HOXA11 plays critical roles in disease progression and response to cytarabine in AML

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Abstract. Lysine methyltransferase 2A (*KMT2A*, also known as *MLL*) translocations (*MLL*-t) are frequently associated with mutations in *RAS* pathway genes in acute myeloid leukemia (AML). Previous findings with a mouse model showed that cooperation of *MLL/AF10* with tyrosine-protein phosphatase non-receptor type 11 (*PTPN11*)^{G503A} accelerated leukemia development, but increased cytarabine (Ara-C) sensitivity of leukemia cells. To identify the genes responsible for reduced survival and Ara-C resistance, transcriptomic profiling between six pairs of mouse *MLL/AF10(OM-LZ)* leukemia cells harboring activating and wild-type *KRAS* or *PTPN11* was compared. A total of 23 differentially expressed genes (DEGs) with >1.5-fold-change between the paired cell lines were identified. The Gene Ontology (GO) terms overrepresented in these 23 DEGs included 'immune system process', 'actin filament binding', 'cellular response to interferon-alpha' and 'sequence-specific DNA'. Among the four genes (*Hoxa11*, PR domain zinc finger protein 5, Iroquois-class homeodomain protein IRX-5 and homeobox protein PKNOX2) mapped to the GO term 'sequence-specific DNA', *HOXA11* upregulation was associated with AML harboring *MLL*-t and *RAS* signaling mutations based on a meta-analysis using data deposited in Oncomine™ and analysis of the clinical samples in the present study. Microarray data revealed that only *Hoxa11* was upregulated in those cells harboring activating *PTPN11*. Functional studies of *Hoxa11* knockdown or overexpression in *MLL/AF10(OM-LZ)* cells revealed that *Hoxa11* expression levels were associated with survival *in vivo* and Ara-C sensitivity/apoptosis *in vitro*. In addition, *Hoxa11* regulated the expression of the apoptosis-related genes, NF-κB inhibitor α, transcription factor p65 and transformation-related protein p53. Furthermore, the results of a meta-analysis using Heuser's AML dataset supported the finding that chemotherapy responders have higher expression levels of *HOXA11*. These results indicated that the expression of *HOXA11* increased cell apoptosis and predicted an improved response to Ara-C in AML.

Introduction

Rearrangements of lysine methyltransferase 2A (*KMT2A*, also known as *MLL*) gene at chromosome 11q23 account for ~10% of all acute leukemia (AL) cases, but are also present in most infant ALs and therapy-associated acute myeloid leukemia (AML), which were previously treated with topoisomerase II inhibitors for other cancers (1).

Although >94 fusion partner genes have been found to fuse with *MLL*, *AF4* (*AFF1*), *AF9* (*MLLT3*), *ENL* (*MLLT1*), *AF10* (*MLLT10*), *ELL* and *AF6* (*MLLT4*) are the most frequent fusion partners found in Als (2). *MLL* translocations (*MLL*-t) confer a poor prognosis in AL, especially *MLL/AF6* and *MLL/AF10* in AML (3,4). *MLL*-t alters MLL methyltransferase activity and leads to dysregulation of *MLL* downstream genes, such as *HOXA7-A10*, which subsequently impairs hematopoietic lineage commitment and induces leukemia development (5,6). In addition to *Hoxa7-Hoxa10* genes, sustained *Hoxa11* expression has been detected in the *MLL/ENL* immortalized myeloid cell line (7). Chromosomal translocation t(7;11)(p15;p15) encoding *NUP98/HOXA11* fusion has been recurrently detected in chronic myeloid leukemia and juvenile myelomonocytic leukemia (8,9). *HOXA11*, *HOXA10*, *HOXA7* and *HOXA4* are downregulated during monocyte-macrophage differentiation in a human leukemic THP-1 cell line (10). In

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contrast to *HOXA7-HOXA10*, the leukemogenic potential of *HOXA11* is not well characterized. In addition to participating in leukemogenesis, the *HOX* family of genes are involved in organ development. In a homeobox swap experiment, it was discovered that *Hoxa10* could partially replace the role of *Hoxa11* in regulating skeletal phenotypes and reproductive tract development (11). However, whether these different *Hoxa* genes are functionally interchangeable or complementary in leukemogenesis is not clear.

Cases of AL with *MLL*-t are frequently found to harbor *RAS* pathway mutations, including *N-/K-RAS* and tyrosine-protein phosphatase non-receptor type 11 (*PTPN11*) activating mutations. The mutation rate of *KRAS* ranges from 7.2~42.4%, whereas that for *NRAS* is 5.3~24.7% and that for *PTPN11* is 1~4.8% (12-15). The impact of *RAS* pathway mutations on *MLL*-t AL is controversial. This is likely due to varied mutant allele frequencies of *RAS* pathway mutations in patients (16,17). We and others have established mouse models with results supporting that cooperation of *MLL*-t with activating *N-/K-RAS* or *PTPN11* mutations accelerate leukemia progression (18-22). Activating *N-/K-RAS* mutations constitutively activate the downstream signaling cascades controlling cell proliferation, apoptosis, differentiation and cell cycle progression (23). Activating *PTPN11* mutations can induce myeloid cell hypersensitivity to growth factors, including granulocyte monocyte-colony stimulating factor and interleukin (IL)-3, and enhance cell cycle progression (22,24,25). We also previously demonstrated that cooperation of *MLL*-t with activating *PTPN11* mutations increased cytarabine (Ara-C) sensitivity in leukemia cells (22).

The underlying mechanism and key downstream players that accelerate leukemia development and Ara-C sensitivity by cooperating mutations have not been clearly illustrated. Thus, in the present study, transcriptomic profiles were compared between mouse *MLL/AF10(OM-LZ)* leukemia cells carrying wild-type and activating *KRAS* or *PTPN11* to identify differentially expressed genes (DEGs) involved in survival and drug sensitivity. One such upregulated DEG, *Hoxa11*, was further investigated to characterize its roles in leukemia cell differentiation, proliferation, survival and Ara-C sensitivity.

Materials and methods

Cell culture. The mouse *MLL/AF10(OM-LZ)* leukemia cell line (12G) and *MLL/AF10(OM-LZ)* cells harboring wild-type or activating *KRAS* (*KRAS^{G12C}*) and wild-type or activating *PTPN11* (*PTPN11^{G503A}*) were generated by the retroviral transduction of genes to 5-fluorouracil (5-FU)-enriched C57BL/6J (B6) mouse bone marrow (BM) cells. The mice were purchased from the National Laboratory Animal Center. These different cell types were either generated in the current study (AKw1G) or in previous studies (AK2G, AK3G, APw1 and APm1) (21,22,26) (Fig. 1A). All of these cell lines expressed the myelomonocytic markers. Mouse leukemia cells were cultured in the RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) with 20% fetal bovine serum (Hyclone; Cytiva), 2 mM L-glutamine, 100 μM 2-mercaptoethanol and 10 ng/ml IL-3 (R&D Systems, Inc.) for maintenance and proliferation analysis.

Microarray analysis and Gene Ontology (GO) enrichment analysis. The total RNA of leukemia cells was prepared using TRIzol® reagent (Gibco; Thermo Fisher Scientific, Inc.). The RNA was amplified, labeled and hybridized to the mouse genome 430A Array chip (12G vs. AK3G and 12G vs. AK2G), 430 2.0 Array chip (12G vs. AK2G and APw-1 vs. APm-1), or Clariom D Array chip (APw-1 vs. APm-1 and AKw1G vs. AK3G) (Affymetrix; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions (this procedure was performed by staff of the Genomic Medicine Research Core Laboratory at Chang Gung Memorial Hospital, Linkou, Taiwan). Microarray data are available at the NCBI Gene Expression Omnibus (GEO) website (https://www.ncbi.nlm.nih.gov/geo/; accession nos. GSE82156 and GSE134586) or can be downloaded from Chang Gung University website (21). Differential Expression Analysis was performed using Transcriptome Analysis Console software version 4.0 (Affymetrix; Thermo Fisher). A heat map was obtained using Cluster version 3.0 (http://bonsai.hgc.jp/~mdehoon/software/cluster/) and Java TreeView version 1.1.6r4 (http://jtreeview.sourceforge.net). DEGs with >1.5-fold-change in paired *MLL/AF10* leukemia cells harboring wild-type and oncogenic *KRAS* or *PTPN11* were then functionally annotated with GO enrichment analysis using online Database for Annotation, Visualization and Integrated Discovery (DAVID) version 6.8 annotation tools (https://david.ncifcrf.gov/). Statistical significance was evaluated using Fisher's exact test and corrected by Bonferroni correction for multiple testing. The GO 'Molecular Function' (MF) or 'Biological Process' (BP) categories with P<0.05 were considered statistically significant.

Validation of HOXA11 expression in patients with AML. To validate the differential expression and drug responsiveness of *HOXA11* in *MLL*-t AML, a meta-analysis was performed using the Oncomine™ database (https://www.oncomine.org/), including Valk leukemia, Wouters leukemia, Balgobind leukemia, and Haferlach leukemia (27-30).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). To evaluate the expression levels of target genes, the total RNA of mouse leukemia cells or AML patient BM cells was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher). The total RNA was reverse transcribed into complementary DNA (cDNA) using random hexamers and SuperScript™ II reverse transcriptase (Thermo Fisher) according to the manufacturer's protocol. qPCR was performed using SYBR-Green PCR master mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) and analyzed by ABI Prism 7900 system (Applied Biosystems; Thermo Fisher Scientific, Inc.). Primer sets were as follows: Mouse *Hoxa11*, 5'-GAAAACCTCGCTTCCTCCGA-3' and 5'-ATAAGGGCA GCGCTTTTTGC-3'; mouse NF-κB inhibitor α (*Nfkbia*), 5'-GAGACCTGGCCTTCCTCAAC-3' and 5'-TCTCGGAGC TCAGGATCACA-3'; mouse transcription factor p65 (*Rela*), 5'-TGGCTACTATGAGGCTGACCT-3' and 5'-TGGTCTGGA TTCGCTGGCTA-3'; mouse transformation-related protein p53 (*Trp53*), 5'-CCTCTCCCCCGCAAAAGAAA-3' and 5'-GGCCCTTCTTGGTCTTCAGG-3'; mouse *Gapdh*, 5'-TTC ACCACCATGGAGAAGGC-3' and 5'-GGCATGGACTGT GGTCATGA-3'; human *HOXA11*, 5'-CGTCTTCCGGCC ACACTGA-3' and 5'-AGACGCTGAAGAAGAACTCCC-3';
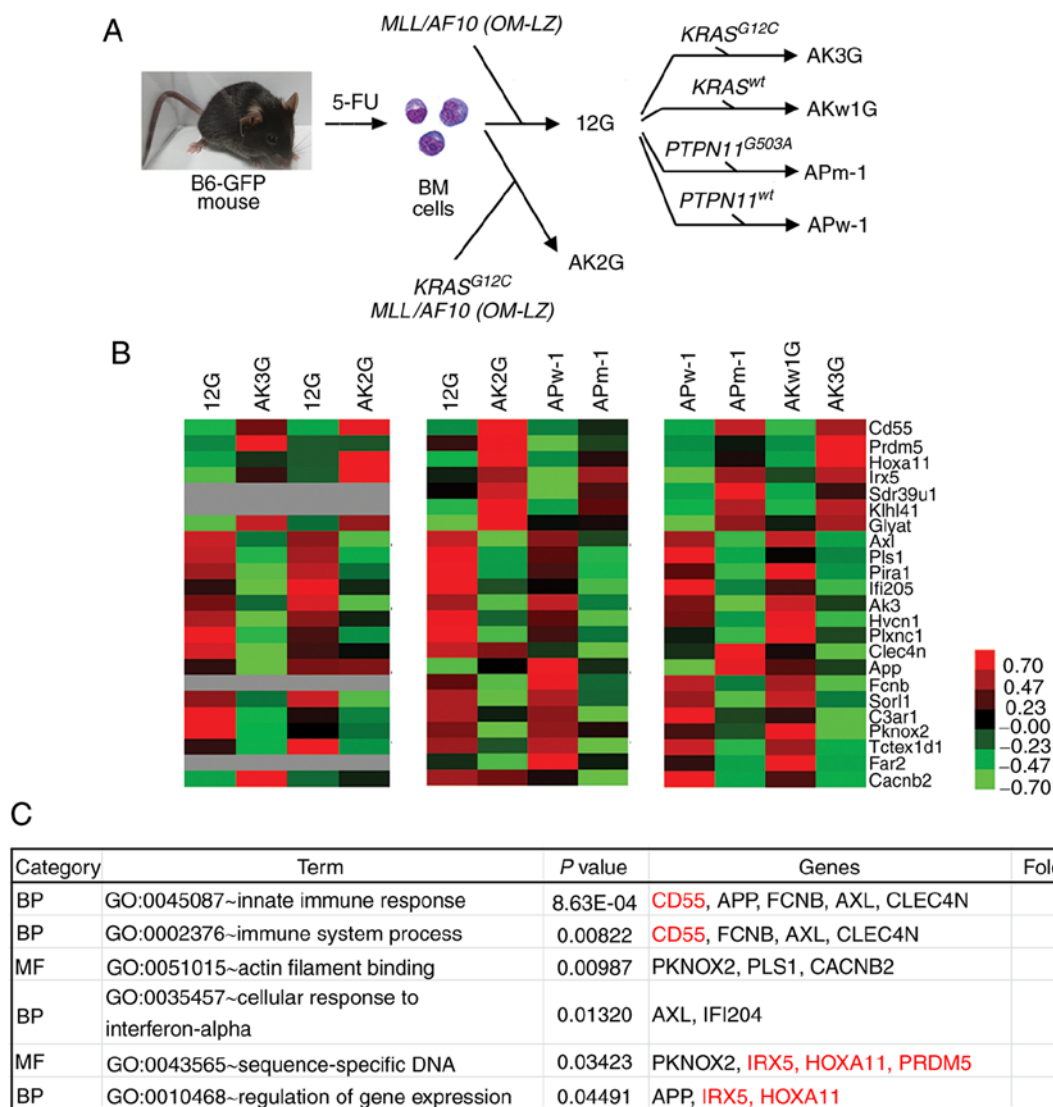
Figure 1. Identification of DEGs between paired *MLL/AF10(OM-LZ)* leukemia cells harboring wild-type and *RAS* pathway mutations. (A) Establishment of immortalized cell lines by retroviral transduction of 5-FU-enriched bone marrow cells with *MLL/AF10(OM-LZ)* alone (12G) or in combination with wild-type *KRAS* (AKw1G) or oncogenic *KRAS*G12C (AK2G, AK3G), and wild-type *PTPN11* (APw-1) or oncogenic *PTPN11*G503A (APm-1). (B) Heat map representing relative gene expression levels of 23 DEGs between paired cell lines based on cDNA microarray data. Different Affymetrix chips were used for the following paired cell lines: 12G vs. AK3G and 12G vs. AK2G (430A); 12G vs. AK2G and APw-1 vs. APm-1 (430_2), APw-1 vs. APm-1 and AKw1G vs. AK3G (Clariom D). Raw values were log2-transformed and centered relative to the median. A heat map was obtained using Cluster version 3.0 and Java TreeView version 1.1.6r4. The color bar depicts the color contrast level of the heat map, in which red and green indicates high and low expression, respectively. Grey indicates genes absent in the Affymetrix 430A chip. (C) Enriched GO terms of 'BP' and 'MF' for the 23 identified DEGs. All GO terms listed in the table show significant enrichment (all P<0.05). Red and black indicate genes that are upregulated or downregulated, respectively, in *MLL/AF10* cell lines harboring *RAS* pathway mutations. DEGs, differentially expressed genes; MLL, lysine methyltransferase 2A; GO, Gene Ontology; BP, biological process; MF, molecular function; *PTPN11*, tyrosine-protein phosphatase non-receptor type 11; BM, bone marrow; 12G, cells with *MLL/AF10(OM-LZ)* alone; AK3G, cells with *MLL/AF10(OM-LZ)* and oncogenic *KRAS*G12C; AKw1G, cells with *MLL/AF10(OM-LZ)* and wild-type *KRAS*; APw-1, cells with *MLL/AF10(OM-LZ)* and wild-type *PTPN11*; APm-1, cells with *MLL/AF10(OM-LZ)* and oncogenic *PTPN11*G503A; *PTPN11*, tyrosine-protein phosphatase non-receptor type 11.

and human *ABL*, 5'-TGGAGATAACACTCTAAGCATAAC TAAAGG-3' and 5'-GATGTAGTTGCTTGGGACCCA-3'. The thermocycling conditions were as follows: 95℃ for 2 min; then 40 cycles of 95℃ for 15 sec and 65℃ for 30 sec. The gene expression levels of mouse genes and human *HOXA11* were normalized against the housekeeping genes *Gapdh* and *ABL*, respectively. Fold-change was calculated using the $2^{-\Delta\Delta Cq}$ method (31). In the present study, leftover BM samples of clinical examination for initial diagnostic work-up of AML were used for gene expression analysis. Samples were obtained from patients admitted to Chang Gung Memorial Hospital (Taipei, Taiwan) between January 2002 and December 2010.

The inclusion criteria were as follows: Adults and children with AML with *MLL*-t. The exclusion criteria were as follows: Non-*MLL*-t AML specimens. Among the 114 cases with *MLL*-t AML (56 men and 58 women, age range 0-84 years, median 27 years), it was determined that eight cases with *MLL/AF10* or *MLL/AF9* had sufficient remaining specimens available for *HOXA11* expression level analysis. Of these eight cases, five had *KRAS* mutations and one had a *PTPN11* mutation.

Western blot analysis. Total cell lysate from 5x10⁶ leukemia cells was prepared by direct lysis of cells with RIPA buffer [20 mM Tris-Cl (pH 7.5) 150 mM NaCl, 1% Triton X-100,

1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 0.5 mM PMSF]. The amount of total protein was assayed using a Pierce® BCA Protein Assay (Pierce; Thermo Fisher Scientific, Inc.). The lysates (20 μg/lane) were electrophoresed on 10% polyacrylamide gel, and subsequently transferred to an Immobilon membrane (EMD Millipore). The membrane was blocked in 5% bovine serum albumin at 4˚C (Sigma-Aldrich; Merck KGaA) for 1 h, and then incubated with primary antibodies at 4˚C overnight against mouse Hoxa11 (1:5,000; cat. no. NBP1-80228; Novus Biologicals, Ltd.), β-actin or Gapdh (1:10,000; cat. nos. sc-47778 or sc-32233; Santa Cruz Biotechnology, Inc.), followed by incubation for 2 h at room temperature with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG secondary antibodies (1:5,000; cat. nos. C04001 or C04003; Croyez Bioscience Co., Ltd.). Western blots were developed with a Western Lightning Plus ECL kit (PerkinElmer, Inc.) and the images were visualized by Analytik Jena™ UVP ChemStudio PLUS and VisionWorks™ software (version 9.0; Analytik Jena US LLC).

In vivo leukemogenesis. The *in vivo* leukemogenic potential of the leukemia cell lines was determined by BM transplantation assay using male B6 mice (n=45, age, 6-8 weeks; weight, 20-24 g). Mice were maintained in pathogen-free devices under a controlled animal housing conditions (temperature 20±3˚C, humidity 60-70% with a 12-h light /dark cycle and access to food and water *ad libitum*). Briefly, leukemia cells (APm1-shV, APm1-shH11-2, 12G-V, and 12G-H11) were intraperitoneally (i.p.) injected into mice (1x10^6 cells/100 μl 1X PBS/mouse; n=10 mice for each leukemia cell line) that had received a sublethal dose of γ-irradiation (5.25 Gy) unless otherwise stated. Mice i.p. injected with normal saline (n=5) served as controls. To monitor leukemia development, peripheral blood (100 μl) was collected weekly for cytologic analysis and complete blood count measurement using Hemavet 950 (Drew Scientific; Erba Diagnostics, Inc.) or BC-5000 (Mindray Medical International Limited) hemocytometers. Mice were sacrificed when moribund (7-15 weeks post-injection). The moribund state was defined as mice displaying leukocytosis, with hunched posture, weakness, shortness of breath and 20% weight loss. Mice were euthanized by an i.p. injection of Zoletil (50 mg/kg) and Rompun (xylazine, 10 mg/kg) or by inhalation of isoflurane (3-5%), followed by cervical dislocation (32). BM, peripheral blood, ascites, organs and tumor masses were collected and weighed.

Gene knockdown by short hairpin RNA (shRNA). To generate stable gene knockdown cell lines, AK3G or APm-1 cells were infected with lentivirus expressing shRNA against *Hoxa11* [The RNAi Consortium (cat. nos. TRCN0000413738 and TRCN0000417739)] at a multiplicity of infection of 1 and selected in RPMI-1640 medium containing puromycin (2.5 μg/ml) for a total of 2 weeks. A third generation system was used. Cells stably transfected with blank lentiviral vector pLKO_025 were used as negative controls. All lentiviruses were obtained from the National RNAi Core Facility at the Institute of Molecular Biology/Genomic Research Center, Academia Sinica (Taiwan).

Ectopic expression of Hoxa11. The full-length *Hoxa11* gene (~1 Kb) was amplified from cDNA of AK3G cells by PCR

using the following primers: 5'-GAAGATCTCCCAAGGTAG CCCAATGATG-3' and 5'-CCGCTCGAGCCAGTAGGC TGGAGCCTTAG-3'. The PCR product was digested with restriction enzymes *Bgl*II and *Xho*I, and was subsequently cloned into the *Bgl*II and *Xho*I sites of the retroviral vector pMSCVpuro (Clontech Laboratories, Inc.). The fidelity of nucleotide sequences of the *Hoxa11* gene was confirmed by Sanger sequencing. Plasmid DNAs of pMSCV-*Hoxa11* and pMSCVpuro were transfected into EcoPack2-293 cells (Invitrogen; Thermo Fisher Scientific, Inc.) to package retroviruses. Viral titer was determined by infection of NIH/3T3 (American Type Culture Collection; ATCC® CRL-1658™), a murine fibroblast cell line, with serial diluted supernatant to generate puromycin-resistant colonies. To generate 12G cell lines with ectopic expression of *Hoxa11* (12G-H11-1 and 12G-H11-2), 12G cells were mixed with retroviruses at a 1:1 ratio and selected in RPMI complete medium containing puromycin (2.5 μg/ml) for a total of 2 weeks. The cells transduced with MSCVpuro retroviruses were used as negative controls (12G-V1 and 12G-V3). The presence of *Hoxa11* gene was confirmed by PCR amplification of the 1-Kb product from the genomic DNA of 12G-H11 cell lines using cloning primers. The fidelity of nucleotide sequences of *Hoxa11* was confirmed by Sanger sequencing.

Phenotypic and Ara-C resistance analyses. For cytologic analysis, cells were cytospinned at 700 x g for 3 min or smeared, air-dried, and stained with Liu reagents (Tonyar Biotech, Inc.) at room temperature. For immunophenotypic analysis, cells were stained at 4˚C for 15 min with phycoerythrin-macrophage-1 antigen (Mac-1), phycoerythrin-CD115 and allophycocyanine-Ki-67 antibodies (cat. nos. RM2804-3, 12-1152-82 and 17-5698-82; eBioscience; Thermo Fisher) followed by flow cytometric analysis using FACSCanto II Cell Analyzer and FACSDiva software version 5.0 (BD Biosciences). For cell proliferation analysis, cells were assessed at indicated time points using Cell Counting Kit-8 (CCK-8/WST-8; Dojindo Molecular Technologies, Inc.) according to the manufacturer's instructions. To determine Ara-C resistance, cells were cultured in RPMI-1640 complete medium and a gradient concentration of Ara-C (0, 128, 320, 640, 1,600, 3,200, 16,000 or 40,000 ng/ml) for 24 h. Cell viability was measured using CCK-8 (incubation time 2 h). To determine apoptotic cell rate, cells were treated with Ara-C (0 or 160 ng/ml) for 24 h, followed by Annexin-V/propidium iodide (Sigma-Aldrich; Merck KGaA) staining in the dark and flow cytometric analysis using a FACSCanto II Cell Analyzer and FACSDiva software version 5.0 (BD Biosciences).

Competitive engraftment and clonal expansion assay. The competitive engraftment and clonal expansion assay was described previously (22,33). Briefly, paired cells (AK3G-shV vs. AK3G-shH11 and 12G-V vs. 12G-H11) were mixed at a ratio of 1:1 and then i.p. injected into B6 mice (1x10^6 cells/mouse). The mice were sacrificed at days 43 and 57. Mice BMs and spleens were collected and used to extract genomic DNA. To amplify 300-bp DNA fragments of the pMSCVpuro vector (from 12G-V) or the region spanning the pMSCVpuro-*Hoxa11* junction (from 12G-H11) from genomic DNA, PCR was performed using the following

primers: MSCV 5' primer 5'-CCCTTGAACCTCCTCGTT CAGCC-3' in combination with MSCV 3' primer 5'-GAG ACGTGCTACTTCCATTTGTC-3' or *Hoxa11* primer 5'-GAG TAGCAGTGGGCCAGATTGC-3'. The PCR products were sequenced using MSCV 5' primer, and the peak height of the 62th nucleotide (C for 12G-H11 and T for 12G-V) was measured. The (C/C+T) peak height ratio was converted to the cell ratio (12G-H11/12G-H11+12G-V) by aligning to the standard curve. The standard curve was generated by assessing the relationship between nucleotide peak height ratios and cell ratios from cell mixtures with mixed paired cells in ratios of 10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9 and 0:10.

Ethics statement. All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by National Institutes of Health (publication no. 85-23, revised 1996) and were carried out according to the protocol approved by the Animal Research Committee of Chang Gung Memorial Hospital (IACUC No. 2014092403; Taoyuan, Taiwan). Human sample collection was conducted in accordance with the Declaration of Helsinki and was approved by the Chang Gung Memorial Hospital Research Ethics Committee (IRB No. 96-1748B). Written informed consent was obtained from all patients. For patients under the age of 18, consent/permission was obtained from the parents/guardians.

Statistical analysis. Statistical analyses were performed using SPSS software version 20.0 (SPSS, Inc). The statistical significance of differences in gene expression levels of the two groups was compared using a Mann-Whitney test. Survival analysis was conducted according to the Kaplan-Meier method, and differences in survival were assessed using the log-rank test. Drug sensitivity was compared using an unpaired two-sample Student's t-test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Identification of DEGs and GO enrichment analysis. Our previous studies demonstrated that mice transplanted with AK2G, AK3G or APm-1 had shorter survival than those transplanted with the 12G and APw-1, respectively (21,22,26). Moreover, APm-1 cells have been reported to be more resistant to daunorubicin, but more sensitive to Ara-C than APw-1 cells (22). To identify genes involved in the acceleration of leukemia development and drug resistance by cooperation of *MLL/AF10* with *RAS* signaling mutations, transcriptomic profiling were compared between 6 paired *MLL/AF10(OM-LZ)* leukemia cells harboring wild-type and oncogenic *KRAS* (12G vs. AK3G, 12G vs. AK2G and AKw1G vs. AK3G) or *PTPN11* (APw-1 vs. APm-1) using cDNA microarray data. A total of 23 DEGs (seven upregulated and 16 downregulated) with >1.5-fold-change were identified in AK3G and APm-1 cell lines compared with 12G/AKw1G and APw-1 cells, respectively (Fig. 1B). The GO terms 'BP' and 'MF' enriched in the 23 DEGs included 'innate immune response', 'immune system process', 'actin filament binding', 'cellular response to interferon-alpha', 'sequence-specific DNA' and 'regulation of gene expression' (Fig. 1C). Among these enriched GO terms, three terms ('innate immune response', 'immune system process' and

'cellular response to interferon-alpha') are related to the immune response; 'actin filament binding' is related to cell protrusion and migration, while the last two terms ('sequence-specific DNA' and 'regulation of gene expression') are related to the regulation of gene expression by transcription factors.

Expression of transcription factors in patients with AML with MLL-t and/or RAS signaling mutations. A total of four genes, *Hoxa11*, PR domain zinc finger protein 5 (*Prdm5*), Iroquois-class homeodomain protein IRX-5 (*Irx5*) and homeobox protein PKNOX2 (*Pknox2*), were mapped to the GO 'BP' term 'sequence-specific DNA'; other than *Pknox2*, these genes were upregulated in the AK3G and APm-1 cell lines according to cDNA microarray data (Fig. 1B). To investigate whether these genes were upregulated in the patients with AML with *MLL*-t, *KRAS* or *PTPN11* mutations, meta-analysis was performed using datasets deposited by Valk (285 AML cases), Wouters (503 AML cases), Haferlach (542 AML cases), and Balgobind (237 childhood AML cases) in the Oncomine™ clinical research data repository for gene expression change. The results revealed that two (Balgobind and Haferlach) of the four AML series showed significant differences ($P < 0.01$) in *HOXA11* expression for patients with AML with or without *MLL*-t (11q23-r) using reporter ID 208493 (Fig. 2A). One (Valk) of the two AML series showed significant differences ($P < 0.05$) in *HOXA11* expression for patients with AML with or without *KRAS* activating mutations (KRASm) (Fig. 2B). In the Balgobind series, there was a trend of higher *HOXA11* expression in patients with AML with *PTPN11* mutations than those without *PTPN11* mutations, but the difference was not statistically significant (Fig. 2C). This is probably due to the low case numbers (n=5). On the other hand, there were no significant differences in *IRX5* and *PRDM5* expression in leukemia cells of patients with AML with or without *MLL*-t, *KRAS* or *PTPN11* mutations (Fig. S1A-F).

HOXA11 expression was also determined in the patients with MLL-t AML included in the present study. The results showed that *MLL/AF10*⁺ patients with AML with *KRAS* or *PTPN11* mutations had higher levels of *HOXA11* expression than patients with wild-type *KRAS* and *PTPN11* genes (Fig. 2D). Similarly, *MLL/AF9*⁺ patients with AML with *KRAS* mutations expressed higher levels of *HOXA11* compared with the patient with wild-type *KRAS* (Fig. 2E). These results supported that cooperation of *MLL*-t with oncogenic *KRAS* or *PTPN11* mutation induces *HOXA11* expression in patients with AML.

Differential expression of Hoxa genes in mouse MLL/AF10 leukemia cells with different RAS signaling mutations. Based on the cDNA microarray data, *Hoxa10* and *Hoxa11* were upregulated (>1.5-fold) in AK3G compared with 12G cells (Fig. 3A). Compared with AKw1G, AK3G cells had higher expression levels (>1.5-fold) of *Hoxa5*, *Hoxa6*, *Hoxa10* and *Hoxa11* (Fig. 3B). Compared with APw-1, only *Hoxa11* was upregulated in APm-1 cells (Fig. 3C). These data suggested that *KRAS* and *PTPN11* mutations had overlapping, but not exactly the same effects on *Hoxa* gene expression in *MLL/AF10* leukemia cells. Conversely, no significant differences in the expression levels of *Hoxb*, *Hoxc*, *Hoxd* and *Meis1* genes were observed in these three pairs of mouse leukemia cell lines (Fig. 3A-C).
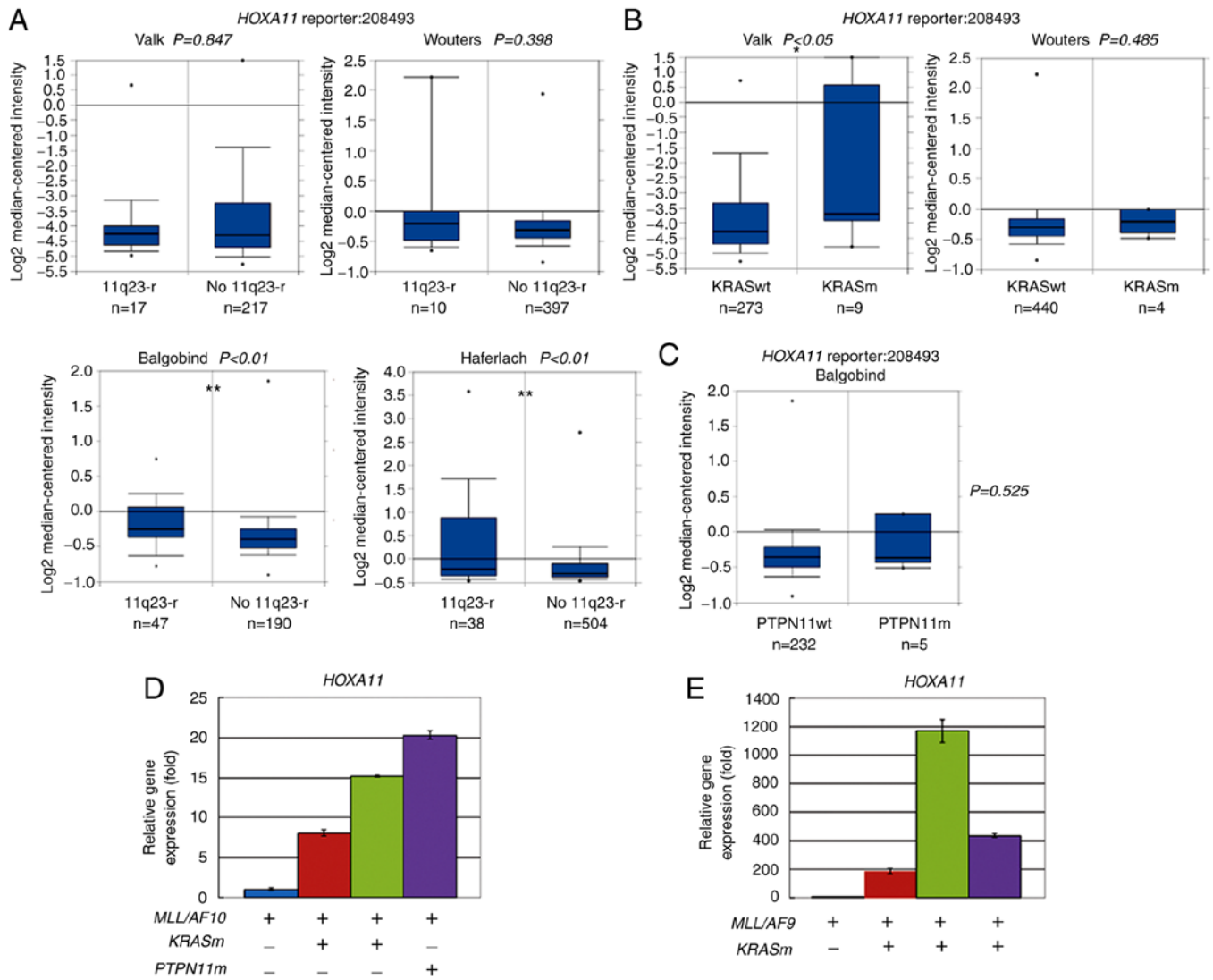
Figure 2. Overexpression of *HOXA11* in AML carrying *MLL*-t, oncogenic *KRAS* or *PTPN11* mutations. (A-C) Meta-analysis of *HOXA11* expression in AML using leukemia database deposited by Valk (n=285), Wouters (n=503), Haferlach (n=542) and Balgobind (n=237, childhood AML) in Oncomine™. Box plots are data of *HOXA11* (reporter ID. 208493) based on cDNA microarray data in patients with AML (A) with 11q23-r or No 11q23-r, (B) patients with KRASm or KRASwt, and (C) patients with PTPN11m or PTPN11wt. Numbers listed at the bottom are case numbers. Center line in box plot represents median value, box limits are 10 and 90th percentiles, and dots represent minimum and maximum values. P-values were determined using an unpaired two-sample Student's t-test. *P<0.05 and **P<0.01. (D and E) Reverse transcription-quantitative PCR analyses were performed to determine relative *HOXA11* expression level in patients with AML carrying (D) *MLL/AF10* or (E) *MLL/AF9* with or without oncogenic KRASm or PTPN11m mutations. Assays were performed in triplicate and data are representative of three independent experiments. Error bars indicate SD of mean. AML, acute myeloid leukemia; MLL, lysine methyltransferase 2A; *MLL*-t, *MLL* translocations; *PTPN11*, tyrosine-protein phosphatase non-receptor type 11; 11q23-r, *MLL* rearrangements; No 11q23-r, wild-type *MLL*; KRASm, oncogenic *KRAS* mutations; KRASwt, wild-type *KRAS*; PTPN11m, oncogenic *PTPN11* mutations; PTPN11wt, wild-type *PTPN11*.

RT-qPCR and western blotting confirmed that the transcriptional and translational levels of *Hoxa11* were increased in *MLL/AF10(OM-LZ)* mouse leukemia cell lines harboring *KRAS* mutation (AK2G, AK3G) and *PTPN11* mutation (APm-1) compared with cells harboring wild-type *KRAS* (12G, AKwlG) or wild-type *PTPN11* (APw-1) (Fig. 3D and E).

Role of Hoxa11 in survival. To determine the role of *Hoxa11* in the leukemogenesis of *MLL/AF10* leukemia cells harboring *RAS* pathway mutations, two lentivirus-based shRNAs that targeted mouse *Hoxa11* gene (shH11-1 and shH11-2) were stably transduced into APm-1 cells. RT-qPCR analysis of the *Hoxa11* knockdown APm-1 (APm-1-shH11-1 and APm-1-shH11-2) cell lines showed that the expression levels of *Hoxa11* were reduced to 86 and 46%, respectively,

compared with the control cell line (APm-1-shV) (Fig. 4A). By combining the fold changes in *Hoxa11* between APw-1 and APm-1 and between APm-1-shV and APm-1-shH11-1, the expression level of *Hoxa11* in APm-1-shH11-2 cells was estimated to be 2.4-fold higher than in APw-1 cells (Figs. 3D and 4A). Western blot analysis further confirmed that the protein levels of *Hoxa11* were decreased in APm-1-shH11 cell lines compared with the APm-1-shV control cell line (Fig. 3E). Mice i.p. injected with APm-1-shH11-2 cells, which had lower *Hoxa11* expression, had significantly longer survival than those injected with APm-1-shV cells (median 64 days vs. 50 days; P<0.01; Fig. 4B and Table I). To further support the role of *Hoxa11* in the survival of leukemic mice, *Hoxa11* overexpressed 12G cells (12G-H11-1 and 12G-H11-2) were generated by transduction of retrovirus-based full-length
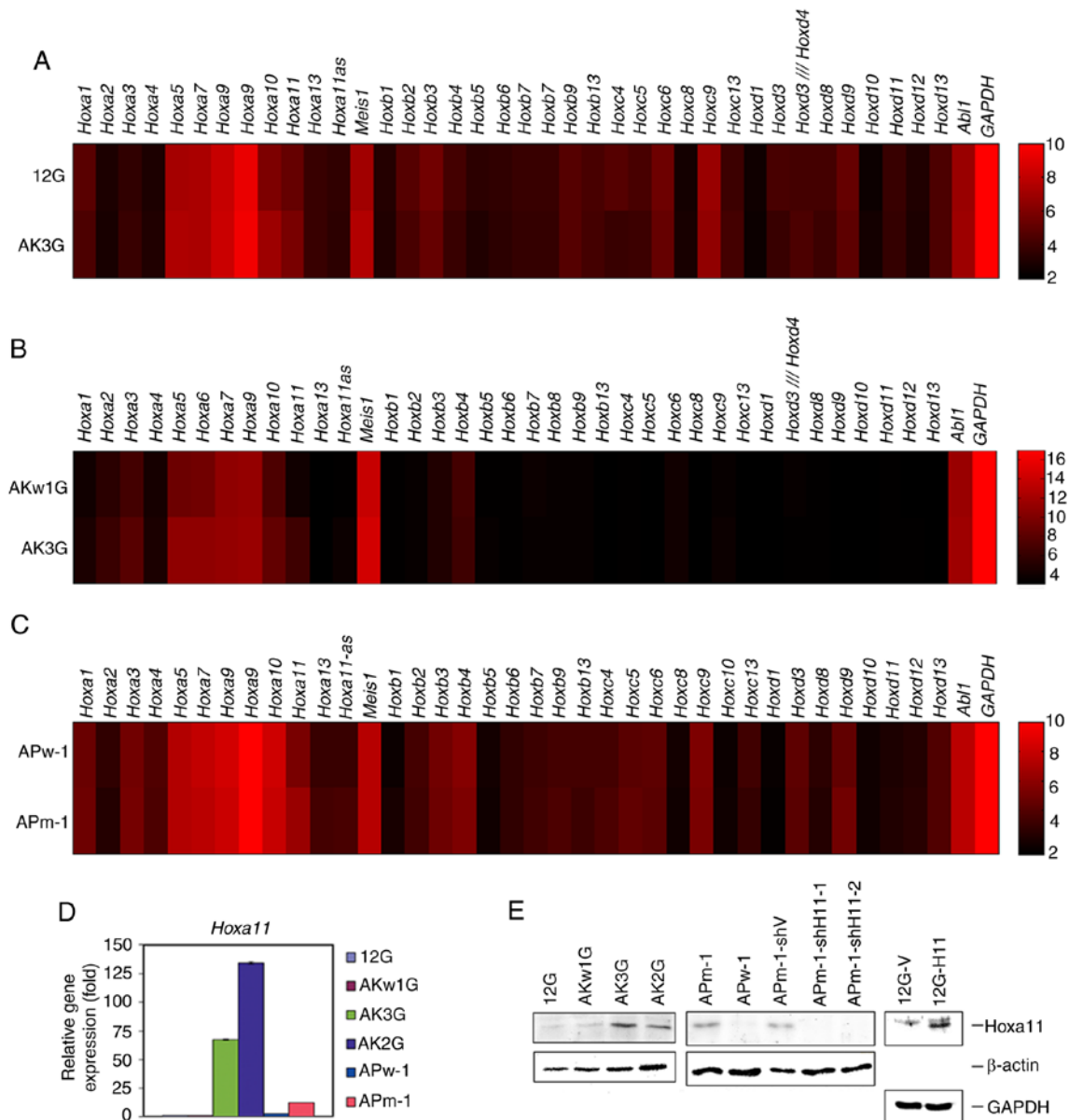
Figure 3. Expression levels of *Hox* clusters A, B, C, D and *Meis1* genes in paired *MLL/AF10* leukemia cells harboring wild-type or oncogenic *RAS* pathway mutations. (A-C) Heat maps of genes based on cDNA microarray data between paired cell lines: (A) 12G and AK3G, (B) AKw1G and AK3G, and (C) APw-1 and APm-1. Raw values are log2-transformed. Red and black indicate high and low levels of gene expression, respectively. Color bar depicts log2-transformed value of genes. (D) Reverse transcription-quantitative PCR analysis was performed to determine the level of *Hoxa11* expression in 12G, AKw1G, AK3G, AK2G, APw-1 and APm-1 cells. Assays were performed in triplicate and data are representative of three independent experiments. Error bars indicate SD. (E) Western blotting was performed to determine the protein level of Hoxa11 in cells of the different cell lines. The detection of β-actin and Gapdh served as a loading control for immunoblot analysis. MLL, lysine methyltransferase 2A; 12G, cells with *MLL/AF10(OM-LZ)* alone; AK3G, cells with *MLL/AF10(OM-LZ)* and oncogenic *KRAS*G12C; AKw1G, cells with *MLL/AF10(OM-LZ)* and wild-type *KRAS*; APw-1, cells with *MLL/AF10(OM-LZ)* and wild-type *PTPN11*; APm-1, cells with *MLL/AF10(OM-LZ)* and oncogenic *PTPN11*G503A; *PTPN11*, tyrosine-protein phosphatase non-receptor type 11.

Hoxa11 gene into 12G cells. The 12G cells transduced with empty retroviruses (12G-V1 and 12G-V3) served as controls. Overexpression of *Hoxa11* in the 12G-H11-1 and 12G-H11-2 cell lines compared with the control 12G-V1 and 12G-V3 cell lines was confirmed by western blotting and RT-qPCR (Figs. 3E and 4C). BM transplantation assay data showed that 12G-H11-1 mice had significantly shorter survival than control 12G-V1 mice (median 76 days vs. 91 days; P<0.001; Fig. 4D and Table I). These results indicated that *Hoxa11* plays a critical role in the survival of leukemic mice induced by *MLL/AF10* leukemia cells and *MLL/AF10* cells harboring activating *PTPN11* mutation.

Compared with the control cell lines, *Hoxa11*-knockdown APm-1 cells and *Hoxa11*-overexpression 12G cells had similar blast-like morphology (Fig. S2A and C, left column), similar percentages of CD115+ cells (Fig. S2A and C, right column), and similar cell proliferation rates *in vitro* (Fig. S2B and D). These results suggested that the role of *Hoxa11* in the acceleration of disease progression in the leukemia mice was not caused by changes in differentiation potential or proliferation rate of the *MLL/AF10* leukemia cells. Flow cytometry analysis of Ki-67, which detects proliferating cells, was performed on the leukemia cells (Mac1+) obtained from BM of 12G-V1 and 12G-H11-1
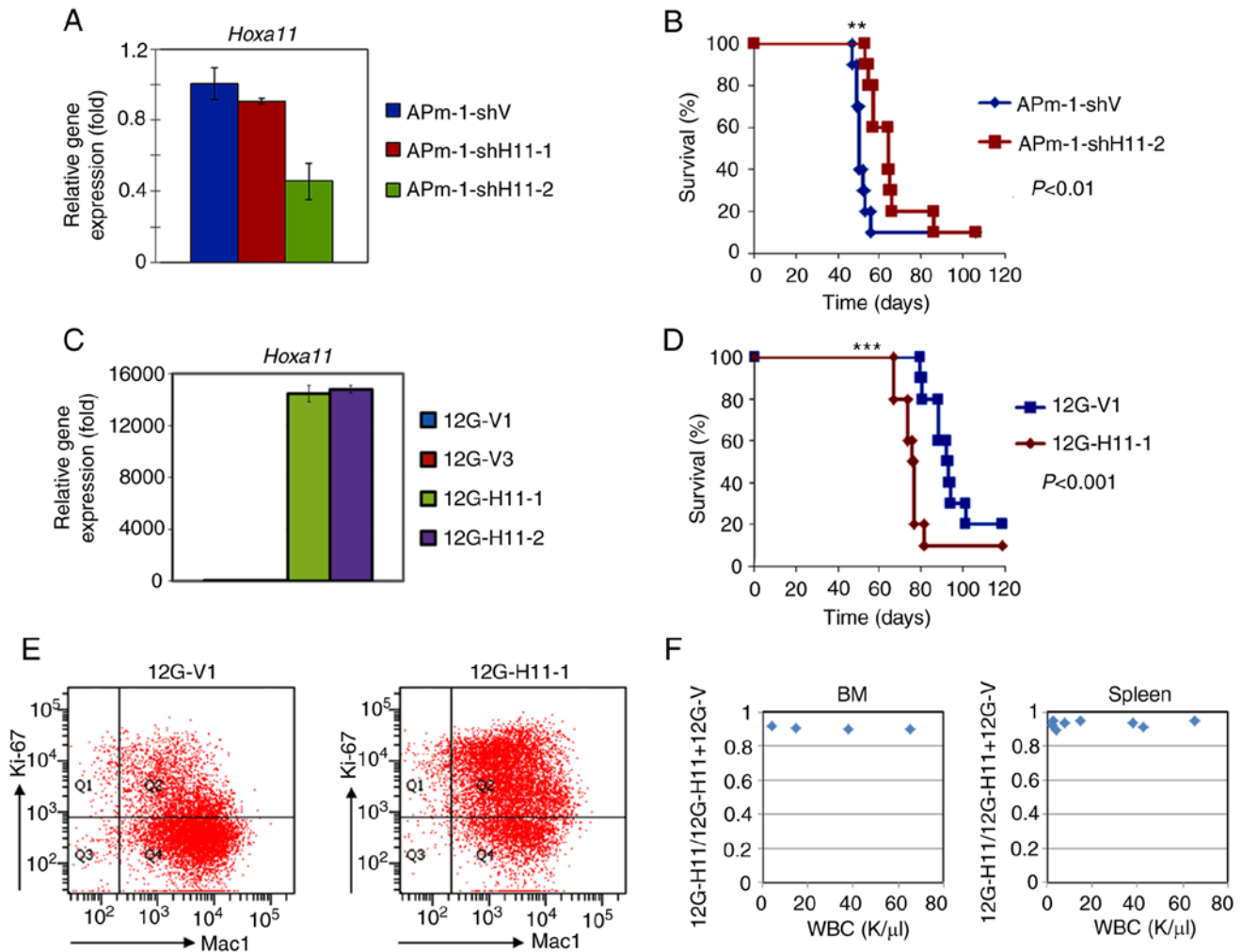
Figure 4. Expression of *Hoxa11* affects survival of *MLL/AF10* leukemia mice. (A and C) Reverse transcription-quantitative PCR analyses were performed to determine *Hoxa11* expression in (A) *Hoxa11*-knockdown APm-1 (APm-1-shH11-1, APm-1-shH11-2) and control (APm-1-shV) cell lines, or in (C) *Hoxa11*-overexpression 12G (12G-H11-1, 12G-H11-2) and control (12G-V1 and 12G-V3) cell lines. Assays were performed in triplicate and data shown are representative of three independent experiments. Error bars indicate SD. (B and D) Survival curves of mice i.p. injected with (B) APm-1-shV or APm-1-shH11-2, and (D) 12G-V1 or 12G-H11-1 cells. Survival analysis was conducted according to the Kaplan-Meier method. **P<0.01 and ***P<0.001 vs. APm-1-shV or 12G-V1 cells. (E) Flow cytometry analyses was performed to determine Ki-67 and Mac-1 expression in the BM cells obtained from 12G-V1 and 12G-H11-1 leukemia mice at moribund stage. Data shown are representative of three mice with similar results. (F) Competitive engraftment and clonal expansion ability between 12G-V1 and 12G-H11-1 cells *in vivo*. 12G-V1 and 12G-H11-1 cells were mixed in a 1:1 ratio and i.p. injected into recipient mice (n=9). The mice were sacrificed at 43 and 57 days post-transplantation (n=2 and n=7, respectively). Allele burden of the 12G-H11-1 clone in BM (left column) or spleen (right column) was determined by PCR-DNA sequencing. MLL, lysine methyltransferase 2A; APm-1, cells with *MLL/AF10(OM-LZ)* and oncogenic *PTPN11*G503A; sh, short hairpin RNA; 12G, cells with *MLL/AF10(OM-LZ)* alone; i.p. intraperitoneally; Mac-1, macrophage-1 antigen; BM, bone marrow; *PTPN11*, tyrosine-protein phosphatase non-receptor type 11.

mice. The results showed that 12G-H11-1 cells were more actively proliferating *in vivo* (Fig. 4E). To investigate whether *Hoxa11* enhanced growth advantage of *MLL/AF10* leukemia cells *in vivo*, competitive engraftment and clonal expansion assays were performed. Compared with the control 12G-V1, 12G-H11-1 cells were more competitive to engraft to and expand in BM and spleen (Figs. 4F and S3A-C). These results suggested that *Hoxa11* promotes disease progression, at least partly, by promoting leukemia cell recruitment and proliferation in their niche.

On the other hand, AK3G cells were also stably transduced with the two lentivirus-based shRNAs. Although the knock-down efficiencies of the shRNAs were significant (Fig. S4), the *Hoxa11* expression levels in AK3G-shH11-2 cells were estimated to be 16.9-fold and 21.7-fold higher than that of 12G and AKw1G cells, respectively, based on the combined RT-qPCR

results (Figs. 3D and S4). Moreover, AK3G cells had higher expression levels of *Hoxa5*, *Hoxa6* and *Hoxa10* compared with AKw1G (Fig. 3B). Due to the high *Hoxa11* expression levels and the possible complementary effect of the *Hoxa5-a10* genes (11), no further assays were performed to assess survival and drug sensitivity of AK3G-shV and AK3G-shH11 cells in this study.

Role of Hoxa11 in Ara-C resistance. To determine whether *Hoxa11* affects Ara-C resistance of *MLL/AF10* leukemia cells harboring *PTPN11*[G503A], APm-1-shV and APm-1-shH11-2 cells were treated with a gradient concentration of Ara-C for 24 h. Compared with APm-1-shV cells, APm-1-shH11-2 cells exhibited significantly higher viability at Ara-C concentrations between 128-1,600 ng/ml (P<0.01; Fig. 5A). The median lethal dose (LD50) of Ara-C for APm-1-shV and APm-1-shH11-2

Table I. Phenotypic characteristics of the mice transplanted with the *Hoxa11*-knockdown APm-1 and *Hoxa11*-overexpression 12G leukemia cells.

A, *Hoxa11*-knockdown APm-1 cells

Features	APm-1-shV	APm1-shH11	P-value
Number of mice, n	10.0	10.0	
Survival median, days, n (range)[a]	50.0 (47.0-56.0)	64.0 (53.0-86.0)	0.00543
WBC, 1×10^9/ml, median (range)[a]	64.2 (58.0-97.6)	85.5 (41.2-207.2)	0.25160
Anemia, n/n (%)[b]	4/4 (100.0)	6/6 (100.0)	1.00000
Thrombocytopenia, n/n (%)[b]	1/4 (25.0)	3/6 (50.0)	0.57100
Ascites, n/n (%)[b]	3/8 (37.5)	0/8 (0.0)	0.20000
Hepatosplenomegaly, n/n (%)	8/8 (100.0)	9/9 (100.0)	1.00000
Myeloid sarcoma, n/n (%)	1/8 (12.5)	1/8 (12.5)	1.00000

B, *Hoxa11*-overexpression 12G cells

Features	12G-V	12G-H11	P-value
Number of mice, n	10.0	10.0	
Survival median, days, n (range)[a]	91.0 (80.0-102.0)	76.0 (67.0-82.0)	0.00023
WBC, 1×10^9/ml, median (range)[a]	151.6 (61.1-354.6)	58.8 (23.3-149.1)	0.07290
Anemia, n/n (%)[b]	1/8 (12.5)	2/5 (40.0)	0.51049
Thrombocytopenia, n/n (%)[b]	2/8 (25.0)	4/7 (57.1)	0.31469
Ascites, n/n (%)[b]	2/9 (22.2)	0/8 (0.0)	0.47059
Hepatosplenomegaly, n/n (%)	8/8 (100.0)	9/9 (100.0)	1.00000
Myeloid sarcoma, n/n (%)	1/9 (22.2)	0/8 (0.0)	1.00000

[a]Student's t-test; [b]Fisher's exact test. MLL, lysine methyltransferase 2A; APm-1, cells with *MLL/AF10(OM-LZ)* and oncogenic *PTPN11^{G503A}*; sh, short hairpin RNA; 12G, cells with *MLL/AF10(OM-LZ)* alone; *PTPN11*, tyrosine-protein phosphatase non-receptor type 11; WBC, white blood cells.

cells was 235 and 315 ng/ml, respectively. By contrast, *Hoxa11*-overexpressing 12G-H11-1 cells showed significantly lower cell viabilities at all tested concentrations of Ara-C (P<0.005; Fig. 5B). The LD50 of Ara-C for 12G-V and 12G-H11 cells was 459 and 224 ng/ml, respectively. Further studies revealed that the apoptosis rate of APm-1-shH11-2 cells was lower than that of APm-1-shV cells before (17.6 vs. 25.4%, respectively) and after (41.3 vs. 45.2%, respectively) Ara-C treatment (160 ng/ml) for 24 h (Fig. 5C). The apoptosis rate of 12G-H11-1 cells was higher than that of 12G-V1 cells before (30.5 vs. 14.9%, respectively) and after Ara-C treatment (57.8 vs. 37.2%, respectively) (Fig. 5D). These results indicated that cooperation of *MLL/AF10* with *PTPN11^{G503A}* upregulated *Hoxa11*, which in turn increased cell apoptosis and rendered cells more sensitive to Ara-C.

As *Hoxa11* encodes a DNA-binding transcription factor and affects apoptosis of leukemia cells, the present study next determined the expression levels of three apoptosis-related genes in *Hoxa11*-knockdown APm-1 and *Hoxa11*-overexpression 12G cells using RT-qPCR analysis. It was found that the silencing of *Hoxa11* significantly increased the expression levels of *Nfkbia*, *Rela* and *Trp53*, whereas the overexpression of *Hoxa11* significantly reduced the expression level of these genes (Fig. 5E). These results suggested that *Hoxa11* induces cell apoptosis, at least partly, via regulation of apoptosis-related gene expression.

To determine whether the expression of *HOXA11* in leukemia cells of patients with AML was associated with chemotherapy drug sensitivity, a meta-analysis was performed using a data set deposited by Heuser consisting of 33 AML cases enrolled in the AML-SHG 01/99 trial, in the Oncomine™ clinical research data repository for gene expression change (34). The results of this analysis showed that responders of chemotherapy or AML induction/consolidation had higher *HOXA11* expression levels than non-responders using reporter AA598674 (Fig. 5F-a and F-b). Collectively, these findings indicated that patients with AML with higher *HOXA11* expression are associated with an improved response to chemotherapy with Ara-C.

Discussion

The present study compared transcriptomic profiling between mouse *MLL/AF10* leukemia cells harboring wild-type and activating *KRAS* or *PTPN11*, and found that *Hoxa7-Hoxa10* were expressed in all *MLL/AF10* leukemia cell lines, whereas *Hoxa11* was only expressed in *MLL/AF10* leukemia cells with activating *KRAS* or *PTPN11* mutations (Fig. 3A-C). Furthermore, a meta-analysis using microarray datasets deposited in Oncomine™ and an analysis of our clinical samples indicated that *HOXA11* is upregulated in *MLL*-t AML with *RAS* signaling mutations. As 29.4~45.8% of cases with *MLL*-t
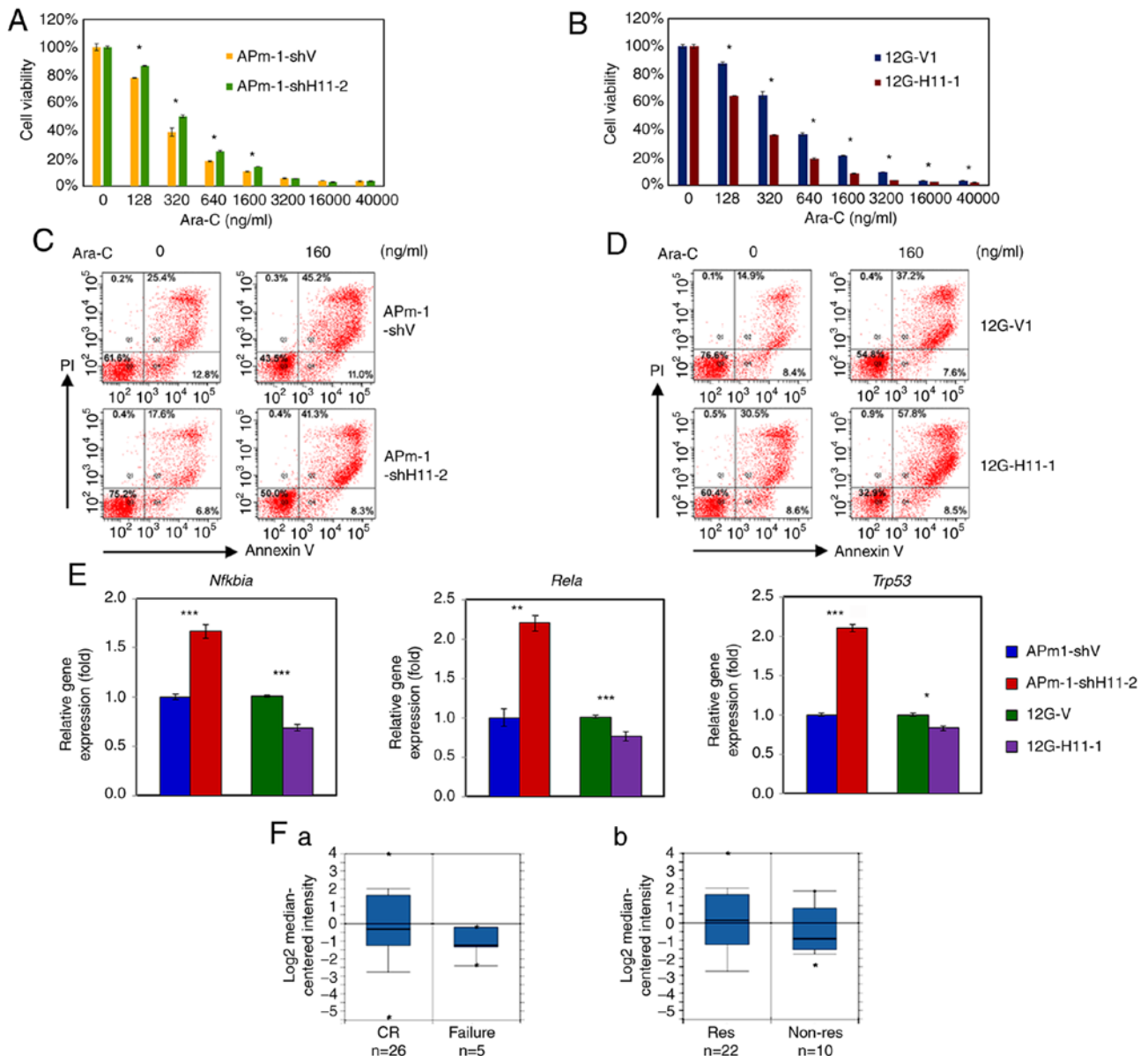
Figure 5. *Hoxa11* expression affects chemotherapy drug resistance. (A-D) Viability assays of (A and C) *Hoxa11*-knockdown APm-1 (APm-1-shH11-2) and control (APm-1-shV) cell lines or (B and D) *Hoxa11*-overexpression 12G (12G-H11-1) and control (12G-V1) cell lines treated with Ara-C at indicated concentrations for 24 h. The viability of leukemia cells was determined by a Cell Counting Kit-8 assay. Assays were performed in triplicate and data are representative of three independent experiments. The error bars indicate SD, and P-values were determined with an unpaired two-sample Student's t-test. (E) The expression levels of the apoptosis-related genes, *Nfkbia*, *Rela* and *Trp53* in *Hoxa11*-knockdown or *Hoxa11*-overexpression *MLL/AF10* leukemia cells were evaluated via reverse transcription-quantitative PCR. *P<0.05, **P<0.01, ***P<0.001 vs. APm-1-shV or 12G-V1 cells. (F) Meta-analysis of *HOXA11* expression in AML using a leukemia database deposited by Heuser (n=33) in Oncomine™. Box plots are the *HOXA11* (reporter ID. AA598674) expression levels based on cDNA microarray data in patients with AML grouped by (F-a) chemotherapy responsiveness and (F-b) AML induction/consolidation response status. Numbers listed at the bottom are the number of cases. The center line in the box plot represents the median, the box limits indicate the 10 and 90th percentiles, and dots represent minimum and maximum values. MLL, lysine methyltransferase 2A; APm-1, cells with *MLL/AF10(OM-LZ)* and oncogenic *PTPN11*G503A; sh, short hairpin RNA; 12G, cells with *MLL/AF10(OM-LZ)* alone; *PTPN11*, tyrosine-protein phosphatase non-receptor type 11; Ara-C, cytarabine; *Nfkbia*, NF-κB inhibitor α; *Rela*, transcription factor p65; *Trp53*, transformation-related protein p53; AML, acute myeloid leukemia; CR, complete remission; Res, responder; Non-Res, non-responder.

AML harbor *N-/K-RAS* or *PTPN11* mutations, this finding may partly explain why *HOXA11* expression is less frequently reported in *MLL*-t AML (12,13).

Data obtained from BM transplantation assay using *Hoxa11*-knockdown or *Hoxa11*-overexpression *MLL/AF10* leukemia cells revealed that the expression levels of *Hoxa11* in leukemia cells was associated with the survival of recipient mice. It was also demonstrated that *Hoxa11* overexpression promoted *MLL/AF10(OM-LZ)* leukemia cells to engraft

and proliferate in BM and spleen (Fig. 4E and F). A similar observation was reported by Sun *et al* (35), which showed that *HOXA11* overexpression promoted cell proliferation and migration, and reduced cell apoptosis in breast cancer.

Based on the present study analyses of *in vitro* cytotoxicity and apoptosis rate of *Hoxa11*-knockdown and *Hoxa11*-overexpression *MLL/AF10(OM-LZ)* leukemia cells showed that *Hoxa11* expression was associated with Ara-C sensitivity and apoptotic cell rate (Fig. 5A-D). In

addition, gene expression analysis revealed that *Hoxa11* induced apoptosis, at least partly, by regulating the expression of apoptosis-related genes, including *Nfkbia*, *Rela* and *Trp53* (Fig. 5E), which is in line with previous findings by Guo *et al* (36), which established Ara-C-resistant human AML OCI-AML2 cell lines. Based on a comparative transcriptomic analysis, they identified *HOXA11* as a DEG between resistant and parent cell lines, and further determined that *HOXA11* promoted Ara-C sensitivity and apoptosis in the cell line (36). Moreover, a meta-analysis of *HOXA11* expression using microarray data deposited by Heuser in the present study supported the findings of an association between *HOXA11* expression and responsiveness of patients with AML treated with chemotherapy or on an AML induction/consolidation regimen (Fig. 5F). These data provided further support that *HOXA11* expression in AML is predictive of an improved response to chemotherapy with Ara-C. The molecular mechanism of *Hoxa11* in the regulation of apoptosis-related genes needs further characterization.

The role of *HOXA11* varies according to cancer type. Epigenetic inactivation of *HOXA11* is a poor prognostic marker and contributes to disease progression in ovarian cancer, non-small cell lung cancer, gastric cancer, urothelial bladder cancer, glioblastoma, renal cell carcinoma and breast cancer (37-43). In these solid tumors, *HOXA11* acts as a functional tumor suppressor; however, the present results showed that upregulation of *Hoxa11* accelerated leukemia development in *MLL/AF10* cooperating *RAS* pathway mutations, suggesting an oncogenic role of *Hoxa11* rather than one of tumor suppression. Further investigation of downstream *Hoxa11* targets and biological pathways will provide an improved understanding of the mechanism underlying the different roles of *Hoxa11* in AML and solid tumors.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JFF was responsible for the conception and design of the present study, data analysis, funding acquisition and writing the original draft. LYS was responsible for providing the resources, analysis and interpretation of data, and wrote, reviewed and edited the manuscript. THY was responsible for data analysis, interpretation and discussion. JFF and LYS confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were carried out according to the protocol approved by the Animal Research Committee of Chang Gung Memorial Hospital (IACUC No. 2014092403; Taoyuan, Taiwan). Human sample collection was conducted in accordance with the Declaration of Helsinki and was approved by the Chang Gung Memorial Hospital Research Ethics Committee (IRB No. 96-1748B). Informed consent was obtained from all patients. For patients under the age of 18, consent/permission was obtained from the parents/guardians.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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