miR129-1 regulates protein phosphatase 1D protein expression under hypoxic conditions in non-small cell lung cancer cells harboring a TP53 mutation

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Abstract. Protein phosphatase 1D (*PPM1D*), which functions as an oncogene, is a known target of the tumor suppressor p53 and is involved in p53-regulated genomic surveillance mechanisms. PPM1D dephosphorylates both p53 and its ubiquitin ligase mouse double minute 2 homolog, as well as the RNA-binding protein (RBM)38, which turns RBM38 from an inducer to inhibitor of *TP53* translation. In addition, RBM38 induces *PPM1D* translation. Hence, the PPM1D-RBM38-p53 axis is important in maintaining genomic integrity and is often altered during tumorigenesis. *TP53*, which encodes p53, is deleted or mutated in >50% of cancer types, including lung cancer. Mutant p53 has been revealed to complex with hypoxia-inducible factor 1α (HIF1α) and upregulate transcription of pro-metastatic genes. However, the mechanism underlying the action of the PPM1D-RBM38-p53 axis in the context of mutant p53 under normoxic and hypoxic conditions is yet to be elucidated. In the present study, using non-small cell lung cancer (NSCLC) cell lines harboring wild-type (A549 cells) or hot-spot mutant (NCI-H1770 and R249WΔ-TP53-A549 cells) *TP53*, it was demonstrated that in cells harboring mutant p53, RBM38 was not the primary regulator of PPM1D translation under hypoxic conditions. Knockdown of *RBM38* in *TP53* mutant cells did not affect the PPM1D protein expression under hypoxic conditions. Instead, in NCI-H1770 cells maintained under normoxic conditions, *PPM1D* was revealed as a target of micro RNA (miR)-129-1-3p, a known tumor suppressor in lung cancer. Hypoxia resulted in the downregulation of miR-129-1-3p expression, and thus, in the downregulation of *PPM1D* messenger RNA (mRNA) translation. In NCI-H1770 cells grown under hypoxic conditions, the transient transfection of miR-129-1-3p mimic, and not control mimic, repressed the expression of a reporter containing wild-type, but not miR-129-1-3p binding mutant, of the *PPM1D* 3'-untranslated region (UTR). Analysis of NSCLC cell lines from the Broad Institute Cancer Cell Encyclopedia and patients with NSCLC from The Cancer Genome Atlas dataset revealed significant co-occurrence of *PPM1D/RBM38* and *PPM1D/HIF1A* mutations. However, there was no significant difference in the overall survival of patients with NSCLC with or without genomic alterations in *TP53*, *RBM38*, *PPM1D* and *HIF1A*. In summary, the current study demonstrated hypoxia-dependent miR-129-1-3p-mediated regulation of PPM1D protein expression in NSCLC cell line harboring mutant *TP53*.

Introduction

Wild-type p53-induced phosphatase 1D or PPM1D (also known as Wip1), a member of the PP2C serine/threonine phosphatase family, functions as an oncogene in multiple different types of cancer, including breast, esophageal, colon, thyroid, pancreatic, gastric, liver, nasopharyngeal, bladder, prostate and ovarian carcinoma (1-5). PPM1D exerts its oncogenic effect primarily via dephosphorylation and increased degradation of p53 protein (6,7). Indeed, *PPM1D* commonly exhibits copy number alterations and is upregulated in different types of cancer, including breast and ovarian carcinoma (2-5). Thus, the p53-PPM1D loop is an important mediator of the role of p53 in genomic surveillance mechanisms.

PPM1D protein upregulation in cancer cells is associated with a corresponding increase in mRNA expression. In fact, it has been revealed that the RNA-binding protein RBM38 or RNPC1 induces translation of *PPM1D* by binding the 3'-untranslated region (UTR) of *PPM1D* (8). Once translated, PPM1D protein dephosphorylates RBM38 at serine 195 residue (8). RBM38 is phosphorylated at the serine 195 residue by glycogen synthase kinase 3β (GSK3β). Dephosphorylated RBM38 forms a complex with the cap-binding protein eukaryotic elongation factor 4E (eIF4E) and prevents this from translating *TP53*

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mRNA (9,10). Phosphorylation by GSK3β inhibits the interaction of RBM38 with eIF4E, resulting in translation of *TP53* mRNA (9,10). Thus, the PPM1D-RBM38-p53 axis is intricately regulated by feedback loops of kinases and phosphatases based on specific cellular context. Indeed, RBM38 is regulated by p53 and E2F1 (11,12). RBM38 can also exert its pro-oncogenic roles by regulating mRNA stability and/or alternative splicing of cyclin-dependent kinase inhibitor 1A (encoding p21), mouse double minute 2 homolog, ELAV-like RNA binding protein 1 (encoding human antigen R), erythrocyte membrane protein band 4.1 and fibroblast growth factor receptor 2 (11,13,14).

The PPM1D-RBM38-p53 axis has been mostly studied in the context of wild-type p53. However, mutations in *TP53* (both null and hot-spot point mutations) are known to exert gain-of-function that in turn regulate both resistance to chemotherapy and metastatic progression (15,16), even though mutant *TP53* is widely pervasive in all tumor types, including non-small cell lung cancer (NSCLC). In the case of T-cell lymphomagenesis, it has been demonstrated that RBM38 functions as a tumor suppressor, and genetic ablation of *RBM38* in a mice model resulted in enhanced mutant *TP53* expression (17). A tumor is subjected to hypoxic conditions both during initial growth and during progression. However, it is not known whether the PPM1D-RBM38-p53 axis functions similarly under normoxic and hypoxic conditions. Given that RBM38 regulates cellular responses to oxidative stress by regulating translation of hypoxia-inducible factor 1α (*HIF1A*) (18), and HIF1α protein forms a complex with mutant p53 protein and induces transcription of extracellular matrix components promoting migration and invasion (19), it may be possible that the PPM1D-RBM38-mutant p53 axis is regulated differently in normoxic and hypoxic conditions. Indeed, microRNAs (miRNAs), a class of small non-coding RNA ~22 nucleotides long, have been shown to differentially regulate cellular response under hypoxic and normoxic conditions (20). miR-129-1-3p and miR-129-1-5p have been shown to function as tumor suppressors in colon, gastric, bladder, and esophageal cancer (21-24). However, to the best of our knowledge, it is unclear if miR-129-1 differentially regulates expression of PPM1D under normoxic or hypoxic conditions. Hence, the objective of the current study was to determine if PPM1D-RBM38-p53 axis is regulated similarly in NSCLC harboring wild-type and mutant p53 gene under the conditions of normoxia and hypoxia.

Materials and methods

Cell culture. A549 and NCI-H1770 cell lines were purchased from the American Type Culture Collection. Both cell lines were cultured in DMEM containing 10% fetal bovine serum and 10 ml/l penicillin/streptomycin (all Thermo Fisher Scientific, Inc.). Cells were incubated in normoxic conditions (21% O_2, 74% N_2 and 5% CO_2) at 37˚C. For growth under hypoxia cells were incubated in a hypoxic chamber (1% O_2, 94% N_2 and 5% CO_2; Jouan SA; Thermo Fisher Scientific, Inc.) at 37˚C for 4 h. During hypoxia induction, HEPES (25 mM final concentration; Thermo Fisher Scientific, Inc.) was added to prevent acidosis (25).

Cell lysate and western blot analysis. At the end of the experimental time point, the medium was removed and the cells were washed twice with ice-cold 1X phosphate-buffered saline (PBS). Cells were then lysed using RIPA buffer (20x volume of cell pellet; Thermo Fisher Scientific, Inc.), centrifuged at 15,000 x g for 15 min at 4˚C, and protein concentration in the extracted whole cell lysate was determined using a bicinchoninic acid assay (Thermo Fisher Scientific, Inc.). A total of 50 μg of lysates per sample were resolved by 10% SDS-PAGE and blotted onto PVDF membranes (Thermo Fisher Scientific, Inc.) and processed for western blot analysis using standard methodologies. Blots were blocked using 5% fat-free milk in TBST buffer (0/1% Tween-20) for 30 min at room temperature before being incubated with the following primary antibodies overnight at 4˚C: p53 (cat. no. 9282; 1:1,000; Cell Signaling Technology, Inc.), HA-Tag (cat. no. 3724; 1:1,500; Cell Signaling Technology, Inc.), PPM1D (cat. no. HPA022277; 1:2,000; Sigma-Aldrich; Merck KGaA), RBM38 (cat. no. ab168455; 1:3,000; Abcam), HIF1α (cat. no. 36169; 1:1,000; Cell Signaling Technology, Inc.), p-p53 (serine 15) (cat. no. 9284; 1:1,000; Cell Signaling Technology, Inc.), GAPDH (cat. no. 5174; 1:4,000; Cell Signaling Technology, Inc.). Blots were probed with anti-GAPDH antibody to ensure equivalent protein loading across samples. After washing thrice with TBST buffer, blots were incubated with HRP-conjugated mouse or rabbit secondary antibody (Thermo Fisher Scientific, Inc.) for 1 h at room temperature. Post-incubation blots were washed thrice with TBST buffer before being developed using Pierce ECL Plus substrate (Thermo Fisher Scientific, Inc.). Each experiment was repeated ≥ three times and densitometry analysis was conducted using ImageJ version 2 software (National Institutes of Health) to determine the relative changes in protein expression under different conditions. Representative blots and quantification results from all experiments are presented in the figures.

miRNA isolation and reverse transcription quantitative (RT-qPCR). Medium was aspirated off and cells were rinsed and scrapped off in ice-cold PBS and centrifuged at 1,000 x g for 5 min at 4˚C. The cell pellet was then used to isolate miRNA using the mirVana miRNA isolation kit (Thermo Fisher Scientific, Inc.). cDNA synthesis was performed using a TaqMan Advanced miRNA cDNA Synthesis kit (Thermo Fisher Scientific, Inc.). Each cDNA sample was pre-amplified using the TaqMan PreAmp Master Mix kit (Thermo Fisher Scientific, Inc.) then used to template the qPCR using the TaqMan Fast Advanced Master Mix and individual TaqMan microRNA assay probes (Thermo Fisher Scientific, Inc.). Thermocycling conditions consisted of an initial denaturation of 20 sec at 95˚C, followed by 40 cycles of 95˚C for 3 sec and 60˚C for 30 sec. qPCR was performed using TaqMan probes (Thermo Fisher Scientific, Inc.) for miR129-1 (Assay ID: 002298; 5'-AAGCCCUUACCCCAAAAAGUAU-3') and *RNU6B* (Assay ID: 001093). RNU6B expression levels were utilized for normalization. Post-normalization relative expression of miR129-1 was calculated using the $2^{-\Delta\Delta Cq}$ method (26). The data were represented as expression in hypoxic conditions relative to normoxia, or expression following transfection of miR129-1 mimic compared with control mimic in hypoxia [mean ± standard error of the mean (SEM)]. The RT-qPCR

for the indicated genes was conducted similarly using TaqMan probes; however, the data were normalized to *GAPDH*. RT-qPCR experiments were run in triplicate.

Plasmid construction. The 3'-UTR of *PPM1D* was amplified from cDNA using the following primer sequences: PPM1D forward, 5'-TGCATCGGGAAATGAGGTT-3' and reverse, 5'-GCCTCCTTCCAGATGACACT-3' and cloned into the pRL-TK-CXCR4 vector (Addgene, Inc.) and called the pRL-TK-*PPM1D* wild-type 3'-UTR plasmid. The miR-129-1 binding site mutant 3'-UTR (nucleotides 292-299 deleted) of *PPM1D* was generated using site-directed mutagenesis (QuickChange II kit; Agilent Technologies, Inc.) and the following primers: Forward, 5'-GAGTCTCTGATACACAGT AATTGTGACAATATGTTTAAAGAAATCAAAAGAATC TATTA-3' and reverse, 5'-TAATAGATTCTTTTGATTTCT TTAAACATATTGTCACAATTACTGTGTATCAGAGAC TC-3' and named the pRL-TK-ΔPPM1D 3'-UTR plasmid. The pGL3 plasmid expressing Firefly luciferase off a CMV promoter was purchased from Promega Corporation. The miR129-1 (hsa-miR-129-1-3p) mimic used was the MISSION® microRNA mimic (cat. no. HMI0159; Sigma-Aldrich; Merck KGaA) and the control mimic used was an oligonucleotide sequence from *Arabidopsis thaliana* (5'-GGUUCGUACGUA CACUGUUCA-3') with no homology to human gene sequences (cat. no. HMC0002; Sigma-Aldrich; Merck KGaA). Lentiviral particles of control short hairpin (sh)RNA (cat. no. sc-108080) and *RBM38* (cat. no. sc-76368-V) were obtained from Santa Cruz Biotechnology, Inc. shRNA targeting the 3'-UTR of *TP53* (V2LHS_93615) was obtained from Open Biosystems. The pCMV-Neo-Bam p53 R248W plasmid (cat. no. 16437) was obtained from Addgene, Inc. and was cloned into pEF-hemagglutinin (HA) vector.

Transfection and transduction. Cells were plated in respective antibiotic-free cell culture DMEM medium. Cells were co-transfected with *Renilla* luciferase reporter plasmids [pRL-TK-*PPM1D* wild-type 3'-UTR or pRL-TK-ΔPPM1D 3'-UTR (nucleotides 292-299 deleted)] and pGL3 plasmid expressing Firefly luciferase, and where indicated along with control or miR129-1 mimic using Polyplus jetPRIME transfection reagent (Polyplus-transfection, SA). For reporter plasmids, cells were seeded in 24-well plates (50,000 cells/well) and co-transfected with 0.5 μg of pRL-TK plasmid(s) and pGL3 plasmid. Luciferase assays were performed 24 h after transfection. miRNA mimics were transfected at a final concentration of 10 nM. Cells were harvested 48 h after transfection with the miRNA mimics.

For transduction, NCI-H1770 cells were transduced with either lentiviral particles containing control shRNA or shRNA targeting RBM38, using polybrene. Cells were selected with puromycin (2 μg/ml) for 2 weeks and successful knockdown was verified via western blot analysis.

A549 cells were first transfected with linearized pEF-HA-*TP53* (R248W) plasmid and selected with G418 (100 μg/ml) for 2 weeks (with addition of fresh media every third day) before being transfected with lentiviral particles containing control shRNA or shRNA targeting 3'-UTR of *TP53* using polybrene. Cells were selected with puromycin (2 μg/ml) for 2 weeks (with addition of fresh media every

alternate day) and successful knockdown of endogenous and overexpression of the mutant TP53 plasmids was verified by western blot analysis using p53 and HA antibodies.

Luciferase assay. After 24 h of transfection, NCI-H1770 cells were washed with ice-cold PBS and then incubated with 100 μl of 1X passive lysis buffer (Promega Corporation). Plates were incubated on a rocker at 37˚C for 30 min. After incubation, 20 μl of lysates were used to perform a luciferase assay using the Dual-Luciferase assay kit (Promega Corporation). Firefly luciferase was used as an internal control and used to normalize relative *Renilla* luciferase expression and expressed as the mean ± SD of three independent experiments.

Data mining. The analysis of The Cancer Genome Atlas (TCGA) (cancer.gov/tcga) data was performed using cBio-Portal for Cancer Genomics (http://cbioportal.org) (27,28). A total of 2,628 patients and 2,855 samples from 7 studies were included (29-35). Analysis was carried out to determine genome amplification, somatic mutations, association with survival and mRNA expression. *In situ* prediction of miRNAs binding to *PPM1D* mRNA was done using TargetScan Human Release 7.1 algorithm (36).

Statistical analysis. Statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software, Inc.). Data were expressed as the mean ± standard deviation of three technical replicates. For RT-qPCR, the data were expressed as the mean ± SEM of three technical replicates. A paired Student's t-test was used to test differences between groups. The log-rank test was used to test whether the difference between overall survival times between two groups was statistically significant. P<0.05 was considered to indicate a statistically significant difference.

Results

PPM1D protein expression is regulated independent of RBM38 expression in NSCLC cell line harboring mutant TP53. The protein expression levels of p53, PPM1D and RBM38 were initially determined in the NSCLC cell line A549 that harbors wild-type *TP53*, and in the NCI-H1770 cell line that harbors the R248W hotspot *TP53* mutation. Although RBM38 and PPM1D expression levels were higher in the NCI-H1770 cells, p53 expression was higher in A549 cells (Fig. 1A). The higher basal expression of p53 observed in NCI-H1770 cells in comparison with the A549 cells was opposite to what is reported by the American Type Culture Collection (atcc.org/en/Documents/Learning_ Center/~/media/5F7B1CCACF724E3398BE56BFBEE3EFE4. ashx). Given that the objective of the present study was to define the regulation of the PPM1D-RBM38-p53 (wild-type/mutant) axis in the cells, this discordance in basal p53 expression was not further investigated.

RBM38 promotes translation of mutant TP53 (9). Mutant p53 protein cooperates with HIF1α to cause transcriptional upregulation of extracellular matrix components (18). RBM38 can also regulate translation of HIF1α (15). Hence, the changes in protein expression of PPM1D, p53, and RBM38 following
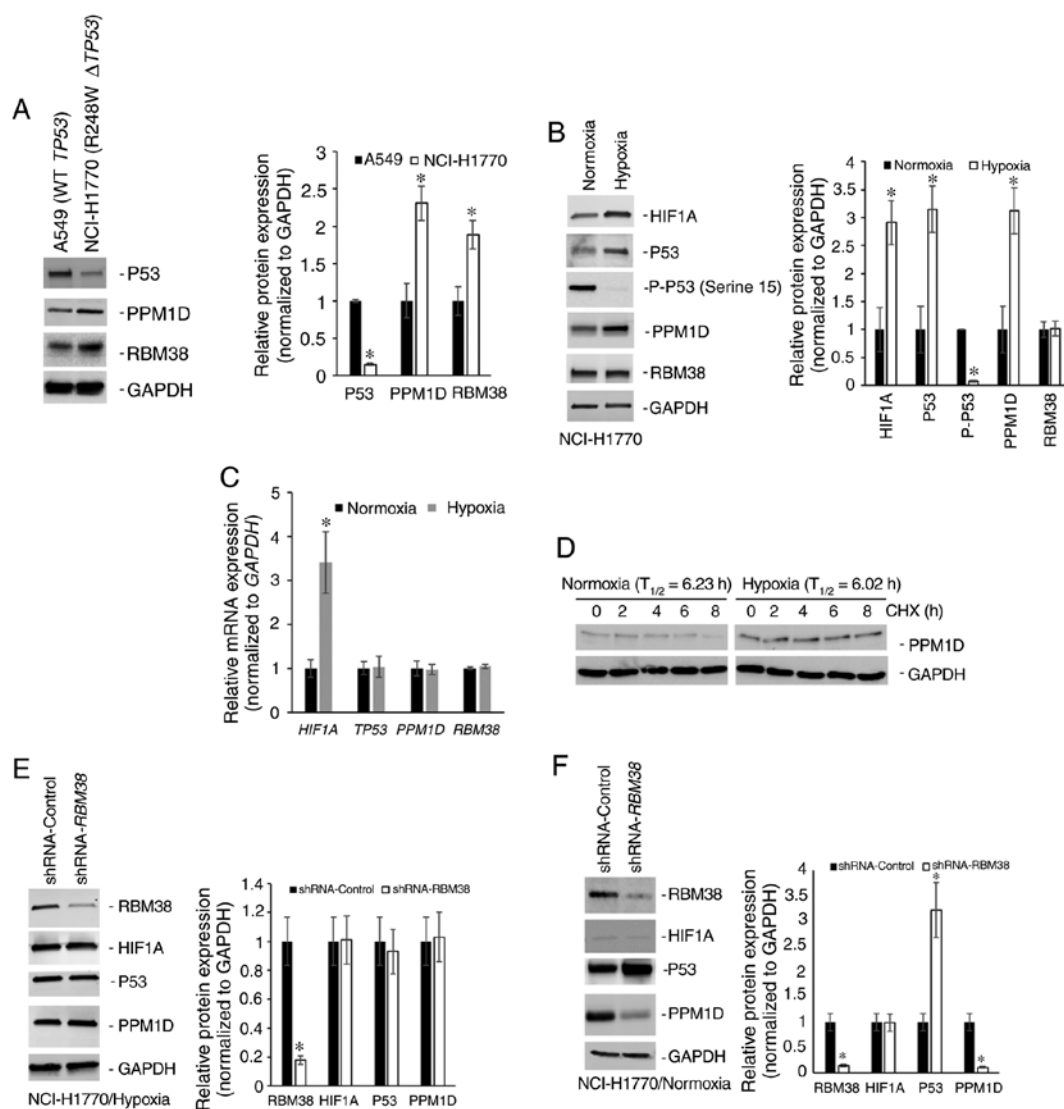
Figure 1. PPM1D protein expression is regulated independently of RBM38 expression in NCI-H1770 cells harboring R248W mutant *TP53*. (A) Western blot analysis of indicated proteins in A549 and NCI-H1770 cell lines maintained under normoxic conditions. The bar graph presents the relative protein expression levels determined by densitometry analysis (n=3) and expressed as the mean ± standard deviation. (B) Western blot analysis of indicated proteins in NCI-H1770 cells maintained under normoxic conditions or subjected to hypoxia for 4 h. The bar graph presents the relative protein expression levels determined by densitometry analysis (n=3) and expressed as the mean ± standard deviation. (C) Relative mRNA expression of indicated genes. Data were normalized to *GAPDH* and expressed as the mean ± standard error of the mean relative to normoxia (n=3). (D) Relative stability of PPM1D protein in normoxic and hypoxic conditions as determined by CHX treatment. $T_{1/2}$ was calculated from three replicates and normalized to GAPDH. Western blot analysis of indicated proteins in NCI-H1770 cells stably transduced with either control shRNA or shRNA targeting RBM38 and maintained under (E) hypoxia for 4 h, or (F) normoxia. Bar graphs in indicate the relative protein expression levels determined by densitometry analysis (n=3) and expressed as the mean ± standard deviation. Blots in each case (A, B, E and F) were probed with anti-GAPDH antibody to confirm equal loading. *P<0.05. mRNA, messenger RNA; $T_{1/2}$, half-life; PPM1D, p53-induced phosphatase 1D; shRNA, short hairpin RNA; RBM38, RNA binding motif protein 38; WT, wild type; CHX, cycloheximide; HIF1A, hypoxia-inducible factor 1A.

induction of hypoxia in the NCI-H1770 cells were investigated. Hypoxia was induced by incubating cells at 1% O_2 for 4 h and confirmed via the induction of HIF1α protein expression. RBM38 protein expression did not change following the induction of hypoxia in these cells; however, both p53 and PPM1D were upregulated following hypoxia (Fig. 1B). Phosphorylation levels of p53 at serine 15 residue decreased following the induction of hypoxia (Fig. 1B), which may have been either due to decreased phosphorylation or increased phosphatase activity, given the increase in PPM1D protein expression. The increase in protein expression of PPM1D and p53 was not due to transcriptional upregulation post-hypoxia, as the steady state mRNA expression level did not differ significantly between

normoxic and hypoxic conditions (Fig. 1C). To determine whether the increased PPM1D protein expression in hypoxia was due to increased stability of the protein, cycloheximide chase experiment was performed, as cycloheximide inhibits translation. PPM1D half-life ($T_{1/2}$) was not significantly different between normoxic (6.23 h) and hypoxic conditions (6.02 h) (Fig. 1D). Cumulatively, these results indicate that PPM1D and p53 protein expression was post-transcriptionally upregulated during hypoxia in the NCI-H1770 cells.

Given that no change was observed in RBM38 protein expression between normoxic and hypoxic conditions, there are two possible explanations for the observed induction of p53 and PPM1D proteins (Fig. 1B). The first one is that the
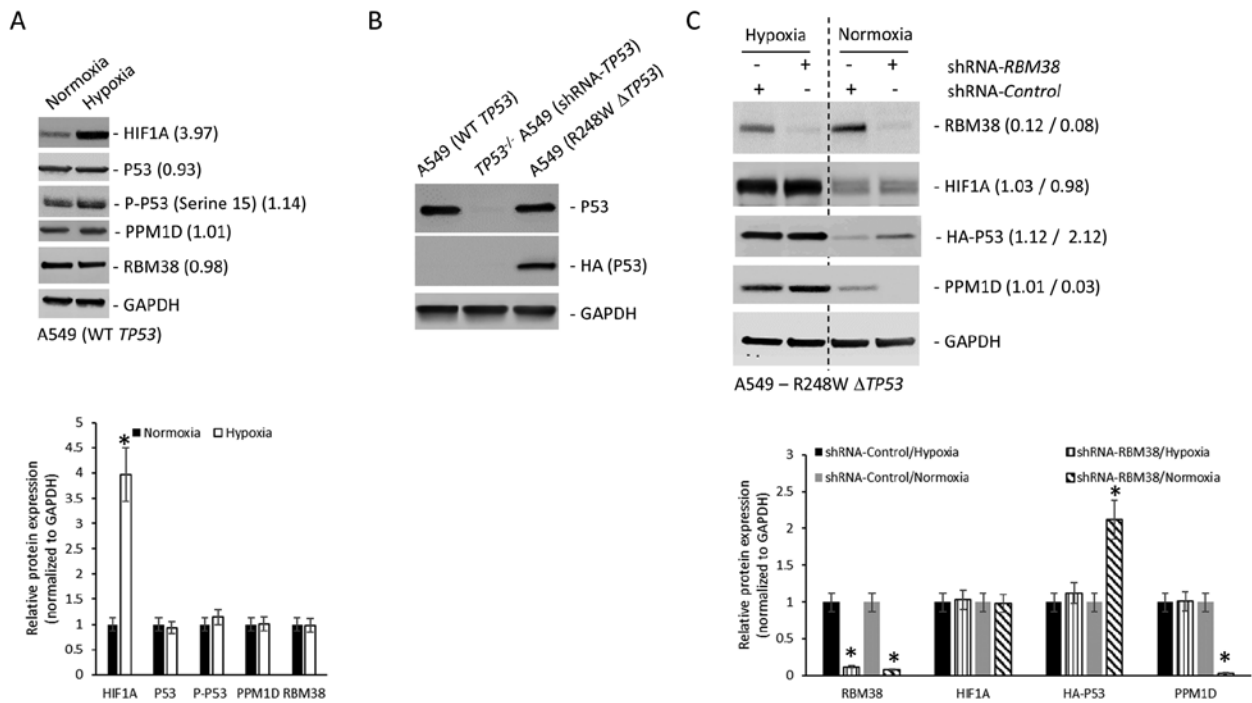
Figure 2. Differential regulation of PPM1D in A549 cells harboring wild-type or mutant TP53. (A) Western blot analysis of indicated proteins in parental A549 cells (harboring wild-type TP53) maintained under normoxic conditions or subjected to hypoxia for 4 h. The bar graph presents the relative expression levels determined by densitometry analysis (n=3) and data are expressed as the mean ± standard deviation. (B) A549 cells were transfected with pEF-HA-tagged R248W TP53 and stable cells were then transduced with shRNA targeting 3'-untranslated region of TP53. Knockdown of endogenous TP53 and expression of the mutant plasmid was verified via western blot analysis. (C) Western blot analysis of indicated proteins in A549-R248W TP53 cells stably transduced with either control shRNA or shRNA targeting RBM38 and maintained under hypoxic conditions for 4 h or under normoxic conditions. The bar graph displays the relative expression levels determined by densitometry analysis (n=3) and expressed as the mean ± standard deviation. Blots in each panel were probed with anti-GAPDH antibody to confirm equal loading. *P<0.05. PPM1D, p53-induced phosphatase 1D; shRNA, short hairpin RNA; RBM38, RNA binding motif protein 38; WT, wild type; p-, phosphorylated; HIF1α, hypoxia-inducible factor 1α; HA, hemagglutinin.

binding of RBM38 to *PPM1D*'s 3'-UTR is increasing under hypoxic conditions, which results in the increased translation of *PPM1D* mRNA, and the translated PPM1D is then dephosphorylating RBM38 protein, inducing the translation of mutant *TP53* mRNA. Alternatively, there is a secondary mechanism of regulating PPM1D expression under hypoxic conditions. To test the first possible explanation, NCI-H1770 variants stably expressing either a control shRNA or shRNA targeting *RBM38* were generated. Successful knockdown was verified by western blot analysis (Fig. 1E). Cells expressing the control or *RBM38* shRNA were subjected to hypoxia. Even though it has been shown before that RBM38 regulates translation of *HIF1A* mRNA (15), knockdown of *RBM38* did not affect HIF1α protein induction (Fig. 1E). This was not surprising given that HIF1α is known to be regulated at multiple different levels. *RBM38* knockdown did not affect the induction in protein expression of PPM1D or mutant p53 (Fig. 1E). However, when the same experiment was repeated under normoxic conditions, knockdown of *RBM38* resulted in a significant decrease in PPM1D protein expression, and an increase in p53 protein expression (Fig. 1F), corroborating findings from other studies performed in normoxic conditions (9,10,17). This indicated that alternate post-transcriptional regulatory mechanism(s) were regulating PPM1D expression under hypoxic conditions.

Differential regulation of PPM1D in A549 cells harboring wild-type or mutant TP53. To determine whether such a potential alternate regulatory mechanism of PPM1D protein

expression operated in NSCLC cells harboring wild-type *TP53*, the A549 cells were subjected to hypoxia. Hypoxia was induced by incubating cells at 1% O_2 for 4 h and confirmed via the induction of HIF1α protein expression (Fig. 2A). The protein expression of RBM38, p53, p-p53 (serine 15) and PPM1D was not significantly different under normoxic and hypoxic conditions (Fig. 2A), indicating a differential response to hypoxia in NSCLC cells harboring wild-type or mutant *TP53*. Given that A549 cells are epithelial cells from a lung tumor mass, whereas the NCI-H1770 cells are neuroendocrine cells derived from lung cancer metastasis in the lymph node, the different origin of A549 and NCI-H1770 was then investigated for the difference observed in A549 and NCI-H1770 cells under hypoxia. A549 cells were transfected with a HA-tagged R428W mutant *TP53* plasmids. Stably selected cells were then transduced with shRNA targeting the 3'-UTR of TP53. Successful knockdown of the endogenous (>90% knockdown was achieved) and overexpression of mutant *TP53* were verified via western blot analysis assays using p53 and HA antibodies (Fig. 2B). To determine whether the regulation of PPM1D protein expression in the *TP53*-mutant A549 cells was such as that observed in NCI-H1770 cells (Fig. 1E and F), variants of the A549-R248W ΔTP53 cells stably expressing either a control shRNA or shRNA targeting *RBM38* were generated. Successful knockdown was verified by western blot analysis (Fig. 2C). Cells expressing the control or *RBM38* shRNA were either maintained under normoxic condition or subjected to hypoxia. As in NCI-H1770 cells, hypoxia
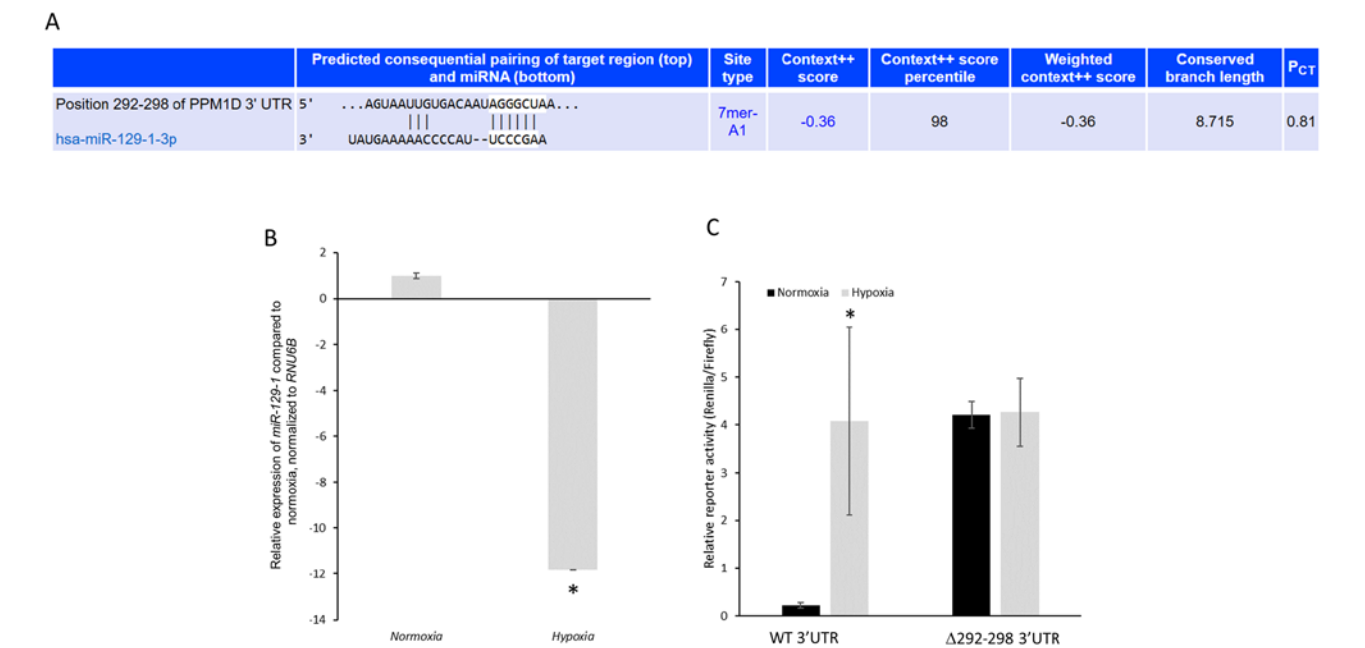
Figure 3. PPM1D is a putative target of miR-129-1-3p. (A) *In situ* prediction of *PPM1D* as a potential target of miR-129-1-3p by TargetScan. (B) Relative expression of miR-129-1 in NCI-H1770 cells grown under normoxic conditions or subjected to hypoxia for 4 h. Data were normalized to *RNU6B* and expressed as the mean ± standard error of the mean. (C) Relative reporter activity of *PPM1D* WT (WT 3'UTR) or miR-129-1-3p binding mutant 3'-UTR (Δ292-298 3'UTR). Data were normalized to Firefly luciferase and expressed as the mean ± standard deviation of the ratio of *Renilla* and Firefly luciferase activity. *P<0.05. UTR, untranslated region; PPM1D, p53-induced phosphatase 1D; miR, microRNA; WT, wild type.

treatment resulted in increased PPM1D and HA-p53 protein expression (Fig. 2C, first vs. third lane). *RBM38* knockdown, as in the NCI-H1770 cells, did not affect the protein expression of PPM1D or mutant p53 under hypoxia (Fig. 2C). However, when the same experiment was repeated under normoxic conditions, knockdown of *RBM38* resulted in significant decrease in PPM1D and increase in mutant p53 protein expression levels (Fig. 2C). Cumulatively, these results confirm that the difference observed in A549 and NCI-H1770 cells under hypoxia was not due to the different cellular origin of the NCI-H1770 and A549 cells. These results also confirm that in NSCLC cells harboring R248W mutant *TP53*, the induction of PPM1D protein expression is independent of RBM38 protein expression.

PPM1D is a putative target of miR-129-1-3p. TargetScan algorithm (36) was used for the prediction of potential microRNAs (miRNAs) targeting *PPM1D* mRNA. There was only one conserved miRNA, miR-129-1-3p, predicted to target nucleotides 292-298 of the 3'-UTR of *PPM1D* mRNA (Fig. 3A). miR-129 has previously been demonstrated to function as a tumor suppressor in lung cancer by regulating cell proliferation and metastatic progression (37,38). Hence, whether *PPM1D* is targeted by miR-129-1-3p in lung cancer cells harboring mutant *TP53* and the effects on PPM1D protein expression under normoxic and hypoxic conditions were investigated.

Primarily, miR-129-1-3p expression levels in NCI-H1770 cells under normoxic and hypoxic conditions was determined. Hypoxia caused an 11.799±0.002-fold decrease in miR-129-1-3p expression compared with normoxic conditions (Fig. 3B). To investigate whether *PPM1D* mRNA is a direct target of miR-129-1-3p, luciferase reporter plasmids harboring either the wild-type 3'-UTR or mutant 3'-UTR (miR-129-1-3p

binding site, nucleotides 292-298, deleted) were generated. These constructs were transfected in the NCI-H1770 cells and their expression was determined under normoxic and hypoxic conditions. The mutant reporter was expressed significantly higher compared with the wild-type reporter under normoxic conditions (4.21±0.28 vs. 0.23±0.06, respectively; P=1.19x10^{-5}) (Fig. 3C). However, following hypoxia induction, both the wild-type and mutant reporters were robustly expressed without any significant difference (4.08±1.97 vs. 4.25±0.71, P=0.89) (Fig. 3C).

PPM1D is targeted by miR-129-1-3p in NCI-H1770 cells under normoxia conditions. The aforementioned results indicate that the decrease in miR-129-1-3p expression under hypoxic conditions may explain the increase in protein expression of PPM1D. To test this hypothesis, control or MIR129-1-3p mimic was transiently transfected in the NCI-H1770 cells and overexpression was confirmed via RT-qPCR (Fig. 4A). The mimic transfected cells were co-transfected with the wild-type and mutant luciferase reporters and subjected to hypoxia. Reporter expression was significantly downregulated in cells transfected with miR-129-1-3p mimic. However, this was not observed with cells transfected with the control mimic (4.18±0.54 vs. 0.26±0.01, respectively; P=3.32x10^{-6}; Fig. 4B). No significant difference was observed in the expression of the mutant reporter following transfection of the miR-129-1-3p mimic (4.47±0.09 vs. 4.27±0.19, P=0.41; Fig. 4B). These results along with those presented in Fig. 2C confirm that miR-129-1-3p is targeting *PPM1D* mRNA in lung cancer cells harboring mutant *TP53* under normoxic conditions.

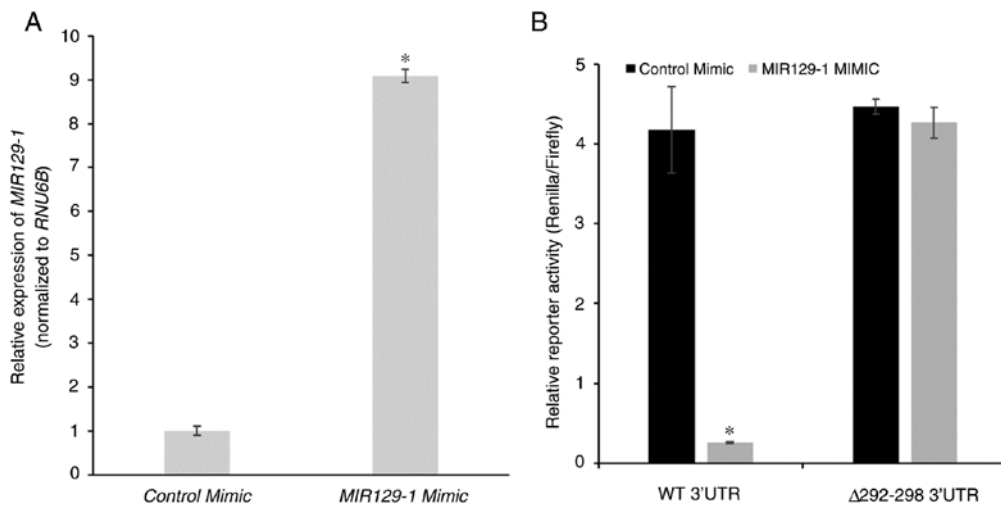*Co-occurrence of PPM1D/RBM38 and PPM1D/HIF1A mutations in patients with NSCLC.* Based on the aforementioned

Figure 4. *PPM1D* is targeted by miR-129-1 in NCI-H1770 cells under normoxic conditions. (A) Relative expression of MIR-129-1in NCI-H1770 cells transiently transfected with either control or miR-129-1 mimic. Transfected cells were treated with hypoxia for 4 h following 48 h of transfection. Data were normalized to *RNU6B* and expressed as the mean ± standard error of the mean. (B) Relative reporter activity of *PPM1D* wild-type or miR-129-1-3p binding mutant 3'-UTR in NCI-H1770 cells transfected with control or miR-129-1 mimic. Data were normalized to Firefly luciferase and expressed as ratio of *Renilla* and Firefly luciferase activity. *P<0.05. UTR, untranslated region; PPM1D, p53-induced phosphatase 1D; miR, microRNA; WT, wild type.
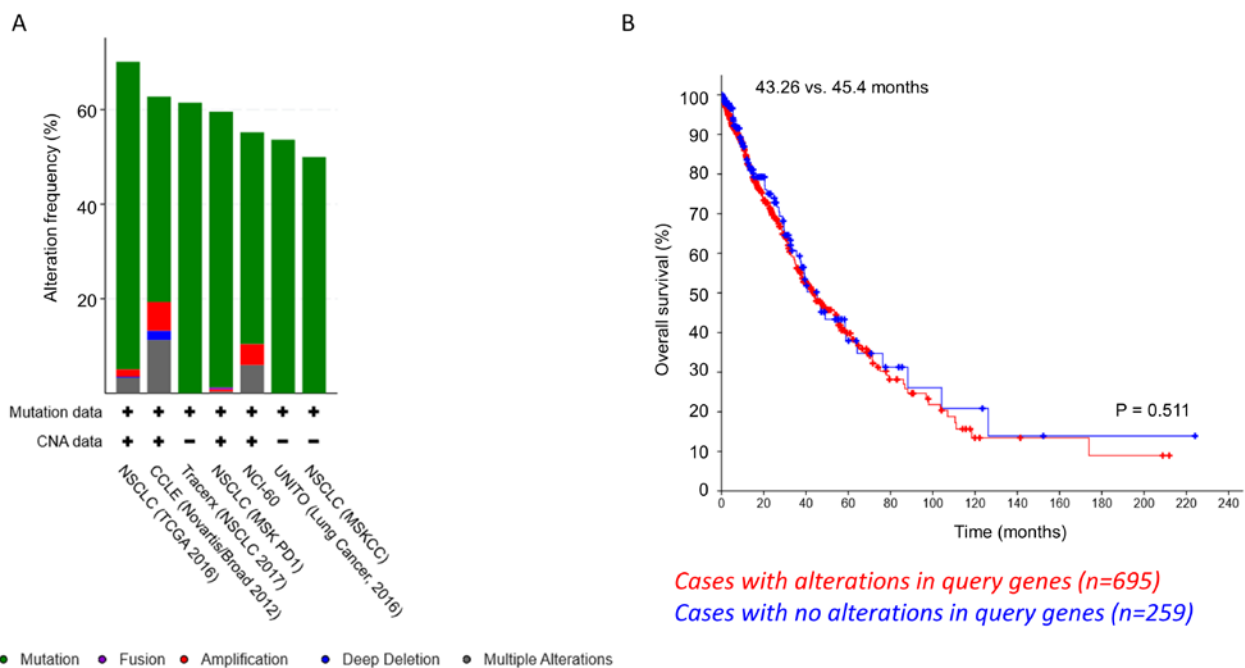

Figure 5. Co-occurrence of *PPM1D/RBM38* and *PPM1D/HIF1A* mutations in patients with NSCLC. (A) Amplification and somatic mutations of TP53, PPM1D, RBM38 and HIF1A in NSCLC cell lines and patients. Data analyzed were retrieved from TCGA and referred to 2,628 patients (2,855 samples) from 7 studies. The analysis based on the cancer studies is presented. (B) Kaplan-Meier overall survival analysis curve did not reveal any significant difference in survival times between patients with NSCLC with (n=695 patients) and without (n=259 patients) genomic amplification or somatic mutation in *TP53*, *PPM1D*, *RBM38* and *HIF1A*. P=0.551. TCGA, The Cancer Genome Atlas; PPM1D, p53-induced phosphatase 1D; shRNA, short hairpin RNA; RBM38, RNA binding motif protein 38; HIF1α, hypoxia-inducible factor 1α; NSCLC, non-small cell lung cancer.

observations, a TCGA dataset on NSCLC samples was analyzed to look at genomic alterations in *PPM1D*, *RBM38*, *TP53*, *MIR129-1-3P* and *HIF1A* in 2,628 patients (2,855 samples) (Fig. S1) (29-35). *TP53* mutations were present in 61% of the cases, whereas *RBM38*, *PPM1D* and *HIF1A* mutations were present in 5, 3 and 4%, respectively. Most of these were point mutations, even though some genomic amplifications and deep deletions were also observed (Fig. 5A).

MIR129-1-3P mutation data were missing among the datasets selected for this study. Notably, when the tendency of co-occurrence of mutations in the aforementioned genes was examined, *PPM1D/HIF1A* and *PPM1D/RBM38* exhibited significant co-occurrence (P=0.016 and 0.029, respectively; Table SI). *RBM38* and *TP53* mutations had a tendency of mutual exclusivity, even though this was not significant. Next, the difference in overall survival of patients with genomic

alterations in *PPM1D*, *RBM38*, *HIF1A*, and *TP53* (n=695) and those without any alterations in these genes (n=259) was investigated. The median survival time was 45.4 months in patients with no alterations vs. 43.26 months in patients with alterations (P=0.511; Log rank t-test; Fig. 5B). These results indicate that in a small cohort of patients with NSCLC, *PPM1D* mutations tend to co-occur with *RBM38* or *HIF1A*; however, not both at the same time.

Discussion

In the present study, miR-129-1-3p, and not RBM38, was revealed to regulate PPM1D protein expression in mutant p53-harboring NSCLC cells under hypoxia. Under normoxic conditions, even though PPM1D expression was higher in the NCI-H1770 cells, PPM1D protein was detectable in A549 cells with wild-type p53. However, high expression of miR-129-1-3p in NCI-H1770 cells maintained under normoxic conditions was detected. miR-129-1-3p expression was downregulated under hypoxia resulting in a further induction of PPM1D protein.

Even though miR-129-1-3p levels were high in normoxic conditions in NCI-H1770 cells, there was not a complete halt in PPM1D translation. One explanation may be that RBM38 is present and is known to induce translation of *PPM1D*. Thus, there may be a homeostatic balance between RBM38 and miR-129-1-3p that results in decreased translation of *PPM1D* under normoxic conditions.

Notably, despite the fact that RBM38 is required for the translation of mutant *TP53* and *PPM1D* (1,6,8,9), no changes in the protein expression levels of p53 or PPM1D in NCI-H1770 cells or mutant A549 following after knockdown of *RBM38* were observed. One argument can be the inefficient knockdown of *RBM38*, allowing the residual protein to bind and translate *PPM1D* and mutant *TP53* mRNAs. However, it is surprising that there was no decrease in PPM1D and p53 protein expression following the 80-90% decrease in RBM38 protein expression. Thus, the mechanism that regulates PPM1D translation in cases where RBM38 is not present needs to be determined. Of note, RBM38 deletion is observed in tumors harboring mutant p53 (39,40). A limitation of the present study was that the mutant A549 cells used were not generated by CRISPR/Cas9-mediated targeted mutation which would have been a more robust model.

Conversely, the expression level of miR-129-1-3p in normal lung epithelial cells and lung cancer cells with wild-type p53 needs to be determined. Even though HIF1α is known to complex with mutant p53 alone to transcriptionally upregulate extracellular matrix component proteins (19), whether that complex is also downregulating miR-129-1-3p expression and consequently inducing PPM1D protein expression is yet to be elucidated. Finally, functional assays need to be performed to define the significance of miR-129-1-3p-mediated PPM1D regulation in NSCLC cells with mutant p53 under hypoxic conditions.

Lung cancer cells, both with wild-type or mutant p53, experience hypoxic conditions, both during initial tumorigenesis and during metastatic progression (41). *PPM1D* is a target of p53 and a key modulator of the p53 genomic surveillance mechanism in normal cells (1,6,7). RBM38 switches from a repressor of translation of p53 according to its phosphorylation by GSK3β (10) and dephosphorylation

by PPM1D (8). Considering that miR-129-1-3p was demonstrated in the present study to regulate PPM1D expression under hypoxic conditions, whether and how miR-129-1-3p expression is regulated during both tumor initiation and progression, and whether it is a direct target of p53 or mutant p53-HIF1α transcriptional complex, remains to be determined.

In summary, a yet undefined mechanism of miR-129-1-3p-mediated regulation of PPM1D protein expression in NSCLC cells with mutant p53 under hypoxic conditions was identified. Whether a similar mechanism exists in other tumor types with wild-type or mutant p53 remains to be determined. In addition, the mechanism by which co-occurring PPM1D/HIF1A and PPM1D/RBM38 mutations affect tumor initiation and disease progression in lung cancer patients would be of interest to investigate.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

HLY and QYL designed the experiments; HWX and QYL performed the experiments; HLY, HWX and QYL analyzed the experimental results. HLY wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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