Inhibiting MDM2-p53 interaction suppresses tumor growth in patient-derived non-small cell lung cancer xenograft models

Josephine Hai[1,2], Shingo Sakashita[1], Ghassan Allo[1], Olga Ludkovski[1], Christine Ng[1], Frances A. Shepherd[1] and Ming-Sound Tsao[1,2]

[1]Princess Margaret Cancer Centre, University Health Network, Toronto, Ontario, Canada and [2]University of Toronto, Department of Medical Biophysics, Toronto, Ontario, Canada

Supplementary Data

Supplementary Materials and Methods:

Silver *in situ* hybridization (SISH)

Silver enhanced *in situ* hybridization (SISH) was performed on 4-µm paraffin sections of tissue microarrays using the BenchMark® XT automated slide processing system (Ventana Medical Systems, Tuscan, AZ).

The dinitrophenol (DNP)-labeled MDM2 DNA repeat-free probe was designed to hybridize to a region of chromosome 12 containing the *MDM2* gene, while the digoxigenin (DIG)-labeled chromosome 12 probe specifically recognizes chromosome 12 centromeric sequences (Ventana Medical Systems, Inc, Tuscan, AZ).

Tissue microarrays (TMA) were constructed from paraffin blocks of formalin-fixed tissues using triplicate 1.5 mm cores of representative areas of each of the frozen banked patient tumor. Formalin-fixed paraffin embedded specimens (4-µm) were prepared by cell conditioning, protease treatment, and denaturation optimized for the intended cohort. The TMA was probed according to the manufacturer's instructions. Briefly, after deparaffinization, samples were treated with cell conditioning 2 (CC2) for four 12 min cycles followed by ISH protease 2 for 12 min. After co-denaturation, MDM2 DNP and Chromosome 12 DIG probes were hybridized at 44°C for 5 hours and washed 3 times at 68°C for 8 min. MDM2 DNP and Chromosome 12 DIG signals were detected using ultraView SISH DNP and ultraView RED ISH DIG detection kits (Ventana Medical Systems, Inc, Tuscan, AZ), respectively, and counterstained with hematoxylin II and bluing reagent.

Tumor specimens were visualized with bright field microscopy. Probe numbers were counted in 30 non-overlapping tumor cells per core and the scores were analyzed to determine the mean MDM2:Chr12 ratio per cell.

Supplementary Table S1. List of human specific primers used in qRT-PCR and DNA sequencing

GADD45	Forward	CCATGCAGGAAGGAAAACTATG
GADD45	Reverse	CCCAAACTATGGCTGCACACT
BAX	Forward	TCCCCCCGAGAGGTCTTTT
BAX	Reverse	CGGCCCCAGTTGAAGTTG
PUMA	Forward	GAAGAGCAAATGAGCCAAACG
PUMA	Reverse	GGAGCAACCGGCAAACG
NOXA	Forward	ATGAATGCACCTTCACATTCCTCT
NOXA	Reverse	TCCAGCAGAGCTGGAAGTCGAGTG T
MDM2	Forward	CTGGCTCTGTGTGTAATAAGGGAG
MDM2	Reverse	CCTGATCCAACCAATCACCTG
MIC-1	Forward	CCATGGTGCTCATTCAAAAGAC
MIC-1	Reverse	GGAAGGACCAGGACTGCTCAT
p21	Forward	TGGGGATGTCCGTCAGAAC
p21	Reverse	GGCGTTTGGAGTGGTAGAAATC
BAT1	Forward	TGCCTCGGCCAAATAGGTT

BAT1	Reverse	CGGTATCAGCAGTTTAAAGATTTTCA
ACTB	Forward	TCCTAAAAGCCACCCCACTTCT
ACTB	Reverse	GGGAGAGGACTGGGCCATT
RPS13	Forward	GTTGCTGTTCGAAAGCATCTTG
RPS13	Reverse	AATATCGAGCCAAACGGTGAA
p53Exon2	Forward	CCAGGTGACCCAGGGTGGA
p53Exon3	Reverse	AGCATCAAATCATCCATTGC
p53Exon2	Forward_nested	TCTCATGCTGGATCCCCACT
p53Exon3	Reverse_nested	AGTCAGAGGACCAGGTCCTC
p53Exon4	Forward	TGAGGACCTGGTCCTCTGAC
p53Exon4	Reverse	AGAGGAATCCCAAAGTTCCA
p53Exon4	Forward_nested	GGGTTGCAGGAGGTGCTTAC
p53Exon4	Reverse_nested	ATACGGCCAGGCATTGAAGT
p53Exon5	Forward	TGTTCACTTGTGCCCTGACT
p53Exon5	Reverse	AGCAATCAGTGAGGAATCAG
p53Exon5	Forward_nested	TTCAACTCTGTGCCCTGACT
p53Exon5	Reverse_nested	CAGCCCTGTCGTCTCTCCAG
p53Exon6	Forward	TGGTTGCCCAGGGTCCCCAG
p53Exon6	Reverse	TGGAGGGCCACTGACAACCA
p53Exon6	Forward_nested	GCCTCTGATTCCTCACTGAT
p53Exon6	Reverse_nested	TTAACCCCTCCTCCCAGAGA
p53Exon7	Forward	TCCCCAAGGCGCACTGGCCTC
p53Exon7	Reverse	GAAGAAATCGGTAAGAGGTGG
p53Exon7	Forward_nested	GTTGTCTCTGACTGTACCACCAT
p53Exon7	Reverse_nested	GGGTCAGCGGCAAGCAGAGGC
p53Exon8	Forward	TTGGGAGTAGATGGAGCCTGG
p53Exon8	Reverse	AGTGTTAGACTGGAAACTTT
p53Exon8	Forward_nested	GGGACAGGTAGGACCCTGATTTC
p53Exon8	Reverse_nested	CCCTTGGTCTCCTCCACCGC
p53Exon9	Forward	CAAGGGTGGTTGGGAGTAGA
p53Exon9	Reverse	GAAAACGGCATTTTGAGTGTT
p53Exon9	Forward_nested	AGGCTGTCAGTGGGGAACAA
p53Exon9	Reverse_nested	TCCACTTGATAAGAGGTCCCA
p53Exon10	Forward	TCTACTAAATCGATGTTGCT
p53Exon10	Reverse	GGATGAGAATGGAATCCTAT
p53Exon10	Forward_nested	CAATTGTAACTTGAACCATC
p53Exon10	Reverse_nested	CTTTCCAACCTAGGAAGGCA
p53Exon11	Forward	AGACCCTCTCACTCATGTGA
p53Exon11	Reverse	TGACGCACACCTATTGCAAG
p53Exon11	Forward_nested	ATCTCTCCTCCCTGCTTCTG
p53Exon11	Reverse_nested	AGGCTGTCAGTGGGAACAA

Supplementary Table S2. Gene mutational status of NSCLC cell lines

			TP53 Status			*KRAS* status	*EGFR* status
Model	**Type**	***MDM2* Amp***	**IHC±**	**DNA Seq**	**Protein**	**Seq**	**Seq**
A549	ADC	-	-	WT	WT	G12S	WT
H1395	ADC	+	+	WT	WT	WT	WT
H157	SQC	-	-	c.892G>T	E298stop	G12R	WT
H1650	ADC	-	-	c.673-2A>G	Splice site	WT	E746_A750del
H358	ADC	-	-	Deletion	Null	G12V	WT
HLC12	ADC	-	-	WT	WT	WT	WT
HLC137	ADC	-	-	c.548C>A	S183stop	WT	E746_A750del
HLC196	ADC	-	+	c.752T>A	I251N	WT	WT
HLC277	ADC	-	-	c.1010G>C	R337P	WT	L858R

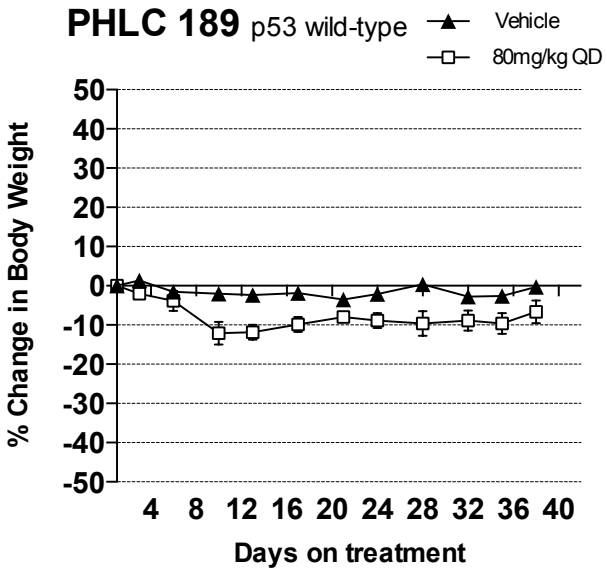
Abbreviations: ADC, adenocarcinoma; SQC, squamous cell carcinoma; Seq, DNA sequencing; IHC, immunohistochemistry; WT, wild-type; amp, amplification; +, positive; -, negative.
* Amplification (+) is defined by SISH ratio≥2 and/or previous reports [1, 2].
± Positive p53 staining is defined as more than 15% of stained tumor cells.

Supplementary Table S3. Gene mutational status of PDX models

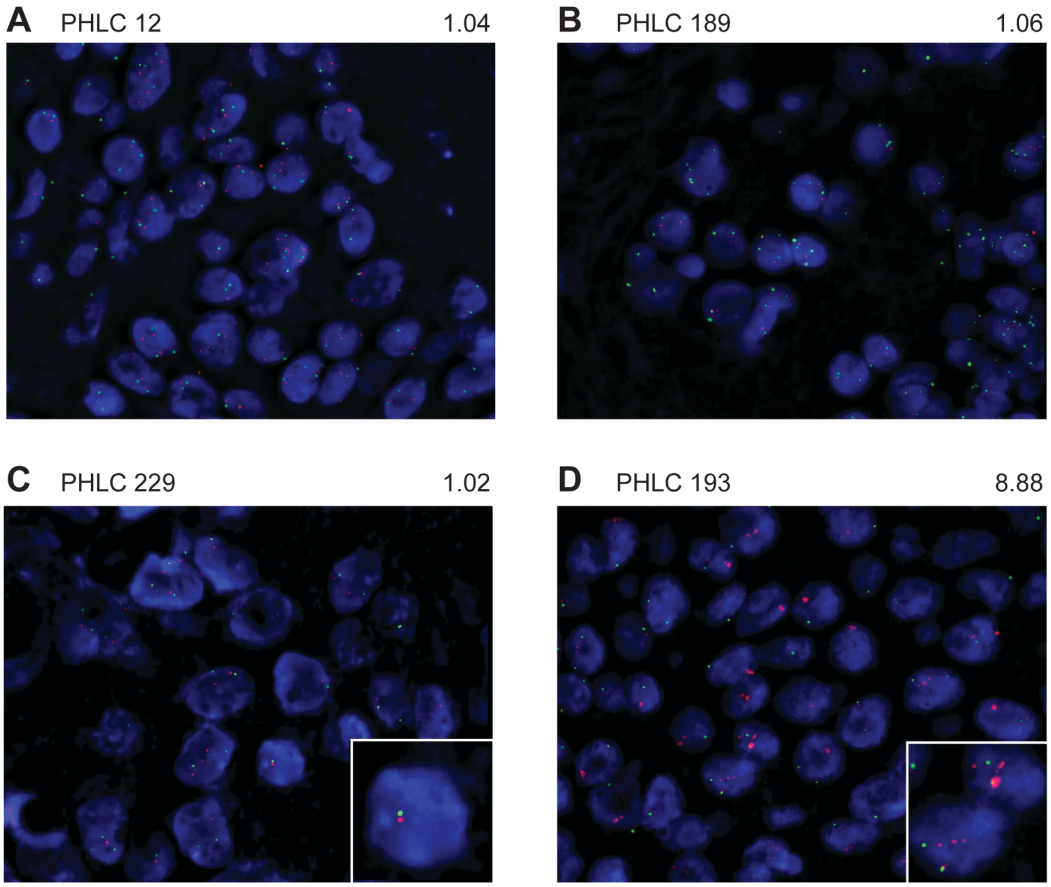
Model		***MDM2* Status**			***TP53* Status**			***KRAS* status**	***EGFR* status**
		Amp* by		**IHC**					
PHLC	**Type**	**SISH**	**FISH**	**(H-Score)**	**IHC±**	**DNA Seq**	**Protein**	**Seq**	**Seq**
12	ADC	-	-	+ (80)	-	WT	WT	G12D	WT
189	ADC	-	-	+ (120)	-	WT	WT	WT	WT
193	SQC	+	+	+ (150)	+	c.848G>A	R283H	WT	WT
229	ADC	-	-	n/a	-	WT	WT	WT	WT

Abbreviations: ADC, adenocarcinoma; SQC, squamous cell carcinoma; Seq, DNA sequencing; IHC, immunohistochemistry; WT, wild-type; amp, amplification; +, positive; -, negative; H-Score, nuclear histological score; SISH, silver *in situ* hybridization assay; FISH, fluorescence *in situ* hybridization; n/a, not available.
* Amplification (+) is defined by SISH and FISH ratio≥2.
± Positive p53 staining is defined as more than 15% of stained tumor cells.

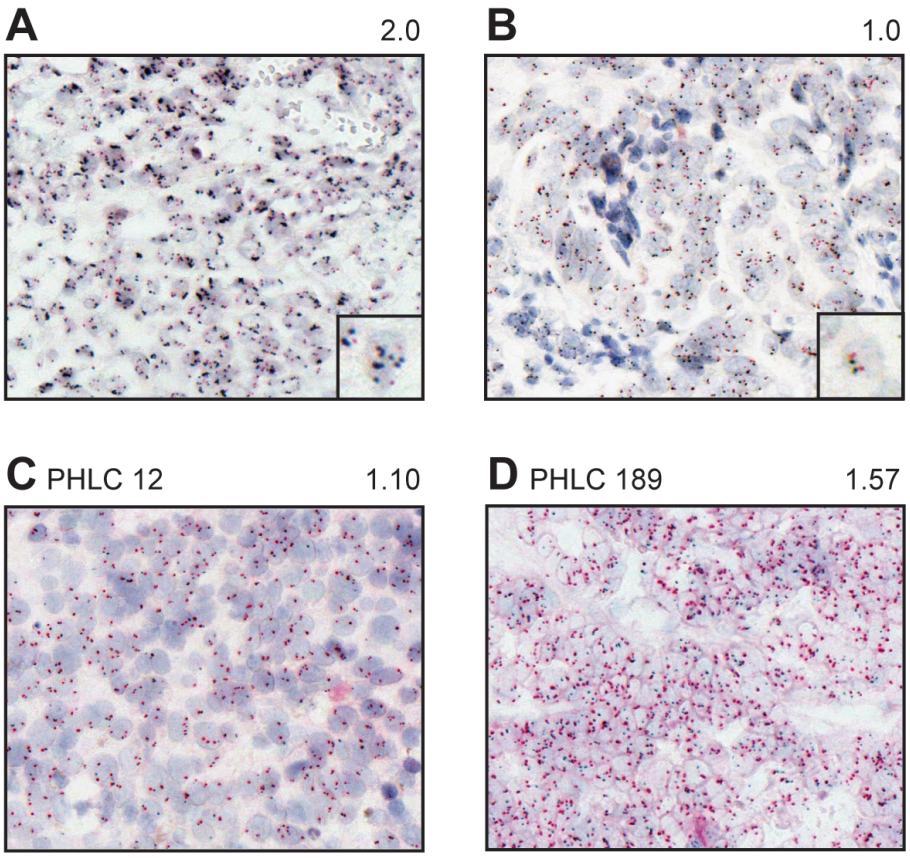
1. Pikor LA, Lockwood WW, Thu KL, et al. YEATS4 is a novel oncogene amplified in non-small cell lung cancer that regulates the p53 pathway. *Cancer Res* 2013;73:7301-7312.
2. Zhao X, Weir BA, LaFramboise T, et al. Homozygous deletions and chromosome amplifications in human lung carcinomas revealed by single nucleotide polymorphism array analysis. *Cancer Res* 2005;65:5561-5570.



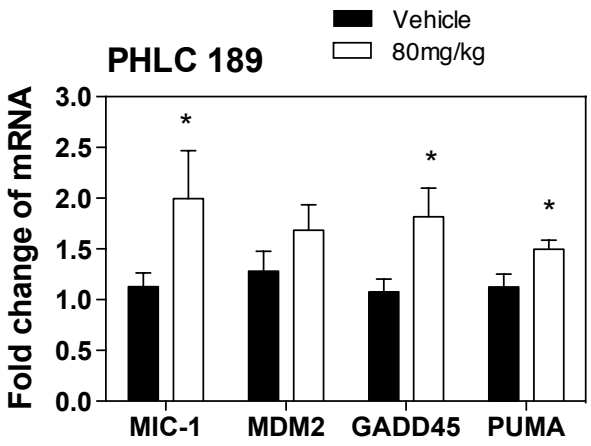
Supplementary Figure S1. Effect of oral administration of RG7388 on animal body weight. Percentage change in mean animal body weights was analyzed. No significant weight loss was observed in treated mice in the first week of daily treatment. A ~9% weight loss in body weight was observed with chronic daily dosing. Mean percentage change and error bars represent SEM.



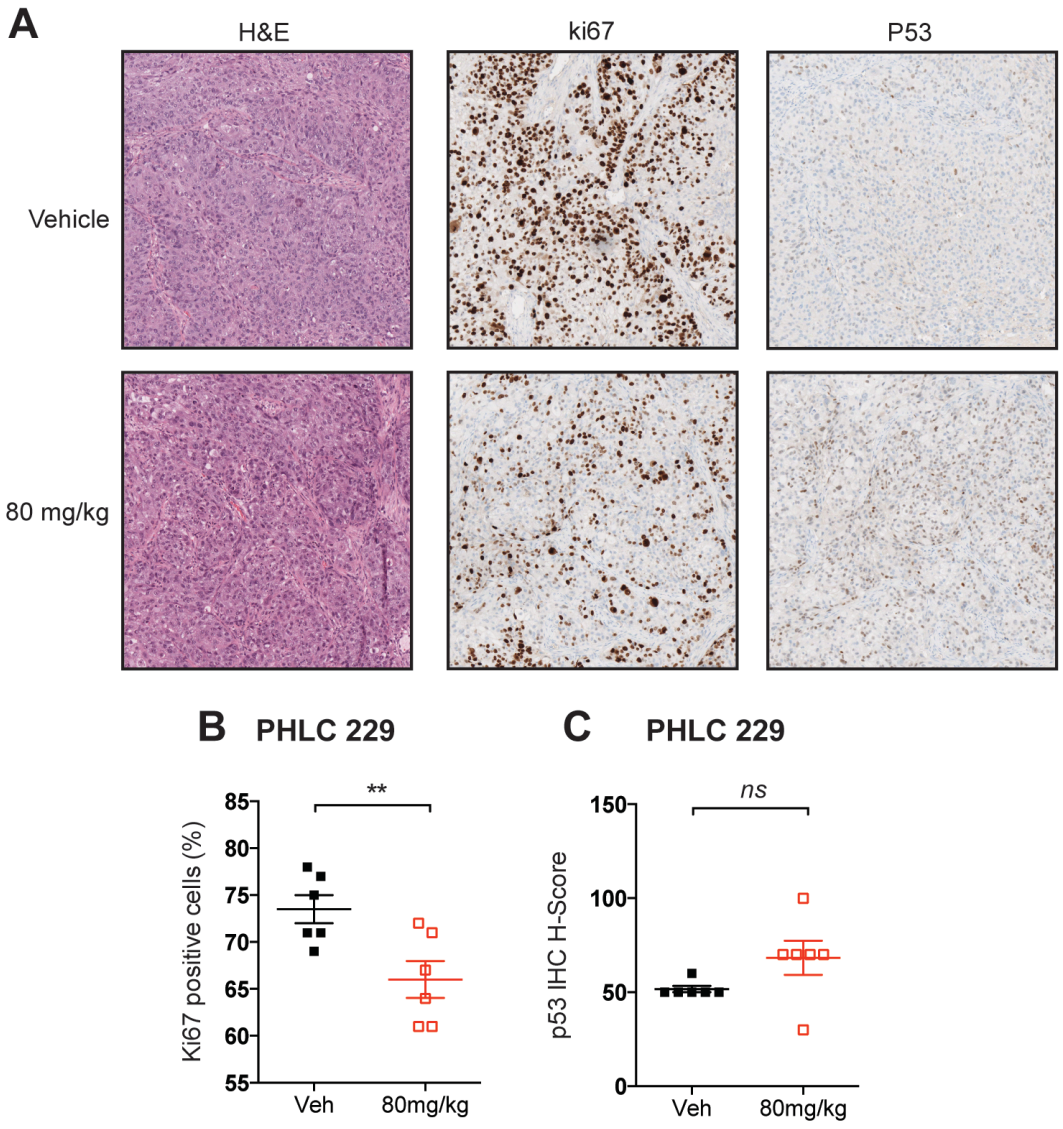
Supplementary Figure S2. Assessment of *MDM2* gene copy number in PDX models using fluorescence *in situ* hybridization (FISH). Representative tumor tissues of PHLC 12 (A), 189 (B), 229 (C) and 193 (D) and their corresponding *MDM2* copy number (Top right: MDM2/CEP12 ratio). (D) Inset shows *MDM2* amplification with discrete clusters of MDM2 signals associated with each centromere signal. Red is *MDM2* and green is *CEP12* probe.



Supplementary Figure S3. Assessment of *MDM2* gene copy number in PHLC 12 and 189 tumors using silver *in situ* hybridization (SISH). Representative tumor tissues with mean *MDM2* copy number of 2.0 (A) and 1.0 (B). PHLC 12 (C) and PHLC 189 (D) with their corresponding *MDM2* copy numbers. Black is *MDM2* and red is Chromosome 12 probe.



Supplementary Figure S4. RG7388 treatment activates p53 pathway in PHLC 189 tumors *in vivo*. Quantitative real-time PCR was performed on total RNA extracted at 24 hours after treatment on PHLC 189 mice to measure transcript levels of indicated genes relative to levels in untreated cells (n=8 mice/group; *, $p \leq 0.05$)



Supplementary Figure S5. RG7388 treatment reduces cell proliferation in PHLC 229 tumors *in vivo*. (A) Representative histologic sections of xenografts from PHLC 229 tumors were immunostained with Ki-67 and p53. (B) The percentage of positive Ki67 cells were quantified in PHLC 229 tumor (n=6/group; 10x magnification). (C) PHLC 229 tumors were immunostained with p53 and scored using H-score criteria.