

Association of FHIT expression and *FHIT* gene hypermethylation with liver cancer risk: a PRISMA-compliant meta-analysis

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Background: There have been suggestions that fragile histidine triad protein (FHIT) expression and *FHIT* gene hypermethylation were crucial to the pathogenesis of liver cancer. However, the conclusions remained unclear because of small sample size, disease subtype, and different detection techniques. Therefore, we performed a meta-analysis to estimate the associations of FHIT expression and *FHIT* gene hypermethylation with liver cancer pathogenesis.

Methods: Studies that were published in electronic databases, such as PubMed, Web of Knowledge, China National Knowledge Infrastructure (CNKI), VIP, and WanFang, were retrieved and selected for the meta-analysis. Relative risk (RR) and 95% confidence interval (CI) were calculated to determine the correlations of FHIT expression and *FHIT* gene hypermethylation with liver cancer pathogenesis with Stata 12.0 software.

Results: A total of 17 papers that evaluated the associations of FHIT expression (14 articles) and *FHIT* gene methylation (3 articles) with liver cancer pathogenesis were included in this meta-analysis. In the overall analysis, the pooled relative risk was 1.93 (95% CI = 1.72–2.17), which indicated a significant association between FHIT low expression and liver cancer risk. According to the results of clinical information, there were significant associations of FHIT expression with TNM-stage (RR = 2.13, 95% CI = 1.72–2.64), tumor size (RR = 1.67, 95% CI = 1.36–2.05), and merger of cirrhosis (RR = 1.34, 95% CI = 1.06–1.69) of liver cancer in the Chinese population. In addition, the *FHIT* gene hypermethylation was significantly associated with the risk of liver cancer (RR = 1.45, 95% CI = 1.08–1.93).

Conclusion: The FHIT expression and hypermethylation of *FHIT* gene were significantly associated with the risk of liver cancer, especially in the Chinese population. Furthermore, the results indicated significant associations between FHIT low expression and TNM-stage, tumor size, and merging of cirrhosis of liver cancer in the Chinese population.

Keywords: FHIT, expression, hypermethylation, liver neoplasms, meta-analysis

Introduction

Liver cancer, one of the most prevalent cancers, is the third leading cause of cancer-related deaths in the world.¹ The incidence of liver cancer has reached one million worldwide every year and has a considerable geographic variation.² The majority of liver cancer patients were found in developing countries such as China, Vietnam, North Korea, and Thailand.³ Although many therapeutic methods of hepatocellular carcinoma were developed, the prognosis was not satisfactory. A study reported that infection of the hepatitis B virus (HBV) and hepatitis C virus (HCV) was the main cause of the high prevalence of liver cancer in Asians.⁴ In addition, many risk factors, such as alcohol drinking, aflatoxin, obesity, diabetes, and tobacco use, and other genetic changes

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have been found to be associated with the development of liver cancer.^{5,6} Damage from liver cirrhosis was often seen during liver cancer. Excessive alcohol drinking was one of the common reasons of liver cirrhosis, which then led to the occurrence of liver cancer. If patients with liver cirrhosis were infected with HCV, they had a high risk of developing liver cancer. At the same time, aflatoxin B1 was another major risk factor for liver cancer due to the common food contamination in developing countries. If aflatoxin B1 was not metabolized completely, it could result in G-T transversions and ultimately induce the occurrence of liver cancer. In addition to these environmental factors, many genetic and epigenetic factors were involved in the initiation, progression, and metastasis of liver cancer.^{7,8} Epigenetic regulations, which consisted of altered gene expression without the change of DNA sequence, played an important role in the normal growth of cells, and its abnormality might lead to the occurrence of tumors.⁹ Therefore, a lot of epigenetic biomarkers, including the change of DNA methylation patterns, histone modifications, and the change of non-coding RNA expression profiles, were researched as the potential targets in the diagnosis, prognosis, and treatment of liver cancer.

FHIT, a member of evolutionary conserved histidine triad (HIT) proteins, belonged to oncosuppressor proteins which induced cell apoptosis. Studies have shown that transfection of *FHIT* cDNA affected the signal transduction of tumor cells and reduced the proliferation of cancer cell lines.^{10,11} The study of FHIT's functions indicated that FHIT exerted its oncosuppressor activity by binding or mediation of fas, caspase-8, and PARP proteins.¹² Deng et al reported that the overexpression of FHIT led to the activation of cell apoptosis factors.¹³ In recent years, several studies have been conducted to explore the association between the change of FHIT epigenetics and cancer risk. For instance, Lin et al found that the frequency of *FHIT* gene hypermethylation in myelodysplastic syndrome patients was higher than in normal persons.¹⁴ Moreover, it has been reported that the loss of FHIT was associated with the risk of gastric cancer, breast cancer, and lung cancer.¹⁵⁻¹⁷ And the aberrant methylation of *FHIT* gene had a significant association with the clinical stage and other clinical features.

Previously, many studies were performed to explore the role of FHIT expression and methylation in the development of liver tumor. The conclusions remained unsatisfactory because of small sample size. Therefore, in order to get more precise conclusions, we aimed to conduct a systematic meta-analysis to clarify the role of FHIT expression and *FHIT* gene methylation in the pathogenesis of liver cancer.

Materials and methods

Study strategy

A literature search was conducted using electronic databases such as PubMed, Web of Science, China National Knowledge Infrastructure (CNKI), VIP, and WanFang. The following terms were used to search the relevant articles: "Fragile histidine triad protein", "FHIT", "Hepatocellular carcinoma", "HCC", "liver cancer", "liver tumor", "liver carcinoma", "methylation", "hypermethylation", "low expression", and "expression". MeSH terms and free-text words were used to search for eligible studies in the literature. The literature retrieval was updated on March, 2017. We checked the relevant reviews and references of included articles in detail and obtained the eligible literature. First, titles and abstracts of articles were read to initially confirm whether these articles were related to the associations of FHIT expression and *FHIT* gene methylation with liver cancer risk. Then, the full text of articles was scanned carefully and the relevant data were extracted.

Inclusion and exclusion criteria

The following inclusion criteria were applied to assess the relevant studies: 1) studies must have detailed diagnostic criteria of liver cancer; 2) studies must contain the data that evaluated the associations of FHIT expression and *FHIT* gene methylation with liver cancer; 3) studies were original research articles in English and Chinese language; and 4) if the data of the publications was the same, the latest study and more accurate data were included. Exclusion criteria were as follows: 1) reviews and meta-analysis were not included in this study; 2) studies that lacked sufficient data to estimate the relative risk (RR) and 95% CI; and 3) studies that contained duplicate data.

Data extraction and methodological assessment

The key components of eligible studies were recorded: publication year, the first author, studied population, type of cancer, cut-off value of immunohistochemical staining, number of patients, detection method of FHIT expression, sample type, frequency of *FHIT* gene methylation in case group and control group, and clinicopathological features. To acquire accurate data, two independent investigators were involved in the extraction of data. The methodological quality of all included studies was assessed using Newcastle-Ottawa Quality Assessment Scale (NOS) table. Studies that got a score of ≥ 6 were included in this meta-analysis.

Statistical analysis

RR and 95% CI were applied to estimate the associations of FHIT expression and *FHIT* gene hypermethylation with the clinical parameters of liver cancer. These clinical features included the TNM-stage, HBsAg, tumor size, liver cirrhosis, and liver capsule of liver cancer. Stata 12.0 software (Stata Corporation, College Station, TX, USA) was applied to calculate the RR and 95% CI. Forest plot and funnel plot were drawn to observe the heterogeneity and publication bias among studies. The chi-square based on Q-test and I^2 test were performed to test the interstudy heterogeneity.¹⁸ Random effects model (DerSimonian and Laird method) and fixed effect model (Mantel-Haenszel method) were used to draw the forest plot and conduct the Q-test.^{19,20} If the absence of heterogeneity among studies was identified, fixed effect model was applied. Otherwise, the random effects model was used. The P -value and I^2 statistic were used to quantitatively estimate the interstudy heterogeneity. The P -value of <0.05 or I^2 statistic of $>50\%$ indicated a significant heterogeneity. Subgroup analysis and random effect model were performed if significant heterogeneity existed. Publication bias was evaluated by Begg's rank correlation test and Egger's weighted regression test, in which $P<0.05$ indicated a significant publication bias.^{21,22} Finally, sensitivity analysis was performed to assess the influence of a single study in the overall RR.

Results

Study characteristics

The process of literature search is summarized in Figure 1. A total of 110 articles were acquired in the initial retrieval by searching electronic databases. About 38 articles were excluded due to duplicate retrieval. Of the remaining 72 articles, 31 were obtained from Chinese databases and 41 publications from English databases. Through reading titles and abstracts, 44 irrelevant articles were excluded. Of the remaining 28 articles, 3 did not have the frequency of FHIT expression in case group and control group, 2 contained duplicate data, the samples of 4 were inappropriate, and 2 publications were about *FHIT* gene methylation and did not have sufficient frequency of *FHIT* gene methylation. Finally, 14 articles with 1,345 controls and 945 cases for FHIT expression were involved, while 3 articles with 75 controls and 124 cases for FHIT methylation were included.^{23–39} According to the extracted information of included studies, the frozen liver cancer tissues and normal or adjacent liver tissues were obtained through surgical operation to extract the DNA and detect the expression of FHIT and methylation of *FHIT* gene. The main characteristics of the eligible studies are shown in Table 1. The scores of methodological assessment of included studies ranged from 6 to 8.

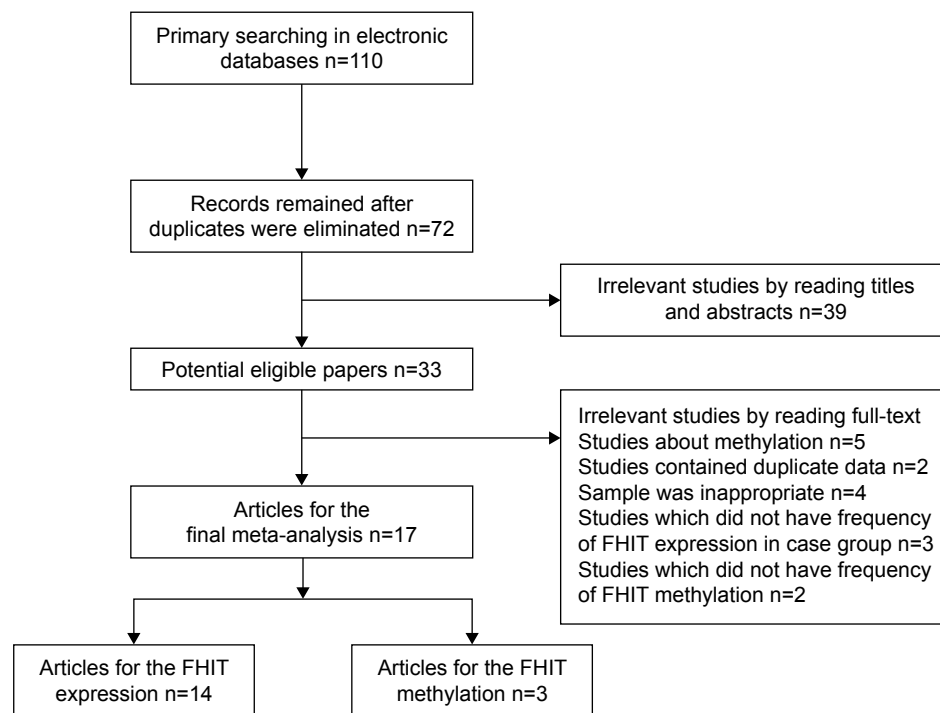


Figure 1 Flow diagram of the eligible literature search.
Abbreviation: FHIT, fragile histidine triad protein.

Table 1 Clinicopathological features of eligible studies

Author	Reference	Year	Country	Race	Histology	Control		Case		Methods	Control materials	Case materials	NOS score	Cut-off (%)
						Positive	Negative	Positive	Negative					
Liu et al	23	2016	China	Asians	LC	64	26	23	67	IHC	Adjacent tissue	HCC tissues	8	Score =0
Lin et al	24	2010	China	Asians	HCC	132	10	68	74	IHC	Adjacent tissue	HCC tissues	8	10
Lin et al	24	2010	China	Asians	HCC	15	0	68	74	IHC	Normal tissue	HCC tissues	6	10
Che et al	25	2008	China	Asians	HCC	8	2	20	30	IHC	Normal tissue	HCC tissues	8	25
Fan et al	26	2008	China	Asians	HCC	52	23	29	46	IHC	Adjacent tissue	HCC tissues	8	5
Liu et al	27	2008	China	Asians	HCC	56	20	75	33	IHC	Adjacent tissue	HCC tissues	8	Score =0
Zhao et al	28	2006	China	Asians	LC	552	55	33	50	IHC	Normal tissue	HCC tissues	6	NR
Liang et al	29	2005	China	Asians	HCC	20	0	32	30	IHC	Normal tissue	HCC tissues	6	5
Liang et al	29	2005	China	Asians	HCC	62	0	32	30	IHC	Adjacent tissue	HCC tissues	8	5
Zheng and Chen	30	2005	China	Asians	HCC	12	0	20	30	IHC	Adjacent tissue	HCC tissues	8	Score =0
Song and Dou	31	2005	China	Asians	LC	10	0	29	41	IHC	Normal tissue	HCC tissues	6	Score =0
Song and Dou	31	2005	China	Asians	LC	10	0	29	41	IHC	Adjacent tissue	HCC tissues	8	Score =0
Nan et al	32	2005	China	Asians	HCC	10	0	19	28	IHC	Normal tissue	HCC tissues	6	5
Nan et al	32	2005	China	Asians	HCC	47	0	19	28	IHC	Adjacent tissue	HCC tissues	8	5
Zekri et al	33	2005	Egypt	Mixed	HCC	10	0	6	17	RT-PCR	Normal tissue	HCC tissues	6	NR
Xu and Yi	34	2004	China	Asians	PHC	10	0	26	20	IHC	Normal tissue	PHC tissues	6	5
Xu and Yi	34	2004	China	Asians	PHC	46	0	26	20	IHC	Adjacent tissue	PHC tissues	8	5
Zhao et al	35	2003	China	Asians	HCC	10	0	54	29	IHC	Normal tissue	HCC tissues	6	5
Zhao et al	35	2003	China	Asians	HCC	83	0	54	29	IHC	Adjacent tissue	HCC tissues	8	5
Yuan et al	36	2000	China	Asians	HCC	10	0	5	5	IHC	Adjacent tissue	HCC tissues	6	NR
Control U M														
Zekri et al	37	2014	Egypt	Mixed	HCC	11	2	10	21	MSP	Normal tissue	HCC tissues	6	NR
Zhang et al	38	2013	China	Asians	HCC	17	31	23	25	MSP	Normal tissue	HCC tissues	7	NR
Sun et al	39	2006	China	Asians	HCC	12	2	13	32	MSP	Normal tissue	HCC tissues	6	NR

Abbreviations: HCC, hepatocellular carcinoma; IHC, immunohistochemical; LC, liver cancer; M, methylation; MSP, methylion-specific PCR; NOS, quality assessment scale; NR, not reported; PHC, primary hepatic carcinoma; U, unmethylation; RT-PCR, reverse transcription polymerase chain reaction.

Association between FHIT expression and clinicopathological features of liver cancer

In total, the pooled results of the meta-analysis showed that persons with a low FHIT expression had a higher risk of liver cancer (RR =1.93, 95% CI =1.72–2.17). In addition, a significant association between FHIT low expression and liver cancer risk (RR =1.91, 95% CI =1.70–2.14) was found in Chinese population. The subgroup analysis based on sample type was conducted due to significant heterogeneity, and the results indicated a decreased heterogeneity and significant associations between FHIT low expression and liver cancer risk both in normal tissue (RR =2.00, 95% CI =1.72–2.32) and in adjacent tissue (RR =1.88, 95% CI =1.58–2.25). Moreover, there were significant associations of FHIT low expression with the TNM-stage (RR =2.13, 95% CI =1.72–2.64), tumor size (RR =1.67, 95% CI =1.36–2.05), and merger of cirrhosis (RR =1.34, 95% CI =1.06–1.69) with liver cancer.

Interestingly, no significant association of FHIT low expression with the hepatitis B surface antigen (HBsAg) was found. According to the results of the TNM-stage, the frequency of FHIT low expression in III–IV stage patients of liver cancer was higher than that in the I–II stage patients of liver cancer. The expression of FHIT might be lower in the later-stage patients than that in the early-stage patients of liver tumor. Furthermore, the frequency of low FHIT expression was higher in the liver cancer tissue that merged with damage from liver cirrhosis. Random effect model was applied due to the existence of significant heterogeneity. No large heterogeneity was found other than the overall meta-analysis of liver cancer risk (Figures 2–5; Table 2).

Association between *FHIT* gene hypermethylation and liver cancer risk

Of all the 3 included studies with 75 controls and 124 cases, there was a significant association between *FHIT* gene

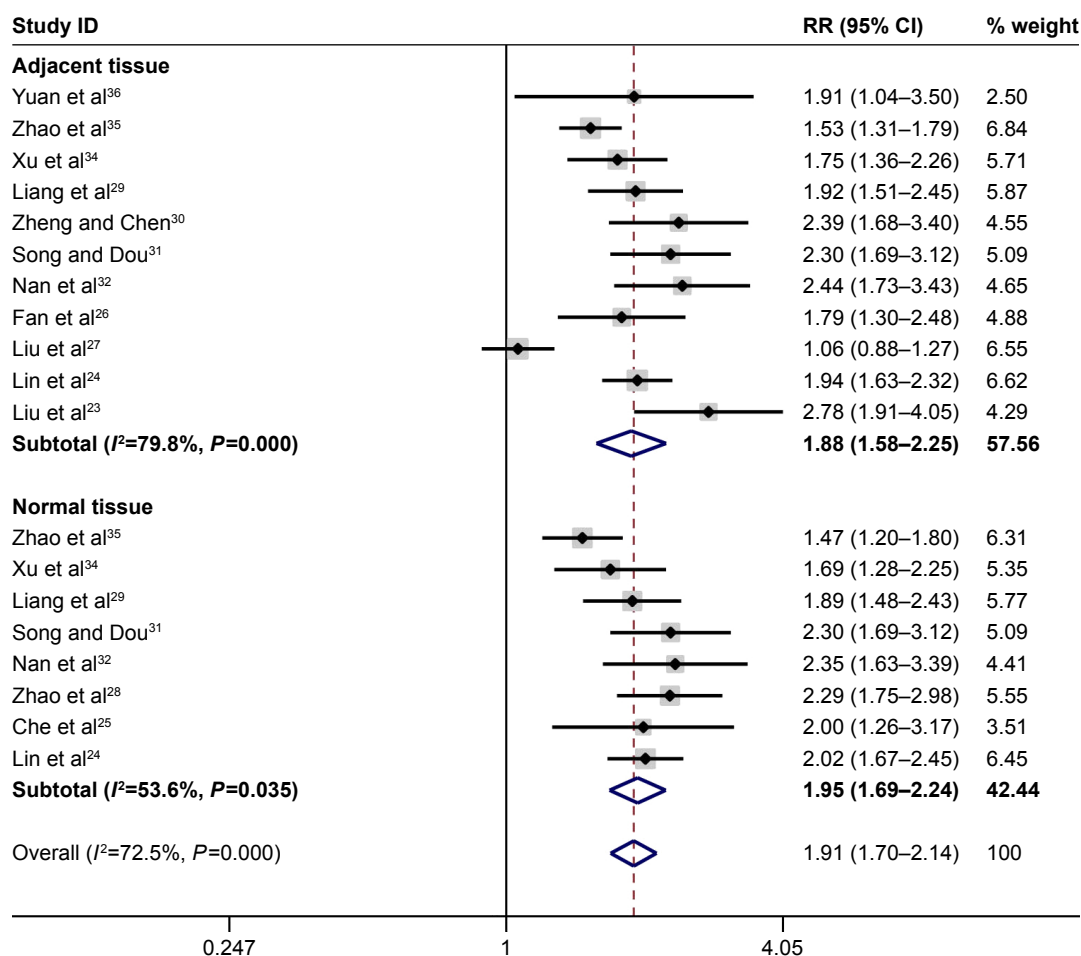


Figure 2 Forest plot of the relationship between FHIT expression and liver cancer risk.

Note: Weights are from random effects analysis.

Abbreviations: CI, confidence interval; FHIT, fragile histidine triad protein; RR, relative risk.

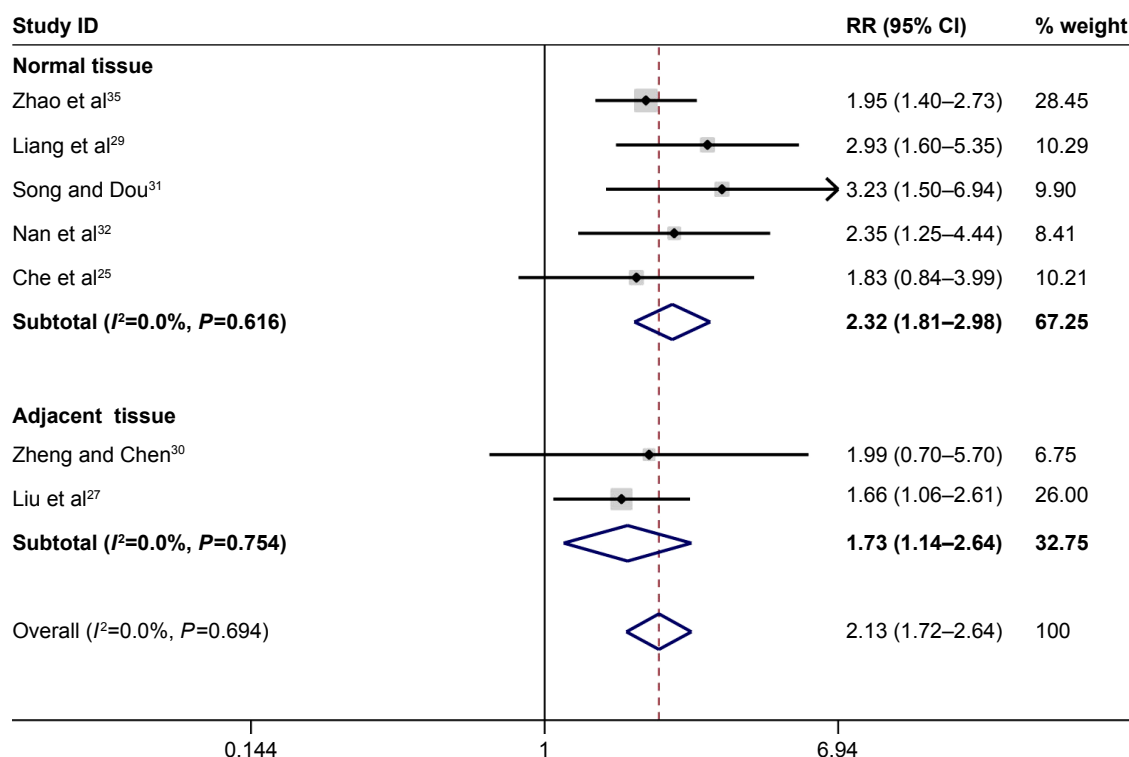


Figure 3 Forest plot of the association between FHIT expression and TNM-stage of liver cancer.

Abbreviations: CI, confidence interval; FHIT, fragile histidine triad protein; RR, relative risk.

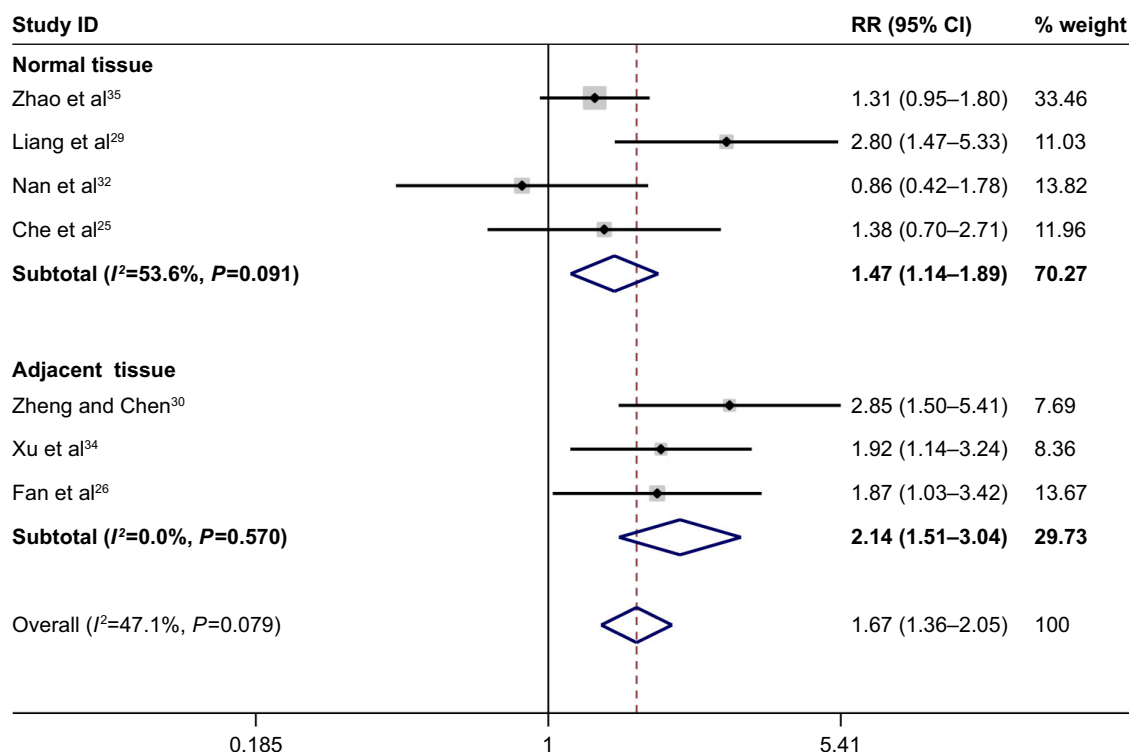


Figure 4 Forest plot of the association between FHIT expression and liver tumor size.

Abbreviations: CI, confidence interval; FHIT, fragile histidine triad protein; RR, relative risk.

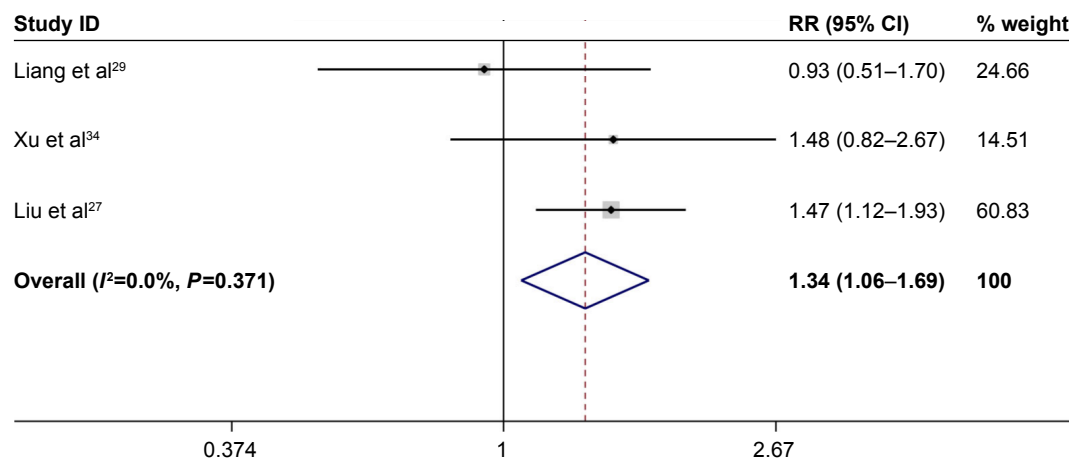


Figure 5 Forest plot of the association between FHIT expression and merging liver cirrhosis into liver cancer.

Abbreviations: CI, confidence interval; FHIT, fragile histidine triad protein; RR, relative risk.

hypermethylation and liver cancer risk (RR =1.45, 95% CI =1.08–1.93, $P<0.05$). No subgroup analysis based on ethnicity and disease type was conducted because little information was available. Furthermore, the results of Q test and the value of I^2 revealed that no heterogeneity was detected in this meta-analysis. And the P -values of Begg's test and Egger's test were all >0.05 , which suggested that the overall RR was stable (Figure 6).

Publication bias and sensitivity analysis

Begg's test and Egger's test were conducted and the results revealed that no significant publication bias was found in the meta-analysis of association of liver cancer pathogenesis with FHIT low expression. In the meta-analysis of FHIT expression, $P<0.05$ of Begg's test indicated a significant publication bias. However, subgroup analysis based on

sample type reduced the publication bias and nonsignificant value was found again. Sensitivity analysis was conducted by removing one study at a time. The pooled RR did not have significant changes, which indicated the stability of the result in this meta-analysis (Figures 7–8).

Discussion

To the best of our knowledge, FHIT protein had a wide range of tumor-suppressive functions such as modulation of the DNA damage, downregulation of oncogene expression, modulation of tumor cells apoptosis, and the growth, cycle, invasion, and metastasis of cancer cells.⁴⁰ FHIT could regulate the metabolism of Ap3A hydrolase and improve the activity of Ap3A which might restrain the growth of normal cells. Thus, the loss of FHIT protein expression might be one of the causes of unlimited reproduction in cancer cells.⁴¹

Table 2 Meta-analysis of the associations of FHIT expression and *FHIT* gene hypermethylation with liver cancer pathogenesis

Clinical features	RR (95% CI)	P-value	Heterogeneity test		Studies (n)	Model	Publication bias	
			I^2 (%)	P-value			Begg's test (P)	Egger's test (P)
FHIT expression (adjacent vs cancer)	1.88 (1.58–2.25)	<0.05	79.80	0	11	Random effects analysis	0.09	0.06
FHIT expression (normal vs cancer)	2 (1.72–2.32)	<0.05	58.10	0.014	9	Random effects analysis	0.25	0.1
Overall liver cancer risk	1.93 (1.72–2.17)	<0.05	72.70	0	20	Random effects analysis	0.02	0.006
TNM stage (I + II vs III + IV)	2.13 (1.72–2.64)	<0.05	0	0.69	7	Fixed effects model	0.76	0.33
HBsAg	1.28 (0.88–1.86)	>0.05	0	0.85	5	Fixed effects model	0.46	0.66
Tumor size (≤ 5 cm vs >5 cm)	1.67 (1.36–2.05)	<0.05	47.1	0.08	7	Fixed effects model	0.76	0.42
Liver capsule	0.95 (0.41–2.22)	>0.05	78.4	0.003	4	Random effects analysis	0.73	0.61
Damage from liver cirrhosis	1.34 (1.06–1.69)	<0.05	0	0.37	3	Fixed effects model	0.3	0.56
Portal venous tumor emboli	0.71 (0.34–1.50)	>0.05	63.5	0.04	4	Random effects analysis	0.31	0.11
FHIT hypermethylation (normal vs cancer)	2.33 (1.85–2.93)	<0.05	0.000	0.422	3	Fixed effects model	0.602	0.25

Abbreviations: CI, confidence interval; FHIT, fragile histidine triad protein; HBsAg, hepatitis B surface antigen; RR, relative risk.

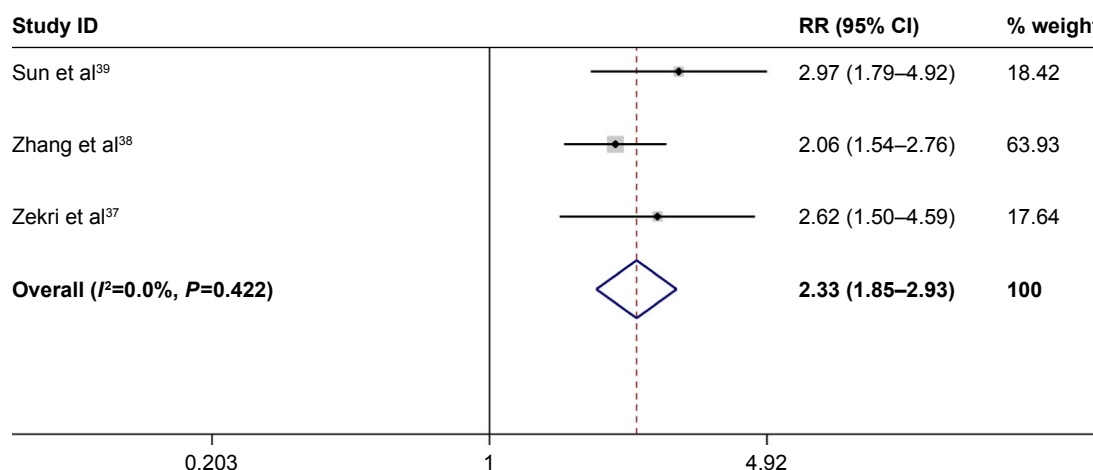


Figure 6 Forest plot of the association between *FHIT* gene hypermethylation and liver cancer risk.

Abbreviations: CI, confidence interval; FHIT, fragile histidine triad protein; RR, relative risk.

In most human normal tissues and cancer tissues, FHIT expression was detected and genetic alternations were often found. In some clinical studies, it has been reported that low FHIT expression had a significant association with the stage of many cancers. The previous study showed that the low expression of FHIT was associated with lymph node metastasis, lymphatic invasion, and the depth of invasion in gastric cancer, which suggested that FHIT expression might contribute significantly to the pathogenesis of gastric cancer.⁴² Moreover, some studies have reported that the change of FHIT low expression and *FHIT* gene hypermethylation had an important role to play in causing risk of lung cancer.^{43,44}

In recent years, several studies have focused on the effect of genetic changes in FHIT protein on the development of hepatocellular carcinoma (HCC). Several studies have

reported that the low expression of FHIT and *FHIT* gene mutations were shown in the HCC cell lines and primary HCC tissues.^{33,36,37} Aberrant FHIT transcripts were found in 39%–70% of HCC patients.⁴⁵ Exon skipping, recognition of cryptic splice site, and use of alternate 5' and 3' splice sites led to the insertions of intronic sequences and further generated these aberrant transcripts.⁴⁶ These transcripts affected the normal expression and function of FHIT protein. From previous studies, these alternations were often identified in the exon 5 and 6, and single-point mutations were very rare.^{37,46,47} In addition, some studies were also conducted to explore the change of FHIT protein expression other than these structural variations of *FHIT* gene. According to our literature search, most studies have focused on the FHIT expression and *FHIT* gene methylation in Chinese population.

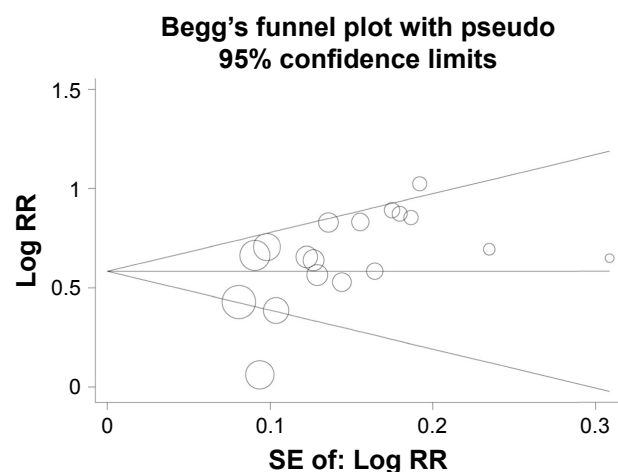


Figure 7 Funnel plot assessing the association of FHIT expression with liver cancer risk.

Abbreviations: FHIT, fragile histidine triad protein; RR, relative risk; SE, standard error.

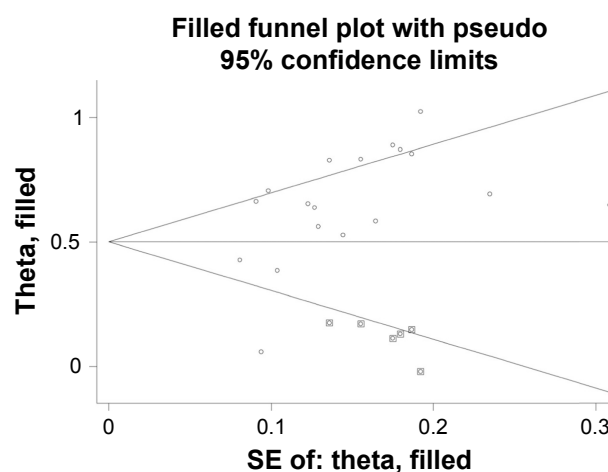


Figure 8 Funnel plot of trim and filling method assessing the association of FHIT expression with liver cancer risk.

Abbreviations: FHIT, fragile histidine triad protein; SE, standard error.

Therefore, a meta-analysis, assessing the associations of FHIT low expression and *FHIT* gene hypermethylation and liver cancer pathogenesis, was performed.

In the present study, 14 studies were included to investigate the correlation between FHIT expression and liver cancer risk and 3 studies were for *FHIT* gene methylation. Although a significant association of FHIT low expression with liver cancer risk was found, significant heterogeneity among studies was found. According to the results of subgroup analysis, the FHIT low expression resulted in a higher risk of liver cancer. At the same time, heterogeneity decreased a lot and no publication bias was found, which indicated a reliable result. In the included studies, Liu et al got an opposite result and other studies obtained the same result.²⁷ Therefore, on the basis of the large sample size in this meta-analysis, we might conclude that there was a significant association between FHIT low expression and liver cancer risk. In the subgroup analysis based on clinical features in liver cancer, the pooled relative risk and 95% CIs suggested that there were significant associations of FHIT low expression with the TNM-stage, liver tumor size, and the merging of liver cirrhosis into liver cancer in Chinese population. Furthermore, in these included studies, Liu et al reported that the FHIT low expression had a higher frequency in the group of liver cirrhosis (patients who had liver cirrhosis and liver cancer).²⁷ The results of Liu et al, Liang et al, Song et al, Nan et al, and Yuan et al indicated that the liver cancer patients of FHIT low expression often had a later stage of TNM.^{27,29,31,32,36} These results demonstrated that the expression of FHIT might be lower in patients with later-stage liver cancer. No significant publication bias and heterogeneity were found in the subgroup analysis based on sample type and clinical characteristics of liver cancer. Currently, complete liver capsule was very important in the diagnosis of liver cancer, which illustrated no infiltration of liver cancer cells. However, our data indicated that the status of FHIT expression was negatively associated with the integrity of the liver capsule. It is noteworthy that the FHIT low expression did not have a significant association with the existence of HBsAg. Thus, although almost 50% of HCC patients were affected with HBV infection, FHIT expression might not be associated with the infection of hepatitis B virus.⁴⁷ In our meta-analysis, the sample of included studies mostly came from the hospital, which might result in some influences in the between-study heterogeneity. Furthermore, the frozen liver tumor tissues and control liver tissues were used to detect the status of FHIT expression and methylation of *FHIT* gene. According to the results of the clinical

features in this meta-analysis, we could also analyze the FHIT expression and *FHIT* gene methylation of liver tumor tissues in different stages. Of importance, the association of clinical features with FHIT expression needed further investigation because of the single population, small sample size, and little clinical information.

Moreover, a significant association between *FHIT* gene hypermethylation and liver cancer risk was found based on a small sample size. On the basis of the results, no heterogeneity and publication bias among studies were found. However, although the results of the meta-analysis were significant and stable, the sample size was still small and little clinical information was available.

In addition, some limitations should be acknowledged in the present meta-analysis. First, cut-off values of immunohistochemistry were inconsistent, and this might reduce the accuracy of the results. Differences in evaluation criterion of staining, immunohistochemistry staining protocols, and cut-off values might lead to the heterogeneity among studies. Second, the studied population of this meta-analysis was mainly Chinese, and therefore, the results of this meta-analysis were mainly adapted to that population. The pathogenesis of liver cancer has a huge difference in different countries and regions. Hence, if we wanted to obtain more representative results all over the world, other studies in other populations and regions must be conducted. Third, the small number of included studies and the small sample size limited the statistical scope of the meta-analysis. Therefore, studies from a variety of countries and ethnicities should be conducted to improve the reliability of the conclusions.

Conclusion

In conclusion, FHIT low expression and *FHIT* gene hypermethylation were significantly associated with liver cancer risk, especially in Chinese population. The data also indicated that significant correlations of FHIT low expression with the TNM-stage, tumor size, and liver cirrhosis into liver cancer in Chinese population were found. Therefore, FHIT expression and *FHIT* gene hypermethylation might be good biomarkers for early diagnosis of liver cancer.

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Disclosure

The authors report no conflicts of interest in this work.

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