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Evaluation of Relationship between Occurrence of Liver Cancer and Methylation of Fragile Histidine Triad (FHIT) and P16 Genes

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

C **Yu Bai**
AB **Yunzhi Shen**
DE **Qiang Yuan**
F **Chengyu Lv**
FG **Qianzhe Xing**

Department of Hepatobiliary Surgery, Third Central Hospital of Tianjin (Tianjin Institute of Hepatobiliary Disease, Tianjin Key Laboratory of Artificial Cell, Artificial Cell Engineering Technology Research Center of Public Health Ministry), Tianjin, P.R. China

Corresponding Author: Yunzhi Shen, e-mail: YunzhiShen2wn@163.com
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Background: We examined the level of fragile histidine triad (FHIT) and p16 gene methylation in patients with hepatocellular carcinoma and explored the relationship to liver cancer.





Material/Methods: There were 56 patients with primary liver cancer who were admitted to the hospital from July 2015 to October 2017 included in the liver cancer group, and 24 non-hepatoma patients (hepatitis A/hepatitis B/hepatitis C, liver cirrhosis, liver fibrosis, and fatty liver, alcoholic liver identified as a control group. Fasting venous blood samples were collected from the 2 groups. Methylation-specific PCR (MSP) was used to detect the methylation of FHIT and p16 genes in the 2 groups. The risk factors for lung cancer were analyzed by logistic regression. In addition, the effects of FHIT and p16 gene methylation on the diagnostic accuracy of liver cancer were calculated.

Results: The incidence of FHIT and p16 methylation in serum from the liver cancer group was 51.8% and 67.9%, respectively. The incidence of FHIT and p16 methylation in the non-hepatoma group was 16.7% and 25.0%. There was a statistical statistically correlated with gender, and the methylation of FHIT and p16 genes ($P < 0.05$). From logistic regression analysis results, methylation of p16 and FHIT genes was an independent risk factor for hepatocellular carcinoma with odds ratio (OR) values of 10.550 (2.313~48.116) and 8.239 (2.386~28.455), respectively.

Conclusions: The methylation of p16 and FHIT genes was an independent risk factor for hepatocellular carcinoma.

MeSH Keywords: **Liver Neoplasms • Methylation • Vitamin D-Binding Protein**

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Background

Hepatocellular carcinoma (HCC) is the third most common cause of cancer death and ranks sixth among the most common malignant diseases and is prevalent in Asia [1]. The incidence of this disease is increasing year by year [2]. According to GLOBOCAN estimates, nearly 782 500 new cases of HCC were diagnosed worldwide in 2012, and about 745 500 patients died of liver cancer. Although the diagnosis and treatment of HCC has greatly improved in recent years, the exact pathogenesis of HCC has not been fully elucidated from the onset of HCC to further development of the disease.

A large number of studies have reported that HCC is related to gene mutation. Epigenetic abnormalities also play a potential role in the pathogenesis of HCC [3]. The activity of tumor suppressor genes and oncogenes has been confirmed as initiating tumorigenesis. Ohta et al. proposed a new tumor suppressor gene in 1996 [4], the fragile histidine triad (FHIT) gene. The decrease of the expression of FHIT protein or even more serious loss of expression, can lead to a decrease of tumor suppressor effect and serious direct loss. It is also highly homologous with histidine triad protein, distributed in most of the normal tissues, across the site of FRA3B, located in the 3q14.2 region of the chromosome. FHIT can block the cell growth cycle and induce cell apoptosis to inhibit the proliferation of tumor cells. The tumor suppressor gene p16 was found in different tumor cell lines in 1994, and the tumor suppressor gene 1 (multiple tumor suppressor-1, MTS1), located on the 9p21 region of chromosome 9, was involved in the regulation of cell cycle, cell growth, and differentiation [5]. When the deletion, methylation, or mutation of FHIT and p16 genes causes the protein to be unable to be expressed, it will cause cell growth to lose control and then cancer is produced in the cell. Therefore, FHIT and p16 genes may be targets in the process of liver cancer; they are valuable markers for diagnosis, treatment, and prognosis.

In this study, in order to investigate the status of FHIT and p16, methylation specific PCR (MSP) was used to detect the methylation of FHIT and p16 genes in the plasma of liver cancer patients and control patient groups to explore the relationship between these 2 genes and liver cancer.

Material and Methods

Twenty-four non-liver cancer patients (hepatitis A (HAV)/HBV/HCV, cirrhosis, liver fibrosis, fatty liver and alcoholic liver) were selected as the control group. In the liver cancer group, 56 patients were included. The median age was 48 years and there were 48 males and 8 females. There were 45 cases of HAV/HBV/HCV infection, 50 cases with cirrhosis, and 45 cases of pathological TNM grade II. In the control group, the median age

was 45 years, and there were 15 males and 9 females; there were 22 cases of HAV/HBV/HCV infection and 7 cases of cirrhosis. All patients were informed and agreed to provide their case information; this clinical trial was approved by the Ethics Committee of Third Central Hospital of Tianjin.

We collected 6 mL fasting venous blood samples from patients in the 2 groups using EDTA anticoagulant tubes and centrifuged samples for 10 minutes at 3000 rev/min, then the upper serum was taken and separated into 1.5 mL EP tube, and saved at -80°C .

Methylation specific PCR (MSP) was performed to determine the methylation status within the promoter region of the FHIT and p16 genes. Genomic DNAs were extracted from serum using Genomic DNA Extraction Kit (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. Then 1 μg DNA was used for analysis of methylation. DNA modification was performed using EZ DNA Methylation Kit (Zymo Research, USA) according to manufacturer's protocol. The bisulfite modified DNA was then used for MSP. According to the literature, the FHIT and p16 gene methylation specific sequence (M) and the non-methylation specific sequence (U) were designed and the primer was synthesized by the Guangzhou Bioengineering Corporation, as shown in Table 1. The PCR amplification was performed using the following conditions: 95°C for 12 minutes, 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, as well as 72°C for 10 minutes. The PCR products were electrophoresed on 3% agarose gel and visualized under UV illumination. For NaHSO_3 treatment of DNA we added sodium hydroxide to the extracted DNA, and after 3-minute denaturation was completed, we added hydroquinone and NaHSO_3 solution ($\text{pH}=5.0$). Amplification was done for methylation/non-methylation of the aforementioned 2 gene promoter sequences that were amplified with PCR using the reaction system of 10 micron SYBY Q PCR, and 0.5 micron each. Primers, 4 μL , treated DNA templates and 5 μL water. Placental DNA treated with methyltransferase and hydrogen sulfite was as positive control (methylation), normal peripheral blood lymphocyte DNA as negative control, H_2O as blank control.

Statistical analysis of data was carried out by SPSS16.0 software. The comparison of all counting data and rate was selected by chi square test, and $P<0.05$ indicated that the difference was statistically significant. In order to screen the risk factors affecting the occurrence of liver cancer, the variables of statistical significance in the single factor analysis were first incorporated into the multifactor unconditional logistic regression model. The variable assignment: non-liver cancer=0, liver cancer=1; p16, FHIT gene unmethylation=0, methylation=1; female=0, male=1.

Table 1. Primers for methylation of FHIT and p16 genes.

Primer	Fragment size (bp)	Primer sequences
FHIT-M	100	Upstream: 5'-GGTTTTTACGCGCTTAGGT-3'
		Downstream: 5'-GCTCATAAAAAGCAAATGCTCC-3'
FHIT-U	100	Upstream: 5'-GGTTTTTATGTGTAGGT-3'
		Downstream: 5'-ACTCATAAAAAACAAATACTCC-3'
p16-M	149	Upstream: 5'-TTATTAGAGGGTGGGGCGGATCGC-3'
		Downstream: 5'-GACCCGAACCGCGACCGTAA-3'
p16-U	151	Upstream: 5'-TTATTAGAGGGTGGGGTGCATTGT-3'
		Downstream: 5'-CAACCCAAACCAACCATAA-3'

Table 2. The comparison of the methylation of FHIT in liver cancer group and non-liver cancer group.

	Case	Methylated	Non-methylated
Liver cancer group	56	29	27
Non-liver cancer group	24	4	20
Total	80	33	47

The comparison of 2 groups, $\chi^2=8.55$, $P<0.01$.

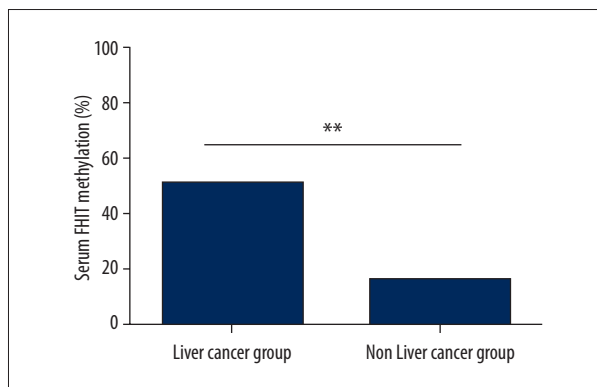


Figure 1. The comparison of the methylation of FHIT in serum of 2 groups. FHIT, fragile histidine triad. The comparison of two groups, ** $P<0.01$.

Results

The methylation of FHIT gene in the liver cancer group and non-hepatoma group was compared using MSP, as shown in Table 2. Out of 56 cases with liver cancer (the HCC group), there were 29 cases of FHIT methylation, of which the methylation rate was 51.8% (29 out of 56), and in the non-liver cancer group (the non-HCC group) the rate was 16.7% (4 out of 24 cases). There was a significant difference in FHIT methylation between the HCC group and the non-HCC group (Figure 1, $\chi^2=8.55$, $P<0.01$).

The p16 gene methylation in the HCC group and the non-HCC group were compared using MSP. As showed in Table 3, 38 patients with p16 methylation in 56 HCC patients were detected, the methylation rate was 67.9% (38 out of 56); 6 patients with p16 methylation in non-HCC group, with the methylation rate of 25% (6 out of 24). The difference of p16 methylation between the two groups was statistically significant (Figure 2, $\chi^2=12.47$, $P<0.01$).

The relationship between single factors and the occurrence of liver cancer was analyzed. As showed in Table 4, liver cancer was not statistically correlated with age and alcohol, but were statistically correlated with gender, and the methylation of FHIT and p16 genes.

Logistic regression analysis liver cancer was set as a dependent variable, and 3 significant factors, sex, FHIT, and p16 methylation, were included in the logistic regression analysis, and the model was established by stepwise regression. As shown in Table 5, methylation of p16 and FHIT genes both were independent risk factors for liver cancer.

Discussion

Liver cancer is a common gastrointestinal cancer diagnosis in the clinic. Its 5-year survival rate is lower than that of other malignant tumors, and only about 10%. At present, the unsatisfactory treatment of liver cancer is mainly because of its characteristics; for example, the disease is developed silently,

Table 3. The Comparison of the methylation of p16 in liver cancer and non-liver cancer groups.

	Case	Methylated	Non-methylated
Liver cancer group	56	38	18
Non-liver cancer group	24	6	18
Total	80	44	36

The comparison of 2 groups, $\chi^2=12.47$, $P<0.01$.

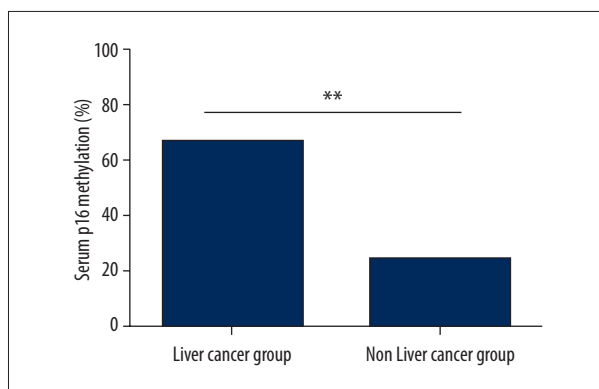


Figure 2. The comparison of the methylation of p16 in serum of 2 groups. The comparison of two groups, ** $P<0.01$.

the early symptoms are not obvious, the development of the disease is rapid which leads to the missed opportunity for the optimal period of surgical treatment, chemotherapy is seriously toxic, and the recurrence rate is extremely high.

The occurrence of liver cancer is a result of a multistep and multifactor interaction, including the interaction of environmental and genetic factors. The factors that have been closely related to liver cancer include hepatitis virus infection, aflatoxin B1 intake, cyanobacteria toxin contaminated drinking water, smoking and alcohol related drinking [6,7]. Therefore, the incidence of HCC in China is ranked fourth in malignant tumors, and the mortality rate is ranked more than second [8]. Thus, the early diagnosis of liver cancer has attracted wide attention. The rapid development of modern medicine has promoted the popularity of imaging studies, such as the extensive application of auxiliary diagnostic techniques such as computed tomography (CT) and B ultrasound, as well as the use of alpha fetoprotein, which all greatly improved the early diagnosis rate of liver cancer; however, there continues to be some limitations. With a deeper study of the molecular biology of cancer, we strive to find an effective approach to the treatment of liver cancer, especially early diagnosis and good prognosis.

Table 4. The analysis of single factor influencing liver cancer.

Variables	Liver cancer group (56)	Control group (24)	χ^2	P
Gender				
Male	48	15	5.41	$P<0.05$
Female	8	9		
Age				
≥ 60	27	8	1.51	$P>0.05$
<60	29	16		
Alcoholic				
Yes	23	11	0.16	$P>0.05$
No	33	13		
FHIT				
Methylated	29	4	8.55	$P<0.01$
Non-methylated	27	20		
p16				
Methylated	38	6	12.47	$P<0.01$
Non-methylated	18	18		

The comparison of multiple single factors.

Table 5. Logistic regression analysis.

Variables	β	SE	Wald χ^2	P	OR	95%CI	
Constants	0.529	0.534	0.981	0.322	0.589		
p16	2.109	0.632	11.121	0.001	10.550	2.313	48.116
FHIT	2.356	0.774	9.261	0.002	8.239	2.386	28.455
Gender	0.425	0.663	0.411	0.521	0.654	0.178	2.395

Analysis of p16, FHIT and gender. FHIT – fragile histidine triad.

Gene involvement in the process of cancer is caused by genetic mechanisms and epigenetic mechanisms; for example, when a tumor suppressor gene is inactivated or a proto oncogene is activated, it can cause genetic abnormalities. DNA methylation is the most widely used marker in epigenetics, which includes the whole process of DNA methyl transferase (DNA methyltransferases, DNMT) catalyzing the formation of methyl cytosine from the 5 carbon atoms. This process can lead to gene silencing, especially the silencing of tumor suppressor genes, which can lead to the occurrence and development of cancer.

FHIT is a tumor suppressor gene found in various types of tumor cells, located in the chromosome 3p14.2 region and it contains the coding function area of the triad protein. Although FHIT belongs to the histidine triad gene family, it is easily broken. Previous studies [9–11] have found that FHIT is involved in the occurrence of kidney cancer, breast cancer, colon cancer, gastric cancer, and liver cancer, and the frequent gene deletion caused by the methylation of the FHIT gene plays an important role in the development of various malignant tumors. Huawen et al. found that the expression of FHIT protein in patients with liver cancer was about 39.18%, followed by the para cancerous tissue group rate of 66.67%. The expression of FHIT protein in the advanced carcinoma tissue group was 93.33%, and the loss of the FHIT protein was more and more serious with the development of HCC, which indicated that deletion of FHIT gene (FHIT methylation) was associated with the occurrence of HCC [12]. On the contrary, Yun et al. detected that the methylation rate of FHIT gene in normal liver tissues was only 14.3%, but the methylation rate of FHIT gene in HCC and HCC cell lines was up to 71.1% and 75%, respectively, but the methylation rate of the FHIT gene in the para cancer tissues was 64.4%, and there was no statistical difference from the methylation rate of the FHIT in the cancer group. This, it is suggested that methylation of FHIT gene is an early event in the occurrence of HCC.

In our study, we detected the methylation of FHIT in the serum of a liver cancer group and a non-liver cancer group by MSP method. The results showed that the methylation rate was 51.8% (29 out of 56) in the liver cancer group, and the methylation rate was 16.7% (4 out of 24) in the non-liver cancer

group, and the methylation of FHIT between the liver cancer group and the non-liver cancer group was significantly different ($P < 0.01$). In our *in vitro* study, the results showed that FHIT was partly methylated in the human hepatoma cell line HepG2 and Hep3B, especially in HepG2, which led to the low expression or even loss of the FHIT gene in HepG2 hepatoma cells, thus promoting the proliferation of HepG2 hepatoma cells.

The p16 gene is the first anticancer gene that has been found to play a direct role in the cell cycle. In research about melanoma cells, Kamb et al. have found that the location of the p16 gene is on the 9p21 region of the chromosome and its total length is about 8.5kb. When the structural area of the p16 gene promoter is methylated, the gene cannot be transcribed, which leads to the deletion of the p16 gene and the deletion of the protein, thereby affecting the failure of the regulation of the whole cell cycle, the malignant proliferation of the cells, and the final formation of the tumor. In addition, the expression product of p16 gene has the function of binding to CDK4/CDK6 competing with cyclin D1, causing long-time stagnation of the cell cycle and playing the role in the negative regulation of cell proliferation [13–15].

It has been reported that the abnormal methylation of the p16 gene is more significant in HCC patients. Early studies found that methylation of the p16 gene is a frequent occurrence in hepatocellular carcinoma. Methylation of the p16 gene can lead to loss expression of mRNA. A recent meta-analysis about the methylation of the p16 gene and the susceptibility to HCC in a total of 23 studies involving 2245 cases showed that the positive rate of p16 gene methylation in HCC tissues was significantly higher than that in normal liver tissue, noncancerous tissue and liver cirrhosis, and that the hypermethylation of the p16 gene was highly closed to the susceptibility to HCC [16,17].

The results of our study were consistent with previous study results. The detection of serum p16 showed that the methylation rate of p16 in the liver cancer group was 67.9% (38 out of 56), and that in the non-liver cancer group it was 25% (6 out of 24), and the difference of the methylation of p16 between the liver cancer group and non-liver cancer group was statistically significant ($P < 0.01$). The occurrence of liver cancer had a statistically

significant correlation with gender, and methylation of FHIT and p16 genes ($P<0.05$). According to logistic regression analysis, the methylation of p16 or FHIT genes was an independent risk factor for the occurrence of liver cancer. This suggests that the methylation of p16 and FHIT genes is involved in the pathogenesis of liver cancer. Nowadays, the possibility of non-invasive detection of peripheral blood for HCC has been explored for use clinically. Peripheral blood methylation analysis may be a powerful tool for studying the pathophysiological basis of DNA abnormalities in cancer.

Conclusions

HCC is one of the most common malignant diseases in China. The past general view was that the central link in the occurrence of liver cancer was genetic change. In recent years, with the deepening of research and the deepening of the understanding of liver cancer, it has also been found that epigenetic changes in the development of liver cancer play an important role. In addition, the methylation of FHIT and p16 genes have been shown to be involved in the development of several cancers [18-20]. In the study about the methylation of tumor

suppressor gene, p16, and FHIT, in the serum of patients with lung cancer, it was found that the methylation of p16 or FHIT genes was the independent risk factor for lung cancer. The combined detection of the methylation of p16, FHIT, and APC can improve the accuracy of the diagnosis of lung cancer, and the related research shows that compared to FHIT, the loss of the expression of p16 gene is most likely to occur in the late stage of malignancy.

At present, with the breakthrough of technology, it is possible to detect DNA methylation in serum, plasma, and other biological samples. This invasive detection is beneficial to early diagnose of the disease, monitoring the disease and evaluating therapeutic effects. In conclusion, the results of serum gene methylation in the liver cancer group and non-liver cancer group in our study showed that the methylation of FHIT or p16 genes was an independent risk factor for the occurrence of liver cancer, and that joint detection could be helpful for diagnosis.

Conflict of interest

None.

References:

- Torre LA, Bray F, Siegel RL et al: Global cancer statistics, 2012. *Cancer J Clin*, 2015; 65: 87–108
- El-Serag HB, Rudolph KL: Hepatocellular carcinoma: Epidemiology and molecular carcinogenesis. *Gastroenterology*, 2007; 132: 2557–76
- Maekawa M, Watanabe Y: Epigenetics: Relations to disease and laboratory findings. *Curr Med Chem*, 2007; 14(25): 2642–53
- Ohta M, Inoue H, Cotticelli MG et al: 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(4): 587–97
- Yuen PW, Man M, Lamk Y et al: Clinicopathological significance of p16 gene expression in the surgical treatment of head and neck squamous cell. *Clin Pathol*, 2002; 55(1): 58–60
- Kiyosawa K, Tanaka E: Characteristics of hepatocellular carcinoma in Japan. *Oncology*, 2002; 62(S1): 5–7
- Hitzfeld BC, Hoger SJ, Dietrich DR: Cyanobacterial toxins: removal during drinking water treatment, and human risk assessment. *Environ Health Perspect*, 2000; 108(S1): 113–22
- Zhang L, Lei Z, Li G et al: Analysis of incidence and mortality of cancer in China Cancer Registry area in 2006. *China Cancer*, 2010; 19(6): 356–65
- Xiao YP, Han CB, Mao XY et al: Relationship between abnormality of FHIT gene and EBV infection in gastric cancer. *World J Gastroenterol*, 2005; 11(21): 3212–16
- Baffa R, Veronese ML, Santoro R et al: Loss of FHIT expression in gastric carcinoma. *Cancer Res*, 1998; 58: 4708–14
- Huiping C, Kristjansdottir S, Bergthorsson JT et al: High frequency of LOH, MSI and abnormal expression of FHIT in gastric cancer. *Eur J Cancer*, 2002; 38: 728–35
- Barnes LD, Garrison PN, Siphrahvili Z et al: FHIT, a putative tumor suppressor in humans is a dinucleoside 5',5'''-P1,P3-triphosphate hydrolase. *Biochemistry*, 1996; 35(36): 11529–35
- Serrano M, Hannon GJ, Beach D: A new regulator motif in cell cycle control causing specific inhibition of cyclin D/CDK4. *Nature*, 1993; 366: 634–35
- Norbori T, Miura K, Wu DJ et al: Deletions of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. *Nature*, 1994; 368: 753–56
- Marx J: A challenge to P16 gene as a major tumor suppressor. *Science*, 1994; 24: 1846
- Shiraz OB, Galehdari H, Yavarian M et al: Possible down regulation of the p16 gene promoter in individuals with hepatocellular carcinoma. *Hepat Mon*, 2011; 11(9): 719–23
- Qu Z, Jiang Y, Li H et al: Detecting abnormal methylation of tumor suppressor genes GSTP1, P16, RIZ1, and RASSF1A in hepatocellular carcinoma and its clinical significance. *Oncol Lett*, 2015; 10: 2553–58
- Shan M, Zhang X, Liu X et al: P16 and p53 play distinct roles in different subtypes of breast cancer. *PLoS One*, 2013; 8(10): e76408
- Zhang C, Ye L, Guan S et al: Autoantibodies against P16 protein-derived peptides may be a potential biomarker for non-small cell lung cancer. *Tumour Biol*, 2014; 35(3): 2047–51
- Wani HA, Beigh MA, Amin S et al: Methylation profile of promoter region of P16 gene in colorectal cancer patients of Kashmir valley. *J Biol Regul Homeost Agents*, 2013; 27(2): 297–307