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

Ke-Jun Nan, Zhi-Ping Ruan, Zhao Jing, Hai-Xia Qin, Hong-Yan Wang, Hui Guo, Rui Xu

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 without 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 to be 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 °C 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 °C 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 °C, rinsed in PBS and incubated for 1 h at 37 °C 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 °C with converter-AP. 5-bromo-4-chloro-3-indolyphosphate/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**

**FHint 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 (χ² = 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)                 |     |            |      |
| ≤50                      | 18  | 10         | 8    | 0.658 |
| >50                      | 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 FHint expression in normal tissues and HCC. A: Yellow granules in normal liver tissues, and para-neoplastic tissues; B: non-stained cytoplasm of tumor cells.
Synthesis cycle for evaluating the malignant feature of tumors, and for process performing an essential function in DNA replication and repair is an auxiliary protein for DNA polymerase delta, which could carcinogens which may induce mutation or canceration. PCNA is an efficacious approach to protect human beings from adverse antagonistic way, apoptosis, determines the cell death. Apoptosis with necrosis, plays an important role in the cell life including cell growth, differentiation and proliferation. The amount of PCNA mRNA varies with DNA life including cell growth, differentiation and proliferation. Apoptosis with necrosis, plays an important role in the cell life including cell growth, differentiation and proliferation. The amount of PCNA mRNA varies with DNA life including cell growth, differentiation and proliferation. Apoptosis with necrosis, plays an important role in the cell life including cell growth, differentiation and proliferation. The amount of PCNA mRNA varies with DNA life including cell growth, differentiation and proliferation. Apoptosis with necrosis, plays an important role in the cell life including cell growth, differentiation and proliferation. The amount of PCNA mRNA varies with DNA life including cell growth, differentiation and proliferation. Apoptosis with necrosis, plays an important role in the cell life including cell growth, differentiation and proliferation. The amount of PCNA mRNA varies with DNA life including cell growth, differentiation and proliferation. Apoptosis with necrosis, plays an important role in the cell life including cell growth, differentiation and proliferation. The amount of PCNA mRNA varies with DNA life including cell growth, differentiation and proliferation. Apoptosis with necrosis, plays an important role in the cell life including cell growth, differentiation and proliferation. The amount of PCNA mRNA varies with DNA life including cell growth, differentiation and proliferation. Apoptosis with necrosis, plays an important role in the cell life including cell growth, differentiation and proliferation. The amount of PCNA mRNA varies with DNA life including cell growth, differentiation and proliferation. Apoptosis with necrosis, plays an important role in the cell life including cell growth, differentiation and proliferation. The amount of PCNA mRNA varies with DNA.
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-induced apoptosis is practiced in a p53-independent apoptotic pathway[27,28]. Furthermore, some more detailed researches have revealed that FHIT-induced apoptosis correlates with mismatch repair deficiency in human advanced colorectal carcinoma. Br J Cancer 2002; 86: 441-445.

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 for malignant tumors.

REFERENCES

1. Ohm A, Inoue H, Cotticelli MG, Kastury K, Baffa R, Palazzo J, Sprichavili Z, Mori M, McCue P, Druck T, Croce CM, Huebner K. The FHIT gene, spanning the chromosome 3p14.2 fragile site and renal carcinoma-associated t(3:8), breakpoint, is abnormal in digestive tract cancers. Cell 1996; 84: 875-879.
2. Qin LX, Tang ZY. The prognostic molecular markers in hepatocellular carcinoma. World J Gastroenterol 2002; 8: 385-392.
3. Hao XP, Willis JE, Pretlow TG, Rao JS, MacLennan GT, Talbot IC, Pretlow TP. Loss of fragile histidine triade expression in colorectal carcinomas and premalignant lesions. Cancer Res 2000; 60: 18-21.
4. Morris GF, Bischoff JR, Mathews MB. Transcriptional activation of the human proliferating-cell nuclear antigen promoter by p53. Proc Natl Acad Sci USA 1996; 93: 895-899.
5. Morris GF, Mathews MB. Regulation of proliferating cell nuclear antigen during the cell cycle. J Biol Chem 1989; 264: 13856-13864.
6. Hall PA, Levison DA, Woods AL, Yu CC, Kellock DB, Watkins JA, Barnes DM, Gillett CE, Camplejohn R, Dover R. Proliferating cell nuclear antigen (PCNA) immunolocalization in paraffin sections: an index of cell proliferation with evidence of deregulated expression in some neoplasms. J Pathol 1990; 162: 285-294.
7. Weber JC, Nakano H, Bachellier P, Oussoultzoglou E, Inoue K, Shimura H, Wolf P, Chenard-Neu MP, Jaek D. Is a proliferation index of cancer cells a reliable prognostic factor after hepatectomy in patients with colorectal liver metastases? Am J Surg 2001; 182: 81-88.
8. al-Sheneber IF, Shibata HR, Sampalis J, Jothy S. Prognostic significance of proliferating cell nuclear antigen expression in colorectal cancer. Cancer 1993; 71: 954-1959.
9. Kerr JF. History of the events leading to the formulation of the apoptosis concept. Toxicology 2002; 181-182: 471-474.
10. Park YN, Chae KJ, Kim YB, Park C, Theise N. Apoptosis and proliferation in hepatocarcinogenesis related to cirrhosis. Cancer 2001; 92: 2733-2738.
11. Hino N, Higashi T, Nouso K, Nakatsukasa H, Tsuji T. Apoptosis and proliferation of human hepatocellular carcinoma. Liver 1996; 16: 123-129.
12. Paiva C, Oshima CT, Lanzoni VP, Forones NM. Apoptosis, PCNA and p53 in hepatocellular carcinoma. Hepatogastroenterology 2002; 49: 1058-1061.
13. Nelson HH, Wiecneck JK, Gunn L, Wain JC, Christiani DC, Kelsey KT. Chromosome 3p14 alterations in lung cancer: evidence that FHIT exon deletion is a target of tobacco carcinogens and asbestos. Cancer Res 1998; 58: 1804-1807.
14. Leung ZZ, Keck-Waggoner C, Zimonjic DB, Thorgersson SS, Popescu NC. Alterations of the FHIT gene in human hepatocellular carcinoma. Cancer Res 2000; 60: 1049-1053.
15. Mascacu M, Martin B, Verdelou J, Meert AP, Ninane V, Sculier JP. Fragile histidine triade protein expression in nonsmall cell lung cancer and correlation with Ki-67 and with p53. Eur Respir J 2003; 21: 753-758.
16. Moro M, Mimori K, Sharshire T, Alder H, Inoue H, Tanaka Y, Sugimachi K, Huebner K, Croce CM. Altered expression of Fhit in carcinoma and precancerous lesions of the esophagus. Cancer Res 2000; 60: 1177-1182.
17. Rocco A, Schandl L, Chen J, Wang H, Tulaszay Z, McMamara D, Malfertheiner P, Ebert MP. Loss of FHIT protein expression correlates with disease progression and poor differentiation in gastric cancer. J Cancer Res Clin Oncol 2003; 129: 84-88.
18. Andachi H, Yashima K, Koda M, Kawaguchi K, Kitamura A, Hosoda A, Kishimori Y, Shiota O, Ito H, Makino M, Kailbra N, Kawasaki H, Murawaki Y. Reduced Fhit expression is associated with mismatch repair deficiency in human advanced colorectal carcinoma. Br J Cancer 2002; 87: 441-445.
19. Takizawa S, Nakagawa K, Nakagawa K, Yasugi T, Fujii T, Kugy U, Tano Y, Yoshikawa H, Taketani Y. Abnormal Fhit expression is an independent poor prognostic factor for cervical cancer. Br J Cancer 2003; 88: 1213-1216.
20. Fouts RL, Sandusky GE, Zhang S, Eckert C, Koch MO, Ulbright TM, Eleb RN, Chong L. Down-regulation of fragile histidine triade expression in prostate carcinoma. Cancer 2003; 97: 1447-1452.
21. Skopolitou AS, Mitselou A, Katsanos K, Alexopoulou V, Tsianos EV. Immunohistochemical expression of Fhit protein in Helicobacter pylori related chronic gastritis, gastric precancerous lesions and gastric carcinoma: correlation with conventional clinicopathologic parameters. J Gastroenterol Hepatol 2003; 15: 515-523.
22. Croce CM, Sozzi G, Huebner K. Role of FHT in human cancer. J Clin Oncol 1999; 17: 1618-1624.
23. Barnes LD, Garrison PN, Sprichavili Z, Guranowski A, Robinson AK, Ingram SW, Croce CM, Ohm A, Huebner K. Fhit, a putative tumor suppressor in humans, is a dinucleoside 5', 5''-P1, P3-triphosphate hydrolase. Biochemistry 1996; 35: 11529-11535.
24. Siprichavili Z, Sozzi G, Barnes LD, MacLennan GT, Robinson AK, Eryomin V, Sard L, Tagliabue E, Grecro A, Fusetti L, Schwartz G, Pierotti MA, Croce CM, Huebner K. Replacement of Fhit in cancer cells suppresses tumorigenicity. Proc Natl Acad Sci USA 1999; 96: 13771-13776.
25. Pace HC, Garrison PN, Robinson AK, Barnes LD, Draganescu A, Rosler A, Blackburn GM, Siprichavili Z, Croce CM, Huebner K, Brenner C. Genetic, biochemical, and crystallographic characterization of Fhit-substrate complexes as the active signaling form of Fhit. Proc Natl Acad Sci USA 1998; 95: 5484-5489.
26. Trappasso F, Krakowiak A, Cesari R, Arkles J, Vendamuri S, Ishii H, Vecchione A, Kuroki T, Bieganowski P, Pace HC, Huebner K, Croce CM, Brenner C. Designed FHIT alleles establish that Fhit-induced apoptosis in cancer cells is limited by substrate binding. Proc Natl Acad Sci USA 2003; 100: 1592-1597.
27. Dunmon K, Ishii H, Vecchione A, Trappasso F, Baldassarre G, Chakrani F, Druck T, Rosato EF, Williams NN, Baffa R, During MJ, Huebner K, Croce CM. Fragile histidine triade expression delays tumor development and induces apoptosis in human pancreatic cancer. Cancer Res 2001; 61: 4827-4836.
28. Ji L, Fang B, Yen N, Fong K, Minna JD, Roth JA. Induction of apoptosis and inhibition of tumorigenicity and tumor growth by adenovirus vector-mediated fragile histidine triade (FHIT) gene overexpression. Cancer Res 1999; 59: 3333-3339.
29. Sard L, Accornero P, Tornielli S, Delia D, Bunone G, Campiglio M, Colombo MP, Gramena M, Croce CM, Pierotti MA, Sozzi G. The tumor-suppressor gene FHIT is involved in the regulation of apoptosis and in cell cycle control. Proc Natl Acad Sci USA 1999; 96: 8489-8492.
30. Gopalakrishnan VK, Banerjee AG, Vishvanatha JK. Effect of FHIT gene replacement on growth, cell cycle and apoptosis in pancreatic cancer cells. Pancreatology 2003; 3: 293-302.