SUPPLEMENTARY APPENDIX

Aberrant let7a/HMGA2 signaling activity with unique clinical phenotype in *JAK2*-mutated myeloproliferative neoplasms

Chih-Cheng Chen,[1,3] Jie-Yu You,[4,5] Jrhau Lung,[2] Cih-En Huang,[1,3] Yi-Yang Chen,[1] Yu-Wei Leu,[6] Hsing-Ying Ho,[1] Chian-Pei Li,[1] Chang-Hsien Lu,[1,3] Kuan-Der Lee,[1,3] Chia-Chen Hsu[1,*] and Jyh-Pyng Gau[5,7*]

[1]Division of Hematology and Oncology, Department of Medicine, Chang Gung Memorial Hospital, Chiayi; [2]Division of Pulmonary and Critical Care Medicine, Department of Medicine, Chang Gung Memorial Hospital, Chiayi; [3]College of Medicine, Chang Gung University, Tao-Yuan; [4]Division of Hematology and Oncology, Department of Medicine, Lotung Poh-Ai Hospital, Yilan; [5]School of Medicine, National Yang-Ming University, Taipei; [6]Department of Life Science and Institute of Molecular Biology, National Chung Cheng University, Chiayi; [7]Division of Hematology, Department of Medicine, Taipei Veterans General Hospital, Taiwan

**C-CH and J-PG contributed equally to this work.*

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Correspondence: jpgau@vghtpe.gov.tw/ chiachen.hsu1008@gmail.com

Supplementary File

Content

Supplementary Methods

Patients

The study involved MPN patients who were followed at the Division of Hematology, Chang-Gung Memorial Hospital, Chiayi, and Veterans General Hospital, Taipei. The patients had stored samples of granulocyte DNA. Adult healthy individuals were also included as the control. All patients and controls provided an informed written consent in accordance with the Declaration of Helsinki for study sample collection as well as permission for the use of DNA for investigational purposes. The study was approved by Institutional Review Board of Chang-Gung Memorial Hospital and Taipei Veterans General Hospital (Taiwan). The diagnosis of MPN fulfilled the 2008 WHO criteria,[1] and post-PV/ET secondary myelofibrosis was diagnosed in accordance with the criteria of International Working Group of Myelofibrosis Research and Treatment.[2] All patients were treated according to institutional guidelines which were based on current recommendations by European LeukemiaNet.[3] Events of major thrombosis and bleeding were recorded according to standard definitions.[4, 5] Thrombosis-free survival (TFS) was measured from the date of diagnosis to the date of first documented major thrombosis, death, or last follow-up. The mean follow-up time was 5.4 ± 4.7 years, and the total time of follow-up was 821.5 patient-years.

Cell lines

Human acute myeloid leukemia (AML) cell lines, KG1a and HL-60, were purchased from Bioresearch Collection and Research Center, Hsinchu, Taiwan. Human erythroleukemia cell line HEL was purchased from ATCC. Human essential thrombocythemia (ET)-transformed AML cell line UKE-1 was purchased from

Coriell Cell Repositories, Coriell Institute (NJ, USA). All cells were maintained according to the distributor's recommendation.

Stable cells co-expressing thrombopoietin receptor MPL and mutated CALR

Considering that mutated CALR needs the presence of thrombopoietin receptor MPL to exert its effect on MPN phenotype,[6, 7] we established stable Ba/F3 cells co-expressing MPL and either type 1 or type 2 CALR mutant to assess the effects of CALR mutation on cellular phenotypes. These cells were generated from mutated CALR constructs (5-bps insertion, INS; 52-bps deletion, DEL, both plasmids were kindly provided by Professor Tony R. Green, University of Cambridge, UK) and MPL (Human TPO expression plasmid, C-Flag, Sino Biological Inc.) co-transfection. Stable colonies were selected and maintained in medium containing G418 (500 μg/ml) and hygromycin (400 μg/ml).

Real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis

RNA isolation, DNA contamination removal, and first-strand cDNA synthesis were carried out as previously described.[8] The expression level of *HMGA2* was measured by qRT-PCR using iTaq™ Universal Probes Supermix (Bio-Rad, Hercules, CA, USA). Detection of the expression level of *LIN28A* was performed by employing SYBR green-based qRT-PCR method. Primers and Taqman probes were designed by Probe FinderTM (Roche, IN, USA; http://www.universalprobelibrary.com). The sequences of all the primers and probes were listed in supplementary Table 1. The annealing temperature for *HMGA2* and *LINA28A* were 62℃ and 55℃, respectively. The β-actin-normalized data were presented as the fold change in gene expression. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method.

To analyze the expression of *let-7a* microRNA and internal control *snoRNA202*, 10 ng of total RNA were reverse transcribed into cDNA using TagMan MicroRNA assays (Applied Biosystem, Carlsbad, CA, USA) under the following conditions: 16℃ for 30 min, 42℃ for 30 min, and 85℃ for 5 min. Each cDNA generated was amplified by quantitative PCR using sequence-specific primers from the TaqMan microRNA assay on a Qiagen Rotor Gene Q system (Qiagen, Hilden, Germany). The reactions were incubated at 95℃ for 10 min, followed by 40 cycles of 95℃ for 15 s and 60℃ for 1 min. We used *snoRNA202* as the internal control for miRNA detection. The relative expression level of *let-7a* was normalized to that of *snoRNA202* microRNA.

Fluorescence in situ hybridization (FISH)

Paraffin cell blocks obtained from PB granulocytes of MPN patients were cut at 5 μm. Slides were deparaffinized in xylene, dehydrated in 100% ethanol and then allowed to dry. A human HMGA2 FITC probe, spanning a 430-kb region located on chromosome 12q15 that covers the whole *HMGA2* gene, was purchased from Abnova. Hybridization was performed following standard protocol. Due to either insufficient sample volume or inadequate quality of sample processing, we did FISH analysis in only 14 HMGA2 (+), 14 HMGA2 (-) and 10 normal control samples. We counted at least 50 cells for each sample.

Western blot analysis

Following treatment in respective experiments, cells were harvested, washed with PBS twice, and then resuspended in RIPA buffer (Thermo Scientific, Carlsbad, CA, USA) with the addition of 1X protease inhibitor (Thermo Scientific), 1X phosphatase inhibitor (Thermo Scientific) and 0.1% SDS (Invitrogen, Carlsbad, CA, USA).

Lysates were incubated on ice for 15-30 min, followed by sonication and centrifugation at 14,000 ×g for 15 min. The supernatants were collected and quantified. Equal amounts of lysate (on a protein basis) were separated by SDS-PAGE, blotted onto polyvinylidene difluoride membranes, conjugated with various specific primary antibodies, and then probed with appropriate secondary antibodies. The immunoreactive bands were detected using the enhanced chemiluminescence method and visualized on Kodak Bio MAX MR film.

Poly (ADP-ribose) polymerase (PARP) is a family of proteins involved in a number of cellular processes, especially programmed cell death,[9] and cleavage of PARP serves as a marker of cells undergoing apoptosis. Moreover, Bcl-2-associated death promoter (BAD) protein is a pro-apoptotic member of the Bcl-2 gene family which is involved in initiating apoptosis by displacing Bax from binding to Bcl-2.[10] Phosphorylation of BAD (*p*-BAD) at Ser112 and Ser136 inhibits apoptotic activity.[11] We used PARP antibody (1:3000, Cell Signaling Technology, Danvers, MA, USA) as well as BAD and *p*-BAD antibodies (1:3000, Cell Signaling Technology) to assess the degree of apoptosis. Primary antibodies against other proteins and their working concentrations were as follows: JAK2 (1:10,000; Abcam, Cambridge, UK), *p*-JAK2 (1:2,000; Cell Signaling Technology), *p*-STAT3 (1:1,000; Cell Signaling Technology), *p*-STAT5 (1:1,000; Cell Signaling Technology), HMGA2 (1:10,000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), LIN28A (1:1,000; Cell Signaling Technology) and β-actin (1:10,000; Sigma-Aldrich, St. Louis, MO, USA). Finally, anti-mouse and anti-rabbit secondary antibodies (both from Jackson ImmunoResearch) were used in a 1/10000 dilution.

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Haematopoietic and Lymphoid Tissues. Lyon, France: International Agency for Research on Cancer, 2008.

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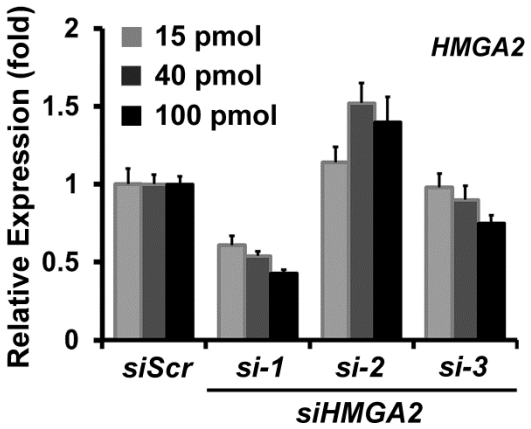
Supplementary Table

Supplementary Table 1. List of primer sequences

Primer	Gene	Sequence(5'-3')	Detection
Ms_HMGA2_F	**Mouse HMGA2 (NM_010441.2)**	AACCTGTGAGCCCTCTCCTAA	**qRT Primers**
Ms_HMGA2_R		GCCGTTTTTCTCCAATGGTC	
Ms_Hmga2 siRNA-1		CCUGUGAGCCCUCUCCUAA[dT][dT]	**KD siRNA**
		UUAGGAGAGGGCUCACAGG[dT][dT]	
Ms_Hmga2 siRNA-2		GCAGCAAGAGCCAACCUGU[dT][dT]	
		ACAGGUUGGCUCUUGCUGC[dT][dT]	
Ms_Hmga2 siRNA-3		CUAUAUUAAUCACUUUGUA[dT][dT]	
		UACAAAGUGAUUAAUAUAG[dT][dT]	
H_HMGA2_F3	**Human HMGA2 (NM_003483)**	AGTCCCTCTAAAGCAGCTCAAAAG	**qRT Primers**
H_HMGA2_R3		GCCATTTCCTAGGTCTGCCTC	
H_HMGA2_Probe		AGAAGCCACTGGAGAAAAACG	
Human_JAK2-V617F_F	**HUMAN JAK2 (NM_004972)**	GGTTTTAAATTATGGAGTATGTT	**V617F**
Human_JAK2-WT_F		GGTTTTAAATTATGGAGTTGTG	**qRT Primers**
Human_JAK2-RT_R		TTTTTCAGATATGTATCTAGTGATCC	
Hu_LIN28A_RT_F	**LIN28A (NM_024674)**	TTGTCTTCTACCCTGCCCTCT	**qRT Primers**
Hu_LIN28A_RT_R		GAACAAGGGATGGAGGGTTTT	
Ms_LIN28A_RT_F	**LIN28A (NM_145833)**	GGAACTTTCCATACCAGATCC	**qRT Primers**
Ms_LIN28A_RT_F		ACCCACCTCGTCTTCATAA	
Hu_ACTB_RT_F	**ACTB (NM_001101)**	CCTGGACTTCGAGCAAGAGATG	**qRT Control**
Hu_ACTB_RT_R		AGGAAGGAAGGCTGGAAGAGTG	
Human_GAPDH_R	**GAPDH (NM_002046)**	TGCCAA ATATGATGACATCAAGAA	**qRT Control**
Human_GAPDH_R		GGAGTGGGTGTCGCTGTTG	
Ms_GAPDH_F	**GAPDH (NM_008085)**	AACTTTGGCATTGTGGAAGG	
Ms_GAPDH_R		ACACATTGGGGGTAGGAACA	
Ms_Scramble siRNA-1	**Scramble Control**	GAUCAUACGUGCGAUCAGA[dT][dT]	**siRNA Control**
		UCUGAUCGCACGUAUGAUC[dT][dT]	

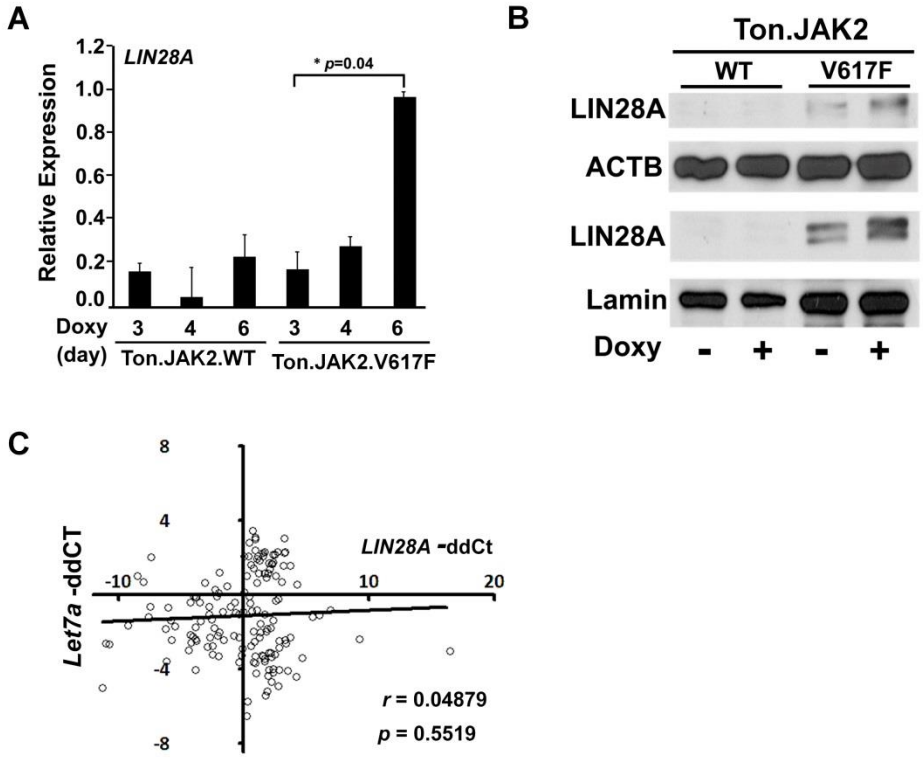
Supplementary Figures

Supplementary Figure 1.



Supplementary Figure 1. Validation of the specificity and sensitivity of *HMGA2* siRNA knockdown efficiency. Three *HMGA2* siRNAs (*si-1*, *si-2* and *si-3*) and scramble control (*siScr*) were purchased from Sigma-Aldrich. Quantitative RT-PCR assay was used to detect the *HMGA2* expression levels at 48 hours after treatment with *HMGA2* siRNAs. The expressional levels of *HMGA2* were initially normalized to internal *GAPDH* control, and the levels of *HMGA2* transcripts of *siScr*-treated cells were set as 1 and used as the comparators. The results indicated a dose-dependent inhibitory effect of *HMGA2* expression by siRNA *si-1* and *si-3* (but not *si-2*). We considered *si-1* as an ideal option and chose it for further experiments. The error bars show the standard deviation (± SD) of three independent experiments.

Supplementary Figure 2.



Supplementary Figure 2. The Expression of LIN28A in stable cells and in clinical samples from patients with MPN. (A) Quantitative RT-PCR was performed after doxycycline induction in transduced Ba/F3 cells for indicated periods of time. The *in vitro* data showed up-regulation of *LIN28A* mRNA in Ton.JAK2.V617F cells 6 days after induced *JAK2*V617F expression. The error bars show the standard deviation (± SD) of three independent experiments. Asterisk indicates statistical significance (t-test; p = 0.04). **(B)** Western blotting was employed to analyze the protein levels in cytosolic (upper 2 panels) and nuclear (lower 2 panels) fractions. Both nuclear and cytosolic LIN28A increased dramatically upon induced expression of mutated JAK2 in Ton.JAK2.V617F cells. Representative data from three independent experiments are presented. **(C)** The expression levels of *LIN28A* and *let-7a* in the peripheral blood granulocytes from patients with MPN showed no apparent correlation between each

other (correlation coefficient r = 0.04879, Pearson's correlation, p = 0.5519). The levels of *LIN28A* and *let-7a* transcripts in the clinical samples were measured by qRT-PCR, and a scatter plot was drawn here.