HIV-TAT-fused FHIT protein functions as a potential pro-apoptotic molecule in hepatocellular carcinoma cells

Gui-Rong YU*†, Wei-Wei QIN*†, Ji-Peng LI††, Wei HUA‡, Yan-Ling MENG§, Rui CHEN*, Bo YAN*, Lei WANG*, Xiang ZHANG*, Lin-Tao JIA*, Jing ZHAO*, Rui ZHANG* and An-Gang YANG*

*State Key Laboratory of Cancer Biology, Department of Biochemistry and Molecular Biology, Fourth Military Medical University, 710032 Xi’an, People’s Republic of China, †Department of Gastrointestinal Surgery, Xijing Hospital, Fourth Military Medical University, 710032 Xi’an, People’s Republic of China, ‡Department of Obstetrics and Gynecology, Xijing Hospital, Fourth Military Medical University, 710032 Xi’an, People’s Republic of China, and §Department of Immunology, Fourth Military Medical University, 710032 Xi’an, People’s Republic of China.

Synopsis
Accumulating evidence has demonstrated that FHIT (fragile histidine triad) is a bona fide tumour suppressor gene in a large fraction of human tumours, including hepatocellular cancer. A virus-based delivery system has been developed to transfer the FHIT gene into many types of cancer cells to inhibit growth or even induce apoptosis. However, a protein-based replacement strategy for FHIT has not been performed in cancer cells. Here, we used HIV-TAT (transactivator of transcription)-derived peptide to transfer the purified FHIT protein into HCC (hepatocellular carcinoma) cells and determine the biological effect of this fusion protein in inducing apoptosis. Affinity chromatography was used to purify TAT peptide-fused human FHIT (TAT–FHIT) protein from BL21 Escherichia coli. Immunofluorescence staining and Western blot analysis were performed to identify the expression and internalization of TAT–FHIT in HCC cells compared with the purified FHIT protein. Our study showed that TAT–FHIT protein can translocate into cancer cells in 1 h after incubation at 37°C. Furthermore, the results of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] assay, Annexin-V staining and Western blotting demonstrated that TAT–FHIT can robustly inhibit growth and induce apoptosis of HCC cells in vitro. In addition, a mechanistic study showed that both exogenous and intrinsic apoptotic pathways were involved in TAT–FHIT-mediated apoptosis and this effect could be attenuated partially by a mitochondrial protector TAT-BH4, indicating that mitochondrion plays a critical role in TAT–FHIT-mediated pro-apoptotic effect in cancer cells. Taken together, our study suggests that TAT–FHIT is a potential pro-apoptotic molecule in HCC cells and strengthen the hypothesis of its therapeutic application against HCC.

Key words: apoptosis, fragile histidine triad (FHIT), hepatocellular cancer, mitochondrion, transactivator of transcription (TAT)

INTRODUCTION

HCC (hepatocellular carcinoma) is an aggressive solid tumour with poor prognosis. Despite aggressive surgical resection and chemotherapy, approx. 50% patients develop recurrent disease. Although many treatment strategies have been developed, the prognosis still remains unsatisfactory. Understanding the molecular mechanism involved in carcinogenesis of hepatocytes is very important for exploring more effective treatment modalities [1,2]. DNA methylation-mediated epigenetic inactivation of tumour suppressive genes has been identified to play an important role in hepatocarcinogenesis [3]. In several recent studies, the CpG islands in the FHIT (fragile histidine triad) promoter region has been demonstrated to be frequently methylated in HCCs, indicating that FHIT may function as a tumour suppressor in the carcinogenesis of liver tissues [3,4]. However, its biological function and mechanism in liver-tissue-derived malignant disease have still not been elucidated clearly.

Previous study has demonstrated that FHIT plays an important role in sustaining the balance of proliferation and apoptosis of tissue homoeostasis [5]. The results from the transgenic
animal model showed that the loss of FHIT could result in malignant diseases, including HCC [6]. In a large-scale detection of multiple types of cancer, it was reported that 60% of primary tumours showed absent or markedly reduced FHIT expression [7]. Moreover, a series of studies showed that the aberrant expression of FHIT existed in the process of hepatocarcinogenesis and it was closely correlated with a significantly higher relapse rate and shorter recurrence time of clinical patients, especially in China [8–10]. Given that the absence or low-level expression of FHIT may be one important cause for carcinogenesis, it is concluded easily that the viral delivery system-mediated expression of FHIT could be an alternative strategy in cancer therapeutics.

Restoration of FHIT by a virus-based gene delivery system has been demonstrated to inhibit growth by arresting cell cycle in G1/S-phase and even induce apoptosis by triggering complicated apoptosis signalling pathways in many types of cancer cells such as breast, lung, prostate, oesophageal, gastric, pancreatic, colorectal, cervical cancer, etc. [7].

Although recombinant viruses have been shown to be very efficient for delivering genes into mammalian cells, they still have limitations for therapeutic implication such as risks of insertional mutagenesis, size limitation of target gene insert and possible immunogenicity [11]. Almost a decade ago, PTD (penetrating transduction peptide)-based delivery was explored to facilitate entry of peptides, proteins, oligonucleotides and even chemotherapeutic agents into mammalian cells in an energy-free manner [11]. Hence manipulation of the HIV-TAT (transactivator of transcription)-derived PTD may be another way to circumvent the virus issue for gene transfer.

Given that the abnormal apoptosis resistance is one characteristic of hepatocellular carcinoma cells and FHIT is a frequently down-regulated molecule in hepatocellular cancer cells [10,12], we hypothesized that the replacement of FHIT could be an alternative strategy to inhibit proliferation and induce apoptosis of hepatocellular cancer cells. In the present study, we purified TAT–FHIT (TAT peptide-fused human FHIT) protein from Escherichia coli. In addition, we used immunofluorescence staining and Western blotting to detect the internalization of TAT–FHIT in hepatocellular cancer cells compared with the purified FHIT protein. We further performed a series of apoptosis-related assays to determine whether TAT–FHIT could induce apoptosis of HCC cells. Moreover, in mechanistic study, by using Western blotting, we analysed the complicated apoptosis signalling pathways triggered by TAT–FHIT, and then used TAT-BH4, a mitochondrial protector, to detect the change of integrity of mitochondrion in the TAT–FHIT-induced apoptosis.

MATERIALS AND METHODS

Cell culture

HepG2 and SMMC-7721 hepatocellular cancer cells were cultured in RPMI 1640 containing 10 mM Hepes (pH 7.5), 2 mM l-glutamine, 5 × 10⁻³ M 2-mercaptoethanol, 1 mM sodium pyruvate, RPMI 1640 amino acids (Sigma) and 10% FBS (fetal bovine serum). Cells were maintained in exponential phase growth in a humidified incubator at 37°C with 5% CO₂/95% air.

Recombinant plasmid constructs

The HIV-TAT sequence was attached to the N-terminus of FHIT by two PCRs with primers P1 and P2 as well as P2 and P3 respectively (forward primer P1: 5′-CTCTGCTGTCACAGGCAGCTGATGGGTGCCGACGTTGC-3′; and P2: 5′-CGCATATGTCAGCTGTAAGAGCTCGTCGTCAAGACGTCGCTG-3′ with an NdeI recognition sequence; reverse primer P3: 5′-CAGTCAGTCAATCCATGCCAGCCAGCAGTGCAGTCTG-3′ with an Xhol recognition sequence). The full-length cDNA of fusion gene TAT–FHIT was identified with NdeI and Xhol cleavage from pMD18T-TAT–FHIT and sequence was subcloned into the expression vector pET32a. In the recombinant pET-32a plasmids, a Trx-tag was fused to the 5′-end of the FHIT or TAT–FHIT protein respectively for the correct folding of the target protein, and a His₆ tag was fused to the 3′-end of the target proteins for the protein purification by using affinity chromatography. Construction procedures of recombinant expression vectors pET32a-TAT–FHIT and pET32a-FHIT are shown in Figure 1(A). pTAT-GFP (green fluorescent protein) vector was constructed as described previously [13].

Expression and purification of recombinant proteins

The recombinant prokaryotic plasmids were transformed into E. coli strain BL21 (DE3) to express the indicated proteins. The bacteria were grown to reach a D₅₆₀ of ~0.5–0.8 prior to induction. To express TAT–FHIT and FHIT, respectively, cells were induced with 0.5 mM IPTG (isopropyl β-D-thiogalactoside) for 5 h. The cells were then harvested, suspended in binding buffer (20 mM Tris/HCl, pH 7.9, and 0.5 M NaCl) and sonicated. The solubilized TAT–FHIT and FHIT fusion protein were purified under native conditions. The fusion proteins were purified by Ni²⁺ affinity chromatography using a chelating Sepharose resin column (Pharmacia). After the column had been washed with 100 mM imidazole in binding buffer, the fusion protein was eluted with 1 M imidazole in binding buffer. The fusion proteins were then concentrated, desalted and buffer exchanged to PBS; the purified fusion proteins were used directly for protein transduction or stored frozen in 10% glycerol at −70°C.

For the transduction of TAT fusion proteins, cells were cultured to 70–80% confluence in 24-well microtitre plates. Before transduction, the culture medium was removed and replaced by 0.5 ml of fresh medium to which TAT fusion proteins at indicated concentrations were then added. After incubation for indicated intervals, the cells were washed with PBS at least four times to avoid possible aggregation of proteins. Cells were then examined directly by fluorescence staining and further used for Western blot analysis and Annexin-V staining assay.

Antibodies for Western blotting and chemical reagents

Protein samples (30 µg each) were separated by SDS/PAGE and then electro-transferred on to nitrocellulose membranes (GE...
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Figure 1 Construction, expression and purification of TAT peptide and FHIT fusion protein in E. coli

(A) Schematic diagram of TAT–FHIT and FHIT coding region. TAT peptides were fused to the N-terminal of FHIT coding region directly. (B) SDS/PAGE analysis of IPTG-induced expression of TAT–FHIT and FHIT proteins in E. coli BL21. M, protein markers; Lane 1, bacterial lysate of uninduced BL21-pET32a-FHIT; Lane 2, bacterial lysate of induced BL21-pET32a-FHIT; Lane 3, bacterial lysate of uninduced BL21-pET32a-TAT–FHIT; Lane 4, bacterial lysate of induced BL21-pET32a-TAT–FHIT. (C) SDS/PAGE analysis of purified TAT–FHIT and FHIT proteins respectively. M, protein marker; Lane 1, supernatant of bacterial lysate of induced BL21-pET32a-FHIT; Lane 2, purified FHIT protein; Lane 3, supernatant of bacterial lysate of induced BL21-pET32a-TAT–FHIT; Lane 4, purified TAT–FHIT protein. (D) Western blot analysis of purified proteins using anti-His antibody. Lane 1, the purified FHIT protein from induced BL21-pET32a-FHIT; Lane 2, the purified TAT–FHIT protein from induced BL21-pET32a-FHIT. (E) Western blot analysis of purified proteins using anti-FHIT antibody. Lane 1, the purified FHIT protein from induced BL21-pET32a-FHIT; Lane 2, the purified TAT–FHIT protein from induced BL21-pET32a-FHIT.

Healthcare. The membranes were allowed to react with primary and secondary antibodies at the optimum dilutions and the immunoreaction signals were detected using an enhanced chemiluminescence kit (Pierce). The scanned images were quantified using Kodak Digital Science one-dimensional software. The antibodies used included FHIT (1:1000 dilution; Cell Signaling Technology), His (1:5000 dilution; Qiagen), β-actin (1:2000 dilution; Sigma), PARP [poly(ADP-ribose) polymerase; 1:500 dilution; Cell Signaling Technology], BID (BH3-interacting domain death agonist; 1:500 dilution; Cell Signaling Technology), Caspase 3, 8 and 9 (1:500 dilution; Cell Signaling Technology). TAT-BH4 peptides, a mitochondrial protector, were purchased from Calbiochem.

Cell viability assay

The cytotoxicity of proteins was determined by standard MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; Sigma] assays as described previously [14]. Briefly, cancer cells were seeded into 96-well plates (2×10^3 cells/well) 24 h before incubation with purified TAT–FHIT protein, and the cell viability was then determined by measuring the absorbance at 570 nm at the indicated time points. Cells treated with PBS or FHIT protein were used as the control. These results are presented as means ± S.D. from two independent experiments.

Annexin-V–FITC staining for apoptosis assay

Cancer cells (3×10^5) were seeded in 35 mm culture dishes with culture medium containing 10% FBS (fetal bovine serum). The following day, the cells were incubated with indicated concentrations of TAT–FHIT or FHIT proteins. At the indicated time points, both floating and attached cells were collected, and the apoptotic cells were detected by Annexin-FITC Apoptosis Detection kit (BD Biosciences). The cells were stained with Annexin-V–FITC according to the supplier’s instructions. Viable and dead cells were detected by flow cytometry.

JC-1 staining for measurement of mitochondrial membrane potential

The fluorescent, lipophilic and cationic probe JC-1 (Beyotime) was employed to measure the mitochondrial membrane potential (ΔΨ_m) of cancer cells according to the manufacturer’s protocol.
Briefly, after indicated treatments, cells were collected and incubated with JC-1 staining solution (5 μg/ml) for 20 min at 37°C. Cells were then rinsed twice with JC-1 staining buffer and the fluorescence intensity of mitochondrial JC-1 monomers (λex = 514 nm, λem = 529 nm) was detected by flow cytometry.

Internalization of the TAT-fused FHIT protein into hepatocellular cancer cells

Cells were plated at 4×10⁵ per well on six-well plates and then treated with purified TAT–FHIT or FHIT proteins. Proteins bound to the cellular surface were removed by incubating with glycine buffer (500 mM NaCl and 0.1 M glycine, pH 2.5) and neutralizing for 5 min with 0.5 M Tris/Cl (pH 7.4), followed by washing with PBS. Immunoblot analysis and immunofluorescence laser scanning microscopy were done as previously described [15].

RESULTS

Expression and purification of TAT–FHIT protein in E. coli

To detect the biological function of FHIT protein in HCC cells, we first generated FHIT protein by using prokaryotic expression system. To directly introduce the purified FHIT protein into cells, we fused an HIV-TAT protein-derived PTD in the N-terminal of FHIT. The fusion protein bearing TAT PTD is known to easily translocate across the plasma membrane [11] and has been useful in treating preclinical models of HCC [16]. Under the optimized condition of IPTG induction, TAT–FHIT and FHIT fusion proteins with a His6 tag were successfully expressed in E. coli transformed with pET32a-TAT–FHIT and pET32a-FHIT respectively (Figure 1A). SDS/PAGE analysis revealed that the significant bands with theoretical molecular mass were induced as shown in Figure 1(B). Solubility analysis showed that both TAT–FHIT and FHIT proteins mainly existed in the supernatant. The recombinant proteins were purified to a single band using Ni²⁺-chelated resin (Figure 1C). Subsequently, Western blot analysis using anti-His6 or anti-FHIT antibody further confirmed the identity of the purified TAT–FHIT and FHIT fusion proteins (Figure 1D).

TAT peptide-mediated internalization of FHIT protein into HCC cells

To demonstrate that the TAT–FHIT protein has a biological activity to transfer into HCC cells, we incubated the purified TAT–FHIT protein with two HCC cell lines respectively and then used immunofluorescence staining to detect whether TAT-peptide could facilitate the passage of FHIT fusion protein into cancer cells. A purified FHIT protein without TAT-peptide was used as a negative control. As shown in Figure 2(A), the result of immunofluorescence staining using a mouse anti-His6 monoclonal antibody revealed that TAT–FHIT fusion protein transduced into cancer cells successfully, whereas the fluorescence signal was absent in cancer cells incubated with the FHIT protein or intact cells.

To further confirm the transduction of TAT–FHIT protein into hepatocellular cancer cells, we incubated the TAT–FHIT and FHIT proteins with HepG2 cells respectively. At 2 h after incubation, the cells were collected and subjected to Western blotting to validate the internalization of TAT–FHIT fusion protein. The results showed that the TAT–FHIT fusion protein could be observed in TAT–FHIT-incubated cells, whereas no signal could be detected in FHIT-incubated cells (Figure 2B). These results suggested that TAT peptide facilitated the transduction of FHIT into hepatocellular cancer cells.

TAT–FHIT inhibited proliferation of human HCC cells by inducing apoptosis of HCC cells

To estimate the effect of TAT–FHIT on viability of HCC cell lines, we added purified TAT–FHIT and FHIT proteins to the cell medium of HepG2 and SMMC-7721 cells for 2 h respectively. And the full cell-culture medium was replaced by the medium containing 10% FBS. As shown in Figure 3(B), TAT–FHIT robustly inhibited the growth of cancer cell lines compared with the FHIT group or mock treatment. We observed the appearance of some HepG2 cells being shrunk, rounded up and detached from plates, indicating the occurrence of cell death. In contrast, the FHIT-incubated or intact cells remained attached to the plates and showed normal morphology (Figure 3A). A similar result was also obtained in SMMC-7721 cells (Figure 3A). This
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Figure 3  Assessment of the cytotoxicity of TAT–FHIT protein on HepG2 and SMMC-7721 hepatocellular cancer cell lines

(A) HepG2 and SMMC-7721 cells were treated with TAT–FHIT or FHIT protein (40 μg/ml) and were observed under an inverted microscope (Olympus BX-60) at 24 h after incubation. Intact cells were used as a control group. (B) Growth curves of TAT–FHIT-incubated cancer cells. Cancer cells were incubated with TAT–FHIT or FHIT protein (40 μg/ml), and then they were assessed by MTT assay at the indicated time points with the mean data from two independent experiments, each carried out in triplicate. Intact cells were used as the control group. (*P < 0.05, **P < 0.01, Student’s t test). (C) Growth curves of TAT–FHIT- or TAT–GFP-incubated cancer cells. HepG2 cells were incubated with TAT–FHIT or TAT–GFP protein (40 μg/ml), and then they were assessed by MTT assay at the indicated time points with the mean data from two independent experiments, each carried out in triplicate. (D) The stability of TAT–FHIT was determined by incubation at 37°C in the cell-free medium. The amount of TAT–FHIT (40 μg/ml) after different incubation periods were determined by MTT assay on HepG2 cells. (E) The t1/2 of TAT–FHIT protein was measured in cancer cells. HepG2 cells were incubated with TAT–FHIT protein (40 μg/ml) at the indicated time points. TAT–FHIT protein was detected by Western blotting. Band intensity was quantified by using Kodak one-dimensional image analysis software and relative expression was normalized by the TAT–FHIT/β-actin ratio. (F) The analysis of Annexin-V staining to assess pro-apoptotic ability of TAT–FHIT in HepG2 and SMMC-7721 cancer cells. At 48 h after incubation with TAT–FHIT or FHIT protein (40 μg/ml), cancer cells were detected by flow cytometry. Intact cells were used as a control group. (G) Analysis of Annexin-V staining to assess pro-apoptotic ability of TAT–FHIT in HepG2 cancer cells with a dose-responsive manner. At 24 h after incubation, cancer cells were detected by flow cytometry. Intact cells were used as a control group.

indicated that TAT–FHIT might inhibit cancer-cell proliferation by inducing apoptosis.

To exclude the non-specific pro-apoptotic effect of TAT–FHIT in hepatocellular cancer cells, we employed a TAT–GFP protein as a control. The PTD domain in TAT–GFP protein could transfer GFP protein into mammalian cells. By performing an MTT assay, we used this protein as a control to determine whether TAT–FHIT protein had a specifically inhibitory effect on the growth of HCC cells. As shown in Figure 3(C), TAT–FHIT protein obviously inhibited the growth of HepG2 cells compared with cells...
of TAT–GFP or mock treatment, while there was no obvious difference between the proliferation of HepG2 cells treated with TAT–GFP and untreated cells. Considering that GFP expression could obstruct the analysis of Annexin-V staining, we did not use this protein as a control for the further study.

The stability of FHIT fusion protein under physiological conditions is required for their potential on the growth of cancer cells. To estimate the stability of TAT–FHIT, we pre-incubated the purified TAT–FHIT protein in cell medium at 37°C for various time periods before testing their biological function determined by MTT assays on HCC cells. The TAT–FHIT protein retained nearly similar activity after incubation in cell medium for up to 24 h (Figure 3D), suggesting that it was stable under these conditions. The analysis of Western blot revealed that the half-life of TAT–FHIT protein in cancer cells was approx. 30 h as shown in Figure 3(E). This result further confirmed that TAT–FHIT was a stable protein in cancer cells and it might exert its inhibitory function on the proliferation of cancer cells for a long time.

To determine the essence of pro-apoptotic function of TAT–FHIT in inhibition of cancer cells, we performed Western blotting to examine the change of apoptosis-associated proteins in HCC cells. The result showed the obvious reduction of pro-caspase 3, a key effector in the downstream of caspase cascade (Figure 4A). And the analysis of Western blot detecting cleaved PARP confirmed the high activation of caspase 3 in TAT–FHIT-incubated cancer cells (Figure 4A).

To further analyse the pro-apoptotic capacity of TAT–FHIT in HCC cells, we performed Annexin-V staining assay to quantitatively measure the percentage of apoptotic cells in TAT–FHIT-treated cells compared with FHIT-treated or intact cancer cells (Figure 3D). The result of Annexin-V–FITC staining revealed that the percentage of apoptotic cells in TAT–FHIT-treated cells was much higher than that in FHIT-treated or intact groups. Moreover, a dose-dependent experiment further confirmed that TAT–FHIT could inhibit the growth of HCC cells by inducing apoptosis (Figure 3E).

**TAT–FHIT-induced apoptosis of HCC cells by triggering both caspase-8- and caspase-9-mediated apoptotic cascades**

According to common knowledge, caspase 8 and caspase 9 represent the functional marker of exogenous and intrinsic apoptotic pathways respectively. To further investigate the detailed mechanism on the pro-apoptotic activity of TAT–FHIT in HCC cells, we used Western blotting to assess the expression pattern of caspase 8 and caspase 9 in HCC cells. In HepG2 cells, the procaspase 8 and procaspase 9 expression levels were decreased in TAT–FHIT-treated cells compared with FHIT-treated cells. Meanwhile, an obvious variation of procaspases expression levels between FHIT-treated and intact cells was not detected (Figure 4B). These suggest that both exogenous and intrinsic apoptotic pathways were involved in TAT–FHIT-induced apoptosis of HCC cells.

**The mitochondrial protector attenuated TAT–FHIT-induced apoptosis in HCC cells**

As we found that both caspase 8 and caspase 9 were cleaved in TAT–FHIT-treated cells and BID, one member of Bcl-2 family, serves as a bridge protein to link exogenous and intrinsic apoptotic pathways, we asked whether tBID (truncated BID) could be detected in TAT–FHIT-treated cells. As shown in Figure 5(A), the obvious cleavage of BID in HepG2 cells was observed in TAT–FHIT-treated cells as compared with FHIT-treated or intact cells. The results indicated that tBID functions in TAT–FHIT-induced apoptosis in HCCs. Given that the formation of tBID resulted from the disruption of the integrity of mitochondrion, we used a mitochondrial protective agent, TAT-BH4, to pre-treat HepG2 cells and further detect the change of apoptotic activation in TAT–FHIT-treated HCCs. As shown in Figure 5(B), the analysis of Annexin-V staining showed that the percentage of apoptotic cells in TAT-BH4-treated group decreased significantly compared with the control group. To further confirm that whether the mitochondrial permeabilization is elicited in TAT–FHIT-treated HCC cells, we performed a JC-1 staining assay to assess the integrity of mitochondrial JC-1 monomers in HepG2 cells compared with that in cells treated with purified FHIT protein, indicating the disruption of mitochondrial integrity. This explained that why mitochondrion-associated Bcl-2 family member BID was cleaved to tBID. All the results demonstrated that the protection of mitochondrial integrity blocked the TAT–FHIT-induced apoptosis and suggested that mitochondria played a critical role in FHIT-mediated apoptosis in HCCs.
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DISCUSSION

The human FHIT gene is located at chromosome 3p14.2, a fragile region frequently perturbed in many types of cancer, including HCC [5,17]. It was reported that obvious reduction or even absence of FHIT existed in approx. 50–65% of HCC cases from China, and it was associated with increasing tumour size and stage [10]. However, a low level expression of FHIT was only detected in a small population of HCC cases from the U.S.A. [18]. A plausible explanation is a low-level exposure to environmental carcinogens in the USA. This is consistent with the data from several established knockout mouse models showing that the loss of FHIT enhanced the sensitivity of multiple organs to carcinogens, indicating that FHIT may function as a defence against environmental carcinogenic agents [19–21]. Even so, there is still no solid evidence to support the notion that the FHIT gene functions in the prevention of hepatocarcinogenesis. Although a recent study has shown that stable expression of FHIT-inhibited proliferation and even induced apoptosis in an HCC cell line, it has been commonly accepted that FHIT restoration by stable transfection in cancer cells has little effect in vitro, unless cells are exposed to stresses such as serum starvation or chemotherapy treatment [22]. Hence it may not be a proper experiment to investigate the biological function of FHIT in HCC cells. The best way to identify the biological effect of FHIT in HCC cells is to transfer FHIT protein into HCC cells directly and detect the response of cancer cells to the ectopic FHIT.

Update of the main studies on the function of FHIT are based on the virus-mediated delivery system to transfer it into cancer cells. Previous studies have also demonstrated that an adenovirus or adeno-associated virus-mediated FHIT replacement could be a promising strategy for the treatment of numerous cancers [23,24]. But to our knowledge, no study has explored the potential of protein-based therapy using FHIT. Given that previous studies have demonstrated that the prokaryotic-expression-derived FHIT protein had the biological enzymatic activity in vitro [25,26], we explored a prokaryotic expression system to obtain the FHIT protein and detect its effect on apoptosis of HCC cells.

Considering that FHIT is a cytoplasmic protein [18], we generated a fusion protein with the TAT-derived PTD domain positioned at the N-terminus of FHIT. Fusion proteins bearing TAT PTD are known to translocate across the plasma membrane and have been useful in the treatment of many malignancies in preclinical models [16]. Since the first time demonstration that the TAT peptide could be used as a potential delivery tool to transduce many biomaterials from the outside to the inside of cultured cells in vitro and even tissue cells in vivo [27], the TAT peptide-based delivery system has been explored widely to transfer protein, nucleotides, chemodrug or even some other molecules with high molecular mass [11]. Recently, a study reported that TAT peptide can be fused with only a functional domain of Met protein and facilitate this domain to exert its effect as well as the entire protein [28]. Zhao’s group [16] even demonstrated that the administration of TAT fusion protein via the tail vein can selectively enrich in tumour tissues and inhibit the growth of human xenograft HCC in nude mice. Here, our observation also showed that TAT–FHIT internalized into HCC cells in 1 h after incubation at 37°C, indicating that our generated TAT–FHIT could be a proper tool to analyse the biological effect of FHIT in hepatocellular cancer cells. And our analysis of apoptosis assay demonstrated that TAT–FHIT was a potential pro-apoptotic molecule in HCC cells.

Although it has been widely accepted that FHIT functions as an oncosuppressor by inducing apoptosis in different types of cancer cells in vitro and in vivo [7], the molecular evidence for how FHIT induces apoptosis of cancer cells has still not been elucidated clearly. In the different cell types, the researchers induced the different or even contracted conclusions in this process. In lung cancer cells, it was reported that the caspase 9-involved intrinsic apoptotic pathway may not be effective in FHIT-overexpressed cells since there was no obvious change in the Bcl-2/Bax ratio, which is regarded as a gold standard to judge the disruption of mitochondrial function in the process of cell death [29]. Meanwhile, the data from Croce’s group [30] showed that caspase-8-triggered exogenous apoptotic pathway did not function in FHIT-mediated apoptosis in breast cancer cells. However, in our experimental
system, we demonstrated that both exogenous and intrinsic apoptotic pathways are involved in FHIT-mediated apoptosis in HCC cells. To further confirm the fidelity of our findings, we detected BID expression, which is recognized as an intracellular link connecting the extrinsic and intrinsic apoptotic pathways. BID is a pro-apoptotic BH3 domain-only member of the Bcl-2 family thought to exert its death-inducing effect by stimulating the release of pro-apoptotic factors such as cytochrome c, AIF (apoptosis-inducing factor) and pro-caspase 9, etc. from mitochondria. Full-length BID in the cytosol is cleaved by activated caspase 8 into a tBID harbouring a C-terminal fragment that translocates into mitochondrion and consequently leads to the release of cytochrome c, resulting in the cascade of apoptosis feedback loop [31,32]. It has been widely accepted that the disruption of exogenous or intrinsic apoptosis signalling pathway could be one obstacle for the therapeutics of apoptosis induction [12]. Previous study has revealed the key role of FHIT in mitochondrial integrity [33]. Mitochondria are at the crossroad of numerous apoptotic pathways that synergize in triggering the morphological transitions underlying the release of pro-apoptotic factors into the cytoplasm [31,32]. Our present study emphasized the implication of TAT–FHIT in the therapeutics of HCC cells.

Taken together, we used the PTD peptide-based TAT–FHIT to demonstrate that FHIT is a potential pro-apoptotic molecule in HCC cells. It can robustly induce apoptosis of HCCs by triggering activation of both exogenous and intrinsic apoptosis pathways. The integrity of mitochondrion plays a critical role in this process. Finally, all of these results strengthen our hypothesis of the therapeutic application of TAT–FHIT against HCC.

AUTHOR CONTRIBUTION
An-Gang Yang and Rui Zhang conceived the project. Gui-Rong Yu and Wei-Wei Qin performed most of the experiments. Ji-Peng Li, Wei Hua and Yan-Ling Meng were responsible for the cell culture. Rui Chen and Bo Yan were responsible for purification of the recombinant proteins. Lei Wang and Xiang Zhang were responsible for the morphological analysis. Lin-Tao Jia, Jing Zhao and Rui Zhang analysed the data. An-Gang Yang and Rui Zhang wrote the paper.

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REFERENCES

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