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• LIVER CANCER •

Expression of fragile histidine triad in primary hepatocellular carcinoma and its relation with cell proliferation and apoptosis

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Abstract

AIM: To evaluate the expression of fragile histidine triad (FHIT) gene protein, product of a candidate tumor suppressor, and to investigate the relationship between FHIT, cell apoptosis and proliferation, and pathological features of primary hepatocellular carcinoma (HCC).

METHODS: Forty-seven HCC and ten normal liver specimens were collected during surgical operation between 2001 and 2003. FHIT and proliferating cell nuclear antigen (PCNA) expression were detected by immunohistochemistry, and apoptotic level was evaluated by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay on the tissue sections.

RESULTS: All normal liver tissues showed a strong expression of FHIT, whereas 28 of 47 (59.6%) carcinomas showed a significant loss or absence of FHIT expression ($P = 0.001$). The proportion of reduced FHIT expression in those carcinomas at stages III-IV (70.6%) and in those with extrahepatic metastasis (86.7%) showed an increasing trend compared with those at stages I-II (30.8%, $P = 0.013$) and those without metastasis (46.9%, $P = 0.010$) respectively. Apoptotic incidence in advanced TNM stage carcinoma and those with positive FHIT expression was higher than that in early stage carcinoma ($P = 0.030$) and in those with negative FHIT expression ($P = 0.044$) respectively. The proliferating potential of hepatocellular carcinoma was associated with FHIT expression ($P = 0.016$) and the aggressive feature ($P = 0.019$). Kaplan-Meier analysis demonstrated that the survival time of these 47 patients correlated with TNM stage, FHIT expression and metastasis.

CONCLUSION: There is marked loss or absence of FHIT expression, as well as abnormal apoptosis-proliferation balance in HCC. FHIT may play an important role in carcinogenesis and development of HCC.

Key words: Hepatocellular carcinoma; Fragile histidine triad protein; Cell proliferation; Apoptosis

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INTRODUCTION

Fragile histidine triad gene has been cloned and is located on 3p14.2[1], which encompasses the most common human fragile site, FRA3B. Alterations of FHIT and loss of its product have been found frequently in several human tumors and tumor-derived cell lines associated with environmental carcinogens. Disturbance of cell number regulation is one of the characteristics of malignant tumors, which could lead to tumor cell proliferation out of control. We have known that a variety of oncogenes, tumor suppressor genes and some modifiers are involved in the regulation mechanism[2]. Human hepatocellular carcinoma is a familiar lethal cancer which is closely related to some carcinogens such as hepatitis B virus infection, dietary aflatoxin and alcohol consumption. It is imperative to speculate on whether FHIT, as a putative tumor suppressor gene, plays a role in the development of hepatocellular carcinoma by participating in the process of apoptosis or cell cycle. From this study, the indices of apoptosis and cell proliferation showed a certain association with the aberrant FHIT expression in hepatocellular carcinoma, which may elucidate one aspect of carcinogenesis of malignant tumors.

MATERIALS AND METHODS

Materials

Forty-seven liver cancer specimens and ten normal liver specimens as controls were obtained from surgical resections in the First Hospital of Xi'an Jiaotong University during 2001 to 2003. The patients included 38 men and 9 women with a mean age of 48.62±10.99 years (range 29-77 years). The pathological types of all specimens were confirmed tobe hepatocellular carcinoma by pathologists in Pathology Department of the First Hospital of Xi'an Jiaotong University. Of these patients, 39 were at grades I and II, 8 at grade III according to Edmondson grading and local invasion or extrahepatic metastasis was observed in 15; and 13 were at stages I-II, 34 at stages III-IV according to the pTNM criteria of UICC. The follow-up for all cases was terminated in April of 2004.

Methods

All surgical specimens were fixed in 10% formaldehyde, embedded in paraffin and cut into 4-μm thick sections. One section of each specimen was stained with H&E and used for histological identification, and the rest were used for immunostaining.

Immunohistochemical analysis for FHIT and PCNA

Slides were deparaffinized in xylene twice for 10 min, rehydrated through graded ethanol to distilled water, incubated for 15 min with 3% hydrogen peroxidase to inhibit endogenous peroxidase activity, and then heated in 0.01 mol/L citrate buffer (pH 6.0) in a microwave oven for 5 min at 100 ℃ for antigen retrieval. After cooled down at room temperature for 30 min, the sections were incubated for 15 min in a blocking solution containing 10%

normal goat serum in PBS [0.01 mol/L phosphate (pH 7.4)] and then incubated for 1 h at 37 ℃ in a humidified chamber with rabbit polyclonal antibody to human FHIT (Zymed Laboratories Inc., South San Francisco, CA) at a dilution of 1:50, followed by incubation for 30 min with goat anti-rabbit IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology). 3,3'-diaminobenzidine was used as the chromogen. Slides were counterstained for 3 min with hematoxylin solution, then dehydrated and coverslipped. Normal liver tissue was used as a positive control, whereas the primary antibody was replaced by PBS for a negative control. PCNA was detected by mouse monoclonal antibody to human PCNA (1:50, Santa Cruz Biotechnology).

Apoptosis detection in situ with TUNEL

Paraffin-embedded sections on polylysine-coated slides were deparaffinized and rehydrated as described in immunohistochemistry method and then incubated for 15 min with 40 mg/L trypsin at 37 ℃, rinsed in PBS and incubated for 1 h at 37 ℃ with TUNEL reaction mixture (buffer solution mixed with terminal deoxynucleotidyl transferase and nucleotide labeled solution; Roche, Germany), rinsed again in PBS and incubated for 30 min at 37 ℃ with converter-AP. 5-bromo-4-chloro-3-indalylphosphate/nitro blue tetrazolium (BCIP/NBT) was used as the chromogen. Slides were counterstained for 5 min with nuclear fast red solution, then dehydrated and coverslipped.

Evaluation of scores

Cells filled with yellow or brown granules in cytoplasm were considered as positive immunostained cells. Both the extent and intensity of immunostaining were considered when FHIT protein expression of a section was scored according to Hao *et al*[3]. The extent of positivity was scored as follows: 0, <5%; 1, 5-25%; 2, 25-50%; 3, 50-75%; and 4, >75% of hepatocytes in respective lesions. The intensity was scored as follows: 0, negative; 1, weak; 2, moderate; and 3, as strong as in normal hepatocytes. The final score was obtained by multiplying the extent of positivity and intensity scores in the range of 0-12. Scores 8-12 were defined as strong staining, scores 0-6 as markedly reduced or lost expression.

PCNA positively immunostained cells were filled with brown granules in nuclei. The apoptotic cells were stained blue in nuclei, whereas the normal nuclei were stained pink. Regardless of the extent or intensity of the PCNA and apoptosis staining, the positive cells were observed and calculated under a light microscope in 5 high power fields (×400). Proliferation index (PI) and apoptosis index (AI), expressed as the ratio of positively stained cells to total cells of the fields, were used to evaluate the proliferation and apoptosis features respectively.

Statistical analysis

Pearson chi-square test and Fisher's exact test (two sided) for trends in proportions were used to assess the association between FHIT expression and pathological indices. Student's *t*-test was adopted to determine the difference between two sample means. Survival time was analyzed by Kaplan-Meier method (SPSS 11.0 for windows). $P<0.05$ was considered statistically significant.

RESULTS

FHIT expression in normal tissues and HCC

All the 10 normal liver tissues and para-neoplastic tissues showed a strong FHIT expression in the cytoplasm of hepatocytes (Figure 1A). Some lymphocytes and fibroblasts were positively stained, also. FHIT was expressed in 19 carcinomas as strongly as or more strongly than in normal, whereas it was expressed negatively in 28 of 47 (59.6%) carcinomas (Figure 1B). The absence of FHIT expression in carcinoma was significant ($\chi^2=11.709$, $P=0.001$).

Relationship between FHIT expression and clinicopathological indices

The proportion of reduced FHIT expression in carcinomas at stages III-IV was significantly higher (24/34) than that at stages I-II (4/13) ($P=0.013$). This proportion in carcinomas with extrahepatic metastasis (13/15) was higher than that in those without metastasis (15/32). No evidence indicated the relation between FHIT expression and other clinicopathological features such as age, sex, histological grade and tumor size (Table 1).

Table 1 Relationship between FHIT expression and clinicopathological indices of HCC

	n	FHIT score		*P*
		-	+	
Age (yr)				
≤45	18	10	8	0.658
>45	29	18	11	
Sex				
Male	38	21	18	0.278
Female	9	7	2	
Histological grade				
I-II	39	21	18	0.119
III	8	7	1	
TNM stage				
I-II	13	4	9	0.013
III-IV	34	24	10	
Tumor size (diameter)				
<50 mm	19	12	7	0.680
≥50 mm	28	16	12	
Extrahepatic metastasis				
Positive	15	13	2	0.010
Negative	32	15	17	

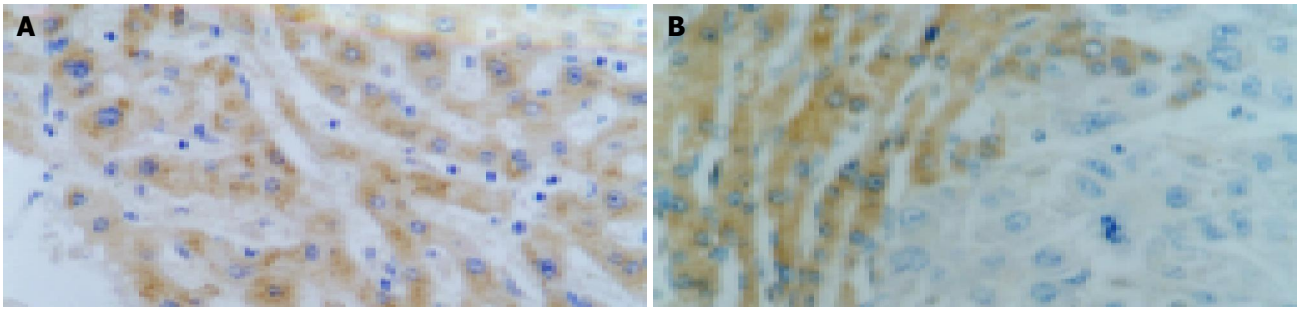

Figure 1 FHIT expression in normal tissues and HCC. A: Yellow granules in normal liver tissues, and para-neoplastic tissues; B: non-stained cytoplasm of tumor cells.

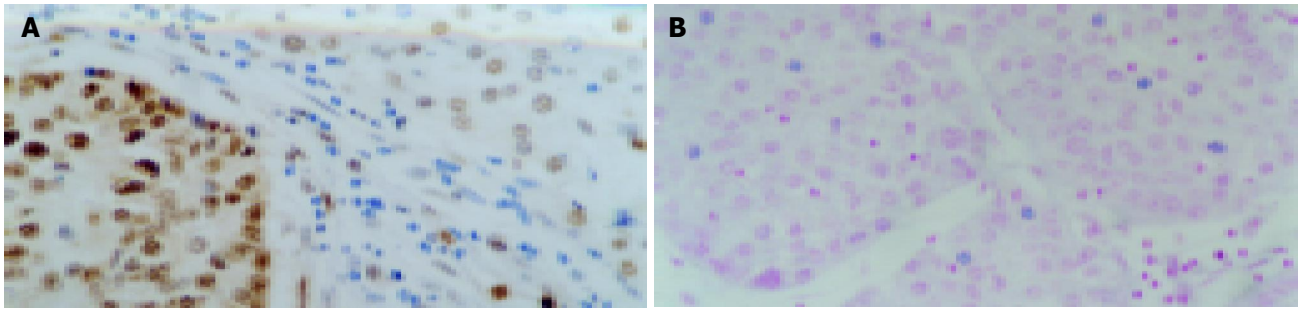

Figure 2 Expression of PCNA in normal and cancer tissues. A: Positive PCNA expression manifested brown granules in nuclei. B: nuclei of apoptic cells were stained blue but normal nuclei were stained pink.

Table 2 Relationship between PI, AI and clinicopathological indices (mean±SD)

	N	AI (%)	*P* value	PI (%)	*P* value
FHIT expression					
Positive	19	9.42±2.85	0.044	28.47±4.22	0.016
Negative	28	8.07±1.63		31.61±4.24	
Histological grade					
I-II	39	8.87±2.38	0.091	30.46±4.46	0.686
III	8	7.38±1.19		29.75±4.74	
TNM stage					
I-II	13	7.46±2.30	0.030	28.85±3.85	0.158
III-IV	34	9.06±2.15		30.91±4.60	
Tumor size (diameter)					
<50 mm	19	8.11±2.03	0.208	29.79±4.85	0.492
⩾50 mm	28	8.96±2.41		30.71±4.23	
Extrahepatic metastasis					
Positive	15	9.00±1.89	0.436	32.53±3.93	0.019
Negative	32	8.44±2.45		29.31±4.38	

Proliferation and apoptosis features in HCC

PCNA was localized in nuclei. Positive PCNA expression manifested brown granules in nuclei (Figure 2A). Nuclei of apoptotic cells were stained blue but normal nuclei were stained pink (Figure 2B).

Table 2 summarizes the relationship between AI, PI and clinicopathological indices. AI showed a similar association with TNM stage and FHIT expression, i.e., those HCCs at stages III-IV and those with positive FHIT expression showed a higher AI compared with those at stages I-II and those with negative FHIT expression ($P<0.05$) respectively. PI showed a relationship with FHIT expression and metastasis ($P<0.05$).

Survival analysis

The survival time of the forty-seven patients undergoing surgical resection varied from 1 to 34 mo. Patients with positive FHIT expression, or those at stages I-II, or those without metastasis had a longer survival time as shown by Kaplan-Meier analysis (data not shown).

DISCUSSION

Unregulation of cell proliferation homeostasis results in unlimited proliferation of malignant cells, which would enlarge the tumor size and cause cancer. However, its opposite antagonistic way, apoptosis, determines the cell death. Apoptosis is an efficacious approach to protect human beings from adverse carcinogens which may induce mutation or canceration. PCNA is an auxiliary protein for DNA polymerase delta, which could perform an essential function in DNA replication and repair process[4]. The amount of PCNA mRNA varies with DNA synthesis cycle[5], and reflects the proliferating activity of cancer cells. Thus, it has been regarded as a proliferation marker for cancer cells[6,7]. The PCNA labeling index is one useful marker for evaluating the malignant feature of tumors, and for predicting recurrence and the prognosis of patients[8].

Apoptosis with necrosis, plays an important role in the cell life including cell growth, differentiation and proliferation[9]. It is a regulation mechanism to maintain the homeostasis of tissues. Malignant tumor tissues lose this regulation and obtain infinite proliferating potential. There is a controversy concerning the relationship between proliferation and apoptosis in cancers[10-12]. Our study showed a significant association between PI, AI and some clinicopathological indices of HCC, such as TNM stage and extrahepatic metastasis.

FHIT gene spans the most active common fragile site in the human genome, FRA3B, which is susceptible to inactivation by environmental carcinogenic factors[13], such as hepatitis B virus infection, dietary aflatoxin and alcohol consumption[14]. Aberrant FHIT expression has been detected in other tumors, including lung[15], esophageal[16], stomach[17], colorectal[18], cervical[19], and prostate[20] carcinomas. Our research demonstrated a high proportion (59.6%) of reduced FHIT expression in HCC, compared with positive expression in all 10 normal liver tissues, as reported previously[14]. Moreover, the aberrant FHIT expression was correlated with TNM stage and extrahepatic metastasis of HCC. The patients with positive FHIT expression showed a longer survival time than those with negative expression. Some findings indicate that loss of FHIT expression might be an early event in the development of human carcinoma[16,21]. Therefore, detection of FHIT expression in malignant tissues by immunohistochemistry may provide some important diagnostic and prognostic information[22].

FHIT gene consists of 10 exons and encodes FHIT protein (M_r 16 800), which consists of 147 amino acids. FHIT protein is a member of histidine triad superfamily and a diadenosine 5', 5'-P1, P3-triphosphate (Ap3A) asymmetrical hydrolase which cleaves Ap3A into adenosine 5'-diphosphate and AMP[23]. Siprashvili *et al*[24] designed a structure similar to FHIT, in which

the middle histidine in histidine triad was replaced by asparagine. This artificial product significantly lost its enzymatic activity but still maintained tumor suppressor function. It has been found that hydrolysis for Ap3A is not required for tumor suppression of FHIT. Researches on the tumor suppression mechanism of FHIT indicate the active suppressor of FHIT might function in the form of FHIT-substrate complex[25,26]. Our study showed a higher AI but a lower PI in carcinomas with positive FHIT expression, suggesting that FHIT might play a role in the regulation of cell proliferation and apoptosis. The conceivable mechanism is that the FHIT-substrate binding complex participates in signal transduction and induces accumulation of cells at S to G2-M phase, and then these arrested cells are cleaned out in manner of apoptosis. This hypothesis is supported by some molecular experiments[27,28]. Furthermore, some more detailed researches have revealed that FHIT-induced apoptosis is practiced in a p53-independent apoptotic pathway[29,30]. This may provide a new method and opportunity in gene therapy for malignant tumors.

Carcinogenesis of HCC is a multi-sequential process involving various oncogenes and tumor suppression genes. Inactivation of FHIT may play a role in the development and progression of HCC. FHIT as a valuable target in gene therapy has a bright future.

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