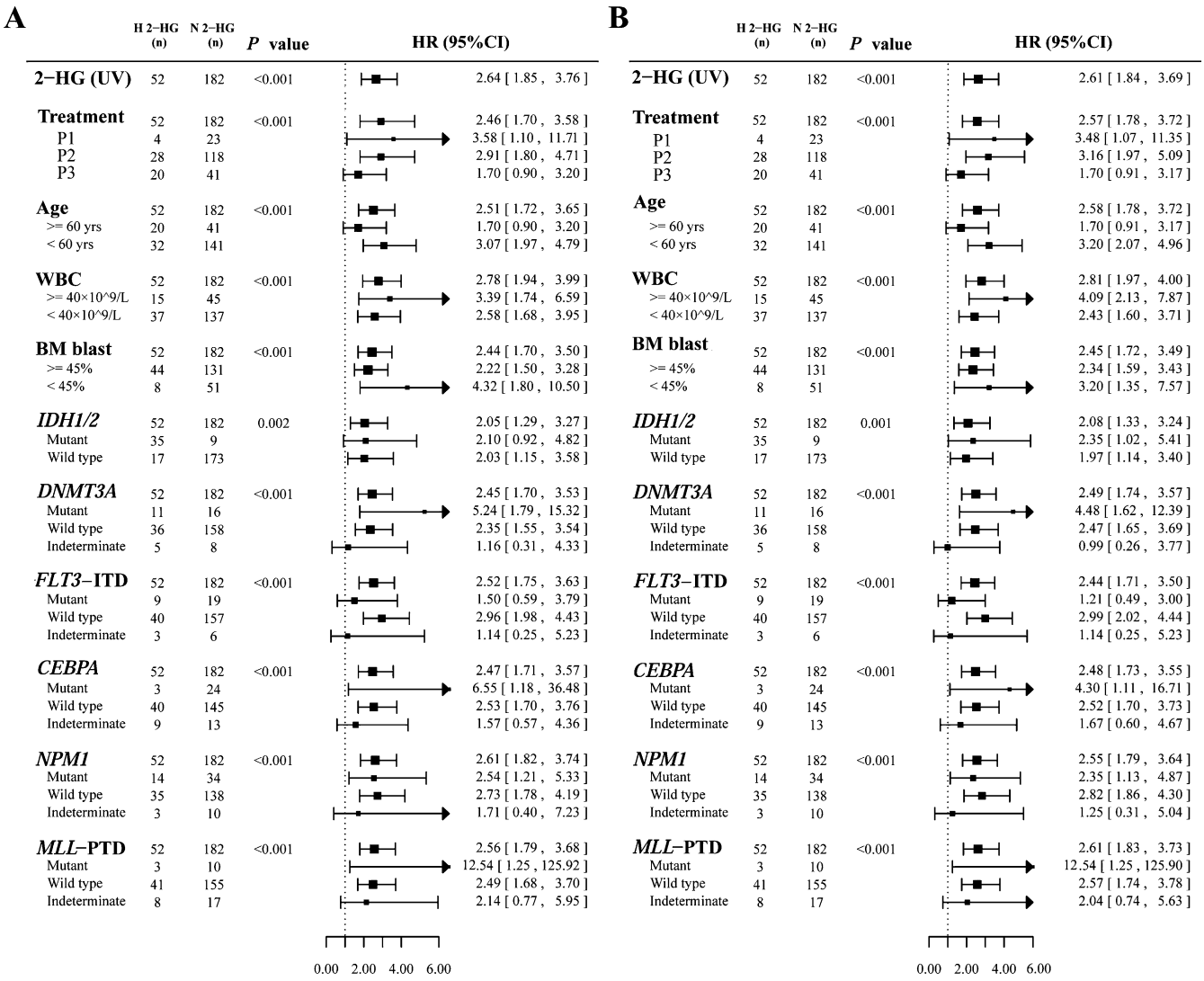

Fig. S2. Hazard ratios (HR) of high 2-HG on overall survival (OS) (*A*) and event free survival (EFS) (*B*) by stratified analysis, respectively. H 2-HG, high 2-HG; N 2-HG, normal 2-HG. Treatment: P1, homoharringtonin-based treatment; P2, DA regimen (Daunorubicin, 45 mg·m^2·d); P3, elderly CN-AML patients who received individualized treatment. Note the *P* values of interaction between altered levels of 2-HG and the other factors are all more than 0.1 (not indicated on the figure). Adjusted "*P* value" of stratification analysis less than 0.05 for the adjusted HR, which is in line with the *P* value for crude HR, indicates this factor was not a significant confounder. In other words, adjustment does not significantly alter the interpretation of the HR of 2-HG. 2-HG (UV) indicates 2-HG in survival univariate analysis (UV) with estimated HR (95% confidence interval) for OS (*A*) or EFS (*B*). On stratifying analyses, nonnormal distribution parameters of BM blasts and WBC were dichotomized based on the first quartile of WBC and third quartile of BM blasts, respectively. For age variable, patients were classified into younger and older groups based on the age in 60 y.

Other Supporting Information Files

Table S1 (DOCX)
Table S2 (DOCX)
Table S3 (DOCX)
Table S4 (DOCX)
Table S5 (DOCX)
Table S6 (DOC)
Table S7 (DOCX)