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

JEN-FEN FU1, LEE-YUNG SHIH2 and TZUNG-HAI YEN3

1Department of Medical Research, Chang Gung Memorial Hospital, Graduate Institute of Clinical Medical Sciences, Chang Gung University, Taoyuan 333; 2Department of Internal Medicine, Division of Hematology-Oncology, Chang Gung Memorial Hospital, Taipei 105; 3Department of Nephrology, Chang Gung Memorial Hospital, Chang Gung University, Taoyuan 333; Taiwan, R.O.C.

Received March 5, 2021; Accepted May 5, 2021

DOI: 10.3892/or.2021.8101

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)G50A 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-xB 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 AIs (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
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 (24,22,25). 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 (KRAS0232) and wild-type or activating PTPN11 (PTPN11P0503) 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 APw1 vs. APm1), or Clariom D Array chip (APw1 vs. APm1 and AKw1G vs. AK2G) (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://www.ncbi.nlm.nih.gov/geo/). 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'-GAAACACCTCTGGCTCTTCCA-3' and 5'-ATAARGGGA CGCGTTTGGTTC-3'; mouse NF-kB inhibitor α (Nfkbia), 5'-GAGACCTGGGCTTCCAC-3' and 5'-TCTCGGAGTCAGGATCACA-3'; mouse transcription factor p65 (Rela), 5'-TGGCTTACTATGAGGGCTGACCT-3' and 5'-TGCTTTGAG CTCGGTGGCTA-3'; mouse transformation-related protein p53 (Trp53), 5'-CCTCTCCGCGGAAAGAAAGA-3' and 5'-GGCCCTTTTGGCTTTCGAG-3'; mouse growth factor inhibitor 1 (Gdf1), 5'-TACACGTCAGGAGAGGC-3' and 5'-GGCATGGACTG TGGTCATG-3'; human HOXA11, 5'-CGCTCTCCGGCCGA CACTGA-3' and 5'-AGACGCTGAAGAGAAGACTCCC-3';
and human ABL, 5'-TGGAGATAACACTCTAGCATACTAAAGG-3' and 5'-GATGTAGTGTGTTGGGACCCA-3'. The thermocycling conditions were as follows: 95°C for 2 min; then 40 cycles of 95°C for 15 sec and 65°C 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.

Western blot analysis. Total cell lysate from 5x10^6 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,
% 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. NB101-2228; Novus Biologicals, Ltd.), β-actin or Gapdh (1:10,000; cat. nos. sc-7778 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 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'-GAAGATCTCCCAAGGTAGCCCAAATGTG-3' and 5'-CCGCCTCAGGCAGTGGAGCCTTAG-3'. The PCR product was digested with restriction enzymes BglII and XhoI, and was subsequently cloned into the BglII and XhoI 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 EcoPack293-293 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 cytopsinned 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 FACSComp 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 FACSComp 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'-CCC TTG AAC CTC CTC GTT CAGTCGACTTCCATTTTGTC-3' or Hoxa11 primer 5'-GAG TAGCAGTGGGCCAGATTCG-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 62th nucleotide (C for 12G-H11 and T for 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-t (11q23-r) using reporter ID 208493 (Fig. 2A). One (Valk) of the two 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 PTEN11 mutations than those without PTEN11 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 PTEN11 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 PTEN11 mutations had higher levels of HOXA11 expression than patients with wild-type KRAS and PTEN11 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 PTEN11 mutation induces HOXA11 expression in patients with AML.

Differential expression of Hoxa genes in mouse MLLAF10 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 PTEN11 mutations had overlapping, but not exactly the same effects on Hoxa gene expression in MLLAF10 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).
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 or KRASm, and 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.

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-shH11-1 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
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 1). 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. 3E, left column), similar percentages of CD115+ cells (Fig. 3E, right column), and similar cell proliferation rates in vitro (Fig. 3E, right column). 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 cells.
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 knockdown 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 PTPN11G503A, 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...
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 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 PTPN11G503A 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 HOX A11 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 HOX A11 expression levels than non-responders using reporter AA598674 (Fig. 5F-a and F-b). Collectively, these findings indicated that patients with AML with higher HOX A11 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 HOX A11 is upregulated in MLL-t AML with RAS signaling mutations. As 29.4~45.8% of cases with MLL-t...
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).
addition, gene expression analysis revealed that HOXA11 induced apoptosis, at least partly, by regulating the expres-
son of apoptosis-related genes, including Nfkbia, Rela
and Trp53 (Fig. 5E), which is in line with previous find-
ings by Guo et al (36), which established Ara-C-resistant
human AML OCI-AML2 cell lines. Based on a compara-
tive 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
lung cancer, gastric cancer, urothelial bladder cancer, glio-
blastoma, 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.

Acknowledgements
We would like to thank Mr. Jun-Wei Huang (Chang Gung
Memorial Hospital, Taoyuan, Taiwan) and Mr. Chih-Shien
Chuang (Chang Gung Memorial Hospital, Taoyuan, Taiwan)
for their technical assistance in all the experiments.

Funding
This work was supported by grants from the Ministry of
Science and Technology, Taiwan (grant no. 107-2320-B-182A013) and Chang Gung Memorial Hospital, Taiwan (grant nos.
CMRPG3E0301-3, CMRPG3E1391-3, CMRPG3G1821 and
CMRPG3J1331).

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.

References
1. Meyer C, Hofmann J, Burmeister T, Gröger D, Park TS,
Emerenciano M, Pombo de Oliveira M, Renneville A, Villareps P,
Macintyre E, et al.: The MLL recombinome of acute leukemias in
2. Meyer C, Burmeister T, Gröger D, Tsaur G, Fechina L,
Renneville A, Sutton R, Venn NC, Emerenciano M, Pombo-de-
Oliveira MS, et al: The MLL recombinome of acute leukemias in
3. Balgobind BV, Raimondi SC, Harbott J, Zimmermann M,
Alonzo TA, Auvriogn A, Beaverloo HB, Chang M, Creutzig U,
Dworzak MN, et al: Novel prognostic subgroups in childhood
11q23/MLL-rearranged acute myeloid leukemia: Results of an
4. Grimwade D, Hills RK, Moorman AV, Walker H, Chatters S,
Goldstone AH, Wheatley K, Harrison CJ, Burnett AK and
National Cancer Research Institute Adult Leukaemia Working
Group: Refinement of cytogenetic classification in acute myeloid
leukemia: Determination of prognostic significance of rare
recurring chromosomal abnormalities among 5876 younger
adult patients treated in the United Kingdom Medical Research
5. Yu BD, Hess JL, Horning SE, Brown GA and Korsmeyer SJ:
Altered Hox expression and segmental identity in Mll-mutant
6. Mohi MG, Williams IR, Dearolf CR, Chan G, Kutok JL,
Prognostic, therapeutic, and mechanistic implications of a mouse
model of leukemia evoked by Shp2 (PTPN11) mutations. Cancer
De Silva I, Moulding DA, Kioussis D, Lappin TR, Brady HJ and
Williams O: Continuous MLL-ENL expression is necessary to
establish a ‘Hox Code’ and maintain immortalization of hemato-
Nakamura T and Ohyashiki K: t(7;11)(p15;p15) Chronic myeloid
leukaemia developed into blastic transformation showing a novel
9. Mizuguchi Y, Fujita N, Taki T, Hayashi Y and Hamamoto K:
Juvenile myelomonocytic leukemia with t(7;11)(p15;q15) and


