ORIGINAL ARTICLE

IFI16 restoration in hepatocellular carcinoma induces tumour inhibition *via* activation of p53 signals and inflammasome

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Abstract

Objective: Interferon-inducible 16 (IFI16) is known to involve in p53-dependent tumour suppression and also the formation of inflammasome, which function, however, remains controversy during carcinogenesis as a pattern recognition receptor for tumour death-derived free DNA. In this study, we investigated the anti-tumour role of IFI16 in hepatocellular carcinoma (HCC).

Materials and methods: Hepatocellular carcinoma tissues (n = 20) and corresponding non-neoplastic tissues (n = 20) were collected to determine the expression of IFI16. After the transfection of pcDNA3.1-IFI16 into Huh7 and SMMC7721 cells in vitro, the influence of IFI16 overexpression on cell vitality, colony formation, apoptosis and migration were analysed. The role effect of IFI16 in vivo was further investigated.

Results: The expression of IFI16 was significantly decreased in tumour tissues and cell lines. Overexpression of IFI16 induced decrease of cell vitality, colony formation and increased apoptosis with impaired ability of migration. Mechanistically, IFI16 could activate p53 at Ser15 to up-regulate the p21^{WAF1/CIP1} level to inhibit tumour growth and migration, which was restored by the p53 inhibitor Pifithrin- α (20 μ mol/L). Moreover, IFI16-induced tumour cell death promoted the recruitment of inflammasome complex to enhance tumour inhibition, but the caspase-1 inhibitor Ac-YVAD-CMK (50 μ mol/L) could suppress this process in HCC. The results in vivo indicated that restored expression of IFI16 in tumour cells effectively promote tumour regression, which could be partly abrogated by the inhibition of activation of p53 signals or induced inflammasome.

Conclusion: IFI16 is a tumour suppressor in HCC *via* activation of p53 signals and inflammasome.

1 | INTRODUCTION

Primary liver cancer is one of the leading causes of cancer-related death worldwide. Hepatocellular carcinoma (HCC) is the most common

type of hepatic malignancies with over 500 000 new cases diagnosed every year and an annual death rate of 250 000 people. Of note, its incidence rate is still increasing, especially in Asia.^{1,2} Patients with cancer are often found to have defected ability of immune defence and are not able to effectively activate immune system to eliminate tumour cells.³ Currently, the vast successful application of anti-programmed

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cell death protein 1 antibody underlines that immunotherapy could be good alternative strategy for the treatment of HCC.⁴

Pathogen-associated molecular patterns (PAMPs) (eg LPS, flagellin, microbial/virus DNA and RNA) and danger-associated molecular patterns (DAMPs) released from damaged host cells (eg ATP, alarmins and host-derived DNA) are danger signals for host immunity and could be sensed by pattern recognition receptors (PRRs) including toll-like receptors, NOD-like receptor, RIG-I-like receptors and AIM2-like receptors.^{5,6} Many immune adjuvants for cancer-therapy are derived from PAMP or DAMP and interact with the deregulated PRRs on immunocytes or tumours,^{7,8} and some have been demonstrated to regulate anti-tumour immunity.^{9,10}

As an AIM2-like receptor, the interferon-inducible 16 (IFI16) protein, on the one hand, can sense dsDNA from some viruses to activate STING-TBK1 pathway for the production of IFN- β for anti-virus defence¹¹ and on the other hand, it has also been uncovered to bind DNA and form an inflammasome—a cytoplasmic protein complex which induces pyroptosis and proteolytic maturation of inflammatory cytokines IL-1 β and IL-18.¹² Therefore, it is critical for the clearance of pathogens or damaged cells. However, the role of IFI16 during carcinogenesis is unclear.

Mounting evidences indicated that IFI16 has been demonstrated to enhance the activation of the known p53 target genes including p21, Hdm2 and bax to induce p53-mediated cell cycle arrest and apoptosis in human cancer cells.^{13,14} But IFI16 can also interact with metastasis-associated protein 1 to down-regulate of oestrogen receptor α , leading to the resistance of tamoxifen-induced growth inhibition.¹⁵ Additionally, although IFI16-induced inflammasome promote the anti-virus immunity, the effects of inflammasome on many types of cancers remain controversy, including gastric cancer, colon cancer and melanoma and seem to be tissue specific.^{16,17}

In this study, we here found that IFI16 was down-regulated in HCC and restored its expression of IFI16 in tumour cells could increase p53-dependent tumour inhibition. Moreover, IFI16-induced inflammasome was found to inhibit HCC growth and metastasis. These data indicated that IFI16 is a tumour suppressor during the development of HCC.

2 | MATERIALS AND METHODS

2.1 | Tumour samples, cell lines and reagents

To estimate the expression of IFI16, HCC tissues ($n = 20$) and corresponding relative-normal tissues ($n = 20$) were collected from Shandong provincial Qianfoshan hospital. According to WHO classifications, all of the patients diagnosed with primary HCC were confirmed by haematoxylin and eosin staining by experienced pathologists. This study was approved by the Research Ethics Committee of Shandong provincial Qianfoshan hospital. All these retrospective specimens were handled and made anonymous according to the ethical and legal standards. Written informed consent was obtained from all of the patients. Besides, the HCC cell lines, HePG-2, Huh-7, Bel-7402, HL-7702, SMMC-7721, and 1 normal human liver cell line, L-02, were purchased from the cell bank of the Chinese academy of sciences (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (Life Technologies, Carlsbad, CA, USA), ampicillin and

streptomycin at 37°C, 5% CO₂ conditions. For the overexpression of IFI16, the overexpression vector pcDNA3.1-IFI16 was conducted by GenePharma (Shanghai, China). Anti-GAPDH, IFI16, p-p53-ser15, p53, p21, adenocarcinoma (ASC), Pro-caspase-1, caspase-1, Pro-IL-1 β , IL-1 β , Pro-IL-18, IL-18 and ki-67 antibodies were obtained from Cell Signaling Tech (Denver, MA, USA) and Abcam (Cambridge, MA, USA). The inhibitor Pifithrin- α (PFT α) and Ac-YVAD-CMK were obtained from Selleck (Houston, TX, USA) and Invivogen (San Diego, CA, USA).

2.2 | Immunohistochemistry

To determine the expression of IFI16, p53 and ki-67 in HCC tissues, 2- μ m-thick, formalin-fixed and paraffin-embedded specimen sections were used. After the slides were incubated in xylene for 5 minutes, 100% ethanol was used for 10 minutes, 95% ethanol for 10 minutes. Antigen unmasking was performed and then the slides were blocked with 3% hydrogen peroxide for 30 minutes at room temperature. Then the primary antibody for IFI16, p53 and ki-67 was incubated the FFPE specimen sections at 4°C overnight and, then the biotinylated horse secondary antibody and streptavidin-horse-radish peroxidase (Zymed Laboratories Inc. South San Francisco, CA, USA) were used for the detection of IFI16 p53 and ki-67. After that, the EnVision Detection System kit (Dako, Glostrup, Denmark) was used for the DAB chromogen followed by nuclear staining using haematoxylin.

2.3 | RNA isolation and qRT-PCR

According to the standard RNA isolation protocol, total RNA from tissues or Huh-7 and SMMC-7721 cells was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Quantitative real-time RT-PCR was performed, and the expression levels of IFI16 were normalized to GAPDH for gene expression. The primer: FP: AGACTGAAGACTGAACCTGAAGA; RP: GAACCCATTGCGGCAACATA.

2.4 | Western blots

According to the manufacturer's instructions, the whole cell protein extracts were prepared and were separated using sodium dodecyl sulphate polyacrylamide gel electrophoresis and 10% gel. Proteins were then transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA), according to the instruction manual. Filters were blocked overnight in 5% w/v low-fat dry milk in 10 mmol/L Tris-HCl, pH 7.5, 0.1 mol/L NaCl and 0.1% Tween-20 and incubated with primary antibodies overnight at 4°C. After washing with TBST buffer, the blots were then incubated with HRP-conjugated secondary antibody for 2 hours at room temperature. After washing with TBST buffer, the blots were visualized using the ECL-Plus reagent (Millipore, Billerica, MA, USA). GAPDH was used as the loading control in the Western blotting.

2.5 | Cell transfection

The Huh-7 and SMMC-7721 cells were seeded into 12-well plates and transfected with pcDNA3.1-IFI16 or negative control for indicated

time at a concentration of 1 ng/mL by Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

2.6 | CCK-8 assay

After transfection of IFI16 in 2 HCC cell lines for indicated time, the cells were harvested and washed with PBS and then cell counting kit-8 (Kumamoto, Japan) mixed with DMEM was used for cell viability assay, and the absorbance was measured at 450 nm by a microplate reader.

2.7 | Scratch wound assay and transwell assay

For the scratch wound assay, the Huh-7 and SMMC-7721 cells were wounded with a plastic tip that was dragged across the cell monolayer upon cells reached confluence. After overexpression of IFI16 for 48 hours, 5 fields were randomly selected and the distances of migrated cells were measured under a light microscope. In Transwell assay, 3×10^4 Huh-7 and SMMC-7721 cells transfected with pcDNA3.1-IFI16 or negative control were in the upper chamber of a non-coated transwell insert. Cells that did not migrate or invade were removed using a cotton swab and were stained by crystal violet and counted under an inverted microscope. Five random views were selected to count the cells.

2.8 | Colony formation assay

The Huh-7 and SMMC-7721 cells with overexpressed expression of IFI16 were harvested and were re-suspended in complete medium containing 10% FBS and were seeded into 12-well plates for 10 days. Using 0.1% crystal violet, cells fixed with methanol for 15 minutes were visualized under a dissection microscope (Olympus, Tokyo, Japan) and colonies consisting of 50 cells or more were counted.

2.9 | Flow cytometer assay

The cells were fixed in cold 70% ethanol at -20°C for 2 hours and then were treated with 10 mg/mL RNase and stained with 2 μL of annexin V mixed with 2 μL of propidium iodide (PI; eBioscience, San Diego, CA, USA) and were used according to the manufacturer's instructions and quantified by flow cytometry on a FACS Calibur instrument.

2.10 | ELISA

According to the manufacturer's instructions, the ELISA kit for IL-1 β , IL-18 and LDH (eBioscience) were used. The absorbency was measured at 450 nm by microplate reader.

2.11 | Tumour model

To investigate the tumour suppressive role of IFI16 in vivo, 2×10^6 SMMC-7721 cells with or without overexpression of IFI16 were subcutaneously injected in rear flank of nude mice (6 per group).

The inhibitor PFT α (1 mg/kg) and Ac-YVAD-CMK (10 mg/kg) were administrated intraperitoneally for 3 times, 5 days apart.

2.12 | Statistical analyses

The Statistical Package for Social Sciences version 16.0 (SPSS 16.0; SPSS Inc., Chicago, IL, USA) and the Prism statistical software package (Version 5.0; Graphpad Software Inc. La Jolla, CA, USA) were used. Unpaired *t*-tests or Mann-Whitney *U* tests were used to compare the 2 groups, and multiple group comparisons were analysed with one-way ANOVA. $P < .05$ was considered statistically significant. All experiments were performed at least 3 times.

3 | RESULTS

3.1 | The expression of IFI16 is down-regulated in hepatocellular carcinoma

To clarify the function of IFI16 during the carcinogenesis of HCC, the clinical expression of IFI16 in patients with primary HCC was analysed. We here collected HCC tissues ($n = 20$) and corresponding non-neoplastic tissues ($n = 20$), and immunohistochemistry analysis of IFI16 indicated that the protein level of IFI16 was significantly lower in tumour samples (tumour) than that in the corresponding non-neoplastic tissue (normal) (Figure 1A). This result was confirmed by WB analysis (Figure 1B). Besides, the gene expression of IFI16 in HCC samples was also determined and we found decreased mRNA expression of IFI16 in HCC tissues (Figure 1C). Moreover, 5 HCC cell lines, HePG-2, Huh-7, Bel-7402, HL-7702 and SMMC-7721, and 1 normal human liver cell line, L-02, were cultured and the expression of IFI16 in HCC cell lines was decreased when compared to that in L-02 cells (Figure 1D). These data implicated that the function of IFI16 might be suppressed during the development of HCC.

3.2 | Restoration of IFI16 level in tumour cells induces apoptosis and impairs ability of migration

We next examined the assumption that restoration of IFI16 could inhibit the growth of HCC cells. Two HCC cell lines with relatively lower expression of IFI16 were used, namely Huh-7 and SMMC-7721. IFI16 was overexpressed in 2 cell lines *via* transfection of pcDNA3.1-IFI16 or negative control and the transfection efficiency was confirmed by the estimation of mRNA and protein level of IFI16 (Figure 2A). Then, we found that forced expression of IFI16 could induce the decrease of cell vitality in 2 HCC cell lines (Figure 2B). The colony formation in cells overexpressed of IFI16 was inhibited (Figure 2C) and the apoptosis was also induced (Figure 2D). Besides, the ability of migration was estimated in Huh-7 and SMMC-7721 cells. After overexpression of IFI16, the ability of migration was impaired when compared to the cells transfected with negative control (Figure 2E,F). The results indicated that restored expression of IFI16 in HCC cells *via* overexpression could effectively inhibit

FIGURE 1 (A) The expression pattern of IFI16 in hepatocellular carcinoma and relatively normal tissues were determined by immunohistochemistry. (B, C) The protein and mRNA level of IFI16 in tumour tissues were determined by WB and Q-PCR. (D) The expression of IFI16 in 5 HCC cell lines, HePG-2, Huh-7, Bel-7402, HL-7702, SMMC-7721, and 1 normal human liver cell line, L-02, were analysed. * $P < .05$, *** $P < .001$, data represent the means $\pm$ SD

the characteristics of HCC including proliferation, apoptosis and migration (Figure 2G).

3.3 | IFI16 activates p53/p21 signals for its anti-tumour effects in vitro

The activation of p53-mediated tumour suppression was reported to be regulated by IFI16,¹³ thus, we further investigated the role of p53 signals in IFI16-induced tumour inhibition. We found that forced expression of IFI16 could promote the expression of p53 and, meanwhile, enhance its activation via phosphorylation at ser15. IFI16 also promoted the activation of p53 target genes p21 which was also a tumour suppressor and elevated the p21 level in Huh-7 and SMMC-7721 cells, which suggested that IFI16 was able to activate p53/p21 pathway for tumour elimination (Figure 3A).

Moreover, to define the anti-tumour role of IFI16/p53 signals, the inhibitor of p53 activation PFT α was used. Although overexpression of IFI16 induced significant apoptosis, the results showed that the treatment of PFT α could render tumour cells resistance to apoptosis (Figure 3B). Correspondingly, the cells treated with PFT α could also resist IFI16 overexpression-induced decreased colony formation (Figure 3C) and an ability of migration (Figure 3D,E). The results

suggested that the activated p53 signal is indispensable for IFI16-induced tumour inhibition (Figure 3F).

3.4 | Inflammasome could be induced by IFI16 to increase apoptosis and impair ability of migration

Apart from the anti-tumour role of p53, the IFI16-induced inflammasome might also participate into this process, because IFI16-induced cell death would release DAMP such as ATP and tumour-derived DNA to activate the recruitment of inflammasome complex, leading to the pyroptosis-related cell death. Moreover, the constitutive level of inflammasome was also reported in cancer.¹⁸ Therefore, we examined the expression of inflammasome-related components in Huh-7 and SMMC-7721 cells and found that increased level of apoptosis-associated speck-like protein containing a CARD (ASC), Pro-caspase-1, caspase-1, Pro-IL-1 β , IL-1 β , Pro-IL-18 and IL-18 in tumour cells with overexpressed expression of IFI16 (Figure 4A). However, as the key molecular in inflammasome, caspase-1 contributes to the production of IL-1 β and IL-18 and the inhibitor of caspase-1, Ac-YVAD-CMK, was found to impair IFI16-activated inflammasome and the constitutive level of inflammasome in 2 cell lines. Subsequently, the function of inflammasome in HCC was determined (Figure 4B-E). The treatment

FIGURE 2 Overexpression of IFI16 (OE-IFI16) inhibits tumour characteristics. (A) The efficiency of transfection of pcDNA3.1-IFI16 or negative control was determined by Q-PCR and WB. (B) The cell vitality was estimated by CCK-8. (C) The colony formation assay was performed to indicate the proliferation of Huh-7 and SMMC-7721 cells. (D) The effects of IFI16 restoration on the apoptosis were also analysed. (E and F) The migrations of 2 tumour cells were determined by Scratch wound assay and transwell assay. (G) The statistical analysis of the colony formation assay, apoptosis and migration. * $P < .05$, ** $P < .01$, *** $P < .001$, data represent the means $\pm$ SD. Abbreviations: NC, negative control; OE, overexpressed

of Ac-YVAD-CMK effectively inhibited IFI16-induced apoptosis and impaired colony formation, and also rescued the ability of migration in Huh-7 and SMMC-7721 cells (Figure 4F). Furthermore, the levels of IL-1 β , IL-18 and LDH were also abrogated by Ac-YVAD-CMK (Figure 4G-I).

3.5 | Overexpression of IFI16 inhibit the tumour growth in vivo

Given the anti-tumour effects of IFI16 in vitro, we next determined the efficiency of IFI16 for tumour inhibition in the xenograft model of human SMMC-7721 and the nude mice were treated with conditional tumour cells with or without overexpression of IFI16. We found that the tumour growth in IFI16-overexpressed group was significantly inhibited with smaller tumour volume (Figure 5A-C). However, the administration of p53 inhibitor and inflammation inhibitor Ac-YVAD-CMK could repress IFI16-induced tumour

inhibition and recover the tumour proliferation. The expression of Ki-67 proliferation index also confirmed this result, along with the results of IFI16 and p53 expression through immunohistochemistry (Figure 5D). Thus, the result in vivo further supported the tumour suppressive role of IFI16 in HCC.

4 | DISCUSSION

Currently, the treatments for HCC are limited including surgery, radiotherapy or chemotherapy, especially for patients with advanced disease who are not eligible for curative hepatectomy or hepatic transplantation. Diagnosis at late stage is the most important reason and the high rate of recurrence also leads to the low 5-year survival of HCC patients with <40%.¹⁹ Thus, understanding the potential mechanisms of HCC pathogenesis is critical for the diagnosis at early stage and effectively new treatment. In this study, we

FIGURE 3 Activation of p53 is required for IFI16-induced tumour inhibition. (A) The levels of p53, p-p53 and p21 in Huh-7 and SMMC-7721 cells were analysed after transfection of pcDNA3.1-IFI16 or negative control. Using the p53 inhibitor Pifithrin- α (PFT α), the apoptosis, proliferation and the ability of migration were determined by (B) flow cytometry, (C) colony formation and (D and E) scratch wound assay and transwell assay. (F) The statistical analysis of the apoptosis, colony formation assay and migration. * $P < .05$, ** $P < .01$, *** $P < .001$, data represent the means $\pm$ SD. Abbreviations: NC, negative control

found that the expression of IFI16 was absent in HCC and restoration of IFI16 in tumour cell would, on the one hand, induce p53/p21-dependent growth inhibition and, on the other hand, activate IFI16-related inflammasome for anti-tumour effects in vitro and vivo.

The function of IFI16 in tumorigenesis has been investigated in several types of cancers, but consistent results have not been confirmed. Immunohistochemical analysis of tissues from 25 breast cancer patients demonstrated that cancer cells showed negative or weaker staining of IFI16 when compared to normal mammary duct epithelium with positive nuclear staining.¹³ However, in ASC, increased expression of IFI16 protein in squamous cell carcinoma was associated with keratinocyte differentiation.²⁰ The expression of IFI16 oral cancer was found to be up-regulated to enhance cell growth by the activation of NF-kappa B signalling which prevented the apoptosis in OSCC cells.²¹ We here found that HCC tissues harboured less level of IFI16 than normal tissues. Forced expression of IFI16 in HCC promoted the apoptosis with decreased cell vitality. The colony formation and migration were also impaired by IFI16 overexpression. Because IFI16 has been demonstrated to activate p53 and induced the expression of

target genes including p21 and bax to induce p53-mediated cell cycle arrest and apoptosis. In this study, we also observed that p53 was activated in IFI16-overexpressed tumour cells with phosphorylation at ser15, which induced the tumour suppressor p21 expression. Similarly, Gariglio et al¹⁴ reported that the expression of IFI16 is lost in head and neck cancer cells, leading to uncontrolled cell proliferation and tumour growth. IFI16 restoration promoted the increase of p53 and p21 levels for G2/M arrest in vitro. Besides, Mazibrada et al²² found that overexpression of IFI16 in tumour cells could significantly reduce the tumour volume of head and neck cancer in vivo, accompanied by decreased tumour neo-vascularization and increased areas of tumour necrosis. Our findings in vivo also demonstrated that IFI16 overexpression in HCC cells could significantly inhibit the tumour growth with decreased tumour volume.

Inflammasomes are multimeric complexes composed of several proteins that promote caspase-1 activation and the synthesis of pro-IL-1 β /IL-18 in response to a variety of physiological and pathogenic stimuli including PAMP and DAMP, which is critical for the clearance of pathogens or damaged cells.²³ However, the role of inflammasome in cancer is unclear. Inflammasome-induced pyroptosis could promote

FIGURE 4 IFI16 also induces the formation of inflammasome for anti-tumour. (A) The protein expression of ASC, pro-caspase-1, caspase-1, pro-IL-1 β , IL-1 β , pro-IL-18 and IL-18 in Huh-7 and SMMC-7721 cells were estimated by WB. After the treatment of inflammasome inhibitor Ac-YVAD-CMK (50 μ mol/L), the apoptosis, proliferation and the ability of migration were determined by (B) flow cytometry, (C) colony formation and (D and E) scratch wound assay and transwell assay. (F) The statistical analysis of the colony formation assay, apoptosis and migration. (G) The levels of IL-1 β , IL-18 and LDH in supernatant of tumour cells were determined by ELISA. * P < .05, ** P < .01, *** P < .001, data represent the means $\pm$ SD. Abbreviations: NC, negative control; LDH, lactate dehydrogenase

the death of tumour cells which results in the release of ATP and sensed by purinergic receptors on the surface of dendritic cell, leading to activation of tumour antigen-specific type-1 T helper cells (Th1) and cytotoxic CD8 T cells for killing tumour cells.^{16,24} Alternatively, the activation of the inflammasome in structural epithelial cells can lead to the activation of Th1 and Th17 cells in an IL-1 β /IL-18-dependent manner.²⁵ However, when IL-12 was absent in tumour, inflammasome-induced

IL-1 β and IL-18 could also promote a Th2-biased response via increased recruitment of suppressive immune cells (eg MDSC and Tregs), which can favour tumour progression.²⁴ In this study, we found that overexpression of IFI16 could activate the inflammasome via caspase-1 to cleave pro-IL-1 β /IL-18, leading to enhance the IL-1 β /IL-18 level. But the inhibitor of caspase-1, Ac-YVAD-CMK, could effectively inhibit IFI16-induced tumour inhibition which suggested that IFI16-inflammasome

FIGURE 5 The anti-tumour role of IFI16 was assessed in vivo. 2×10^6 SMMC-7721 cells with or without overexpression of IFI16 were subcutaneously injected in rear flank of nude mice (6 per group). The inhibitor Ac-YVAD-CMK (10 mg/kg) and Pifithrin- α (PFT α) (1 mg/kg) were administrated intraperitoneally. (A-C) The mean tumour size (mm³) was analysed. (D) The expressions of IFI16, p53 and Ki-67 proliferation index were estimated by immunohistochemistry. * $P < .05$ vs NC, # $P < .05$ vs OE, data represent the means $\pm$ SD. Abbreviations: NC, negative control; OE, overexpressed

contributed to the increased apoptosis, decreased colony formation and migration.

Taken together, we found that IFI16 restoration in vitro in HCC cell lines induced cell apoptosis and impaired its ability of colony formation and migration in p53- and caspase-1-dependent manner. Moreover, IFI16-related inflammasome functioned as anti-tumour role in HCC. The results in vivo confirmed the tumour suppressive role of IFI16 in HCC.

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DISCLOSURE STATEMENT

No competing financial interests exist.

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