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Original Article

Nutlin-3-induced redistribution of chromatin-bound IFI16 in human hepatocellular carcinoma cells *in vitro* is associated with p53 activation

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Aim: Interferon-γ inducible protein 16 (IFI16), a DNA sensor for DNA double-strand break (DSB), is expressed in most human hepatocellular carcinoma cell (HCC) lines. In this study we investigated the re-localization of chromatin-bound IFI16 by Nutlin-3, a DNA damage agent, in HCC cells *in vitro*, and the potential mechanisms.
Methods: Human HCC SMMC-7721 (wild-type *TP53*), Huh-7 (mutant *TP53*), Hep3B (null *TP53*) and normal fetal liver L02 cell lines were examined. DSB damage in HCC cells was detected via γH2AX expression and foci formation assay. The expression of *IFI16* and *IFNB* mRNA was measured using RT-PCR, and subcellular localization and expression of the IFI16 protein were detected using chromatin fractionation, Western blot analysis, and fluorescence microscopy.
Results: Treatment of SMMC-7721 cells with Nutlin-3 (10 μmol/L) or etoposide (40 μmol/L) induced significant DSB damage. In SMMC-7721 cells, Nutlin-3 significantly increased the expression levels of *IFI16* and *IFNB* mRNA, and partially redistributed chromatin-bound IFI16 protein to the cytoplasm. These effects were blocked by pretreatment with pifithrin-α, a p53 inhibitor. Furthermore, Nutlin-3 did not induce ectopic expression of IFI16 protein in Huh-7 and Hep3B cells. Moreover, the association of IFI16 with chromatin and Nutlin-3-induced changes in localization were not detected in L02 cells.
Conclusion: Nutlin-3 regulates the subcellular localization of IFI16 in HCC cells *in vitro* in a p53-dependent manner.

Keywords: IFI16; DNA double-strand break; Nutlin-3; etoposide; pifithrin-α; p53; human hepatocellular carcinoma; SMMC-7721 cell; Huh-7 cell; Hep3B cell

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Introduction

Hepatocellular carcinoma (HCC) is a global health problem usually associated with an inactive form of p53[1]. Murine double minute 2 (MDM2), a p53-selective E3 ligase, negatively controls p53 function by ubiquitination and degradation and ultimately inhibits *TP53* transcription and translation[2]. Restoration of p53 activation by antagonizing MDM2 might offer a new therapeutic strategy.

Nutlin-3, a MDM2 antagonist, disrupts the interaction between p53 and MDM2 and dissociates p53 to bind to other C-terminal modifiers such as interferon-γ inducible protein 16 (IFI16)[3]. IFI16 belongs to the PYHIN family[4], which contains a pyrin domain (PYD) at the N-terminus and two C-terminal HIN200 domains, HIN-A and HIN-B, which can sense double-stranded DNA (dsDNA)[3]. Meanwhile, the IFI16 HIN-A and HIN-B domains interact with the C-terminus and the core DNA binding region of p53, respectively[3]. The role of *IFI16* is more diverse than that of a traditional interferon-inducible gene[5]. First, IFI16 regulates cell proliferation[6] and cell cycle[7] and inhibits cell growth as observed in breast cancer[8], head

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and neck squamous cell carcinoma[9], and prostate cancer[10]. Second, IFI16 contributes to the suppression of viral replication and the promotion of viral clearance to control HBV[11] or Herpes viruses[12] infection. Third, IFI16, one of the AIM2-like receptors (ALRs), acts as a DNA sensor and triggers innate immune response leading to IFN-β production[13] or inflammasome formation[14]. Additionally, IFI16 is involved in DNA double-strand break (DSB) repair[15], autophagy[16], cellular senescence[17, 18], and autoimmune disease such as systemic lupus erythematosus (SLE)[19]. IFI16 is expressed in most human HCC cell lines and tissues but not in healthy adult liver cells[18]. IFI16 triggers innate immune responses to suppress HBV/HCV replication and promote viral clearance[11, 20]. Our previous hypothesis showed that IFI16 mis-localization may be a contributing factor to HCC progression[21]. However, the role of IFI16 subcellular localization is still unclear in HCC chemotherapy.

The present study focused on the relationship between the re-localization of chromatin-bound IFI16 and Nutlin-3 in HCC chemotherapy and the mechanisms underlying the wild-type p53-induced IFI16 re-localization.

Materials and methods

Cell lines and agents

SMMC-7721 (wild-type *TP53*), Huh-7 (mutant *TP53*), Hep3B (null *TP53*) and normal fetal liver L02 cell lines were generous gifts from Prof Cong LIU of the West China Second University Hospital/West China Women's and Children's Hospital.

Nutlin-3, Etoposide, and Pifithrin-α (PFT-α) were purchased from Sigma-Aldrich Technology Company and stored frozen as a 20 mmol/L stock solution in DMSO (Sigma, USA).

Cell culture and treatment

The cultivation medium contains DMEM (Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA), 100 U/mL penicillin and 100 μg/mL streptomycin. The cells were cultured at 37 °C under a 5% CO_2 atmosphere.

The Nut group of SMMC-7721, Huh-7, Hep3B and L02 cells were cultured with 10 μmol/L Nutlin-3 for 48 h[22, 23]. The PFT group of SMMC-7721 cells was treated with 20 μmol/L PFT-α for 48 h. The PFT+Nut group of SMMC-7721 cells were pretreated with PFT-α (20 μmol/L) for 12 h[24] and then exposed to Nutlin-3 (10 μmol/L) for 36 h together with PFT-α. The Eto group of SMMC-7721 cells was cultured with 40 μmol/L Etoposide for 48 h[25], which was used as the positive control of DNA DSB damage. The final concentrations of the tested compounds were prepared by diluting the stock solutions in DMEM. DMEM containing 0.1% DMSO was used as a control.

Real time PCR (RT-PCR)

SMMC-7721 cells were seeded in 6-well plates (3×10^5 cells/well) and treated as described above. Then, total RNA was isolated with TRIzol (Invitrogen) and reverse transcribed using a Prime Script™ Kit (TaKaRa). The RT-PCR was performed on a 7500 Real-Time PCR System (Applied Biosystems, USA). The specific human primers used were as follows (Table 1, forward and reverse).

Table 1. Primer sequences.

IFI16	5′-GAAGTGCCAGCGTAACTCCTA-3′
	5′-TACCTCAAACACCCCATTCAC-3′
IFNB1	5′-TCCACTACAGCTCTTTCCATGA-3′
	5′-AGTATTCAAGCCTCCCATTCAA-3′
TP53	5′-TAGTGTGGTGGTGCCCTATGAG-3′
	5′-AGTGTGATGATGGTGAGGATGG-3′
ACTB	5′-GGCATCCACGAAACTACCTTCA-3′
	5′-GTGATCTCCTTCTGCATCCTGTC-3′

The TP53 and ACTB gene were used as the positive and internal control, respectively. The RT-PCR was performed as follows: 40 cycles of 95 °C for 5 s and 60 °C for 30 s. All samples were analyzed in triplicate on the same plate.

Chromatin fractionation and Western blot analysis

SMMC-7721 and L02 cells were seeded in 6-well plates (3×10^5 cells/well), treated as described above, and fractionated to obtain the chromatin[26]. Whole-cell extracts were directly prepared in an SDS sample buffer (50 mmol/L Tris-HCl pH 6.8, 1% SDS, 10% glycerol, 5% β-ME, 0.01% bromophenol blue). The primary antibodies were rabbit anti-γH2AX polyclonal antibody (bs-3185R, Bioss, diluted 1:200), mouse anti-IFI16 monoclonal antibody (ab50004, Abcam, diluted 1:1000), rabbit anti-H2b polyclonal antibody (BS1657, Bioworld, diluted 1:500), and β-actin antibody (BA2305, Boster, diluted 1:500). The secondary antibodies, goat anti-mouse IgG-HRP (sc-2005, diluted 1:5000) and goat anti-rabbit IgG-HRP (sc-2004, diluted 1:5000), were purchased from Santa Cruz Biotechnology. H2b and β-actin served as the quality controls for nuclear fraction and cytoplasmic fraction, respectively.

Immunofluorescence (IF) assay

SMMC-7721, Huh-7, Hep3B, and L02 cells were cultured for 48 h on glass coverslips in 24-well plates (2×10^5 cells/well) with or without Nutlin-3 treatment. The PFT and PFT+Nut group of SMMC-7721 cells were treated as described above. The samples were fixed, permeabilized, blocked, and then incubated for 1 h with rabbit anti-γH2AX polyclonal antibody (bs-3185R, Bioss, diluted 1:200) or mouse anti-IFI16 monoclonal antibody (ab50004, Abcam, diluted 1:1000) at 37 °C and then with goat anti-mouse FITC-conjugated secondary antibody (F0257, Sigma, diluted 1:100) or sheep anti-rabbit Cy3-conjugated secondary antibody (C2306, Sigma, diluted 1:100) at 37 °C for 1 h. Cells were counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (10 μg/mL) (Sigma, USA). Images were captured via fluorescence microscopy (Olympus BX51).

Statistical analysis

The data are presented as the mean±SD. The means were analyzed using one-way ANOVA to compare between the groups. Differences with $P<0.05$ were considered statistically

significant. All statistical tests were performed using SPSS 18.0 (SPSS, Chicago, IL, USA).

Results

Nutlin-3 causes DNA DSB damage in SMMC-7721 cells

γH2AX, the phosphorylated H2AX (at Ser[139]), is a sensitive marker of DNA DSB[27]. To examine the impact of Nutlin-3 on DNA DSB damage, we incubated SMMC-7721 cells with Nutlin-3 (10 μmol/L) or Etoposide (40 μmol/L), an agent capable of inducing DSB, for 48 h and preformed Western blots to assess the expression level of γH2AX. We observed that Nutlin-3 or Etoposide increased the expression level of γH2AX, consistent with Etoposide inducing DSB (Figure 1A). These results suggest that Nutlin-3, like Etoposide, caused DNA DSB damage and triggered H2AX phosphorylation at Ser[139] in SMMC-7721 cells.

γH2AX foci on mitotic chromosomes represent repaired lesions or unrepaired DNA breaks[28]. We next sought to establish whether the observed Nutlin-3-induced activation of H2AX phosphorylation was indicative of γH2AX foci formation. As expected, treatment of SMMC-7721 cells with Etoposide or Nutlin-3 was observed to induce γH2AX foci formation (Figure 1B). Taken together, Nutlin-3, like Etoposide, caused DNA DSB damage in SMMC-7721 cells, characterized by H2AX phosphorylation (at Ser[139]) and γH2AX foci formation.

Nutlin-3 induces the chromatin-bound protein IFI16 to partially localize in the cytoplasm of SMMC-7721 cells

IFI16 is a member of the DNA sensors involved in DNA DSB repair[15]. First, because *IFI16* is regulated at the transcriptional and post-transcriptional level[29], we preformed RT-PCR to determine the expression level of *IFI16* mRNA. We treated SMMC-7721 cells with PFT-α, a p53 transcriptional inhibitor[30], for 48 h to test TP53 mRNA levels as a positive control. These data showed that Nutlin-3 significantly increased the expression level of *IFI16* mRNA (2.58 fold, $P<0.05$) (Figure 2A). These results suggest that Nutlin-3 promoted *IFI16* expression at the transcriptional level.

As the IFI16-HIN200 domain contains a DNA binding region at the C-terminus, we then extracted the chromatin fractions[26] and used Western blots to investigate the association of IFI16 with chromatin and the expression level of the IFI16 protein. However, we detected that Nutlin-3 down-regulated the expression level of the IFI16 protein in SMMC-7721 cells (Figure 2B).

Next, we sought to establish whether the observed decrease in IFI16 levels was due to its subcellular localization. Interestingly, IFI16 was detected in only the chromatin-binding fraction of control cells, suggesting that it is a chromatin-bound protein (Figure 2B). We have previously confirmed that IFI16 is mainly localized in the nucleus of SMMC-7721 cells[31]. However, IFI16 was partially detected in the cytoplasm of Nutlin-3-treated cells (Figure 2B). Nuclear IFI16 is induced in the cytoplasm of stratified squamous epithelia in response to UVB exposure and acts as a mechanism of auto-antigen processing in SLE[19]. Meanwhile, endogenous IFI16 released by apoptotic cells acts as a novel alarmin, binding to neighbor cells and propagating the damaged-signal[32]. In addition, nuclear IFI16 is relocalized to the cytoplasm leading to proteasomal degradation by infection with HSV-1[33]. According to the results that Nutlin-3 up-regulates *IFI16* mRNA and down-regulates IFI16 protein levels, we proposed that IFI16 might be partially degraded in the cytoplasm or released into the extracellular milieu. These results indicate that IFI16 distribution is dynamic in response to Nutlin-3 treatment in SMMC-7721 cells.

Nutlin-3 increases the expression level of *IFNB1* mRNA

In our previous study, we have shown that Nutlin-3 causes apoptosis in HCC cells with different *TP53* genotypes[23]. It

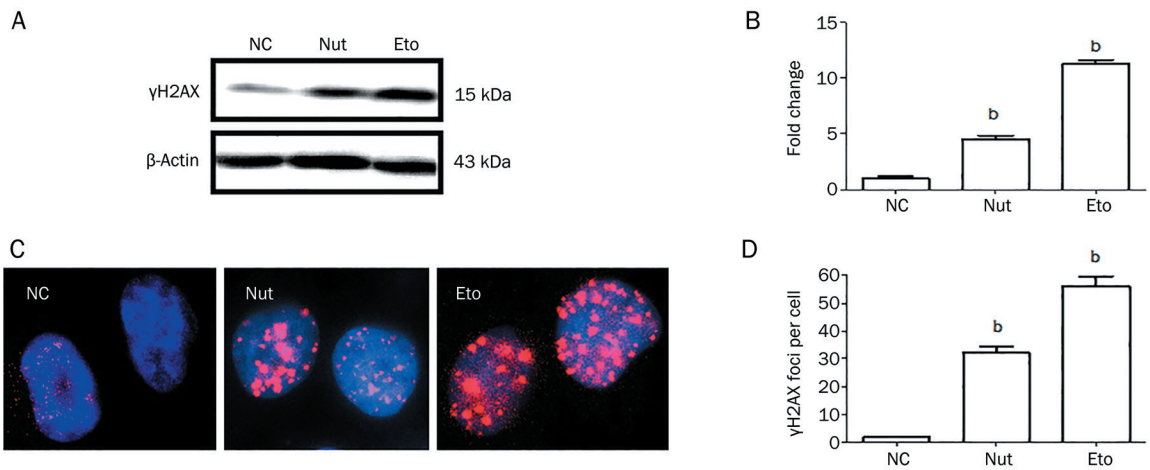

Figure 1. Nutlin-3 causes DNA DSB damage in SMMC-7721 cells. (A) Nutlin-3 increased γH2AX expression level. β-Actin served as the loading control. (B) The relative expression levels of γH2AX. The value represents the mean±SD derived from triplicate tests. $^bP<0.05$ *vs* NC group. (C) Representative images of γH2AX foci formation (1000×). SMMC-7721 cells were treated with 0.1% DMSO, Etoposide (40 μmol/L), or Nutlin-3 (10 μmol/L) for 48 h and analyzed for γH2AX (red). Nuclei were counter-stained with DAPI (blue). The images were merged using Image-Pro plus 6.0. (D) Statistical analysis of the number of γH2AX foci. Data are shown as the mean±SD ($n=3$). $^bP<0.05$ *vs* NC group.

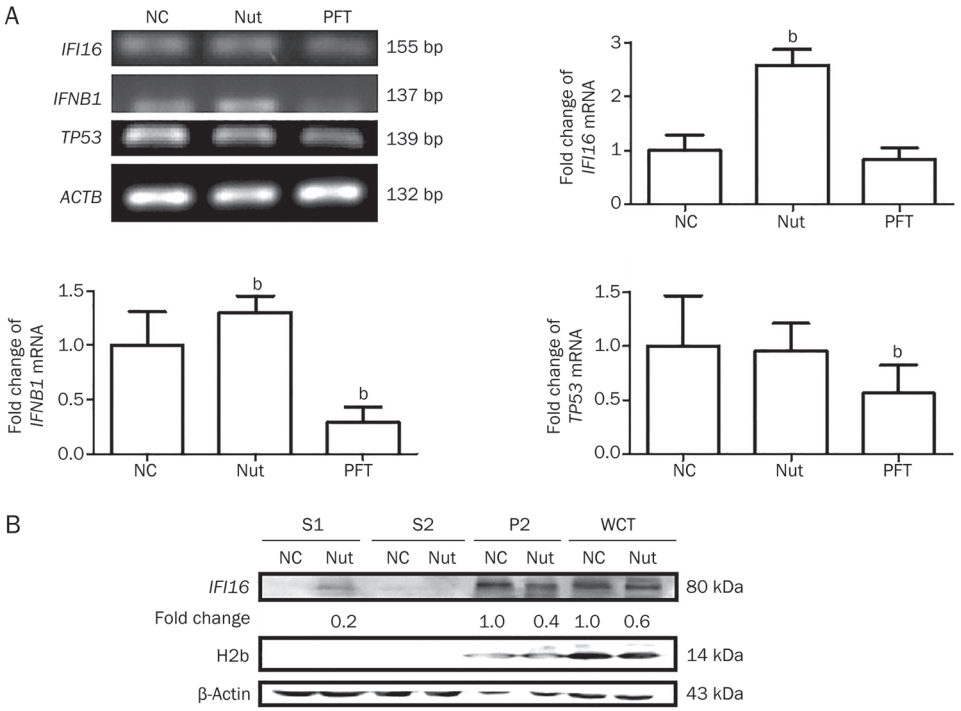

Figure 2. Nutlin-3 induces the chromatin-bound protein IFI16 to partially localize to the cytoplasm of SMMC-7721 cells and increases the expression level of *IFNB1* mRNA. (A) Nutlin-3 increased the expression level of *IFI16* mRNA. The left panel shows representative gels of *IFI16* and *IFNB1*. The TP53 and ACTB gene were used as the positive and internal control, respectively. The right graph shows the relative expression levels of *IFI16* and *IFNB1* mRNA. The value represents the mean±SD (n=3). $^bP<0.05$ *vs* NC group. (B) Chromatin fractions were analyzed via Western blot in SMMC-7721 cells. H2b and β-actin served as the quality control for the nuclear fraction and the cytoplasmic fraction, respectively. S1, cytoplasmic proteins; S2, soluble nuclear proteins; P2, chromatin-enriched sediment; WCE, whole-cell extracts.

has been reported that IFI16 cytoplasmic accumulation can maximize innate immune system sensitivity in cancer chemotherapies[34]. To determine whether the ectopic expression of IFI16 triggered IFN-β production in the cytoplasm of Nutlin-3-treated cells, we investigated the level of *IFNB1* mRNA using RT-PCR analysis. The *TP53* mRNA in PFT-treated cells was used as the positive control (Figure 2A). Compared with the NC group, the expression level of *IFNB1* mRNA was increased in Nutlin-3-treated SMMC-7721 cells (1.30 fold, $P<0.05$) (Figure 2A). These data suggest that the ectopic expression of IFI16 might trigger the expression of *IFNB1* at the transcriptional level in SMMC-7721 cells.

Nutlin-3 causes the ectopic expression of IFI16 in HCC cells in a p53-dependent manner

During DNA DSB damage, p300 acetylates p53[35] and IFI16-NLS leading to IFI16 cytoplasmic localization[34]. To clarify whether IFI16 re-localization is a p53-dependent response by Nutlin-3, we used PFT-α to pre-treat SMMC-7721 cells (wild-type *TP53*) for 12 h to inhibit p53 activity and then exposed the cells to Nutlin-3 together with PFT-α for 36 h at the same conditions. After treatment, we performed IF analysis with anti-IFI16. Consistent with the Western blot results, IFI16 was distributed in only the nucleus of SMMC-7721 cells, but in the nucleus and cytoplasm of Nutlin-3-treated cells (Figure 3A). As expected, we detected IFI16 in only the nucleus of PFT and PFT+Nut group cells (Figure 3A), suggesting that IFI16 re-localization was not detected in p53-inhibited SMMC-7721 cells.

IFI16 is also a chromatin-bound protein in Huh-7 (mutant TP53) and Hep3B (null *TP53*) cells[21]. To clarify whether the change in IFI16 localization is associated with p53 status in HCC cells, we used Huh-7 and Hep3B cells to perform IF analysis with the anti-IFI16. We detected IFI16 in only the nucleus of Huh-7 and Hep3B cells (Figure 3A). Interestingly, IFI16 re-localization was not detected in Huh-7 or Hep3B cells treated with Nutlin-3 (Figure 3A). Taken together, these results show that Nutlin-3-induced IFI16 redistribution occurs in a p53-dependent manner (Figure 3B).

IFI16 re-localization is a unique response in SMMC-7721 cells

To clarify whether Nutlin-3 causes IFI16 re-localization in normal liver cells, we used the L02 cells to perform IF analysis with an IFI16 antibody. Surprisingly, IFI16 re-localization was not detected in Nutlin-3-treated L02 cells (Figure 4A). This result suggests that Nutlin-3 does not change IFI16 subcellular localization in L02 cells with wild-type *TP53*.

Next, we investigated the association of IFI16 with chromatin using Western blot analysis. Interestingly, IFI16 was found in the cytoplasm and nucleoplasm, revealing that it is not a chromatin-bound protein in L02 cells. Consistent with the IF analysis (Figure 4A), Nutlin-3 did not change IFI16 localiza-

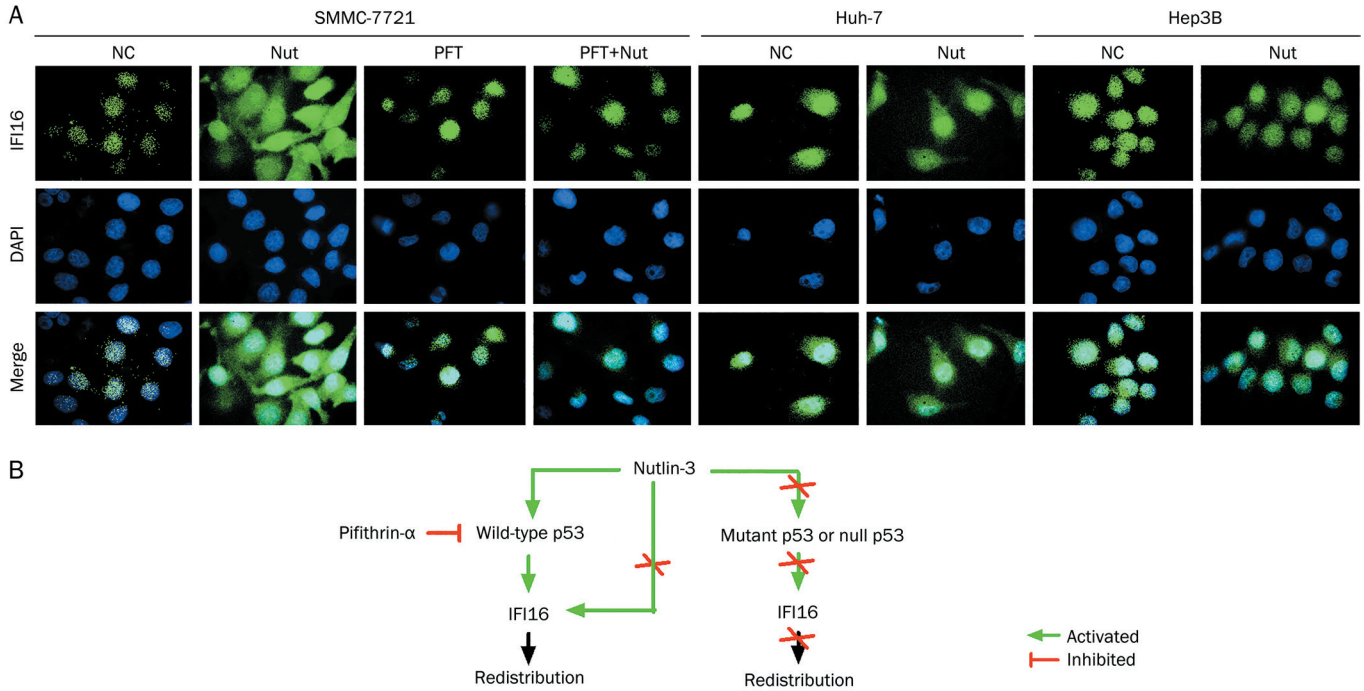

Figure 3. Nutlin-3 causes IFI16 ectopic expression in a p53-dependent manner. (A) IFI16 subcellular localization was detected in HCC cells with different *TP53* genotypes using fluorescence microscopy (1000×). Cells were treated as described above and stained for IFI16 (green). Nuclei were counter-stained with DAPI (blue). Images were merged using Image-Pro plus 6.0. (B) Proposed model for IFI16 subcellular localization regulated by Nutlin-3 in a p53-dependent manner in HCC cells.

tion (Figure 4B). Together, these results show that the re-localization of IFI16, a chromatin-bound protein, is a unique response in Nutlin-3-treated SMMC-7721 cells.

Discussion

The effect of Nutlin-3 on HCC with mainly wild-type p53 are already known[22, 23]. In this article, we demonstrated a link between Nutlin-3, a DNA damage agent[36], and IFI16, a DNA sensor, through wild-type p53 activation in HCC cells. Nutlin-3 treatment increased the expression level of *IFI16* and *IFNB1* mRNA, and partially triggered chromatin-bound IFI16 redistribution to the cytoplasm of SMMC-7721 cells in a p53-dependent manner but not in L02 cells. This study provides new insight into the relationship between Nutlin-3 and the re-localization of chromatin-bound IFI16 in HCC therapy.

IFI16 is involved in liver cancer progression. *IFI16* is up-regulated in chronic HCV and/or HBV liver tissue, suggesting that elevated IFI16 blocks viral replication and promotes viral clearance[11, 37]. In the present study, we also showed that Nutlin-3 triggers *IFI16* mRNA expression in SMMC-7721 cells. Therefore, we proposed that increased IFI16 expression in HCC cells by Nutlin-3 is similar to acute liver graft rejection[38] and virus-infected liver cells[39], which might reflect hepatic reversion to fetal status after liver injury.

IFI16 was predominantly in the nucleus[34]. We have confirmed that IFI16 is a chromatin-bound protein in HCC cells but not in L02 cells[21]. Recently, a study clearly demonstrated that the IFI16-HIN domains recognize the dsDNA sugar-phosphate backbone in non-sequence-specific manner and that the PYD domain inhibits the HIN:DNA interaction, even though it has no DNA-binding capacity[40]. For example, the PYD and HIN domains of AIM2, the other ALRs, form an intra-molecular complex in an auto-inhibited 'resting' state in HEK293T cells[40]. In the present study, we found that the subcellular localization of IFI16 was partly changed in response to Nutlin-3 treatment in SMMC-7721 cells. Consistently, some studies reported that IFI16 localization is involved in the DNA damage pathway by DNA-damaging agents such as IR[15] and UVB[19] in some cancer cells. The strong preference of IFI16 protein binding to supercoiled DNA and cruciform structures suggests that it may play an important role in DNA damage recognition during the repair response[41]. Further studies need to be conducted to address the mechanism of Nutlin-3 and re-localization of IFI16 in normal fetal liver cells.

IFI16 localization is important for its biological activities. IFI16 sensing of pathogenic or host-damaged DNA[42] depends on the localization of the sensor and the DNA target, which is consistent with a two-signal model of innate immunity[43]. Therefore, re-localization of IFI16, a scaffold protein, may be a means for its activation. For example, IFI16 acts as a DNA sensor in the nucleus and redistributes to cytoplasm and forms inflammasomes upon KSHV[14], EBV[44], and HSV-1[33] infection. IFI16 cytoplasmic accumulation can also benefit innate immunity in cancer chemotherapies[34]. In the present study, we detected the increased expression level of *IFNB1* mRNA in Nutlin-3-treated SMMC-7721 cells, which promotes the anti-

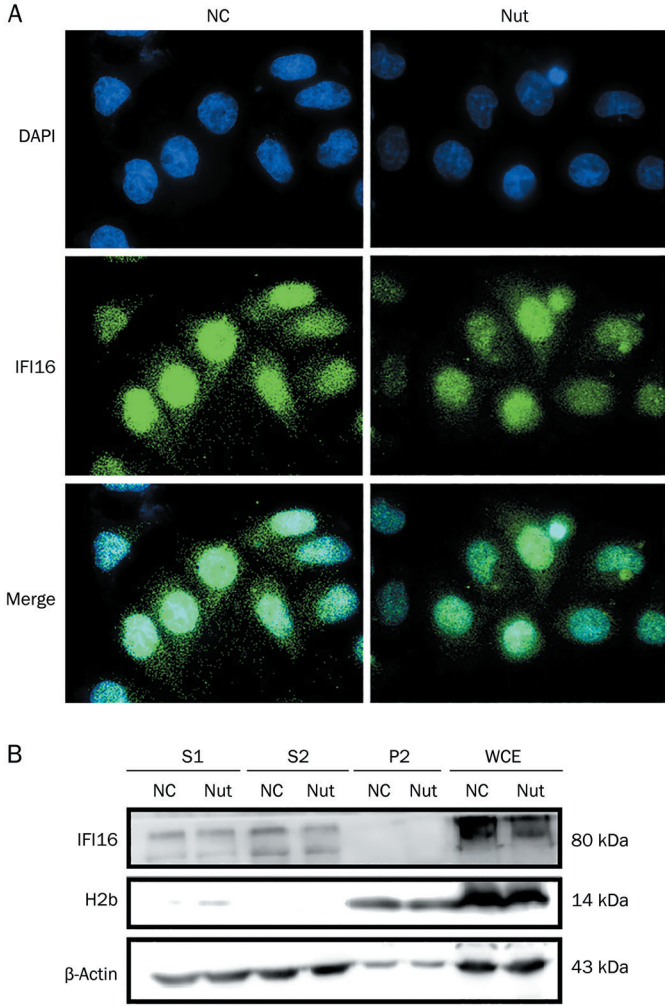

Figure 4. IFI16 subcellular localization regulated by Nutlin-3 in L02 cells. (A) IFI16 subcellular localization was detected in L02 cells using fluorescence microscopy (1000×). Cells were treated as described above and stained for IFI16 (green). Nuclei were counter-stained with DAPI (blue). Images were merged using Image-Pro plus 6.0. (B) Chromatin fractions were analyzed via Western blot in L02 cells. H2b and β-actin served as the quality control for the nuclear fraction and the cytoplasmic fraction, respectively. S1, cytoplasmic proteins; S2, soluble nuclear proteins; P2, chromatin-enriched sediment; WCE, whole-cell extracts.

inflammatory activation of IFI16[45].

The function of the injury-activated innate immune system is similar to the two sides of a coin[46]. According to the danger hypothesis[47], over-expressed IFI16 protein released in the extracellular niche acts as a novel alarmin propagating the damaged-signal[32]. For example, nuclear IFI16 delocalizes to the cytoplasm and then exists in the extracellular environment circulating as a new danger signal in response to UV or infected DNA, leading to impaired endothelial cells through high-affinity membrane binding in autoimmune diseases[32].

Mechanically, we found that the IFI16 re-localization induced by Nutlin-3 treatment was involved in the p53 pathway but not in L02 cells. Consistent with our results, a study reported that IFI16-NLS acetylation by p300, a histone acetyltransferase, promotes IFI16 cytoplasmic localization[34]. p300 binds to and activates p53 in response to DNA-damaged agents[48]. Novel anticancer agents target p53 activation, a critical cellular pathway. Loss of IFI16 results in deregulation of p53-mediated apoptosis in breast cancer[8]. Consistently, over-expressed IFI16 has no effect on cell growth in p53-inactived oral squamous cell carcinoma[49]. Our previous hypothesis also proposed that IFI16 mis-localization may be a contributing factor to HCC progression[21]. If we are able to redistribute chromatin-bounding IFI16 into the cytoplasm with activated-p53 restoration, we may offer an alternative for HCC therapy.

In recent years, some studies have focused on the role of IFI16 and targeted the nucleolus for cancer therapy. However, IFI16, a multifunctional protein, remains relatively poorly characterized in HCC. Therefore, further studies are needed to examine the location-function relationship for IFI16 in HCC chemotherapy.

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Author contribution

Xin-li SHI, Ming-yuan LI, and Bao-ning WANG designed the research; Xin-li SHI performed the experiments; Xin-li SHI wrote the manuscript with contribution from other authors.

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