RESEARCH COMMUNICATION

Aberrant FHIT Expression is Linked to Bladder Carcinogenesis and Apoptosis

Yi Han[1,2], Zhe Zhang[1], Guo-jun Zhang[3] Kun-feng Guo[1], Guang-yi Shan[1], Chui-ze Kong[1*]

Abstract

    The fragile histidine triad gene (FHIT) functions as tumor suppressor in many epithelial cell types. Although the exact mechanisms remain unclear, it is apparent that in its absence, cell cycle homeostasis is often perturbed resulting in the development of soft tissue tumors. Here, we investigated the role of FHIT expression in bladder carcinogenesis and progression using immunohistochemistry. Bladder carcinoma tissue and the 5637 cell line were also studied for FHIT expression by RT-PCR and Western blotting, respectively. FHIT was found to be expressed in carcinoma and adjacent normal tissues at both mRNA and protein levels, but the 17kDa FHIT was lower in tumors (P<0.05), this being confirmed immunohistochemically. There was a negative correlation between FHIT expression and histological grade of bladder transitional cell carcinoma (P<0.05), but no clear relationship with clinical stage or relapse (P>0.05). Overexpression of FHIT could induce apoptosis in bladder carcinoma 5637cells, which could be enhanced by adding adriamycin (ADR). These findings suggest important roles of FHIT in bladder cancer development and provide support for the feasibility of FHIT-based gene therapy.

Keywords: FHIT - bladder carcinogenesis - apoptosis - adriamycin

Introduction

    The bladder is a common site for cancer development in the urinary tract. Urinary bladder cancer ranks ninth in worldwide cancer incidence; it is the seventh most common malignancy in men and 17th in women. In the United States and Western Europe, the lifetime risk is about 1 in 25 and 1 in 80 for white males and females, respectively. Furthermore, approximately 145,000 patients die from this disease worldwide per year (Greenlee et al., 2000; Parkin, 2008; Ploeg et al., 2009). Majority of bladder tumors originate from transitional epithelium or urothelium, a multi-layered epithelium without squamous cells, which covers the inside of this organ (Naik et al., 2011).

    Fragile Histidine Triad (FHIT) gene is located at 3p14.2 (Ohta et al., 1996) and its gene product, FHIT protein, is a diadenosine triphosphate (Ap3A) hydrolase belonging to the histidine triad superfamily of nucleotide-binding proteins (Huang et al., 1995; Barnes et al., 1996). FHIT is a tumor suppressor gene vital to the maintenance of proper cell growth and division. Although the exact mechanism of tumor suppression by FHIT remains unclear, it is apparent that in its absence, cell cycle homeostasis is often perturbed resulting in the development of soft tissue tumors. First identified in digestive tract cancers,

FHIT gene expression was discovered to be absent as a consequence of deletions in the coding region of the gene (Ohta et al., 1996; Smith et al., 2007). Numerous studies have shown the FHIT gene is a frequent target of deletions associated with abnormal RNA and protein expression in primary tumors and cell lines of lung, esophageal, head and neck, cervical and breast cancer (Kastury et al., 1996; Negrini et al., 1996; Virgilio et al., 1996). Tumor suppressor function of FHIT protein is attributable, at least in part, to the induction of apoptosis (Sard et al., 1999, Ji et al., 1999; Roz et al., 2002). Stable exogenous FHIT expression in FHIT-negative lung cancer cells resulted in inhibition of tumor cell growth (Sard et al., 1999). Moreover, transient transfection with adenoviral vectors encoding FHIT protein also inhibited tumorigenicity in nude mice. These findings imply that the loss of FHIT protein is associated with tumorigenesis. This can be the basis for the gene therapy using FHIT in cancer patients. In general, restoring tumor suppressor gene function alone is insufficient to induce apoptosis of all tumor cells. Not all of the cells transferred with FHIT gene and overexpressing FHIT protein are going to die, but some subpopulation of the cells can survive despite FHIT expression. Thus, combination treatments with a tumor suppressor gene and an apoptosis inducer, such as chemotherapeutics, may be required. However, the effect of FHIT expression on

*[1]Department of Urology, The First Hospital of China Medical University, [2]Department of Urology, Shenyang Red Cross Hospital, [3]Department of Hematology, Shengjing Hospital of China Medical University, Shenyang, China   *For correspondence: kongchuize_cmuu@yahoo.com*

apoptosis induced by chemotherapeutic agents and its intracellular mechanism is poorly understood.

Taken together, it is suggested that FHIT protein might have a negative regulation of tumor progression. In this study, to the roles of FHIT expression in the bladder carcinogenesis and subsequent progression, we examined the expression of FHIT mRNA and protein in bladder cancer, the adjacent normal tissues, and carcinoma cell line 5637. Additionally, its expression in tissues was compared to clinicopathological parameters of carcinomas. The effect of FHIT gene expression on ADR-induced apoptosis in bladder cancer cells was also investigated. These results suggest that ADR-induced apoptosis may be enhanced by FHIT expression in bladder cancer cells.

Materials and Methods

Cell culture

The human bladder carcinoma cell line 5637 comes from Cell bank of Chinese Academy of Sciences, Shanghai, China. 5637 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100μg/mL streptomycin, in a humidified atmosphere of 5% CO_2 at 37°C. After overnight culture, cells were treated with 12.5μg/ml of Adriamycine (ADR, Pharmacia) for 24 hours and then were harvested to exam Apoptosis assay.

Construction of the FHIT vector

The eukaryotic expression vector, pCDNA3.1(+), containing a neomycin resistance gene was purchased (Promega). We constructed a plasmid vector expressing FHIT. In brief, a DNA fragment containing the FHIT cDNA was obtained by PCR amplification of the human FHIT cDNA. The PCR-amplified product was introduced into the EcoR V -digested pCDNA3.1(+) vector, and recombinant clones containing the FHIT gene were sequenced (Figure 2A).

Establishment of stably transfected cell lines

5637 cells were transfected with empty vector pCDNA3.1(+) or plasmid pCDNA3.1(+)-FHIT using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Cells were plated in RPMI supplemented with 10% FBS and 400 μg/mL G418 (Gibco-BRL). Individual G418-resistant colonies were isolated after 2 weeks of selection and expanded in the presence of G418. Two stable FHIT-reexpressing clones were isolated, and they were mixed together to eliminate the interclonal bias.

RT-PCR

Total RNA was extracted from 5637 comes or tissues using Trizol (QIAGEN, Germany) according to the manufacturer's protocol. Total RNA was subjected to cDNA synthesis using the AMV transcriptase and random primers (Takara). Oligonucleotide primers for PCR were sense, 5'-TGCTCCTTGCTGCATTGAT-3' and anti-sense, 5'- TGCGTATTCTACTCCCTCTGG-3' for FHIT (149bp, 2025-2173, NM_016373.2), and sense, 5'-AGTTGCGTTACACCCTTTCTTG-3' and anti-sense,

5'- TCACCTTCACCGTTCCAGTTT-3' for β-actin (150bp, 1227-1376, NM_001101.3). PCR conditions were denaturation at 95 oC for 7 minutes, followed by 33 cycles of denaturation at 95 oC for 30 seconds, annealing for 30 seconds, and extension at 72 oC for 30 seconds. As a termination step, the extension time of the last cycle was increased to 10minutes. The amplicons were electrophoresed in 2% agarose gel.

Western blot

Denatured protein was separated on an SDS-polyacrylamide gel and transferred to Hybond membrane (Amersham), which was then blocked overnight in 5% skim milk in TBST. For immunobloting, the membrane was incubated for overnight at 4oC with the rabbit antibody against FHIT (ab53074, abcam, Algeria; 1:1000). Then, it was rinsed by TBST and incubated with anti-rabbit IgG conjugated to horseradish peroxidase (DAKO, Carpinteria, CA93013, USA, 1:1000) for 2 hours. Bands were visualized by ECL-Plus detection reagents (Amersham). After that, membrane was washed with WB Stripping Solution (Pierce) for 15 minutes and treated as described above except anti-tubulin antibody (T-3526, Sigma, 1:1000) as an internal control.

Subjects

Fresh bladder carcinomas and paired normal tissues from the surgical resection were collected from the First Affiliated Hospital. None of patients underwent chemotherapy, radiotherapy treatment before operation. Patients' consent for the research use of tumor tissue was obtained, and the research protocol was approved by Ethical Committee at China Medical University.

Immunohistochemistry

Consecutive sections were deparaffinised with xylene, dehydrated with alcohol, and subjected to antigen retrieval by irradiating in target retrieval solution (TRS, DAKO, USA) for 15 minutes with microwave oven. The sections were quenched with 3% hydrogen peroxide in absolute methanol for 20 minutes to block endogenous peroxidase activity. Five percent bovine serum albumin was then applied for 5 minutes to prevent non-specific binding. The sections were incubated with the rabbit antibody against FHIT (1:100) from Abcam for overnight at 4oC, then treated with the anti-rabbit conjugated to horseradish peroxidase (DAKO) antibodies for 2 hours. After each treatment, the slides were washed with TBST three times for 1 minute. Binding sites were visualized with 3, 3'-diaminobenzidine. After counterstained with Mayer's hematoxylin, the sections were dehydrated, cleared and mounted. Omission of the primary antibody was used as a negative control. In brief, each entire slide was evaluated by light microscopy. First, an intensity score was assigned, which represented the average intensity of positive tumor cells (0, none; 1, weak, 2, intermediate; and 3, strong). Next, a proportion score was assigned, which represented the estimated proportion of positive-staining tumor cells (0, none; 1, < 10%; 2, 10%to 25%; 3, 25% to 50%; 4, 50% to 75%; and 5, > 75%). The proportion and intensity scores were then added to obtain a total score S,

high expression+(10<S<15), low expression±(5<S<9), and none-(0<S<4). All slides were viewed and judged independently by two pathologists according to this multiplied scale at different times without knowledge of clinical outcome and FHIT status.

Colony-forming experiment

100 cells were resuspended in 10mL RPMI 1640 medium, and seeded in the dish and allowed to adhere. After two week, the colony formation can be seen, then terminated culture, and fixed in cold 10mL methanol. The dish with cells was added by Giemsa and incubated at room temperature for 30 minutes. The rate of colony forming (%) = (average clone number / inoculated cell number) × 100%.

Proliferation assay

Cell viability was measured by a 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. MTT solution was added to cells in 96 well plates to the final concentration of 0.5 mg/ml, and cells were incubated at 37°C for 4 hr. After removing culture media, 150 μL of dimethyl sulfoxide (DMSO) was added, and the optical density of each well was read at 590 nm.

Apoptosis assay by flow cytometry

The 5×10^6 cells were collected, washed by PBS twice and fixed in cold 10mL ethanol for overnight. And then, cells were washed by PBS. The tube with cells was added by PI to 50 μg/mL and incubated at 4°C in the dark for 30 minutes. Finally, FACS was employed to examine the PI signal for apoptotic rate.

Acridine orange fluorescence staining to detect cell apoptosis

Single-cell suspension from each group was prepared by concentration. The acridine orange dye 5μl was added into 95μl of cell suspension, and the mixture was checked up on the slide under fluorescence microscope. Fluorescence microscope used in the absorption wavelength of 405nm, emission wavelength of 570nm.

Statistical analysis

Statistical evaluation was performed using Spearman's correlation test to analyze the rank data and Fisher's exact test to compare the different rates. SPSS 10.0 software was applied to analyze all data and P<0.05 was considered statistically significant.

Results

FHIT expression in bladder carcinoma tissue samples and cell line 5637

Among 78 cases of frozen bladder samples, FHIT mRNA level was greater in the adjacent normal tissues than in carcinoma (P<0.05, Figure 1A), and in addition, RT-PCR detected 40 cases (51.3%) for positive expression. 17kDa FHIT protein bands were weaker in carcinoma than matched adjacent normal tissues (P<0.05, Figure 1B), and Western blot results showed that 78 cases of bladder transitional cell carcinoma in 40 cases (51.3%)
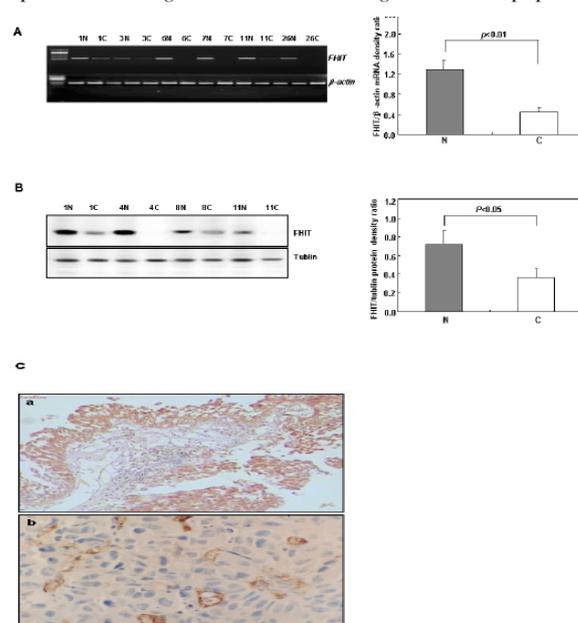

Figure 1. FHIT Expression in Bladder Carcinoma and Matched Normal Tissues. FHIT cDNA was amplified by RT-PCR with β-actin as an internal control and densometric analysis showed FHIT mRNA level was higher in carcinoma than paired normal tissues (p < 0.05, A). There was also an obvious increase of FHIT expression (17kDa) in the tissue lysates of bladder carcinoma compared with matched normal tissues with tubulin (50kDa) as an internal control (p < 0.05, B). FHIT was expressed in carcinoma (a) and paired normal tissues using FHIT antibody by immunohistochemistry (C). Note: N, normal tissues; C, cancer
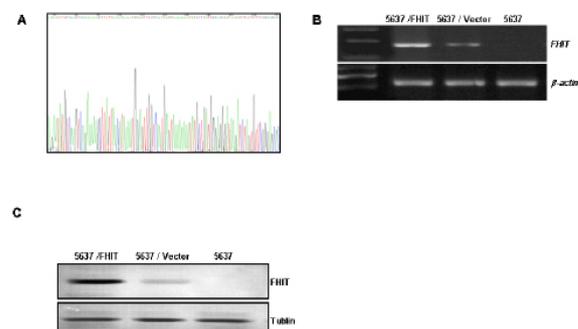

Figure 2. Establishment of pCDNA3.1(+)- FHIT Transfected Cell Line 5637. Recombinant clones containing the FHIT gene were sequenced (A). After transfection of pCDNA3.1(+)- FHIT, FHIT expression became strong in 5637 cells by RT-PCR (B) and Western blot (C).

were positive. The FHIT mRNA and protein was not detected to express in bladder carcinoma cell line 5637 (Figure 2B and 2C).

As shown in Figure 1C, FHIT protein distributed in the cytoplasm of bladder carcinoma cells. FHIT expression was detectable in bladder adjacent normal tissues (88.5%, 23/26), and primary carcinoma (46.2%, 36/78), respectively. According to its frequency and density, the FHIT expression was statistically lower in bladder carcinoma than adjacent normal tissues (P<0.01, Table 1).

The relationship between FHIT expression and clinicopathological parameters of bladder carcinomas

The FHIT expression of bladder cancer was

Table 1. Relationship Between FHIT Expression by IHC and Clinicopathological Features of Bladder Carcinomas

linicopathological features	n	FHIT -	expression by IHC +	PR (%)	p value
Groups					<0.01
Bladder cancer	78	42	36	46.2	
Normal tissue	26	3	23	88.5	
UICC staging					<0.05
I	21	6	15	71.4	
II	33	16	17	51.5	
III	24	20	4	16.6	
Tumor Staging					>0.05
Tis-T1	32	15	17	53.1	
T2-T4	46	27	19	41.3	
Recurrence					>0.05
-		52	25	27	51.9
+		26	17	9	34.6

PR, positive rate; Tis, carcinoma in situ; T1, involvement of the lamina propria and submucosa; T2, muscularis propria and subserosa; T3, serosa; T4, invasion through the serosa; UICC, Union Internationale Contre le Cancer
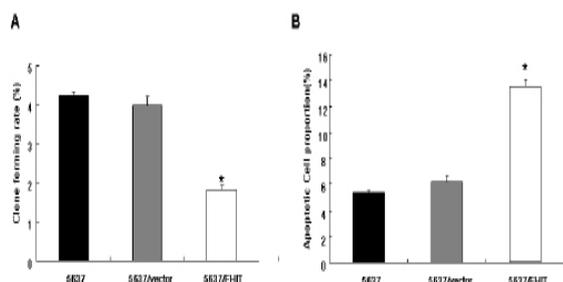

Figure 3. FHIT Overexpression Induces Apoptosis of Bladder Carcinoma Cells. The transfectants showed lower clone forming rate in comparison with control (A). There was an apoptosis-induced effect of FHIT in 5637 transfectant, evidenced by FACS assay (B). Note: * p < 0.05, compared with 5637 and 5637/vector. Results are representative of 3 different experiments, and data is expressed as mean ± SE

significantly lower than normal tissue adjacent to cancer by the use of immunohistochemistry, Western blot, RT-PCR methods. As summarized in Table 1 and Table 2, there was a negative correlation between FHIT expression and UICC staging of bladder carcinomas (P<0.05). In contrast, the relationship of FHIT was not linked to TNM staging of bladder carcinomas or recurrence (P> 0.05).

Effects of FHIT over-expression on growth and apoptosis level of bladder carcinoma 5637cells

After transfected with pcDNA3.1(+)-FHIT, 5637 cells overexpressed FHIT at both mRNA and protein levels (Figure 2B and 2C). There was a lower growth of the FHIT transfectants than the maternal cells, evidenced by Colony-forming experiments (P< 0.05, Figure 3A). The colony forming rate of 5637 cell transfected by pcDNA3.1(+)-FHIT(named 5637/FHIT) was significantly reduced. 5637 Group, 5637/vector group and 5637/FHIT group are separately colony forming efficiency4.22%, 3.96% and1.82%. There was a high level of apoptosis in 5637 after FHIT transfection, evidenced by Acridine orange fluorescence staining (P< 0.05, Figure 3B).

Table 2. Relationship Between FHIT Expression by Western Blot and Clinicopathological Features of Bladder Carcinomas

linicopathological features	n	FHIT expression by Western Blot -	+	PR (%)	p value
Groups					<0.05
Bladder cancer	78	38	40	51.3	
Normal tissue	26	2	24	92.3	
UICC staging					
I	21	6	15	71.4	
II	33	12	21	63.6	
III	24	20	4	16.6	
Tumor Staging					
Tis-T1	32	13	19	59.3	
T2-T4	46	25	21	45.7	

PR, positive rate; Tis, carcinoma in situ; T1, involvement of the lamina propria and submucosa; T2, muscularis propria and subserosa; T3, serosa; T4, invasion through the serosa
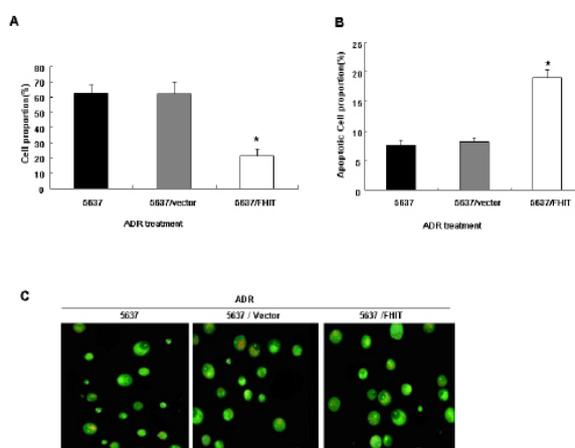

Figure 4. Overexpression of FHIT-induced Apoptosis was Enhanced by ADR in Bladder Carcinoma Cells. Growth inhibition was significantly enhanced in FHIT transfection group than the other two cell lines (A). The apoptosis rate of ADR treatment was significantly increased in FHIT transfection group by FACS assay (B). After ADR treatment, the morphological changes of apoptosis in 5637/FHIT group were more obvious by Acridine orange fluorescence staining (C). Note: * p < 0.05, compared with 5637 and 5637/vector. Results are representative of 3 different experiments, and data is expressed as mean ± SE

ADR enhances overexpression of FHIT-induced apoptosis in bladder carcinoma 5637cells

MTT results showed that 12.5μg/ml adriamycin (ADR) have value-added inhibitory effect for growth in three kinds cells, and cell growth proportion was 62.7% in the 5637 group, 61.9% in 5637/vector group, and 21.8% in 5637/FHIT group. And growth inhibition was significantly enhanced in FHIT transfection group than the other two cell lines (P< 0.05, Figure 4A). The flow cytometry apoptotic rate in each group showed significant increased in FHIT transfection group, compared with the other two control groups (P< 0.05, Figure 4B). After ADR treatment, the fluorescence can be seen under the microscope. Three groups were showed typical apoptotic morphology, cell number reduction, and there were more obvious morphological changes of apoptosis in 5637/FHIT group (Figure 4C)..

Discussion

Here, it was not found that 5637 bladder cancer cells expressed FHIT mRNA and protein. We for the first time examined FHIT expression in bladder carcinoma samples by immunohistochemistry, Western blot or RT-PCR. It was found that FHIT protein was mainly localized in the cytoplasm of bladder carcinoma cells by virtue of their specific histomorphological features and topographic location by immunohistochemistry. Additionally, we detected a negative correlation of FHIT expression with UICC staging of bladder carcinomas. However, we have not found the relationship between FHIT and TNM staging of bladder carcinomas or recurrence.

Gene therapy strategies using tumor suppressor genes have been applied to human cancers (Roth et al., 2003). A body of evidence indicates that FHIT is a well-known commonly mutated tumor suppressor gene in various human cancers, including esophageal, head and neck, cervical and breast cancer and lung cancer. Previous reports have shown that FHIT protein has antitumor activity in vitro and in vivo (Ji et al., 1999; Sard et al., 1999; Roz et al., 2002). Many previous studies have reported that viral FHIT gene delivery resulted in apoptosis in many cancer cell lines (Ji et al., 1999, Ishii et al., 2001, Dumon et al., 2001, Roz et al., 2002, Sevignani et al., 2003). In contrast, we used stably transfected 5637 ladder cancer cells with plasmid vectors encoding FHIT protein. The difference of spontaneous apoptosis between stable FHIT-transfected and mock-transfected cells was obvious. Our results provide evidence that FHIT overexpression could reduce 5637 bladder cancer cells proliferation, and induce apoptosis. This is different from a previous study showing that most stably FHIT-transfected clones do not show altered growth characteristics (Siprashvili et al., 1997). But this contrasts with the findings of another previous study showing that stably FHIT gene-transfected NCI-H460 cells could increase spontaneous apoptosis compared to that of mock-transfected cells(Sard et al., 1999). One factor, which could contribute to this discrepancy, might be different cell lines used in each experiment. However, the mechanisms of its cell-specific characteristics should be further investigated. It has been well documented that FHIT protein enhances paclitaxel-induced apoptosis in lung cancer cells (Kim et al., 2006). Here, we found that ADR-induced apoptosis was enhanced by restoration of FHIT gene function. The finding may imply that FHIT protein is required for enhanced ADR-induced apoptosis. With these studies in mind, it is likely that there are several competing factors involved in the regulation FHIT gene expression and that each may influence FHIT transcription and transcript stability to different degrees based on cell type and lineage.

To the best of our knowledge, this study is the first to describe the possible role of FHIT protein in enhanced ADR-induced apoptosis of bladder cancer cells. However, our data do not demonstrate a direct connection between modulations of FHIT signaling. These require further detailed study.

Acknowledgements

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