DDX31 Regulates the p53-HDM2 Pathway and rRNA Gene Transcription through Its Interaction with NPM1 in Renal Cell Carcinomas

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Abstract

Studies of renal cell carcinoma (RCC) have led to the development of new molecular-targeted drugs but its oncogenic origins remain poorly understood. Here, we report the identification and critical roles in renal carcinogenesis for DDX31, a novel nucleolar protein upregulated in the vast majority of human RCC. Immunohistochemical overexpression of DDX31 was an independent prognostic factor for patients with RCC. RNA interference (RNAi)-mediated attenuation of DDX31 in RCC cells significantly suppressed outgrowth, whereas ectopic DDX31 overexpression in human 293 kidney cells drove their proliferation. Endogenous DDX31 interacted and colocalized with nucleophosmin (NPM1) in the nucleoli of RCC cells, and attenuation of DDX31 or NPM1 expression decreased pre-ribosomal RNA biogenesis. Notably, in DDX31-attenuated cells, NPM1 was translocated from nucleolus to the nucleoplasm or cytoplasm where it bound to HDM2. As a result, HDM2 binding to p53 was reduced, causing p53 stabilization with concomitant G1 phase cell-cycle arrest and apoptosis. Taken together, our findings define a mechanism through which control of the DDX31–NPM1 complex is likely to play critical roles in renal carcinogenesis. Cancer Res; 72(22): 5867–77. ©2012 AACR.

Introduction

Renal cell carcinoma (RCC) is the most common cancer of the kidney (1, 2), and up to 30% of patients with RCC present with metastatic disease (3, 4). At an early stage, RCC can be cured by surgical resection, which is the most effective treatment for localized RCC tumors (5). Moreover, cytokine therapies, such as interleukin-2 or IFN-α, are widely used as a first-line treatment for metastatic disease. However, these treatments can be associated with severe toxicity (6–9). In addition to these anticancer drugs, several molecular target drugs, such as anti-VEGF antibody (bevacizumab) and small-molecule inhibitors (sunitinib, sorafenib, axitinib, temsirolimus, and everolimus) have been recommended as treatment options for metastatic RCC and have been shown to prolong survival (10–13). However, such treatments have also resulted in severe side effects (14–16). The prognostic factors that determine the treatment outcome in patients with metastatic RCC have been identified according to the status of the patient (17). These markers have been widely used to determine the best treatment approach and, as a result, may improve survival. At present there are no new relevant molecular markers that have been identified to predict the prognosis of RCC (18). Therefore, it is highly important to develop a new molecular target agent(s) against RCC as well as prognostic molecular markers.

The ’omics’ technologies, including transcriptomics, genomics, and proteomics, provide a detailed characterization of individual cancers that should help improve clinical strategies for neoplastic diseases through the development of novel drugs, as well as providing the basis for personalized treatment (19). Hence, these technologies have helped identify the diagnostic and therapeutic targets for cancer and understand the detailed molecular mechanism of carcinogenesis. To this end, we analyzed the expression profiling of clear cell RCC (ccRCC), which is a major histologic type of RCC (the Gene Expression Omnibus database accession number: GSE39364; ref. 20), and identified and characterized DEAD (Asp-Glu-Ala-Asp) box polypeptide 31 (DDX31), a novel member of the DEAD box protein family.

DDX31 is the human homologue of Saccharomyces cerevisiae DEAD box protein 7 (Dbp7), which was originally identified in a Basic Local Alignment Search Tool (BLAST) search of the Saccharomyces cerevisiae Genome Database (Stanford...
University, Stanford, CA) as a new ATP-dependent RNA helicase (21). Dhp7 has been shown to play an important role in the assembly of pre-ribosomal particles during the biogenesis of the 60S ribosomal subunit (21). However, to date, no reports have characterized the biologic function of DDX31, the human homologue of Dhp7, and the significance of its transactivation in clinical RCC progression.

Here, we identify and characterize DDX31 as a clinical prognostic factor and a potential therapeutic target, and provide evidence that DDX31 is involved in regulating ribosomal RNA (rRNA) gene transcription and the p53–HDM2 pathway in renal carcinogenesis.

**Materials and Methods**

**Cell lines and specimens**

ACHN, Caki-1, Caki-2, 769-P, HK-2, HEK293, and COS-7 cells were obtained from the American Type Culture Collection between 1994 and 2012. OS-RC-2 was provided by the RIKEN BioResource Center (Tsukuba, Japan) in 2005. KC-12, KMRC-1, KMRC-20, and YCR-1 cells were kindly provided by Dr. Taro Shuin (Department of Urology, Kochi Medical School, Kochi University, Kochi, Japan) in 2006. All cells were cultured under conditions recommended by their respective depositors. We monitored the cell morphology of these cell lines by microscopy and confirmed that they had maintained their morphologic states in comparison with the original morphologic images. No mycoplasma contamination was detected in cultures of all of these cell lines using a Mycoplasma Detection Kit (Roche) in 2011. Surgically resected RCC tissue samples and their corresponding clinical information were acquired from The University of Tokushima Hospital (Tokushima, Japan) after obtaining written informed consent. This study, as well as the use of all clinical materials described above, was approved by the Ethical Committee of The University of Tokushima.

**Plasmids**

Plasmids used in this study are described in Supplementary Materials and Methods.

**Quantitative and semiquantitative reverse transcription PCR**

The extraction of total RNA from cultured cells and RCC clinical tissues, reverse transcription, and subsequent PCR amplification were conducted as previously described (20). β-Actin was used as a quantitative control. The sequences of each primer set are described in Supplementary Table S1.

**Generation of a specific anti-DDX31 polyclonal antibody**

The recombinant DDX31 protein was expressed and purified as previously reported (22) and subsequently inoculated into rabbits (Scrum Inc.). The immune sera were then purified on antigen affinity columns using Affi-gel 10 gel (Bio-Rad).

**Antibodies**

Antibodies used are described as follow; the anti-DDX31 polyclonal antibody (see above; 1:100), anti-NPM1 monoclonal antibody (FC-61991, Invitrogen 32-5200; 1:1,000), anti-HA rat monoclonal antibody (3F10, Roche 11867423001; 1:1,000), anti-FLAG M2 monoclonal antibody (M2, Sigma-Aldrich F3165; 1:5,000), β-actin (AC-15, Sigma-Aldrich A5441; 1:5,000), anti-p53 (DO-7, Dako M7001; 1:200), anti-p53 (FL-393, Santa Cruz sc-6243; 1:1,000), anti-p21 (Abcam, ab-7860; 1:200), anti-HDM2 (2A10, Abcam, ab16895; 1:500), or anti-PARP (Cell Signaling Technology #9542; 1:200).

**Western blot analysis**

SDS-PAGE and immunoblotting were conducted as described previously (23). Full description for this analysis is described in Supplementary Materials and Methods.

**Immunohistochemical staining**

Preparation of frozen sections (5 μm) of RCCs and normal kidney tissues and immunohistochemical staining were conducted as described previously (2), with anti-DDX31 antibody (1:500), and classified the DDX31 expression levels on the stained tissues based on a scoring method as follows; The percentage of DDX31-positive cancer cells was scored on a scale of 0 to 2 (0: <5%, 1: 5–49%, and 2: >50%). Moreover, the intensity of DDX31 or NPM1 staining was semiquantitatively evaluated by 3 independent experienced pathologists without prior knowledge of clinicopathologic data based on the following scoring system: 0: no staining, 1: low, 2: moderate, and 3: high. Cases were accepted as positive only if all reviewers independently defined them as such. In addition, the product of the DDX31 staining score was divided into the following 2 groups: 3 ≤ and > 4.

**RNA interference experiments**

We conducted RNA interference (RNAi) experiments as previously described (24, 25). The detailed Materials and Methods of this experiment are described in Supplementary Materials and Methods.

**Establishment of stable transfectants**

Mock (no insert) or pCAGGS-DDX31-HA expression vectors were transfected into HEK293 cells as previously described (20, 26). Following experiments are described in Supplementary Materials and Methods.

**Measurement of pre-rRNA biogenesis**

The amount of 45S pre-ribosomal RNA (pre-rRNA) in total RNA extracted was analyzed as described previously (27). Full description of this analysis is described in Supplementary Materials and Methods.

**Immunocytochemical staining analysis**

The methodology for this analysis is described in Supplementary Materials and Methods.

**Immunoprecipitation and mass spectrometry**

DDX31-9 and Mock-3 stable transfectant cells were lysed as described previously (26). Liquid chromatography/mass spectrometry and data acquisition were conducted as previously described (28).
Coimmunoprecipitation assay

Coimmunoprecipitation assay was conducted as described in Supplementary Materials and Methods.

p53 In vivo ubiquitination assay

The methodology for this assay is described in Supplementary Materials and Methods.

Cell-cycle analysis

We conducted flow cytometry with analysis gates to remove debris and doubles after transfection of siDDX31 or siEGFP, as previously described (23). The DNA content of 10,000 cells was analyzed by FACS Calibur and CellQuest software (BD Biosciences).

Statistical analysis

Progression-free survival curves were estimated using the Kaplan–Meier method. The statistical significance of a relationship between the clinical outcome and DDX31 expression was assessed using the trend log-rank test. Cox proportional hazards analysis was used to identify significant prognostic clinical factors and to test for an independent contribution of DDX31 expression to progression-free survival. To examine potential confounding factors, age was treated as a continuous variable, and gender, nuclear grade, T stage, M stage, and the DDX31 expression score were treated as ordinal variables. Statistical significance was calculated using Student t test to evaluate cell proliferation or gene expression. A difference of P < 0.05 was considered statistically significant.

Results

Upregulation of DDX31 in renal cell carcinomas

We verified that DDX31 was upregulated in 7 of 9 ccRCC cases compared with the corresponding normal kidney, and in 8 of 9 ccRCC cell lines compared with normal human kidney by quantitative RT-PCR (qRT-PCR; Fig. 1A and B). To investigate

Figure 1. DDX31 expression in RCCs. A and B, expression of the DDX31 gene in 9 RCC tissues (T), the adjacent normal kidney (N), and 9 RCC cell lines and a normal kidney as determined by quantitative RT-PCR (qRT-PCR). β-Actin served as a quantitative internal control. C, DDX31 protein expression in RCC cell lines and HK-2 cell line as determined by Western blot. W, p53 wild-type; M, p53 mutant. D, genomic structure of 3 DDX31 splicing variants (V1, V2, and V3). White and black triangles indicate the initiation and stop codons, respectively. Arrows indicate the primer set that detects each of the 3 variants. The number above each box indicates the exon number. E, expression pattern of each of the three DDX31 splicing variants in three RCC cell lines and a normal human kidney examined by semiquantitative RT-PCR.
DDX31 expression at the protein level, we generated an anti-DDX31 polyclonal antibody and confirmed that this antibody specifically recognized exogenously expressed DDX31 protein in HEK293 cells as well as endogenous DDX31 in Caki-1 cells in Western blot analyses (Supplementary Fig. S1A). The Western blot results accorded well with those of the qRT-PCR results in most of the tested RCC cell lines and a normal human kidney epithelial cell line, HK-2 (Fig. 1C). Furthermore, northern blot analysis showed that the DDX31 transcript was weakly or undetectably expressed in normal human organs (data not shown). In the NCBI database, cDNA sequences corresponding to 3 transcript variants, denoted DDX31V1, DDX31V2, and DDX31V3, consist of 2555, 2328, and 2337 nucleotides that encode 851, 775, and 778 amino acids, respectively. These 3 transcriptional variants contain 20, 20, and 18 exons, respectively, as shown in Fig. 1D. The V2 variant has a unique exon 19, which is 58 bp longer at the 5′ end than exon 19 of V1 and V3, resulting in a frameshift within exon 19 and an early stop codon. The V3 variant lacks exons 17 and 18 and has a unique exon 16, which is 35 bp shorter at the 5′ end than exon 16 of V1 and V2 (Fig. 1D). Subsequent semiquantitative RT-PCR confirmed that the DDX31V1 variant had the highest expression in ccRCC cells (Fig. 1E). Therefore, we focused on elucidating the biologic roles of the DDX31V1 transcript due to its predominant expression in cancer cells.

Association of higher DDX31 expression with poorer prognosis

To investigate the detailed expression pattern of the DDX31 protein and characterize its biologic functions, we conducted immunohistochemical staining of 70 ccRCC specimens with the anti-DDX31 antibody, and classified the DDX31 expression levels on the stained tissues based on a scoring method (see Materials and Methods). DDX31 was observed in the nuclei of many RCC specimens, although the staining patterns and intensities varied across individual cases (Fig. 2A), whereas no staining was detected in normal kidney tissues (Fig. 2B). Next, to investigate the clinicopathologic significance of DDX31 expression in RCC tissues, we analyzed the relationship between the calculated immunohistochemical DDX31 scores and the clinicopathologic variables of 70 ccRCC specimens (Supplementary Table S2). We used a Cox proportional hazards regression analysis to examine the association between DDX31 expression and other clinical and pathologic variables to predict progression-free survival. Both univariate and multivariate analyses revealed that DDX31 expression was a significant and independent prognostic factor for patients with RCC (P = 0.0062 and P = 0.014, respectively; HR: 1.8 per score; Table 1). A Kaplan–Meier survival analysis with the log-rank significance test also showed that high DDX31 expression (score ≥ 4) was associated with a significantly worse progression-free survival than those with low DDX31 protein expression (Score ≤ 3; Fig. 2C, log-rank, P = 0.00002). These results suggested that DDX31 overexpression was indicative of worse outcomes in RCC.

Effects of DDX31 expression on cell growth

To investigate the growth promoting role of DDX31 in RCC cells, we knocked down the expression of endogenous DDX31 in Caki-1 (Fig. 3A and B) and KMRC-1 (Fig. 3C and D) using RNAi techniques. qRT-PCR showed significant DDX31 knockdown in cells transfected with DDX31 shRNAs or siRNAs.
(\#1 and \#2), but not the controls (shEGFP or siEGFP). In concordance with the knockdown effect, MTT assays clearly revealed significant growth suppression in the 2 cell lines by both \#1 and \#2, compared with the controls (shEGFP or siEGFP; Fig. 3B and D). In addition, a colony formation assay also confirmed that introducing both shRNA-DDX31 constructs remarkably suppressed the growth of Caki-1 cells compared with shEGFP-transfected cells (shEGFP: \#1 or \#2 = 100%: 56% or 18%; Fig. 3E). To further confirm the growth promoting effects of DDX31 overexpression, we established HEK293 cell derivatives that stably express exogenous DDX31 (DDX31-1, -8, -9, and -10). Western blot analysis with an anti-HA antibody confirmed the high expression of DDX31 protein in these 4 clones (Fig. 3F). Subsequent MTT assays showed that all 4 DDX31-stable derivative cells (DDX31-1, -8, -9, and -10) grew significantly faster than the mock-transfected controls (Mock-1, -2, and -3; Fig. 3G; \(P = 0.022\)). Together, these findings strongly imply that DDX31 plays an important role in cell proliferation of RCC.

**DDX31 interacts with NPM1**

Because the biologic functions of DDX31 are unknown, we searched for DDX31-interacting protein(s) using immunoprecipitation and 2-dimensional image converted analysis of liquid chromatography and mass spectrometry (2DICAL) analyses (28). Nucleophosmin (NPM1), a member of the nucleoplasmin superfamily (29), was identified as a candidate DDX31-interacting protein. We first investigated the expression levels of NPM1 in RCCs by qRT-PCR, Western blot, and immunohistochemistry analyses and found that NPM1 was highly expressed in most of the RCC specimens and cell lines (Supplementary Fig. S1B–S1D). To validate this interaction, HA-tagged DDX31 (DDX31-HA) and FLAG-tagged NPM1 (NPM1-FLAG) were co-transfected into COS-7 cells, and then the proteins were immunoprecipitated with an anti-FLAG or anti-HA antibodies. Subsequent Western blot analysis with the indicated antibodies showed that exogenous NPM1 co-precipitated with DDX31 (Fig. 4A). In addition, we confirmed that endogenous DDX31 co-precipitated with endogenous NPM1 in KMRC-1 cells (Fig. 4B). Next, we examined the effects of knocking down NPM1 on the growth of RCC cell line KMRC-1, and found that NPM1 depletion resulted in a significant decrease in cell viability of KMRC-1 cells (Fig. 4C), although this effect was not comparable with siDDX31. These findings suggest that NPM1 also has important roles in the growth of ccRCC cells.

**DDX31 is involved in rRNA gene transcription**

Dbp7 (Dead-box protein 7), the yeast homologue of DDX31, reportedly plays an important role in the assembly of preribosomal particles during the biogenesis of the 60S ribosomal subunit (21). In addition, NPM1 is also a nucleoprotein that has been implicated in ribosome biogenesis (27, 30). Particularly, a previous report showed that NPM1 depletion reduced the transcription rate of the ribosomal RNA (rRNA) gene (27). Therefore, to examine the effects of DDX31 or NPM1 knockdown on the transcription rate of rRNA, we monitored the amount of pre-rRNA by qRT-PCR. DDX31 expression did not affect NPM1 knocking down in KMRC-1 cells (Fig. 4D), but knocking down of DDX31, NPM1, or both significantly reduced the expression of the pre-rRNA RNA45S subunits (Fig. 4D and E). Conversely, we confirmed that pre-rRNA synthesis was significantly increased in DDX31 stable transfectants (Fig. 4F). These findings suggest that the DDX31–NPM1 complex plays a crucial role in rRNA gene transcription.

**DDX31 regulates the p53 stabilization through its interaction with NPM1 in the nucleoli**

It has been reported that the redistribution of NPM1 upon DNA damage, such as UV damage, leads to an interaction with HDM2, and that this binding could affect p53 stabilization and activity by inhibiting HDM2–p53 complex formation, indicating that NPM1 acts as a negative regulator of HDM2–p53 interaction (31). Moreover, we found that a reduced level of pre-rRNA biogenesis (Fig. 4E) weakly correlated with decreased cell growth (Fig. 3) in DDX31- or NPM1-depleted RCC cells. Therefore, we hypothesized that DDX31 regulates p53–HDM2 pathway through its interaction with NPM1 in RCC.

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**Table 1. The association of DDX31 expression with other clinical and pathologic variables**

<table>
<thead>
<tr>
<th>Variables</th>
<th>(P)</th>
<th>HR (95% CI)</th>
<th>(P)</th>
<th>HR (95% CI)</th>
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<tr>
<td>Age</td>
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<tr>
<td>Gender</td>
<td>0.89</td>
<td>1.13 (0.218–5.83)</td>
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<td>Nuclear grade</td>
<td>0.036</td>
<td>3.51 (1.09–11.36)</td>
<td>0.13</td>
<td>3.53 (0.71–17.69)</td>
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<tr>
<td>Score</td>
<td>0.0062</td>
<td>1.72 (1.17–2.52)</td>
<td>0.014</td>
<td>1.8 (1.13–2.87)</td>
</tr>
<tr>
<td>T stage</td>
<td>0.041</td>
<td>2.45 (1.04–5.79)</td>
<td>0.16</td>
<td>1.98 (0.76–5.15)</td>
</tr>
<tr>
<td>N stage</td>
<td>Not calculated</td>
<td></td>
<td>0.029</td>
<td>7.40 (1.23–44.3)</td>
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<tr>
<td>M stage</td>
<td>0.0008</td>
<td>16.13 (3.16–82.30)</td>
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</table>

Note: Cox proportional hazard regression analysis was used to test for prediction of the progression-free survival. Univariate analysis showed that nuclear grade, T stage, M stage, and DDX31 scores were significant predictors of progression (\(P = 0.036, 0.041, 0.0008, \) and 0.0062, respectively).

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DDX31 Regulates the p53 Activity in RCC Cells
We first examined the effects of knocking down DDX31 on the nuclear localization of NPM1 in Caki-1 cells by immunocytochemistry with anti-DDX31 or anti-NPM1 antibodies. We observed that DDX31 and NPM1 colocalized in the nucleoli of Caki-1 cells transfected with siEGFP as a control (Fig. 5A; siEGFP), whereas translocation of endogenous NPM1 from the nucleoli to the nucleoplasm or cytoplasm was observed in DDX31-depleted cells (Fig. 5A; siDDX31). On the other hand, NPM1 knockdown did not affect the nucleolar localization of DDX31 (Supplementary Fig. S2A). Moreover, translocation of endogenous nucleolin, which is also an abundant nucleoprotein, from the nucleoli to the nucleoplasm or cytoplasm was not observed in DDX31-depleted cells (Fig. 5A; siDDX31). On the other hand, NPM1 knockdown did not affect the nucleolar localization of DDX31. These findings suggest the possibility that DDX31 specifically interacts with NPM1, and thereby inhibits its translocation from the nucleoli to the nucleoplasm or cytoplasm in RCC cells.

Next, we examined the expression level of p53 protein upon siRNA-mediated knockdown of DDX31. The expression of p53 was clearly upregulated at the protein level upon DDX31 knockdown in both Caki-1 and KMRC-1 cells (Fig. 5B), but the steady-state levels of p53 mRNA remained constant in cells treated with DDX31-siRNA (Supplementary Fig. S3A). On the other hand, p21, a p53 downstream target, was clearly upregulated at the protein and transcriptional levels at 72 hours after siDDX31 transfection (Fig. 5B, Supplementary Fig. S3B and S3C). Moreover, we confirmed that DDX31 depletion did not affect NPM1 protein expression levels (Fig. 5C). Subsequently, we investigated whether NPM1 could bind to endogenous HDM2 when DDX31 expression was knocked down by siRNA, and found that endogenous NPM1 and HDM2 formed a protein complex in DDX31-depleted cells, whereas the formation of p53–HDM2 complex was clearly decreased (Fig. 5D).

Because HDM2 regulates p53 turnover via its E3 ligase activity, we hypothesized that DDX31 deficiency inhibits p53 degradation by reduction of its HDM2-mediated ubiquitination. We transfected KMRC-1 cells with plasmids expressing ubiquitin and p53 proteins and transfected these cells with siDDX31 or siEGFP as a control. Subsequent immunoprecipitation and immunoblotting analyses revealed that the p53 polyubiquitination was clearly decreased in DDX31-depleted cells compared with that in siEGFP-transfected cells under the presence of MG132, which is a 26S proteasome inhibitor (Fig. 5E). These results show that DDX31 inhibition possibly prevents HDM2-mediated p53 polyubiquitination.
DDX31 deficiency induced p53-dependent G1 arrest and apoptosis

Next, to examine the effects of DDX31 ablation on the perturbation of cell cycle of RCC cells, we conducted fluorescence-activated cell sorting (FACS) analysis. A clear increased population of G0–G1 cells at 96 hours after siDDX31 transfection (siEGFP: siDDX31 = 69.71%: 79.01%) was observed (Fig. 6A). We detected upregulation of p53 at protein level, but not at mRNA, whereas upregulation of p21 occurred at both protein and mRNA levels (Fig. 6B and C). We also observed an increased sub-G1 population from 96 hours (siEGFP: siDDX31 = 3.45%: 16.52%; Supplementary Fig. S4A) to 120 hours (siEGFP: siDDX31 = 8.52%: 28.54%; Fig. 6D). In addition, we detected a cleaved PARP at 120 hours (Fig. 6E), indicating an induction of G1 arrest and subsequent apoptosis by DDX31 depletion. More importantly, siRNA-mediated p53 knockdown reversed the effect of DDX31 silencing on cell growth, indicating that DDX31 deficiency led to apoptosis in a p53-dependent manner (Fig. 6F). On the other hand, in p53-mutant KMRC-20 cells, which do not express p53 protein due to a 5 bp insertion within exon 5, DDX31 knockdown did not affect p21 expression level and growth reduction (Supplementary Fig. S4B and S4C) compared with that in p53-wildtype KMRC-1 cells (Fig. 6B, C). Thus, DDX31 levels are relevant to the p53-mediated cellular responses in RCC cells.

Discussion

Through an expression profiling analysis of ccRCC, we identified the upregulation of DDX31 in the majority of ccRCC cases but its minimal expression in normal human tissues (data not shown). RNAi-mediated knockdown of DDX31 drastically suppressed RCC cell growth, whereas introducing DDX31 into HEK293 cells enhanced cellular proliferation. Moreover, clinicopathologic evidence through immunohistochemistry using 70 ccRCC specimens showed that RCC cases...
strongly expressing DDX31 had shorter progression-free survival periods than those with negative or weak DDX31 expression. These results strongly suggest that DDX31 overexpression is critically involved in renal carcinogenesis.

The DDX31 gene encodes a putative 851 amino acid protein with a predicted ATP-dependent RNA helicase domain that is conserved among the DEAD-box protein family. It has been reported that Dbp7, the Saccharomyces cerevisiae homologue of DDX31 (21% identity and 72% similarity), localizes to the nucleolus and is required for optimal vegetative growth (21). Dbp7 depletion results in reduced amounts of the 60S ribosome subunits and the accumulation of halfmer polysomes (21). Thus, Dbp7 is likely involved in the assembly of pre-ribosomal particles during the biogenesis of the 60S ribosomal subunit (21, 32), but the pathophysiologic functions of human DDX31 in RCC cells are unknown. Here, we showed that DDX31 interacts and co-localizes with the nucleophosphoprotein NPM1 in the nucleoli of RCC cells. Moreover, siRNA-mediated knockdown of DDX31 or NPM1 expression led to a significant reduction in cell growth, suggesting that this protein complex plays an important role in the cell growth of RCC.

Accumulating evidence indicates that NPM1 is implicated in multiple functions, including ribosomal biogenesis (28, 30), centrosome duplication (33), and regulation of p53 stabilization (34–38). Therefore, we first examined whether depleting DDX31 or NPM1 affects pre-rRNA synthesis and found that there was a significant reduction in cell growth in DDX31- or NPM1-depleted RCC cells, whereas a significant enhancement of pre-rRNA synthesis was observed in DDX31-overexpressed cells. These findings suggest that DDX31–NPM1 complex formation in the nucleoli plays a crucial role in rRNA gene transcription in renal carcinogenesis.

Previous reports have indicated that cellular stress causes NPM1 to undergo nucleoplasmic translocation and associate with HDM2, and the formation of this complex leads to the stabilization of the p53 protein (31). In addition, NPM1 also regulates basal levels of the p53 protein in unstressed cells through its interaction with HDM2 (31), indicating that NPM1 negatively regulates the p53–HDM2 interaction. Thus, the nucleoli to nucleoplasmic translocalization of NPM1 resulted in p53 activation after cellular or nucleolar stress, but it is unclear how NPM1 is anchored in the nucleoli of RCC cells.
which highly expresses DDX31, even after doxorubicin-induced cellular stress (Supplementary Fig. S5A). On the other hand, in normal epithelial kidney cell HK-2, which does not show DDX31 protein at detectable levels, NPM1 translocated to the nucleoplasm with treatment of doxorubicin, but was localized in the nucleoli without doxorubicin (Supplementary Fig. S5B). Nucleolar stress is often caused by disruption of ribosomal biogenesis, which in turn can be caused by malfunction of nucleolar proteins (39–41) and chemotherapeutic drugs such as doxorubicin (42). As shown in Fig. 4E, DDX31 depletion caused a reduction in rRNA gene transcription, which is a key step of ribosome biogenesis. Our results suggest the possibility that nucleolar stress such as malfunction of rRNA gene transcription induced by DDX31 depletion resulted in translocation of the NPM1 from the nucleoli to the nucleoplasm or cytoplasm in DDX31-depleted RCC cells, whereas nuclear stress such as doxorubicin treatment caused the translocation of NPM1 to the nucleoplasm of normal kidney cells.

Next, we investigated the effects of DDX31 depletion on the p53–HDM2 pathway in RCC cells. Decreased rRNA gene transcription caused by DDX31 depletion resulted in translocation of NPM1 from the nucleoli to the nucleoplasm, and NPM1 bound to HDM2 and results in TP53 accumulation (depletion of DDX31). On the other hand, DDX31 plays an important role in rRNA gene transcription and regulates the p53–HDM2 pathway through its interaction with NPM1 in the nucleoli of RCC cells (overexpression of DDX31).
depleted RCC cells, although further analysis of the DDX31–NPM1 functions will be necessary. Together, we showed that DDX31 captures NPM1 in the nucleoli, which prevents NPM1–HDM2 complex formation in the nucleoplasm and subsequent enhancement of p53 ubiquitination and degradation, resulting in p53 downregulation in RCC cells (Fig. 6F, overexpression of DDX31). To date, mutations in the p53 gene have only been rarely observed in RCC specimens and cell lines (46, 47). Hence, our results may propose a new mechanism of p53 inactivation in RCC cells.

In conclusion, our findings show that DDX31 likely plays a significant role in renal carcinogenesis by regulating the p53–HDM2 pathway and rRNA gene transcription via its interaction with NPM1. We believe that DDX31 could be a useful prognostic marker for RCC and inhibiting DDX31–NPM1 complex formation is a promising therapeutic target for RCC, especially in patients with p53 protein-intact RCC.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References
DDX31 Regulates the p53 Activity in RCC Cells


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