이학석사 학위논문

간암에서 RNA-binding protein, NONO의 분자적 기능 규명

Identification of the molecular function of RNA-binding protein, NONO, in hepatocellular carcinoma

울산대학교 대학원

의 과 학 과

주 진 성

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이 논문을 이학석사학위 논문으로 제출함

2020년 2월

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주 진 성

주진성의 이학석사학위 논문을 인준함

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Abstract

Hepatocellular carcinoma (HCC) has a high incidence and high mortality rates worldwide. However, patients with HCC have limited targeted therapeutic options and the therapies are unsatisfied. Previously published studies have reported that RNA-binding proteins (RBPs) function as oncogenes in tumorigenesis. NONO has been reported to exhibit a variety of molecular roles in human cancers. However, its function in HCC has not yet been completely identified. In the current study, we identified how NONO has a role as an oncogene with molecular function. First, we determined that NONO expression was increased in HCC through the use of diverse, patient-derived HCC datasets and tissues. We found that NONO regulates the expression of genes related to cell cycle control and DNA damage response by using gene expression profiling. Next, our findings suggest that inhibition of NONO expression can reduce the proliferation of HCC cells. We focused on FOXM1 which regulates the progression of DNA replication and the transcription of cell cycle genes in the various NONO-regulated genes. We identified the NONO binding site on FOXM1 and found that it binds directly to the RNA and protein of FOXM1 to regulate the expression and function of FOXM1. The NONO-FOXM1 axis was also found to have a positive correlation and which was found to be significant for predicting the patient survival prognosis. We found that NONO was associated with the DNA damage response and identified the potential for NONO to increase the sensitivity of IR therapy in HCC. In conclusion, our data determined that NONO could regulate HCC proliferation by regulating the expression of genes related to the cell cycle and the DNA damage response and, therefore, significantly predict patient survival in those with HCC.

Keywords: RNA-binding protein; NONO; Hepatocellular carcinoma

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Introduction

Liver cancer is the sixth most prevalent cancer worldwide and the fourth most common cause of cancer-related death. Hepatocellular carcinoma (HCC) comprises approximately 85% of primary liver cancers.[1]1 Surgical resection and transplantation are eligible therapeutic strategies for the early stage of HCC patients due to the late occurrence of their symptoms.[2] However, these curative treatments cannot be applied in all patients with early stage HCC because of poor baseline hepatic function, shortage of donor organs, and difficulty location of the tumors.[3] Therefore, alternative treatments such as transarterial chemoembolization, radioembolization, external beam radiotherapy, and even systemic therapies have also been used in such cases. Because of the tumor biology and de novo carcinogenesis in cirrhotic liver, most patients with early stage HCC experienced tumor recurrences even after curative treatments during the follow-up periods.[4] Therefore, further studies are still necessary to improve the oncologic outcomes of HCC patients.

In previously published studies, RNA binding proteins (RBPs) have been reported to contribute to the regulation of diverse molecular functions such as mRNA stability, splicing, and translation efficiency.[5] RBPs have revealed more than 1500 of these molecules according to the analysis of human genome data[6], although the mechanisms are still uncovered in tumorigenesis. Analyses of RBPs expression in HCC revealed that RBPs dysregulated and that RBPs dysregulation is associated with poor patient survival outcomes. Among RBPs, negative elongation factor E (NELFE) has been reported to be associated with an HCC poor prognosis and to promote HCC progression by regulating the MYC pathway.[7] NONO (non-POU domain-containing, octamer-binding) is an RNA binding protein that mostly exists in the nucleus and is involved in transcriptional regulation and RNA processing.[8-11] Previous studies have reported that NONO promotes tumorigenesis in human cancer through diverse molecular functions, such as regulating apoptosis or inducing drug resistance.[12-15] However, the functional role of HCC is not yet clear. Modulating RBPs, including NONO, has the potential to be used as a new therapeutic strategy.

In the current study, we found that NONO was upregulated in HCC compared with non-tumor. NONO functions as an oncogene that regulates HCC cell proliferation. NONO directly binds the FOXM1 RNA or protein to function as oncogene.

Materials and Methods

Data processing

The gene expression data set was downloaded from GEO (Gene Expression Omnibus, GSE14520; GSE39791; GSE87630), TCGA. All of the data were normalized using the quintile normalization method in the R-program and were used for analysis.

Survival Analysis

Indicated patient data were used in the survival analyses. The association of NONO and FOXM1 expression states, i.e. rank-ordered and split into high or low, relative to patient survival was tested using the log-rank test and the results were visualized using Kaplan–Meier survival plots.

Cell lines

HepG2 Cells, Hep3B, and SK-hep1 cells were maintained in DMEM (HyClone) supplemented with 10% Fetal bovine serum and 1% Antibiotic-Antimycotic (Gibco). SNU-449 cells were grown in RPMI 1640 (HyClone) medium supplemented with 10% Fetal bovine serum and 1% Antibiotic-Antimycotic (Gibco). All of the cell lines were grown at 5% CO_2 and 37 ℃.

Immunoblotting

Cell lysates were prepared with RIPA buffer (Intron) containing Halt^{TM} protease&phosphatase inhibitor cocktail (ThermoScientific). Protein lysates were separated on 8%, 10%, and 12% SDS-PAGE, and were then transferred to a PVDF membrane. Protein detection was performed using anti-NONO (05-950; Millipore, #A300-582A (587A); Bethyl, anti-FOXM1 (#3948; #20459, Cell signaling), anti-Cyclin B1 (#1223115, Cell signaling), anti-β-actin (#4967, Cell signaling), anti-r-H2AX (NB100-384, Novus).

RNA-Immunoprecipitation

RNA-Immunoprecipitation (RNA-IP) was performed according to the manufacturer's Active Motif Magnetic Chromatin Immunoprecipitation kit (#53024) protocol. SNU-449 cells were cultured to 90% confluency in 15cm plates. Cells were cross-linked with 1% formaldehyde and washed 1X PBS. The cells were then lysed in lysis buffer containing protease inhibitor and RNase inhibitor. Cross-linked nucleus lysates were subjected to six sets of sonication on ice. Each sets consisted of 20 seconds of sonication with a 30-second rest on ice between each of the sets. The digested chromatin was

immunoprecipitated overnight with primary NONO antibody at 4 ℃. Normal rabbit anti-IgG was used in parallel as a control. The samples were pulled down with magnetic beads and with reversal of cross-linked at 65 ℃ for 1.5h. RNA purification was then performed using the easy-BLUE^{TM} Total RNA extraction kit (Intron) according to the manufacturer's instructions. Quantification of the amount of immunoprecipitated RNA was performed with FOXM1 primer (HS 01073586_m1, IDT)

Lentiviral generation using short hairpin RNA transfections and infection

pLKO.1 plasmid of shNONO (TRCN0000286628, TRCN0000074562, TRCN0000294049, Sigma Aldrich) was purchased from Sigma. Lentivirus was produced by transfection into lenti-X 293T cells with lentiviral vectors psPAX2 and pMD2.G using Lipofectamine® 3000 Reagent (Invitrogen). Supernatants containing lentivirus were collected 48h after transfection. HCC cell lines were infected in the presence of polybrene (8μg/ml) and were selected with puromycin for three days.

Quantitative PCR

The total RNA was extracted using the easy-BLUE^{TM} Total RNA extraction kit (Intron) according to the manufacturer's instructions. Quantitative PCR was performed with gene-specific TaqMan primer and SensiFAST$^{™}$ Probe Hi-ROX One-Step Kit (Bioline) for gene expression analysis. Each gene was normalized against human PPIA. The primer sequences are as follows: PPIA (Hs0419421_s1, ABI); NONO (Hs, PT.58.25447000, IDT); POLE2 (Hs00160277_m1, ABI); FOXM1 (Hs 01073586_m1, ABI); CCND1 (Hs00765553_m1, ABI); TOP2A (Hs03063307_m1, ABI); CDC2 (Hs00176469_m1, ABI); MSH5 (Hs00159268_m1, ABI); and BRCA1 (Hs00173237_m1, ABI).

Cell viability assay

$3x10^3$ HCC cells were plated in triplicate 96-well plates and were incubated for 96h. Cell viability was measured using the CCK8 assay (Dogen) following the manufacturer's protocol. CCK8 assay reagent was added onto the plates and incubated at 37 ℃ for 1h. Then the absorbance was measured at 450nm.

Colony formation assay

For the colony formation assay, $1x10^3$ HepG2 and SNU-449 cells were seeded in 6-well plates at 37℃. Cells were cultured for 14 days. Cells were washed with 1X PBS and fixed using methanol for 20 min. After fixation, the cells had 0.05% crystal violet staining for 1h and were washed with dH_2O. Then the colonies were manually counted.

Chromatin immunoprecipitation assay

SNU-449 cells were cultured to 90% confluency in 10 cm plates. Cells were cross-linked with 1% formaldehyde for 10 min, after which ChIP assay was performed with a Pierce Magnetic ChIP kit (#26157, Pierce) according to the manufacturer's instructions. The digested chromatin was immunoprecipitated overnight with anti-NONO(5ug) at 4 ℃. Normal rabbit IgG antibody was used as a control. The purified chromatin was analyzed using quantitative reverse transcriptase PCR. The CCNB1 promoter primer sequences are as follows: forward 5'-CGCGATCGCCCTGGAAACGCA-3', reverse 5'-CCCAGCAGAAACCAACAGCCGT-3'.

Cycle threshold (Ct) values were normalized to the 10% input sample.

Cell cycle analysis

For cell cycle analysis, cells were cultured to 80% confluency in 10 cm plates. The cells were washed with 1X PBS and trypsinized followed by the addition of 1X PBS. The cells were then fixed with 75% EtOH and stained with propidium iodide (Sigma Aldrich), adding RNase A at 4 ℃ for 1h in the dark. PI-stained cells were analyzed on the CytoFLEX Flow Cytometer (Beckman Coulter). Flow cytometry data were analyzed using CytExpert software.

Immunofluorescence

For the detection of DNA damage-induced foci of γ-H2AX, $5x10^4$ HepG2 cells were seeded in glass-bottom cell culture dishes (NEST). Cells were fixed with 4% paraformaldehyde solution and permeabilized with 0.2% Triton-X 100 for 10 min at room temperature. After blocking with 1% BSA, samples were incubated overnight with primary antibodies at 4 ℃ and were washed with 1X Tween20/PBS(0.1%). Samples were incubated with Alexa Fluor antibodies for 1h in the dark, followed by adding with DAPI(1:5000). Images were acquired using a laser scanning confocal microscope.

Microarray

Microarray analysis was performed as previously described. The total RNA was isolated from the indicated cell lines using a mirVana RNA isolation kit (Ambion, Inc). 1μg of total RNA was used for labeling and hybridization, according to the manufacturer's instructions (#AMLIL1791, Ambion, Inc.). The labeled samples were processed with the bead chip which was washed and scanned with an Illumina Bead Array Reader (Illumina, Inc.). The data were normalized using the quantile normalization method in the Linear Models for Microarray Data (LIMMA) package in the R-program.

Luciferase assay

To construct the FOXM1 promoter reporter plasmid, the FOXM1 promoter region (-2312bp) was inserted into pGL3 basic vector. Cells were transfected with the indicated reporter genes and plasmids using Lipofectamine® 3000 Reagent (Invitrogen) according to the manufacturer's protocols. After 24 hours, cells were harvested for measurement of their luciferase activity using the Dual-Glo® Luciferase Assay System (E2940; Promega) in accordance with the manufacturer's instructions, and which was normalized to that of Renilla.

Immunoprecipitation

Cells were cultured in 15cm plates until 90% confluency. Cells were washed with 1X PBS and lysed in RIPA buffer containing Halt™ protease&phosphatase inhibitor cocktail (ThermoScientific). Cell lysates were centrifuged at 13,000 rpm for 30min. For the pull-down, cell lysates were pre-cleared with protein A/G beads and subsequently incubated for 4h with protein A/G beads covalently coupled with NONO antibody and Normal rabbit IgG as the control. The precipitates were washed five times with cell lysis buffer and then resuspended 2X loading dye buffer. Eluted samples and whole-cell lysates were separated by SDS-PAGE followed by western blot.

Immunohistochemistry

The paraffin blocks were deparaffinized using xylene and rehydrated with ethanol. 10 mM sodium citrate buffer, pH 6.0 was used for antigen retrieval for 10 min in a microwave. 0.3% Hydrogen peroxide was used for peroxidase activity inhibition. Primary NONO antibody (#A300-582A (587A), Bethyl) was diluted at 1:000 in TBS (1% BSA). The slides were incubated with anti-NONO (#A300-582A (587A) diluted in TBS (1% BSA) at 4 ℃ overnight. Then, the slides were washed and incubated with HRP-conjugated secondary antibody (VECTOR Lab) at room temperature for 1h. Visualization of proteins was performed with DAB (VECTOR Lab), and hematoxylin was used as counterstaining.

Results

NONO is upregulated and correlated with the poor prognosis of patients in HCC.

To identify the functional roles of NONO in HCC, we examined the NONO expression in independent HCC datasets. In the dataset from TCGA, the GEO dataset, NONO expression was higher in tumor tissue compared with normal tissue (Figure. 1a). TCGA data also reveals that there is genetic alteration in HCC (Figure. 1b). We then identified the protein level of NONO in seven pairs of HCC and paired normal tissue. NONO expression was significantly higher in tumor tissues compared with normal tissues (Figure. 2a and b). We also validated the NONO expression with HCC samples using the western blot. This result is coincident with the HCC dataset (Figure. 2c). We then examined whether a high NONO expression level is associated with poor patient survival outcomes in those with HCC. We found that high NONO expression was correlated with poorer patient survival (Figure. 3). These results demonstrate that NONO is highly expressed in HCC and its expression is associated with poor patient prognosis in HCC.

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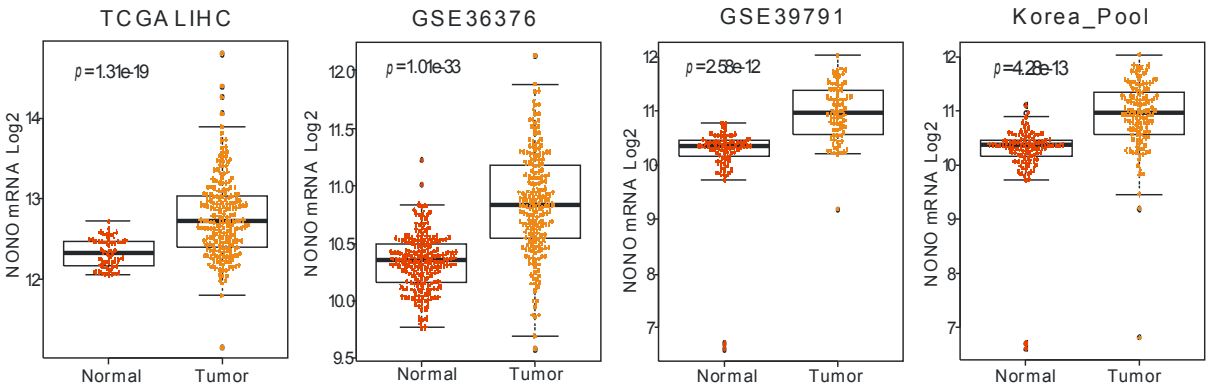

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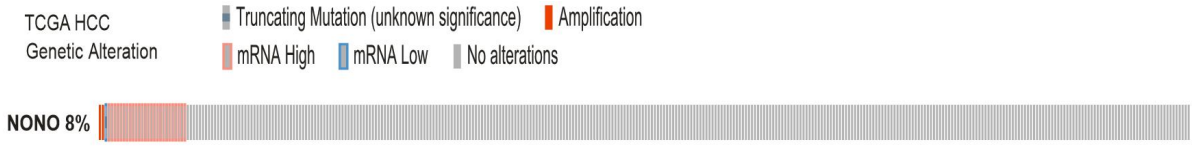
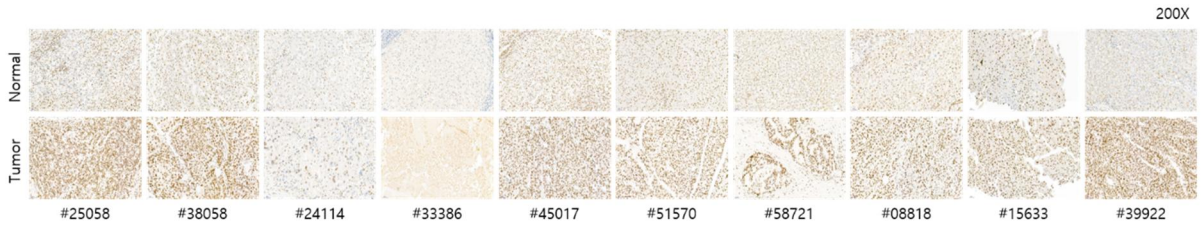


Figure. 1 NONO mRNA expression in HCC patient data.

(a) NONO mRNA expression (Log2) level in HCC and normal tissues. (b) Gene alteration of NONO in the TCGA-HCC cohort.

a



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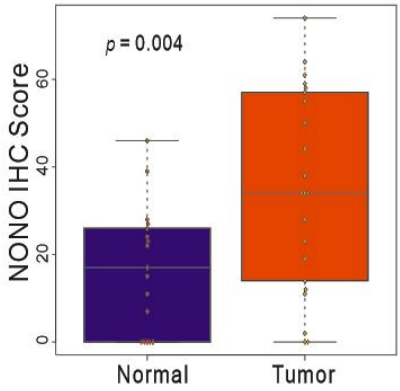

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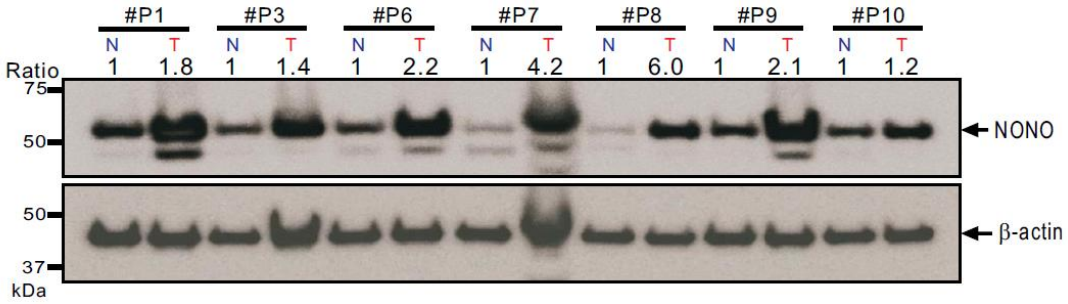


Figure. 2 NONO protein level in HCC patient tissues.

(a) Normal and tumor tissue from HCC patients was stained with NONO antibody. (b) Different staining intensity and quantification of the staining results are shown between normal and tumor tissue. (c) Western blot analysis of NONO expression in tissues from HCC patient.

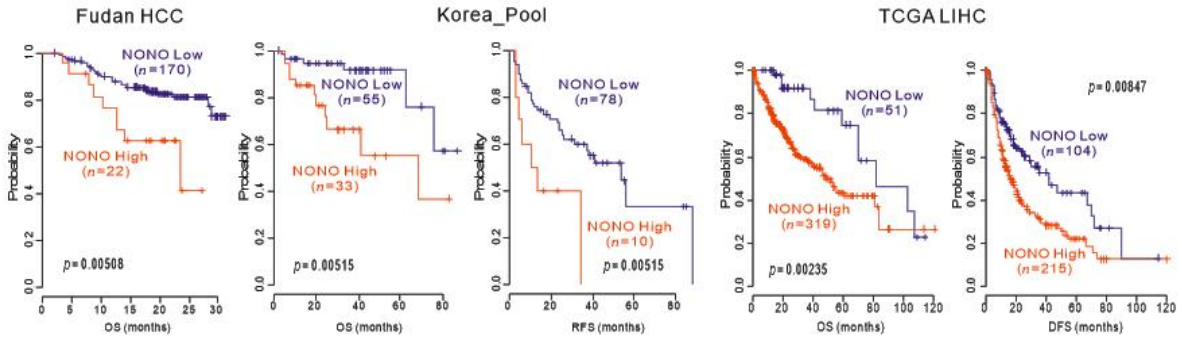


Figure. 3 Kaplan-Meier survival analysis of the HCC datasets based on the segmentation value of NONO.

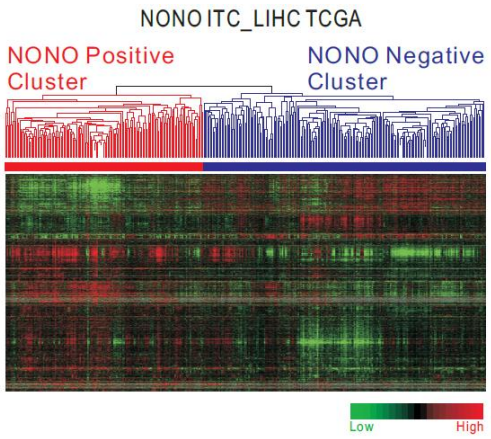
HCC cohorts (HCC patients from Fudan University, China (GSE14520); HCC patients from Korea University (GSE39791, and GSE87630); TCGA-LIHC) were dichotomized by relatively high or low NONO expression and were considered for plotting. The differences between these two groups were statistically significant in OS and DFS. OS, overall survival; DFS, disease-free survival

NONO regulated cell cycle regulator and DNA damage response genes.

Since NONO is expressed in cancer, we determined which genes are correlated with NONO. We performed correlation analysis using Pearson's coefficient values. The TCGA LIHC dataset was divided into NONO positive clusters and NONO negative clusters based on the results of the correlation analysis (Figure. 4a). Survival analysis shows that the NONO positive cluster has poorer outcomes than the NONO negative cluster (Figure. 4b). To identify the gene pathway involved in NONO, we conducted the canonical pathway analysis. These findings indicated that NONO related to DNA damage response, ATM signaling, cell cycle control of chromosomal replication, and G2/M DNA damage checkpoint regulation (Figure. 4c).

To identify the downstream target of NONO in HCC, we generated a gene expression profile with NONO-knockdown Hep3B and SNU-449 cells. The expression profile shows that 1095 mRNAs were differentially expressed in response to NONO silencing in both HCC cell lines (Figure. 5a). We found that cell cycle control genes such as PLK4, CENPE, CDK2 and DNA damage response genes such as RAD54L, POLQ (Figure. 5b). Canonical pathway analysis from the Gene expression profile indicated that 1095 genes were associated with the DNA damage response and the cell cycle checkpoint (Figure. 5c). To validate that NONO regulates cell cycle regulation and DNA damage response genes, we examined the mRNA expression using qRT-PCR in NONO-silenced cells. We observed that NONO decreases the expression of cell cycle regulators and DNA damage response genes at the RNA level (Figure. 6a). NONO reduced genes associated with DNA damage response and cell cycles, such as FOXM1 and CCNB1, at the protein level (Figure. 6b). These findings suggest that NONO regulates the expression of genes related to cell cycle control and DNA damage response.

a



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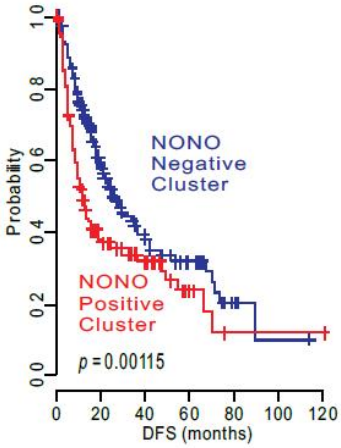

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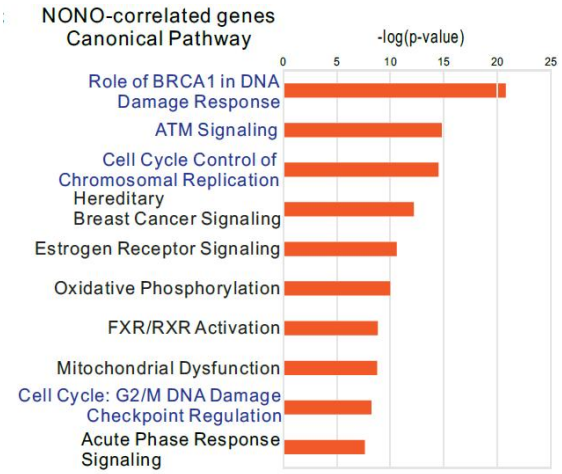


Figure. 4 NONO affects the patient prognosis and enhances the DNA damage response and cell cycle in the TCGA-LIHC dataset.

(a) NONO-correlated genes expression are shown in the heatmap. The TCGA-LIHC cohort was divided into NONO-positive or NONO-negative clusters using Pearson's coefficient values (R value ≥ +0.2 is NONO-positive cluster, ≥ -0.2 is NONO-negative cluster). (b) Kaplan-Meier plots of OS or DFS in TCGA-LIHC were predicted using the NONO-positive and NONO-negative cluster gene signature. (c) Canonical pathway analysis of NONO-correlated genes. OS, overall survival; DFS, disease-free survival

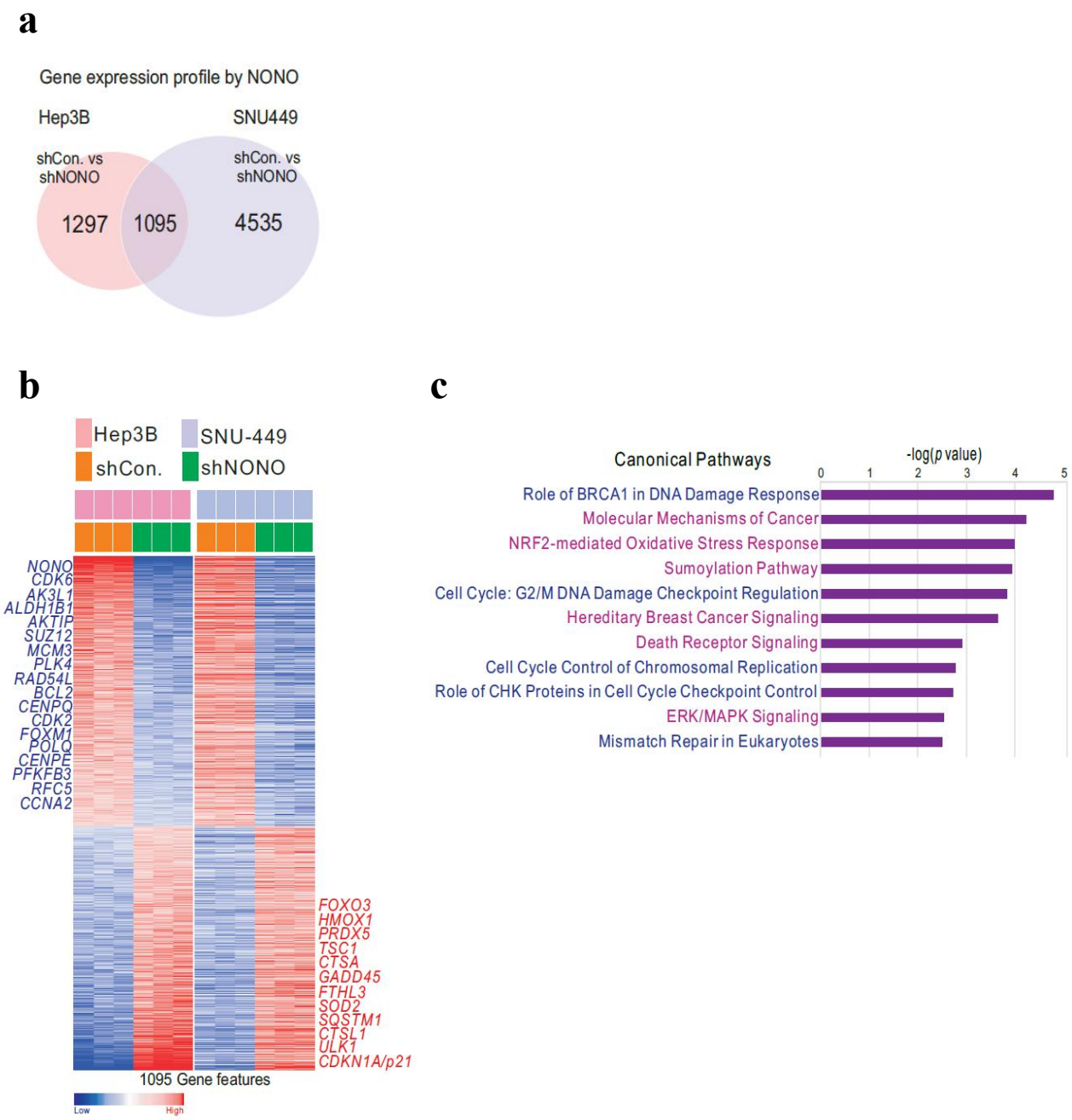
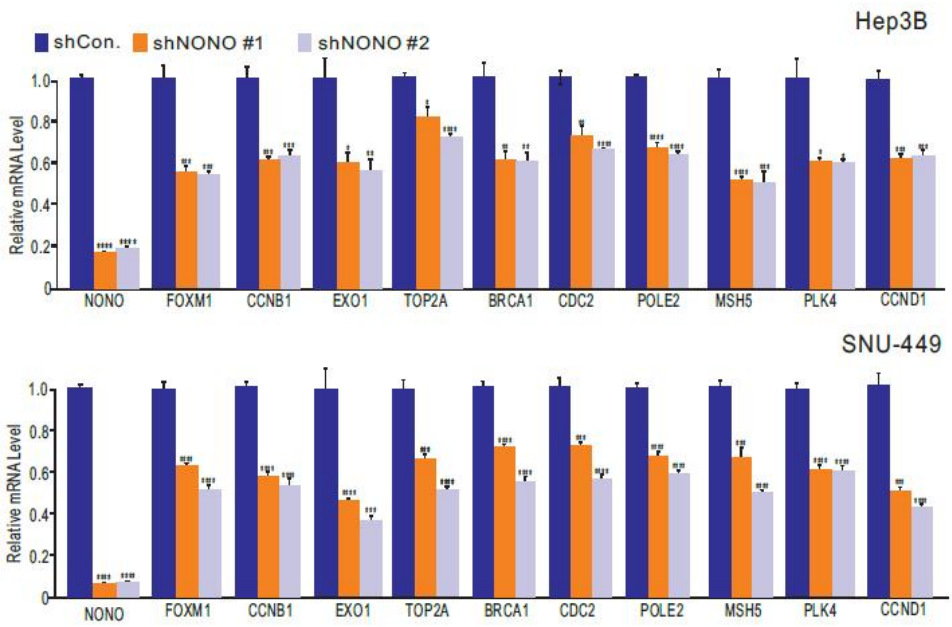


Figure. 5 NONO regulates the DNA damage response and cell cycle-associated genes in HCC.

(a) Gene expression signatures specific to inhibition of NONO expression via shNONO in HCC cell lines. Genes in the Venn diagram were selected by using Class Comparison Analysis from the BRB array tool ($p<0.001$). (b) Gene expression profiling is shown in the matrix format. Red and blue show relatively high and low expression, as indicated in the scale bar (log2-transformed scale). (c) Canonical pathway analysis of the genes differentially expressed after NONO inhibition.

a



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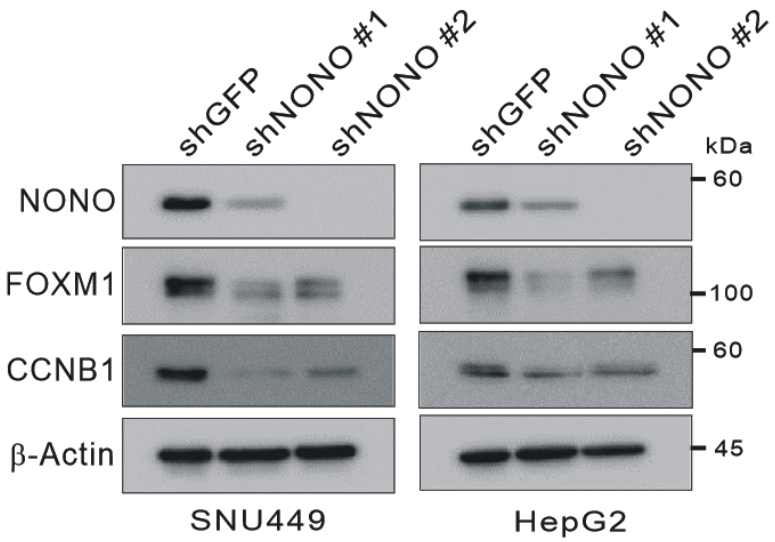


Figure. 6 Gene expression by depletion of NONO.

(a) q-RT PCR analysis of the DNA damage response and cell cycle-associated genes by NONO silencing in Hep3B and SNU-449 cells. (b) HCC cells were infected with the indicated lentivirus and were analyzed for NONO using western blot. All results are means ± standard deviations (SDs) from three-independent replicates, i.e. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, and ****$p < 0.001$.

NONO is involved in HCC cell growth and the cell cycle.

To investigate whether NONO influences HCC cell growth, we performed a cell proliferation assay. HCC cell lines, including SNU-449 and HepG2, were infected with shNONO when using the lentivirus system that knocked down NONO expression (Figure. 7a). Knockdown of NONO in HCC cells inhibited HCC cell proliferation (Figure. 7b). Depletion of NONO decreased colony formation in SNU-449 and HepG2 cells (Figure. 7c).

The gene expression profile has shown that NONO regulates cell cycle regulator genes. To examine whether cell cycle regulation is associated with NONO in HCC cell lines, we created NONO-silenced Hep3B cell lines using lentivirus. Propidium iodide staining showed that knockdown of NONO induced the sub-G1 phase. Furthermore, inhibition of NONO is contributed to reduce the S and G2/M phase (Figure. 8). These results demonstrated that NONO regulated the cancer cell proliferation and cell cycle as an oncogene in HCC.

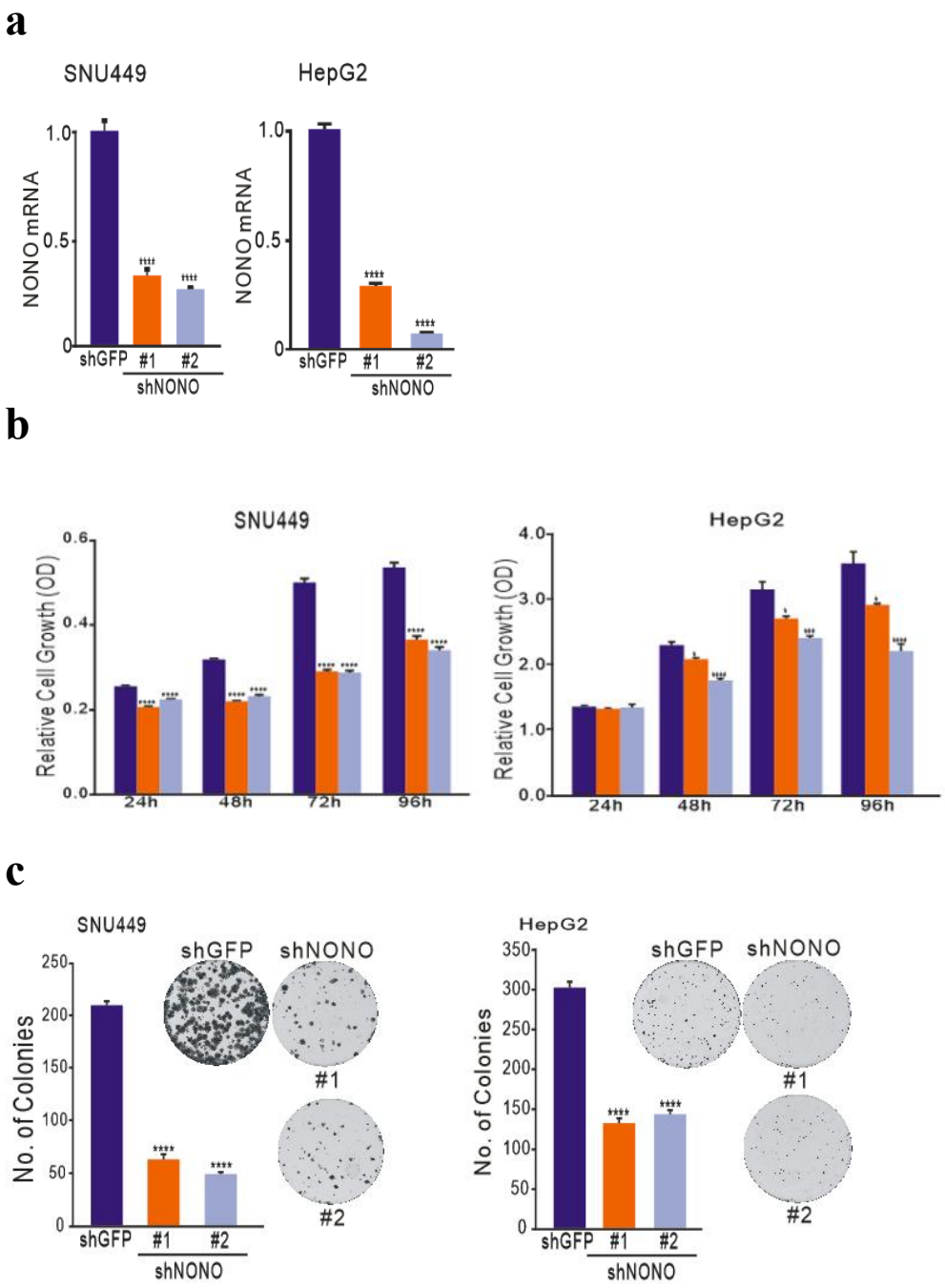
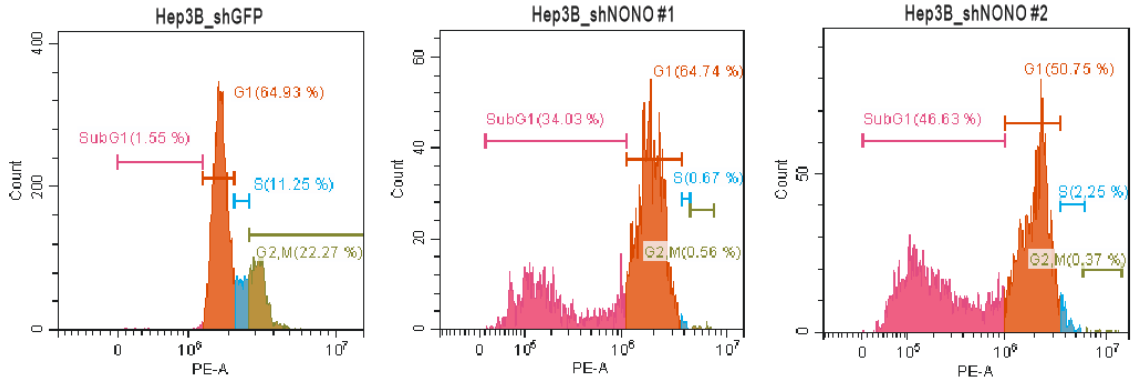


Figure. 7 NONO activates the proliferation of HCC cells.

(a) NONO knockdown efficiency was verified at the mRNA levels in HCC cells by qRT-PCR. (b) The proliferation of HCC cells infected with the indicated lentivirus was analyzed using the CCK8 assay. (c) Representative image and quantification of the colony formation assay by NONO knockdown in HCC cells. All results are means ± standard deviations (SDs) from three-independent replicates (*$p < 0.05$, **$p < 0.01$, ***$p < 0.005$ and ****$p < 0.001$).



Hep3B

Cell Cyle	shGFP	shNONO #1	shNONO #2
Sub-G1	1.55	34.03	46.63
G1	64.93	64.74	50.75
S	11.25	0.67	2.25
G2/M	22.27	0.56	0.37

Figure. 8 Cell cycle alteration of HCC cells by NONO expression.

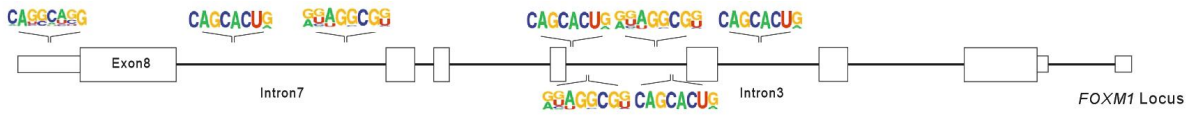
The cell cycle of Hep3B cells infected with lentivirus was analyzed by FACS.

NONO interacts FOXM1 by directly binding.

We found NONO regulated the DNA damage response and cell cycle regulator genes, including FOXM1 (Figure. 5). Among the genes found in these experiments, we focused on FOXM1 as a downstream target of NONO. FOXM1 functions as a transcription factor that regulates the transcription of cell cycle genes involved in the progression of DNA replication and mitosis.[16-19]

To identify how NONO regulates FOXM1 gene expression, we examined the direct binding of NONO with FOXM1 RNA or protein. Previous studies have suggested that NONO directly binds to multiple regions of RNA.[20] We found that the FOXM1 locus has eight NONO binding sites, including 3' UTR (Figure. 9a). To validate the binding site of NONO, we performed RNA-IP with HCC cells. NONO directly binds to FOXM1 RNA but not to CCNB1, the downstream target gene of FOXM1 (Figure. 9b). As FOXM1 functions as a transcription factor at the protein level, we investigated whether NONO influences the transcriptional activity of FOXM1. An IP experiment reveals that NONO directly interacted with FOXM1 (Figure. 10a). Furthermore, NONO-FOXM1 complex regulated promoter activity by recruiting the CCNB1 promoter, which is a target of FOXM1 (Figure. 10b). To determine whether NONO functions on the FOXM1 promoter to regulate FOXM1 expression, we performed a luciferase assay on the FOXM1 promoter. NONO expression activated FOXM1 promoter activity in HCC (Figure. 10c). These results suggest that NONO regulates FOXM1 expression by directly binding RNA and protein and influences the FOXM1 transcription factor function.

a



Motif	*FOXM1* Locus
CAGGCAGG	Chr12:2,857,796~2,857,803
GGAGGCGG	Chr12:2,862,105~2,862,112 Chr12:2,867,588~2,867,595 Chr12:2,867,904~2,867,911
CAGCACUG	Chr12:2,860,252~2,860,259 Chr12:2,866,902~2,866,909 Chr12:2,868,501~2,868,508 Chr12:2,870,914~2,870,921

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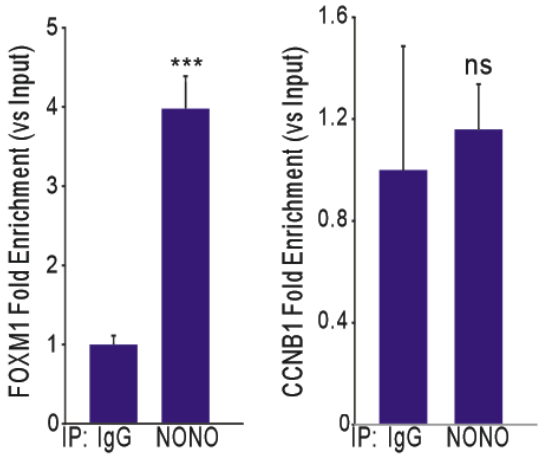


Figure. 9 NONO binding sites in the FOXM1 RNA.

(a) Alignment of the FOXM1 locus sequence. (b) RNA-IP was performed with anti-NONO in SNU-449 cells. After RNA-IP, the cells were analyzed by qRT-PCR with the indicated probes. All of the results are means ± standard deviations (SDs) from three-independent replicates (*$p < 0.05$, **$p < 0.01$, ***$p < 0.005$ and ****$p < 0.001$).

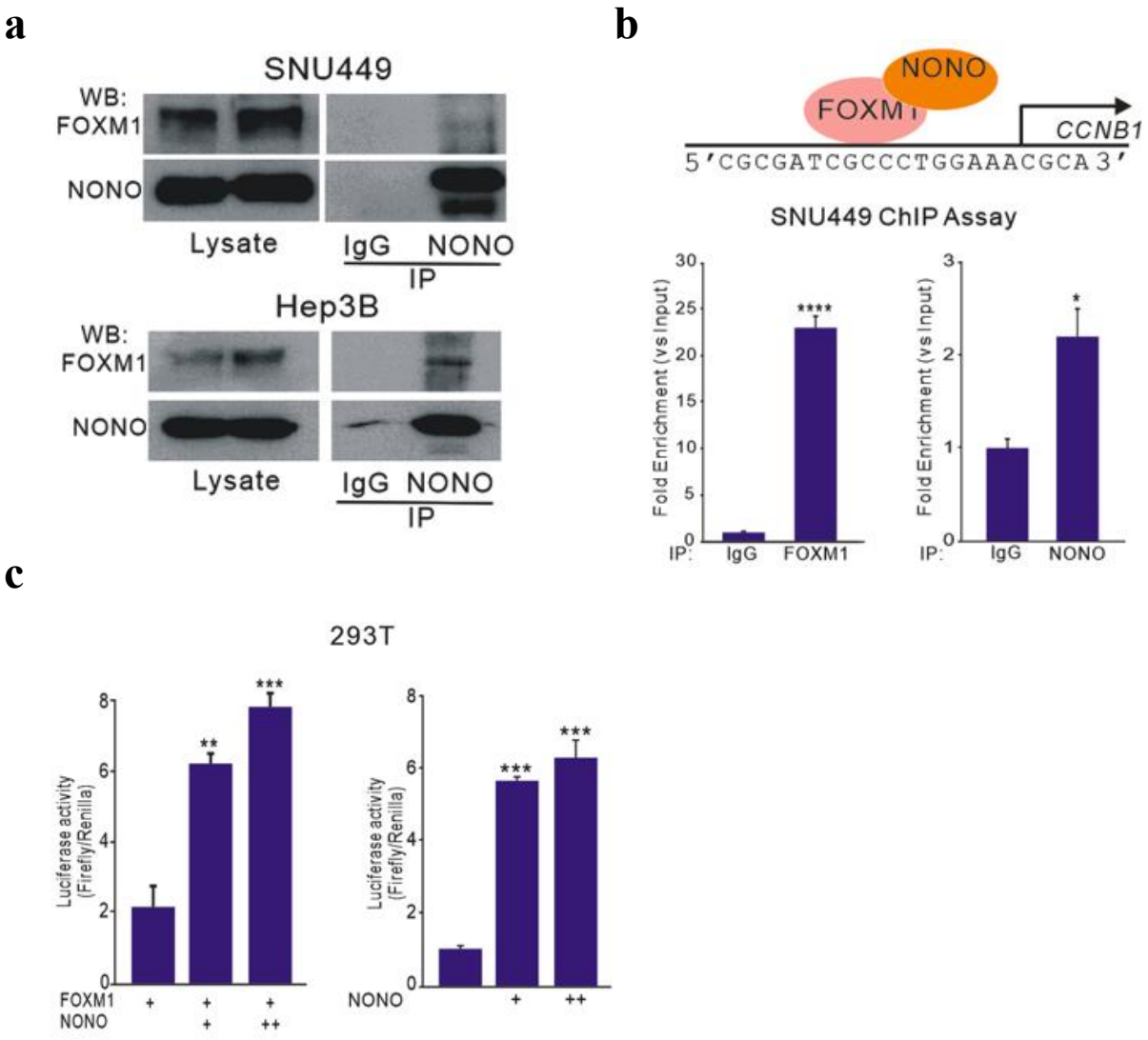


Figure. 10 NONO interacts directly with the FOXM1 to regulate the function of FOXM1 as a transcription factor.

(a) HCC cells were lysed and immunoprecipitated with IgG and NONO antibodies. The precipitates were analyzed using western blot with NONO and FOXM1 antibodies. (b) Schematic of the CCNB1 promoter region. ChIP assay was performed in SNU-449 cells using NONO and FOXM1 antibodies. Recruitment of the NONO-FOXM1 complex to the CCNB1 promoter was analyzed using primers specific to this promoter. IgG was used as an internal control. (c) NONO expression mediated FOXM1 promoter activity, based on the luciferase reporter assay of the FOXM1 promoter in SNU-449 cells. All results are means ± standard deviations (SDs) from three-independent replicates (*$p < 0.05$, **$p < 0.01$, ***$p < 0.005$ and ****$p < 0.001$).

NONO -FOXM1 has clinical relevance in HCC as it shares the function.

NONO is associated with the clinical prognosis in HCC patients, and FOXM1 is regulated by NONO, including gene expression and function. To investigate the clinical relevance of FOXM1 in HCC, we analyzed diverse datasets of HCC patients. We found that FOXM1 expression is positively correlated with NONO in patients and in cell lines data (Figure. 11a). Survival analysis of dichotomized NONO and FOXM1 expression confirmed that higher NONO and FOXM1 expression levels are related to poorer clinical outcomes in HCC (Figure. 11b).

Since NONO and FOXM1 influx`1ence downstream genes as transcriptional regulators. We investigated whether NONO and FOXM1 share the gene signature. We compared the regulated gene signatures by silencing of the both genes in HCC data. We identified 207 genes as downstream targets of both factors, suggesting that the biological functions of NONO might depend on FOXM1 (Figure. 12a). To understand the gene network that is common to both factors, we analyzed 207 gene signatures using the Ingenuity Pathway Analysis. This result shows that the common gene signatures is associated with Cellular assembly, DNA replication, and repair (Figure. 12b). Then we examined the clinical relevance of these signatures by applying a previously established prediction strategy that employs multiple. different algorithms. These gene signatures were associated with patient outcomes and disease recurrence in HCC patients when determined using the predicted outcomes of various classifiers.[21] We found that patients with knockdown signatures (KD signatures) show a better prognosis in HCC datasets (Figure. 12c and d). These findings suggest that NONO is functionally related to FOXM1 and can predict the clinical outcomes in HCC.

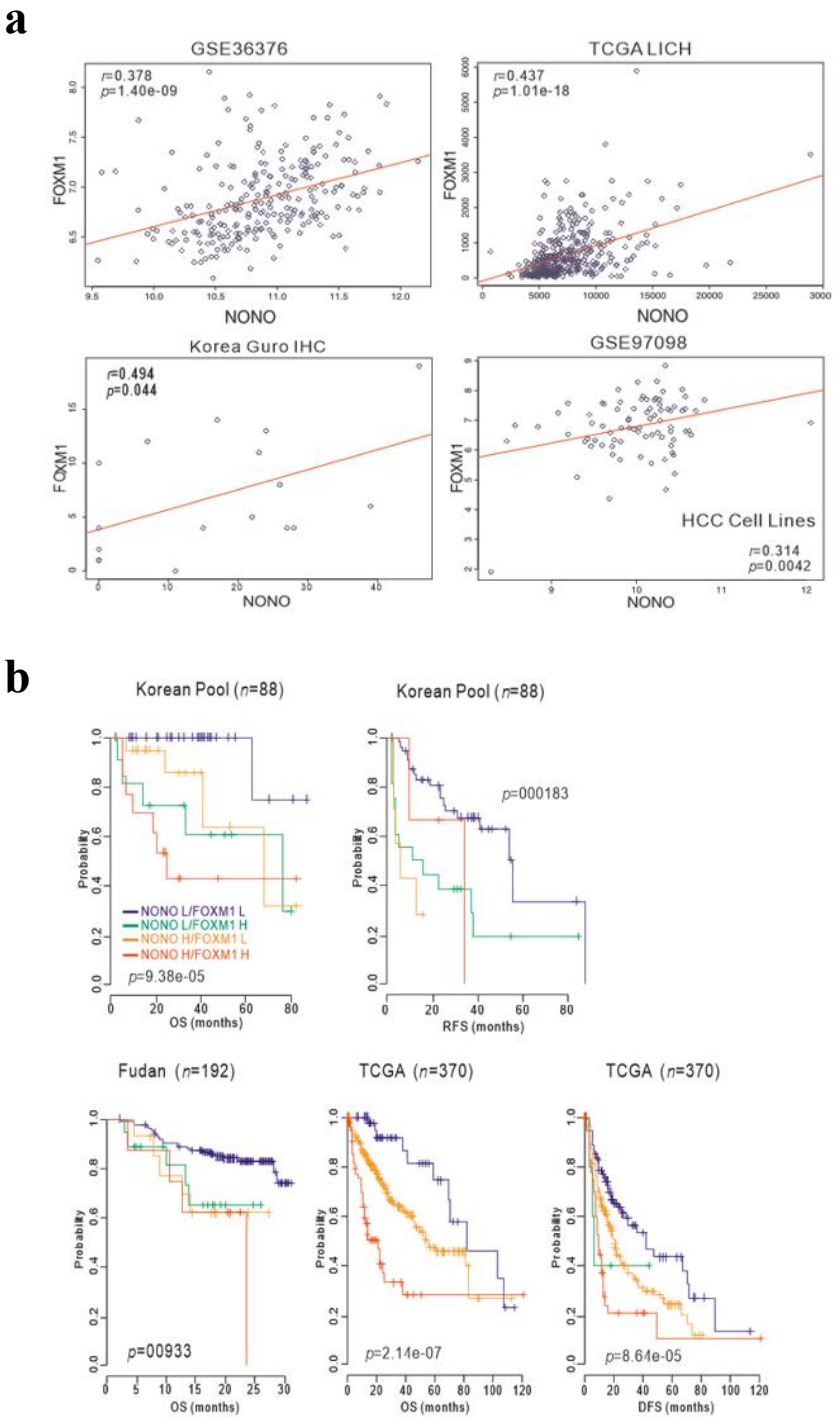
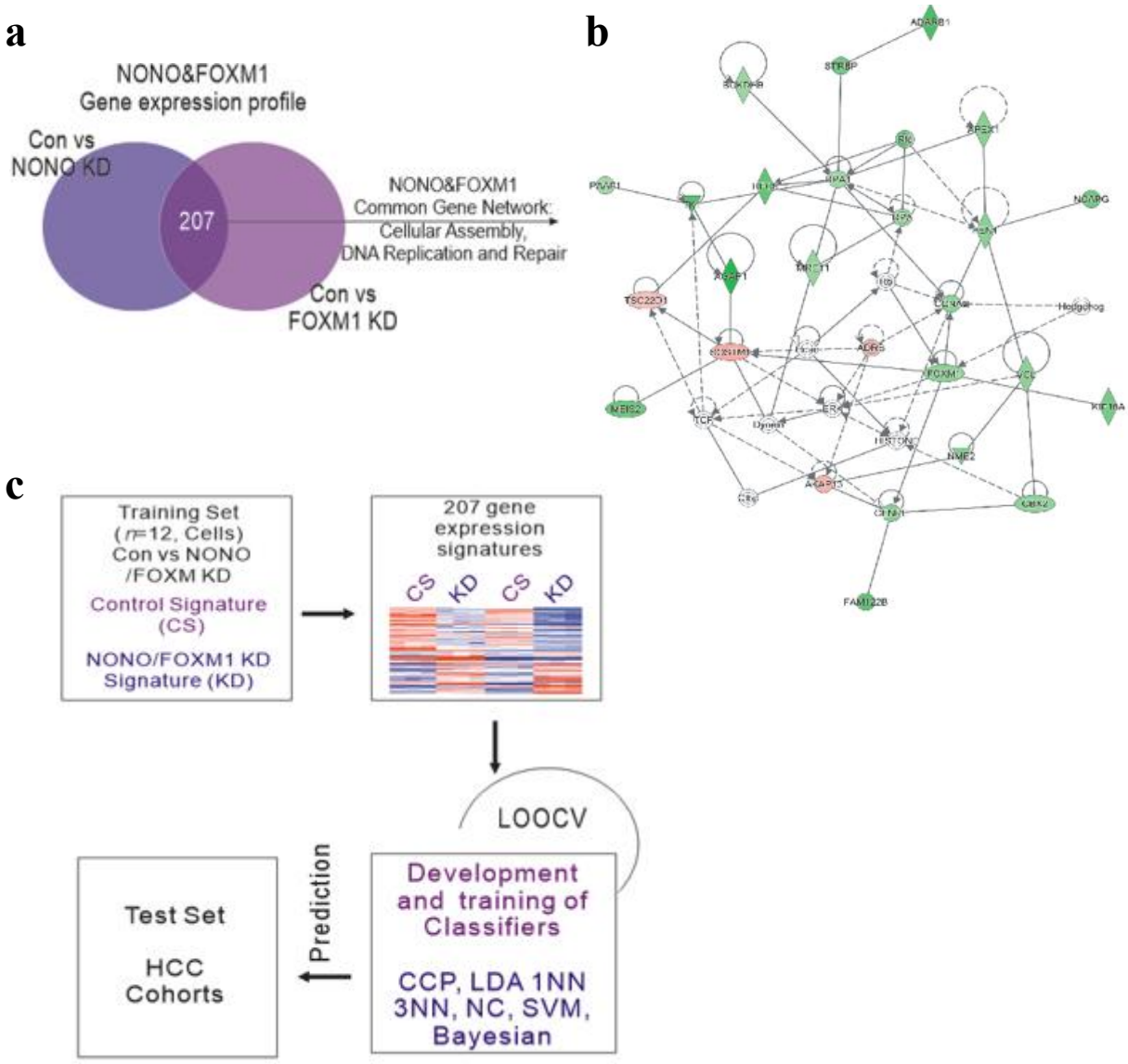


Figure. 11 NONO-FOXM1 is clinically associated with HCC.

(a) Correlation of NONO and FOXM1 gene expression in the indicated HCC patient cohorts. Scatter plots of NONO and FOXM2 in the HCC cohorts are shown. (b) Indicated patient cohorts were segmented by the expression of both NONO and FOXM1. A log-rank test was used to analyze the survival prognosis following NONO and FOXM1 expression.


a
NONO&FOXM1
Gene expression profile
Con vs
NONO KD
207
NONO&FOXM1
Common Gene Network:
Cellular Assembly,
DNA Replication and Repair
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Training Set
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Con vs NONO
/FOXM KD
Control Signature
(CS)
NONO/FOXM1 KD
Signature (KD)
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expression
signatures
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LOOCV
Development
and training of
Classifiers
CCP, LDA 1NN
3NN, NC, SVM,
Bayesian
Prediction
Test Set
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d
TCGA-LICH
LDA
KD
CS
p =0.00309
Probability
OS (months)
TCGA-LICH
CCP
KD
CS
p =0.0035
Probability
DFS (months)
Fudan
3NN
KD
CS
p =0.0364
Probability
OS (months)
Korea_Pool
Bayesian
KD
CS
p =0.0496
Probability
OS (months)

Figure. 12 Prediction of the survival prognosis following NONO and FOXM1 expression using the prediction model.

(a) Gene expression signature specific to the depletion of NONO or FOXM1 expression by shRNA, respectively, in HCC cells. Genes in the Venn diagram were selected by using a two-sample Student's t-test ($P < 0.001$; more than 1.5 fold). The blue and purple circles represent genes whose expression patterns are significantly related to the inhibition of NONO or FOXM1, respectively. (b) Genes expressed differently by inhibition of NONO and FOXM1 were analyzed by Ingenuity Pathway Analysis (IPA). (c) Schematic diagram of the prediction model generation and evaluation of predicted outcomes based on a shared gene expression signature of NONO and FOXM1 in HCC cells. A shared gene expression signature was used to form a series of classifiers that estimated the probability of how much the expression patterns of HCC patients were similar to the shared signature; control signature (CS) vs. knockdown signature (KS). (d) Kaplan-Meier plots of OS or DFS HCC patients in the indicated cohorts were predicted using the gene expression signature as a classifier. The differences between groups were significant as indicated (log-rank test): LDA, linear discriminator analysis; CCP, compound covariate predictor; 1NN, one nearest neighbor; 3NN, three nearest neighbors; NC, nearest centroid; SVM, support vector machines; OS, overall survival; and DFS, disease-free survival.

NONO is associated with DNA damage response in HCC.

We found NONO regulated DNA damage response genes using microarray analysis (Figure. 5). We examined whether the NONO expression in HCC was related with the DNA damage response. DNA damage by ionizing radiation (IR) and doxorubicin induced an increase in expression of NONO and FOXM1 (Figure. 13a). In addition, the lower NONO expression induced more gamma H2AX foci formation (Figure. 13b). To identify the therapeutic potential of NONO in radiation therapy, we performed a colony formation assay using DNA damage-induced HCC cell lines. We found that NONO-silenced cells became sensitive to IR (Figure. 14). This result indicates that NONO is available for new therapeutic strategy that will increase the efficiency of radiation therapy.

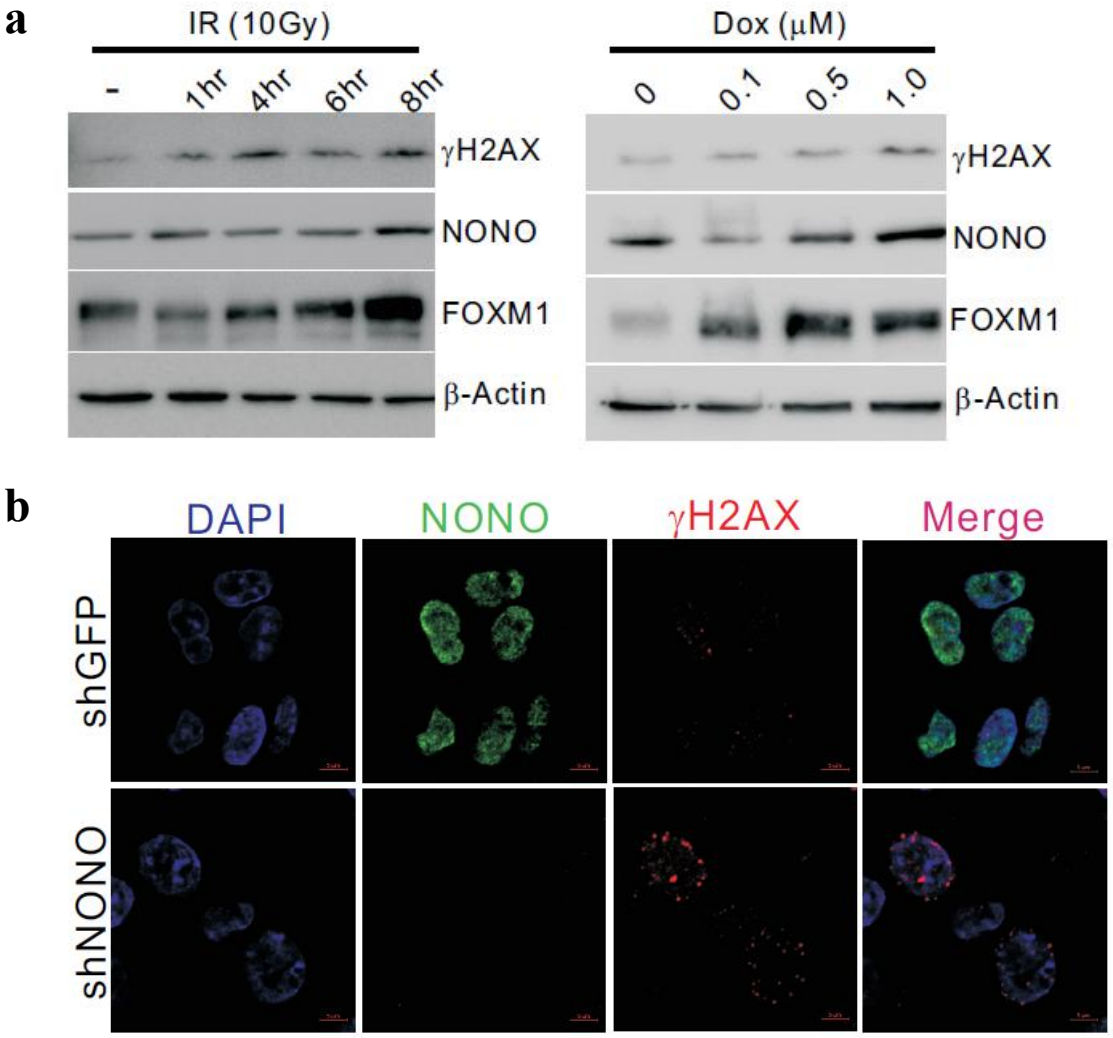

Figure. 13 NONO is involved in the DNA damage response.

(a) Hep3B cells were treated with IR (10Gy) and Dox (0, 0.1, 0.5, 1 μM) to induce DNA damage. Expression of the indicated proteins was assessed using western blot. (b) HepG2 cells were infected with shGFP and shNONO lentivirus. Cells were fixed and immunostained with indicated antibodies followed by confocal microscopic analysis.

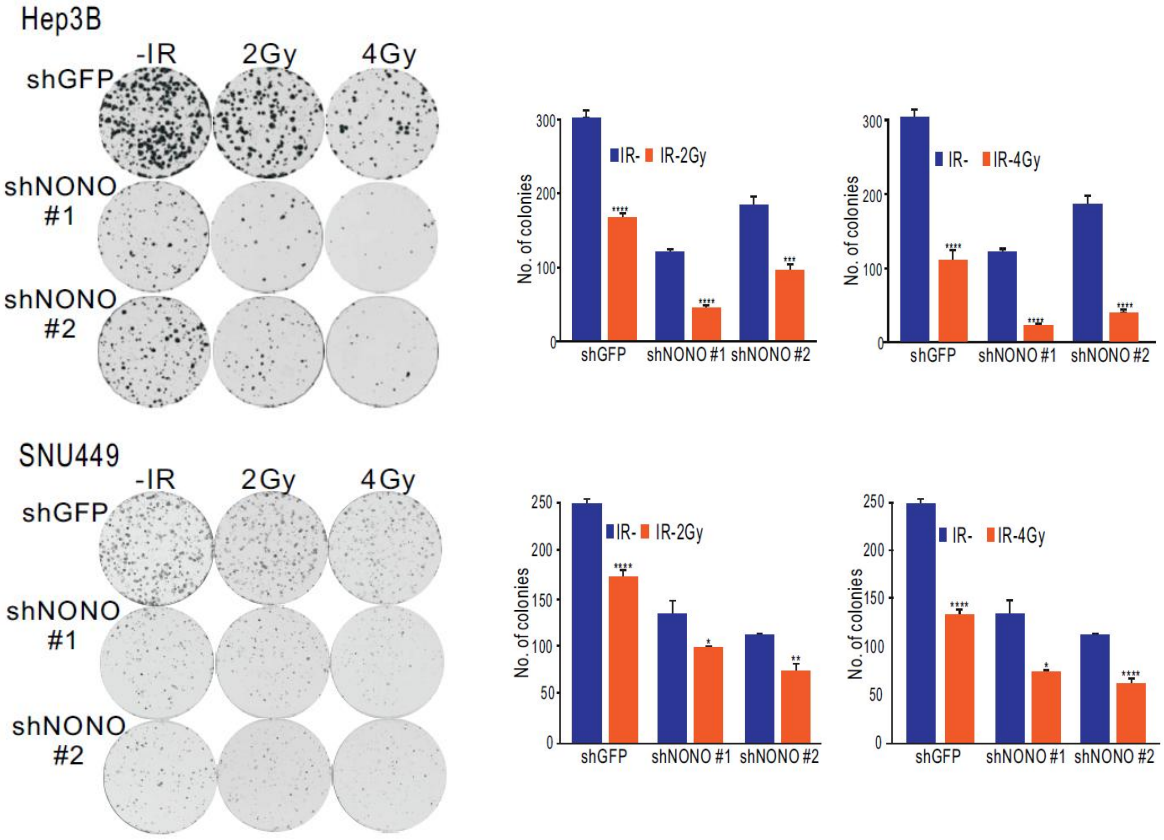

Figure. 14 NONO increases the sensitivity of HCC cells to IR treatment.

Representative image and quantification of the colony formation assay by NONO knockdown and IR treatment (2Gy, 4Gy) in HCC cells. All of the results are means ± standard deviations (SDs) from three-independent replicates, i.e. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, and ****$p < 0.001$.

Discussion

Several RBPs are highly upregulated in solid tumors and have been uncovered to be a driver of tumorigenesis.[22-24] RBPs interact with thousands of RNAs, resulting in the production of abnormal proteins in cancer. These proteins are highly stable, efficiently translated, and abundantly present in cells.[25] Therefore, abnormal expression of RBPs is the cause of transcriptional alteration and which suggests that RBPs have oncogenic properties. In this study, we found that RNA-binding protein NONO expression was significantly upregulated in HCC. Inhibition of NONO decreased HCC cell growth and induced an abnormal cell cycle. NONO is associated with genes such as DNA damage response and cell cycle. Previous studies have shown that NONO has various molecular functions in human cancer.

Upstream regulators of NONO have been defined as Ets-1 and CRTC/LINC004732.[26] We identified that NONO regulated DNA damage response and cell cycle genes as downstream targets. Among these genes, NONO interacted with FOXM1 by direct binding. We focused on the NONO-FOXM1 axis because FOXM1 has been known to regulate the DNA damage response and cell cycle-related genes as the transcription factor. In a previous study, FOXM1 was overexpressed in various human cancers.[27-30] FOXM1 is known to regulate the transcription of genes such as XRCC1 and BRCA2 involved in the homologous recombination repair of DNA double-strand breaks.[31-33] Furthermore, depletion of FOXM1 leads to impaired DNA repair and this impairment may result in cell cycle arrest of cancer cells.[18] Our present results suggest that the oncogenic potential of NONO is dependent on FOXM1 expression (Figure. 3, 4, and 5). NONO alters the mechanism of FOXM1 by directly binding to the FOXM1 promoter, RNA, and protein. Targeting NONO can be an effective strategy to block the oncogenic properties of FOXM1 and increase the sensitivity to radiation therapy.

The development of small molecular inhibitors targeting RBPs could be a new therapeutic strategy for human cancer. However, a number of RBPs have difficulty in drug development because of the lack of well-defined binding pockets for drug binding. To inhibit the oncogene function, small molecular antagonists that inhibit the RNA binding activity of MSI2 have been reported in previous studies.[34] Based on the investigation of the NONO protein structure, the development of small molecular inhibitors that suppress NONO RNA binding activity can be used as a treatment for HCC. Methods of inhibiting NONO include siRNA and antisense RNA, and these methods have been adopted in various clinical trials. Interfering with RBP and RNA interactions such as small molecular inhibitor or oligonucleotide can also be used as a method of blocking NONO.[35] The treatment with a combination of inhibition of NONO activity and radiation therapy may be a better therapeutic strategy.

Early-stage HCC patients have limited therapeutic strategies, including radiation therapy. Radiation therapy throughout the liver has limited the therapeutic efficiency of HCC due to low patient tolerance. Radiation-induced liver disease also reduces the sensitivity of HCC to radiation therapy.[36,37] Recently, diverse methods have been developed to reduce these side effects, but the resistance to radiation therapy still has problems. Previous studies have demonstrated that RBPs induce radioresistance by affecting cellular processes in cancers.[38] We found that NONO modulates the DNA damage response of HCC. Inhibition of NONO induced the accumulation of γ-H2AX which promotes double-strand break repair. These findings demonstrate that targeting the NONO expression can increase the sensitivity of radiotherapy of HCC. Previous studies have reported that the resistance of HCC radiotherapy is associated with DNA damage and repair.[39,40] Radiotherapy can be used in combination with other therapies, such as immunotherapy, for HCC. However, immunotherapy has approximately a 20% response rate. Radiotherapy has been reported as the most appropriate method to overcome the problems of immunotherapy.[41-43] The sensitivity of radiotherapy by NONO targeting could significantly enhance the therapeutic effect of immunotherapy.

Conclusion

We found that NONO expression was upregulated in HCC and has significance in predicting the prognosis of HCC patients. Gene expression profiling revealed that NONO regulates the DNA damage response and cell cycle-related gene expression. In addition, our findings suggest that the proliferation of HCC cells can be regulated by regulating NONO expression. NONO regulates FOXM1 expression and function by binding directly to FOXM1 RNA and protein. Our data show that new functions of NONO and the relationship between NONO and FOXM are now uncovered. Furthermore, we confirmed that the gamma-H2AX level, a DNA damage marker, increased when NONO expression was suppressed. Our findings provide a basis for understanding cancer cell proliferation and radiotherapy efficiency by NONO and exploring new strategies for improving HCC therapy.

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국문요약

간암 (HCC)은 전 세계적으로 발병률과 사망률이 높다. HCC 환자는 제한된 표적 치료 방법을 이용할 수 있으며, 치료 효과는 만족스럽지 못하다. 이전에 발표된 연구는 RNA-binding proteins (RBPs)이 종양 형성에서 종양 유전자로서 기능한다고 보고하였다. NONO는 인간 암에서 다양한 분자적 역할을 하는 것으로 보고되었다. 그러나 HCC에서의 기능은 아직 명확하게 확인되지 않았다. 현재의 연구에서 우리는 NONO가 분자적 기능을 가진 종양 유전자로서 어떻게 역할을 하는지 확인했다. 첫째, 우리는 다양한 환자 유래 HCC 데이터 세트 및 조직을 사용하여 NONO의 발현이 HCC에서 증가되어 있는 것을 확인하였다. NONO 유전자 발현 프로파일링을 사용하여 NONO가 세포주기 제어 및 DNA 손상 반응 관련 유전자들의 발현을 조절하는 것을 확인하였다. 그리고 NONO의 발현을 억제함으로써 HCC 세포의 증식을 감소시킬 수 있었다. 우리는 NONO가 조절하는 여러 가지 유전자 중에서, DNA 복제와 세포주기 유전자들의 전사를 조절하는 FOXM1에 초점을 맞추었다. 우리는 FOXM1에서 NONO의 결합 부위를 확인하였고 FOXM1의 RNA와 단백질에 직접적으로 결합하여 FOXM1의 발현과 기능을 조절하는 것을 확인하였다. NONO-FOXM1 축이 서로 양의 상관관계를 가지는 것을 확인했고 이는 환자 생존 예후를 예측하는데 유의한 것으로 밝혀졌다. 우리는 NONO가 DNA 손상 반응과 연관이 있음을 찾았고 NONO가 방사선 치료의 민감도를 증가시킬 수 있는 가능성을 확인하였다. 결론적으로, 이 데이터들은 NONO가 세포주기 및 DNA 손상 반응과 관련된 유전자의 발현을 조절함으로써 HCC의 증식을 조절할 수 있고, 따라서 HCC 환자에서 환자 생존을 유의하게 예측할 수 있다는 것을 주장한다.

중심 단어: RNA-결합 단백질; NONO; 간암