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p16 Methylation was associated with the development, age, hepatic viruses infection of hepatocellular carcinoma, and p16 expression had a poor survival

A systematic meta-analysis (PRISMA)

Xueyou Lv, MM, Guoliang Ye, MD, Xinjun Zhang, MD*, Tao Huang, MD*

Abstract

Background: Loss of tumor suppressor gene *p16* expression via promoter methylation has been reported in hepatocellular carcinoma (HCC). This meta-analysis was conducted to evaluate the correlation between p16 methylation and HCC. Additionally, we also analyzed the potential prognostic role of p16 methylation, expression or alteration-associated HCC.

Methods: Online databases based on the Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) guideline were performed to analyze the role of *p16* gene in HCC. The combined odds ratios (ORs) or hazard ratios (HRs) and their 95% confidence intervals (95% CIs) were summarized.

Results: Final 3105 HCCs and 808 non-tumor controls (chronic hepatitis and liver cirrhosis) were performed in this meta-analysis. *p16* promoter methylation in HCC was significantly higher than in chronic hepatitis and chronic hepatitis in tissue and blood samples. In addition, *p16* promoter methylation was notably higher in patients >50 years' old than in patients aged <50 years, and it was higher in hepatitis B virus (HBV) or hepatitis C virus (HCV)-positive HCC than in hepatic viruses-negative HCC. However, *p16* promoter methylation was not correlated with sex, cirrhosis, tumor differentiation, clinical stage. No association was found between *p16* methylation or alteration and the prognosis of patients with HCC in overall survival (OS) and disease-free survival (DFS). Although *p16* expression was significantly correlated with a poor prognosis in OS and DFS (*P* < .05)

Conclusions: Our results indicate that *p16* methylation was linked to the development, age, HBV, and HCV infection of HCC. *p16* methylation or alteration was not associated with the prognosis, but *p16* expression was linked to a poor survival.

Abbreviations: 95% CI = 95% confidence interval, DFS = disease-free survival, HBV = hepatitis B virus, HCC = hepatocellular carcinoma, HCV = hepatitis C virus, HR = hazard ratio, MSP = methylation-specific PCR, OR = odds ratio, OS = overall survival, PRISMA = the preferred reporting items for systematic reviews and meta-analyses, TSG = tumor suppressor gene.

Keywords: disease-free survival, hepatocellular carcinoma, overall survival, *p16*

* Correspondence: Dr. Xinjun Zhang and Dr. Tao Huang, the Affiliated Hospital of Ningbo University, Ningbo, Zhejiang 315020, People's Republic of China (e-mails: zxj14@163.com, huangtao433@163.com)

1. Introduction

Hepatocellular carcinoma (HCC) is the sixth most common malignant disease and the second most common cause of cancer-related deaths.[1] HCC is a prevalent malignancy in Asia, and the incidence of this disease is increasing.[2] According to GLOBO-CAN estimates, approximately 782,500 new cases with HCC were clinically diagnosed, with an estimated 745,500 deaths owing to HCC in 2012 worldwide.[1] Chronic infections with hepatic viruses, such as hepatitis B virus (HBV) and hepatitis C virus (HCV), have been found to be associated with HCC.[3,4] However, the underlying molecular genetic events in hepato-carcinogenesis remain unclear.

Studies have shown that DNA methylation, a key epigenetic mechanism, contributes to the initiation and progression of various types of human carcinomas.[5–7] Aberrant promoter methylation of tumor suppressor genes (TSGs), such as *P14* or O6-methylguanine-DNA methyltransferase, is reported to be involved in HCC development.[8] Located on human chromosome 9p21, the *p16* gene, a family of regulators of the cell cycle, consisting of 3 exons and 2 introns, is a cyclin-dependent kinase inhibitor and plays a key role in cell cycle regulation.[9,10] The loss of *p16* expression through methylation has

revealed that *p16* acts as a TSG, suggesting the frequent inactivation of this gene in human tumors.[11–13] Promoter methylation of the *p16* gene is frequently reported in HCC.[14–16] The downregulation of *p16* expression through promoter methylation has been reported in HCC by some studies.[17–19] Data from the cBioportal (The Cancer Genome Atlas dataset) were used to explore the role of gene alteration, such as mutation, deletion, or amplification.[20]

However, the clinical effect of *p16* promoter methylation in HCC remains to be determined. For example, Zhang et al, 2014[15] reported that a significant relationship was observed between *p16* promoter methylation and HBV infection of HCC patients. No correlation was found between *p16* promoter methylation and HBV infection of HCC patients by Narimatsu et al, 2004.[21] Therefore, we determined this meta-analysis to evaluate the association between *p16* promoter methylation and HCC in cancer versus chronic hepatitis and liver cirrhosis. In addition, we evaluated the correlation of *p16* promoter methylation with clinicopathological features, including age, sex, tumor differentiation, clinical stage, cirrhosis, HBV, and HCV infection. Finally, we analyzed the prognostic role of *p16* methylation, expression, or alteration in OS and DFS.
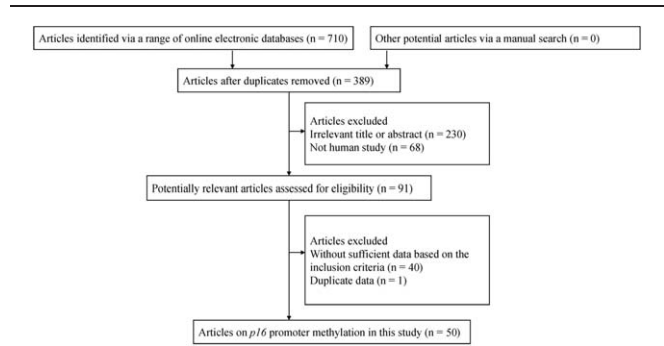
Figure 1. Flow chart of the study selection in the meta-analysis.

2. Materials and methods

2.1. Search strategy

The PubMed, Cochrane Library, Embase, and EBSCO databases were systemically searched to find the relevant articles published up to December 15th, 2016. We used the following key words

Figure 2. Forest plot of the correlation between *p16* promoter methylation and hepatocellular carcinoma in cancer vs. chronic hepatitis (tissue: OR=7.42, 95% CI=3.09–17.78, *P*<.001; blood: OR=12.94, 95% CI=2.29–73.02, *P*=.004). CI=confidence interval, OR=odds ratio.

Figure 3. Forest plot of the correlation between *p16* promoter methylation and hepatocellular carcinoma in cancer vs. liver cirrhosi (tissue: OR=4.87, 95% CI= 3.49–6.78, P<.001; blood: OR=6.44, 95% CI=1.16–35.66, P=.033). CI=confidence interval, OR=odds ratio.
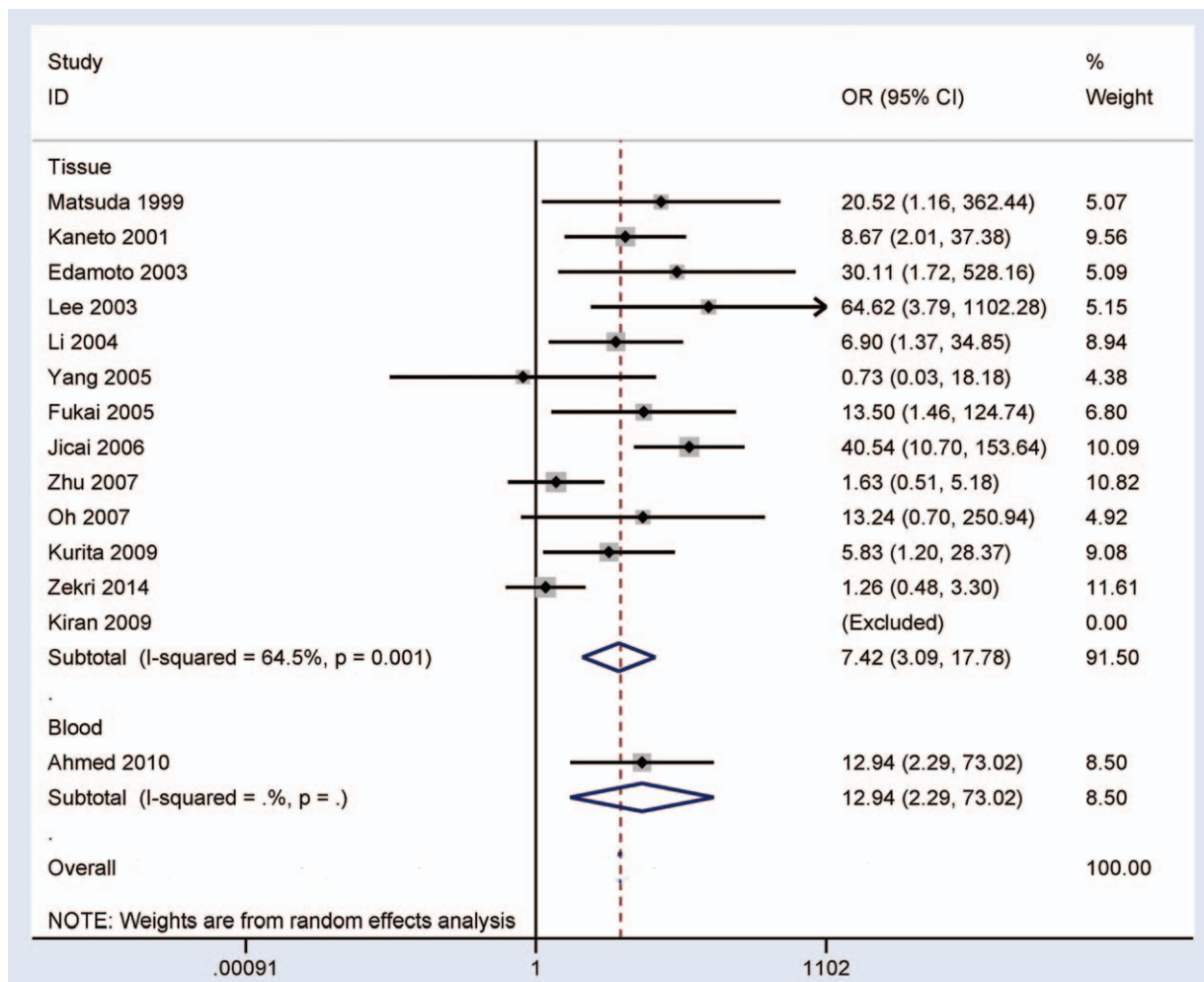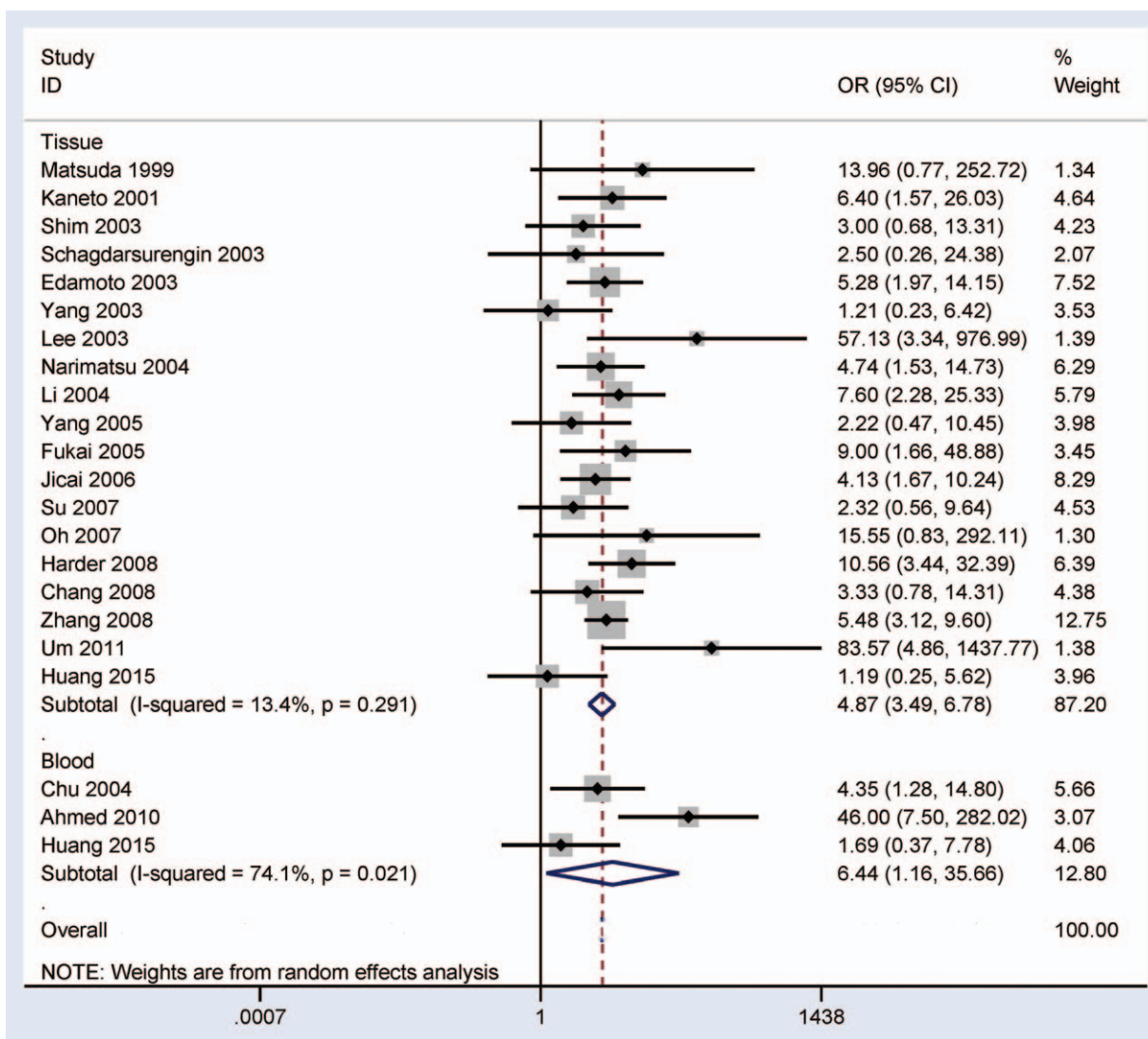
and terms during the search: (p16 OR INK4A OR CDKN2A OR cyclin-dependent kinase inhibitor 2A) AND (liver OR hepato-cellular OR hepatic) AND (cancer OR tumor OR neoplasm OR carcinoma) AND (methylation OR epigene*). Moreover, we performed a manual search of the reference lists from the eligible articles to obtain other potential studies.

2.2. Eligibility criteria

The eligible studies were identified in the present meta-analysis if they met a set of inclusion criteria as follows: patients with HCC were confirmed by histopathologic information; promoter meth-ylation of the *p16* gene was conducted in primary tumor of HCC patients; the methods of methylation included methylation-specific PCR (MSP) and quantitative detection; studies provided data with respect to the correlation between *p16* promoter methylation and HCC in cancer versus nontumor controls (chronic hepatitis and liver cirrhosis); studies should provide sufficient information to analyze the correlation of *p16* promoter methylation with clinicopathological features of HCC patients; studies provided sufficient information on the prognosis in overall survival (OS) and

disease-free survival (DFS) if possible; only studies published in English were included in the meta-analysis. Only the study with larger population or more detailed information was used when multiple articles were published from the same sample data.

2.3. Ethical review

Although this study was not primary research involving human participants, our study was a secondary analysis regarding human subject data published in the public domain.

2.4. Data extraction

Two authors (XL and GY) independently reviewed the potential articles and extracted the following information from the included studies: first author's last name, year of publication, country, race, age, clinical stage, detection methods of methylation, level of methylation, the number of study subjects, expression information, OS, DFS, and clinicopathological features (age: >50 vs. ≤50 years; sex: male vs. female; tumor differentiation: poor vs. well/moderate; clinical stage: stage 3–4 vs. stage 1–2; virus infection: HBV or HCV
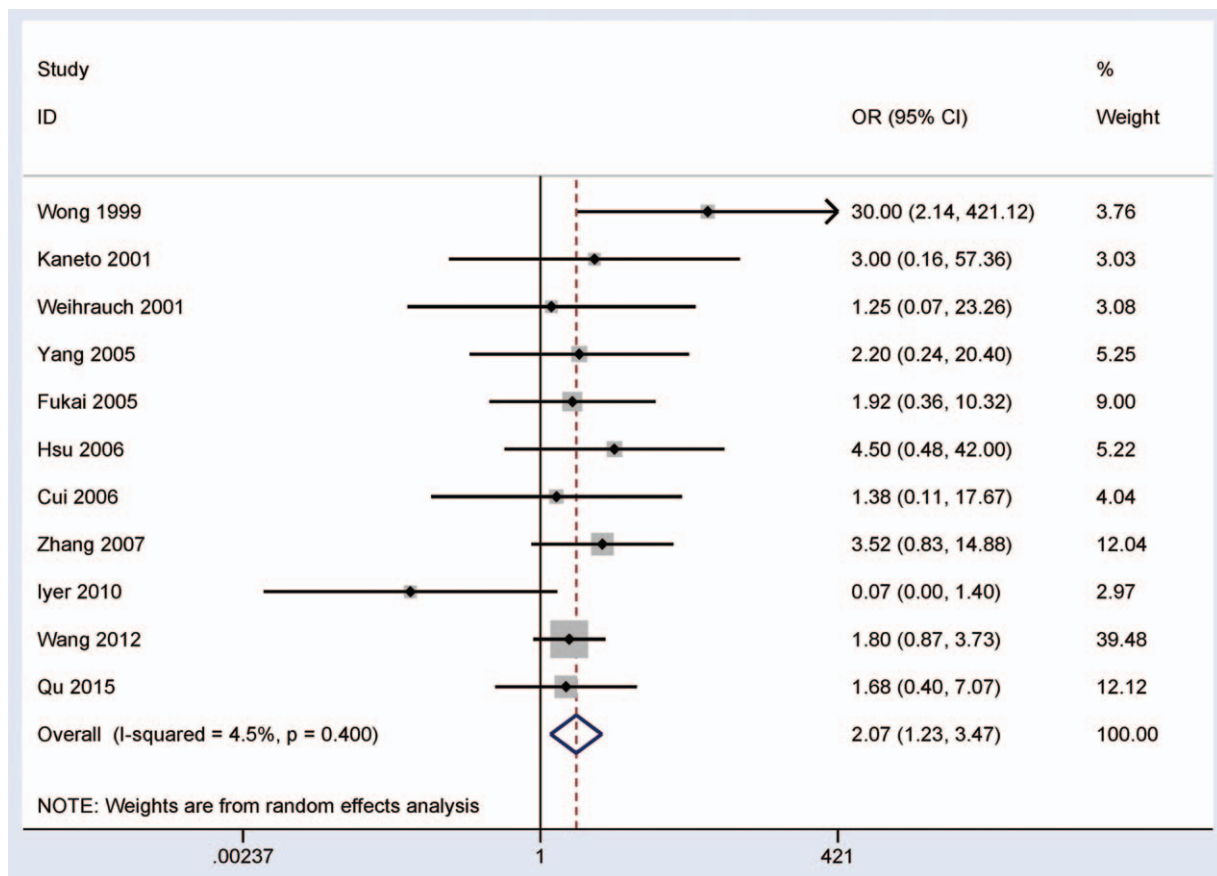
Figure 4. Forest plot of the correlation showing the pooled OR of *p16* promoter methylation with age factor of hepatocellular carcinoma (>50 vs. ≤50 years: OR = 2.07, 95% CI = 1.23–3.47, *P* = .006). CI = confidence interval, OR = odds ratio.

vs. negative virus infection; cirrhosis: yes vs. no). Any disagreements were resolved by all authors' discussion.

2.5. Statistical analysis

The meta-analysis was accomplished using the Stata software (version 12.0, Stata Corporation, College Station, TX). The combined odds ratios (ORs) and their 95% confidence intervals (95% CIs) were calculated to estimate the relationship between HCC and nontumor controls, and the correlation of *p16* promoter methylation with clinicopathological characteristics of patients with HCC. The pooled hazard ratios (HRs) and their 95% CIs were also calculated to evaluate the prognostic effect of *p16* methylation, expression, or alteration in OS or DFS if possible. Heterogeneity among the included studies was measured based on the Cochran's Q test.[22] The random-effects model was chosen in the meta-analysis.[23,24] When an obvious evidence of heterogeneity was found in this meta-analysis, a sensitivity analysis was conducted to evaluate the change of the pooled OR by deleting an individual study for the results with more than five studies.[25] Egger test was used to detect the potential publication bias for the results with >9 studies.[26]

## 3. Results

3.1. Characteristics of the eligible studies

According to the above selection criteria, a total of 50 eligible articles published between 1999 and 2015[14–19,21,27–69]

were included in this final meta-analysis (Fig. 1), including 2279 patients with HCC and 808 nontumor controls (chronic hepatitis and liver cirrhosis). Of these eligible studies, 14 studies evaluated the correlation between *p16* promoter methylation and HCC in cancer versus chronic hepatitis.[18,30,34,36,38,43,44,46,50,51,55,58,64,68] Twenty-one articles reported the available data on the correlation between *p16* promoter methylation and HCC in cancer versus liver cirrhosis.[17,18,21,29,33,34,39, 40,42,43,46,50,51,54,55,57–60,64,68] A total of 38 studies involving 1726 patients with HCC assessed the relationship of *p16* promoter methylation with clinicopathological characteristics of HCC,[14–19,21,27,28,31,32,35,37–39,41,44–53,56–59, 61–67,69] including age, sex, tumor differentiation, clinical stage, cirrhosis, HBV, and HCV infection. Three studies involving 125 HCCs reported the survival information in OS or DFS.[28,43,51] The baseline characteristics of the included studies are summarized in Table S1.

3.2. Association between p16 promoter methylation and HCC in cancer versus nontumor controls

When HCC was compared to chronic hepatitis, the result of tissue samples showed that the frequency of *p16* promoter methylation in HCC was significantly higher than in chronic hepatitis (OR = 7.42, 95% CI = 3.09–17.78, *P* < .001), including 525 HCC patients and 252 patients with chronic hepatitis (Fig. 2). The relationship between *p16* promoter methylation and HCC was found in the blood of 1 study (OR = 12.94, 95% CI = 2.29–73.02, *P* = .004) (Fig. 2).
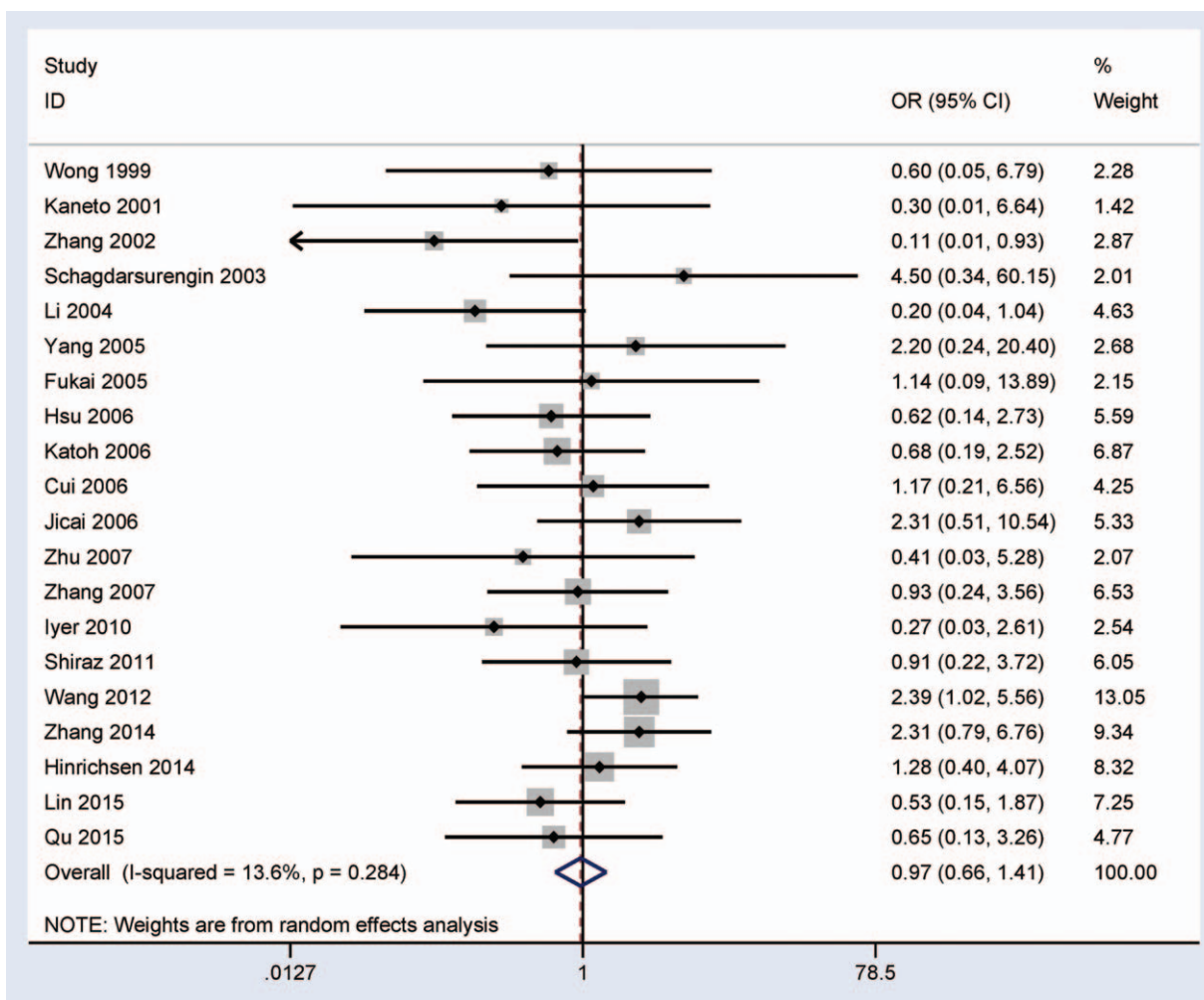
Figure 5. Forest plot of the correlation showing the pooled OR of *p16* promoter methylation with gender of hepatocellular carcinoma (male vs. female: OR = 0.97, 95% CI = 0.66–1.41, *P* = .869). CI = confidence interval, OR = odds ratio.

When HCC was compared to liver cirrhosis, the results showed that *p16* promoter methylation was correlated with HCC in tissue and blood samples (tissue: OR = 4.87, 95% CI = 3.49–6.78, *P* < .001; blood: OR = 6.44, 95% CI = 1.16–35.66, *P* = .033) (Fig. 3), including 19 studies of the tissue with 865 HCCs and 486 patients with liver cirrhosis, and 3 studies of the blood with 102 HCCs and 53 cases of liver cirrhosis. The analyses should be cautious because of small sample sizes in the blood.

3.3. Association between p16 promoter methylation and age of HCC

The result involving 11 studies with 416 HCC patients demonstrated that *p16* promoter methylation was correlated with age of patients with HCC (>50 vs. ≤50 years: OR = 2.07, 95% CI = 1.23–3.47, *P* = .006) (Fig. 4).

3.4. Association between p16 promoter methylation and gender of HCC

No significant correlation was found between *p16* promoter methylation and sex of HCC (OR = 0.97, 95% CI = 0.66–1.41, *P* = .869), including 20 studies with 908 HCC patients (Fig. 5).

3.5. Association between p16 promoter methylation and tumor differentiation of HCC

No association between *p16* promoter methylation and tumor differentiation of HCC was found among 20 studies with 886 patients with HCC (OR = 0.97, 95% CI = 0.67–1.42, *P* = .888) (Fig. 6).

3.6. Association between p16 promoter methylation and tumor stage of HCC

No association was observed between *p16* promoter methylation and clinical stage of HCC (OR = 1.19, 95% CI = 0.61–2.34, *P* = 0.606) (Fig. 7), including 11 studies with 520 HCCs.

3.7. Association between p16 promoter methylation and HBV infection of HCC

When HCCs with only HBV infection were compared to HCCs without virus infection, the result showed that promoter methylation of the *p16* gene was significantly higher in HCC with HBV infection than in HCC without hepatitis virus infection (OR = 2.14, 95% CI = 1.22–3.72, *P* = .008) (Fig. 8), including 28 studies with 876 patients.
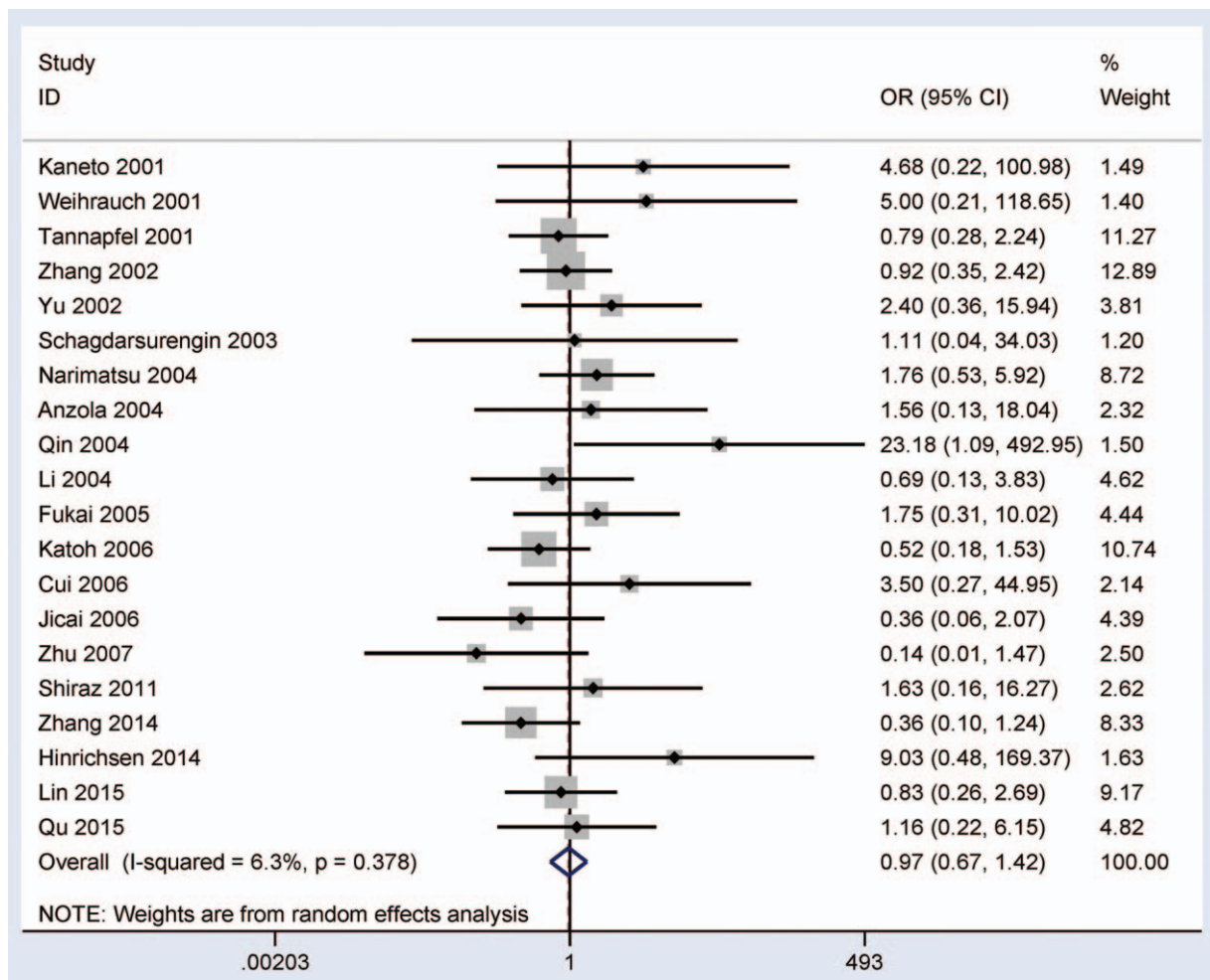
Figure 6. Forest plot of the association showing the pooled OR of *p16* promoter methylation with tumor differentiation of hepatocellular carcinoma (poor vs. well/moderate: OR=0.97, 95% CI=0.67–1.42, *P*=.888). CI=confidence interval, OR=odds ratio.

3.8. Association between p16 promoter methylation and HCV infection of HCC

When HCCs with only HCV infection were compared to HCCs without virus infection, data analysis included 17 studies with 508 cases, the result showed that the combined OR was 2.24 (95% CI=1.24–4.05, *P*=.008) (Fig. 9).

3.9. Association between p16 promoter methylation and cirrhosis of HCC

When HCCs with cirrhosis were compared to HCCs without cirrhosis, the pooled OR showed no significant relationship between *p16* promoter methylation and cirrhosis of HCC (OR=1.31, 95% CI=0.81–2.11, *P*=.27) (Fig. 10).

3.10. Sensitivity analysis

When substantial heterogeneity was detected in cancer versus chronic hepatitis, in relation to clinical stage, HBV, and HCV infection (*P*<.1), the sensitivity analyses were carried out to determine the influence of the overall results by excluding 1 study (Figures S1 and S2 , http://links.lww.com/MD/B880).

When HCC was compared to chronic hepatitis, we removed 2 studies (Zero et al, 2014, in Egypt[30] and Zhu et al, 2007, in

China),[44] and recalculated the overall OR (OR=12.79, 95% CI=6.67–24.53, *P*<.001), with a significantly decreased heterogeneity (*P*=.382).

When advanced stage HCC (stage 3–4) was compared to early stage HCC (stage 1–2), one study (Katoh et al, 2006)[48] was removed, and the recalculated OR was 1.42 (95% CI= 0.78–2.57). The result showed that no significant heterogeneity was found (*P*=.150).

In the comparison of HCC cases with HBV infection and HCC cases without hepatitis virus infection, we deleted 2 studies (Zhang et al, 2014, in China[15] and Jicai et al, 2006, in China),[46] and recalculated the overall OR (OR=1.42, 95% CI=0.97–2.07), with no evidence of heterogeneity (*P*=.382). Based on the omission of this study by Hinrichsen et al, 2014, in Germany,[14] the pooled OR between *p16* promoter methylation and HCV infection status was 2.65 (95% CI=1.53–4.59), resulting in no heterogeneity (*P*=.268).

3.11. Publication bias

Egger test was performed to evaluate the possible publication bias in HCC versus chronic hepatitis and liver cirrhosis, and in relation to age, sex, tumor differentiation, clinical stage, cirrhosis, HBV, and HCV infection. No obvious evidence of publication bias was noted in HCC versus chronic hepatitis and liver
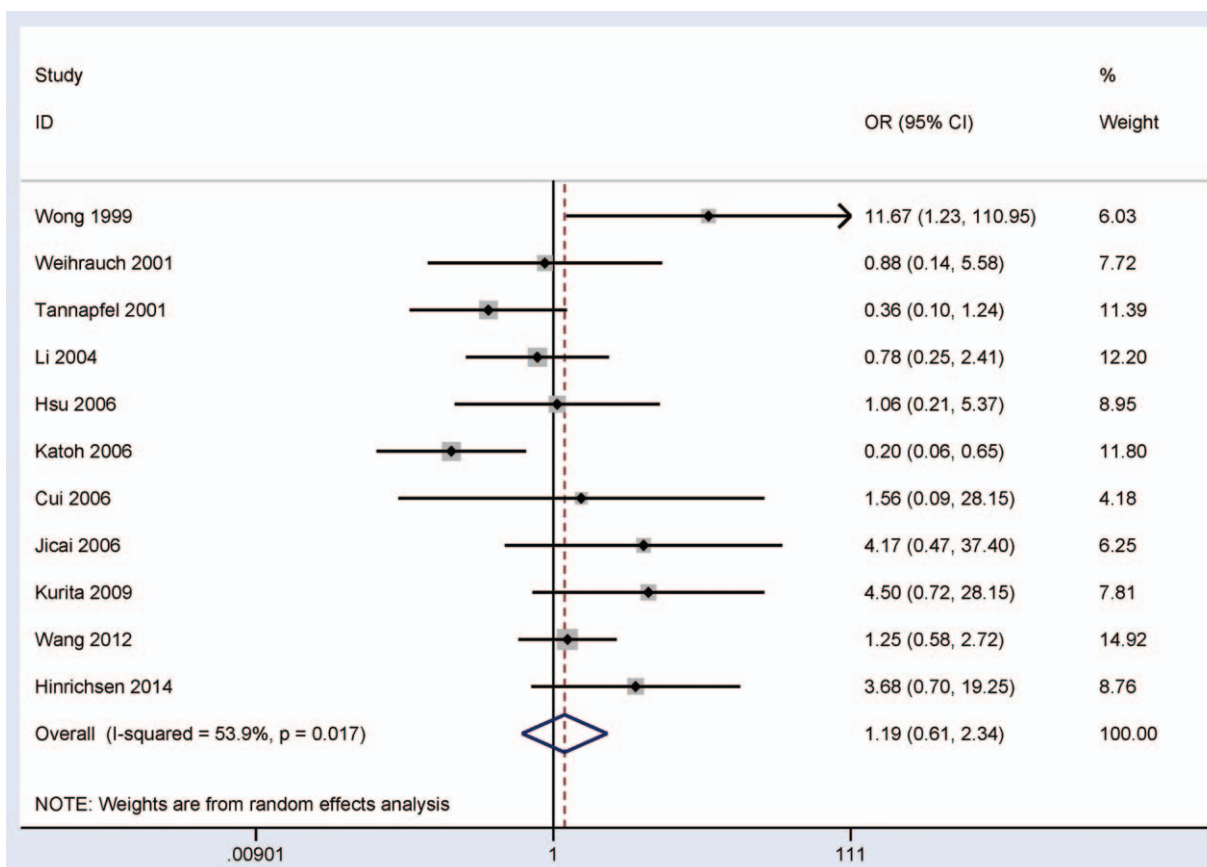
Figure 7. Forest plot of the association showing the pooled OR of *p16* promoter methylation with clinical stage of hepatocellular carcinoma (stage 3–4 vs. stage 1–2: OR = 1.19, 95% CI = 0.61–2.34, P = .606). CI = confidence interval, OR = odds ratio.

cirrhosis, and in relation to age, clinical stage, cirrhosis, HBV, and HCV infection (*P* > .05) (Figures S3 and S4 , http://links.lww.com/MD/B880). A slight publication bias was measured between *p16* promoter methylation and sex, and tumor differentiation (*P* = .036 and *P* = .019) (Figures S3 and S4 , http://links.lww.com/MD/B880).

3.12. Prognostic role of p16 promoter methylation in OS and DFS

No correlation was reported between *p16* promoter methylation and OS,[28,43] and DFS of patients with HCC.[28,43,51]

3.13. Prognostic role of p16 expression form Gene Expression Profiling Interactive Analysis

The association between *p16* expression and OS or DFS was analyzed from Gene Expression Profiling Interactive Analysis[70] among 384 HCC patients. The results showed that *p16* expression was correlated with a poor prognosis in OS and DFS (*P* < 0.05) (Figs. 11 and 12).

3.14. Prognostic role of p16 alteration from the Cancer Genome Atlas Research Network

The data from the Cancer Genome Atlas Research Network were finally analyzed to assess the relationship between *p16* alteration

and the prognosis in OS and DFS among 442 HCC patients.[20,71] *p16* alteration had a frequency of 8% among 366 sequenced HCCs. No correlation was found between *p16* alteration and HCC in terms of OS and DFS (*P* > .05) (Figures S5 and S6 , http://links.lww.com/MD/B880).

4. Discussion

Epigenomic regulation of genes involves in 2 major molecular mechanisms, the hypermethylation of TSGs, and hypomethylation of oncogenes, which may play important roles in cancer carcinogenesis and progression.[72–74] The downregulation of TSGs expression via promoter methylation within CpG islands has been indicated as a key molecular mechanism in cancer.[75,76] Some studies suggest that promoter methylation of the *p16* gene is closely associated with its expression, with the loss of *p16* expression in HCC, which may play a key role in HCC development.[19,21,51,52,66] However, the exact correlation underlying *p16* promoter methylation-associated HCC is still unclear. This meta-analysis was performed to estimate the clinical effect of *p16* promoter methylation in patients with HCC.

In the current meta-analysis, 4 studies showed that no significant correlation was found between HCC and chronic hepatitis.[30,43,44,50] *P16* promoter methylation had a same frequency in HCC and chronic hepatitis.[36] The other studies showed that *p16* promoter methylation in HCC was significantly higher than in chronic hepatitis.[18,38,46,51,55,58,64,68] Nine studies
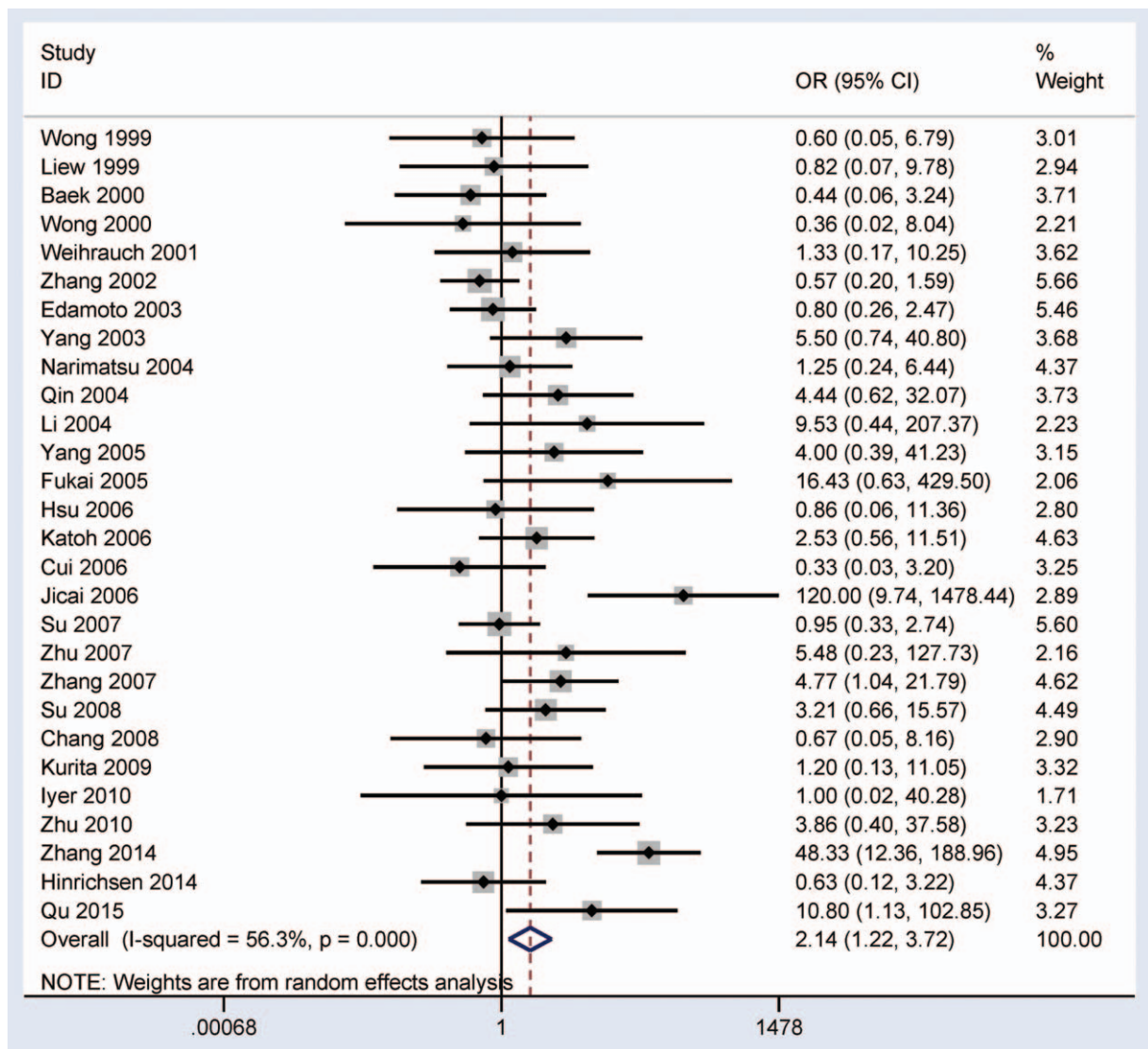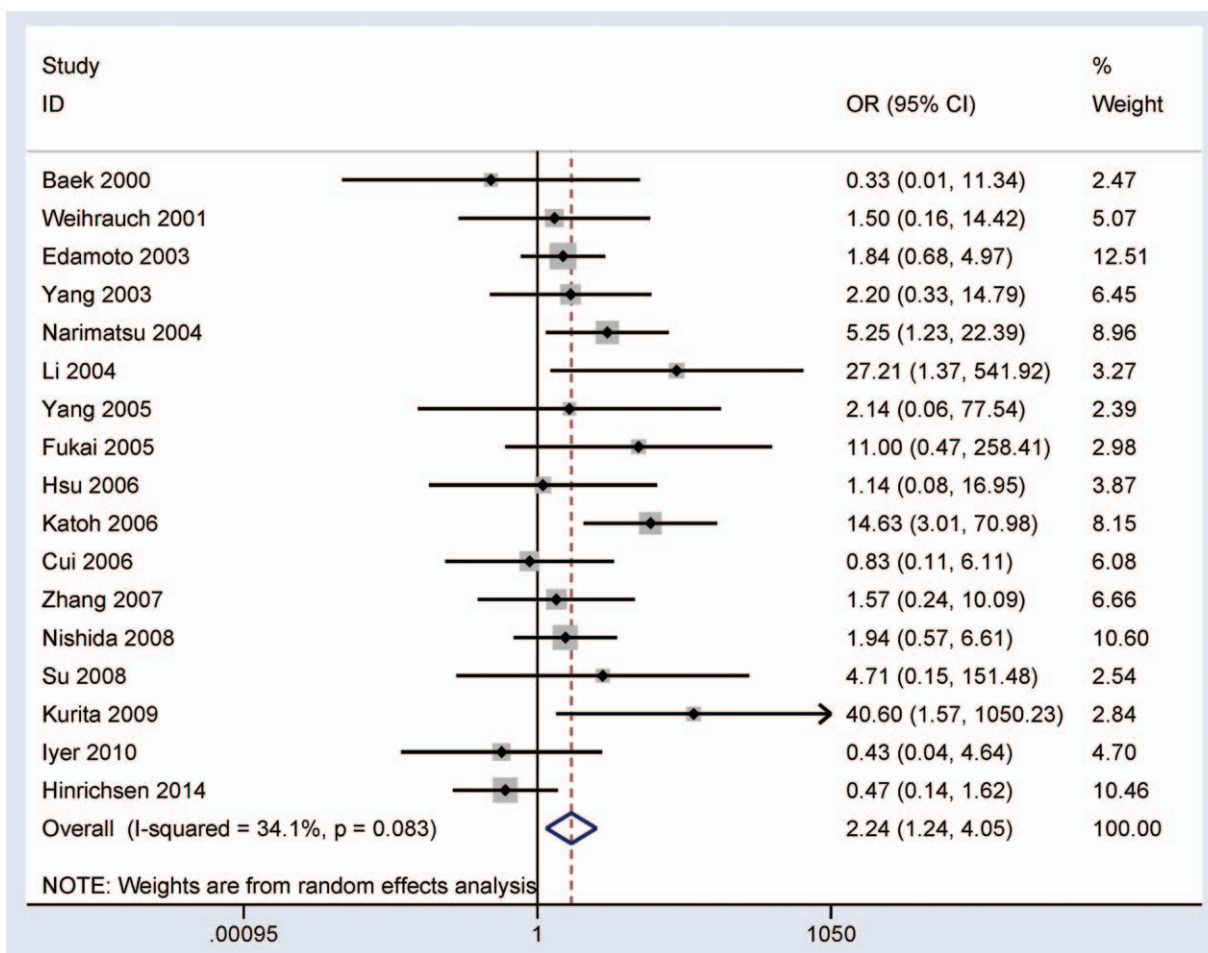
Figure 8. Forest plot of the association showing the pooled OR of *p16* promoter methylation with hepatitis B virus (HBV) infection of hepatocellular carcinoma (HCC) (HBV-positive vs. hepatic viruses-negative HCC: OR=2.14, 95% CI=1.22–3.72, *P*=.008). CI=confidence interval, OR=odds ratio.

showed no correlation between HCC and liver cirrhosis,[17,29,39,43,50,57,59,60,68] and the remaining studies showed that *p16* promoter methylation had a significantly higher level in HCC than in liver cirrhosis.[18,21,33,40,42,46,51,55,58,64] The combined results of tissue samples showed that *p16* promoter methylation in HCC was notably higher than in chronic hepatitis and liver cirrhosis, suggesting that *p16* promoter methylation was closely correlated with HCC development. Additionally, we also found that *p16* promoter methylation was correlated with HCC in the blood, which indicated that *p16* promoter methylation may distinguish HCC and benign lesions as a noninvasive biomarker using blood samples. Based on small sample sizes in the blood, additional studies with large population are needed to confirm its diagnostic effect in blood samples.

Next, we evaluated the correlation between *p16* promoter methylation and clinicopathological features of HCC. One study showed a significantly positive correlation between *p16* promoter methylation and age.[69] Although no significant correlation between *p16* promoter methylation and age was observed among other studies.[18,19,27,32,37,45,47,49,50,64] The current result involving a larger HCC patients demonstrated that *p16* promoter methylation was positively associated with age factor. Additionally, no significant correlation was found between *p16* expression/alteration and age factor of HCC from the cBioportal database (data not shown). We found that *p16* promoter methylation was not correlated with sex, cirrhosis, tumor differentiation, and clinical stage. Meanwhile, studies reported that *p16* promoter methylation was not associated with the prognosis of HCC in OS[28,43] and DFS.[28,43,51] No significant relationship was observed between *p16* methylation and the prognosis of patients with HCC. The above analyses revealed that *p16* promoter methylation was not correlated with HCC progression, metastasis, and prognosis. We used the cBioportal database to explore the potential prognostic effect of *p16* gene alteration (mutation and deep deletion) in HCC, which showed that no correlation was found between *p16* alteration and the survival of HCC patients. Although *p16* expression was significantly linked to a worse prognosis of HCC in OS and DFS. Other mechanisms such as allelic loss or amplification were

Figure 9. Forest plot of the association showing the pooled OR of *p16* promoter methylation with hepatitis C virus (HCV) infection of hepatocellular carcinoma (HCC) (HCV-positive vs. hepatic viruses-negative HCC: OR=2.24, 95% CI=1.24–4.05, *P*=.008). CI=confidence interval, OR=odds ratio.

not very clear, which may cause *p16* gene expression. And *p16* expression influenced the survival of HCC.

p16 promoter methylation was significantly associated with HBV infection among four studies.[15,27,45,46] Other studies shown no association between *p16* promoter methylation and HBV infection of HCC cases.[14,17–19,21,35,37–39,41,44,47–52,57,58,62,65–67,69] Four studies reported that *p16* promoter methylation was closely correlated with HCV infection in HCC.[21,38,48,51] No correlation among the remaining studies was observed between *p16* promoter methylation and HCV infection of HCC.[14,16,18,19,37,41,45,47,49,50,57,58,66] Our results involving lager patients (876 HBV-related HCC cases and 508 HCV-related HCC cases) showed that promoter methylation of the *p16* gene was significantly correlated with HBV or HCV infection, and it was more frequent in HBV-positive or HCV-positive HCC than in hepatic viruses-negative HCC.

There was obvious evidence of heterogeneity in cancer versus chronic hepatitis, in relation to clinical stage, HBV, and HCV infection (*P* < .1). Thus, the sensitivity analyses were conducted in our study. Two studies[30,44] (MSP method) were removed in cancer versus chronic hepatitis, and 1 study[48] (MSP method) was excluded in relation to clinical stage. In addition, 2 studies were removed[15,46] (MSP method) in HBV-positive HCC versus hepatic viruses-negative HCC, and 1 study[14] (MSP method) was

deleted in HCV-positive HCC versus hepatic viruses-negative HCC. The combined OR was not significantly changed, with no evidence of heterogeneity (*P* > .1). The sensitivity analyses revealed that our study was stable and credible. The detailed reasons of significant heterogeneity were not clear, perhaps because of the use of inappropriate or different conditions of MSP in detection of *p16* methylation.

A previous meta-analysis only reported that *p16* methylation was significantly higher in HCC than in normal or adjacent tissues by Zhang et al, 2016.[77] The present results compare favorably with the previous meta-analysis by Zang et al, 2011,[78] which also reported that *p16* promoter methylation was correlated with HCC risk in cancer versus liver cirrhosis. The number of study population included in our meta-analysis (n= 1351 tissues) was significantly larger than in the previous meta-analysis (n=408 tissues). Additionally, this previous meta-analysis did not analyze the correlation of *p16* promoter methylation between HCC and chronic hepatitis.

The present meta-analysis had some limitations. First, our study mainly included Asian population, and other ethnic groups, such as the white and mixed populations, were small. Second, a slight publication bias was detected in relation to sex and tumor differentiation. Studies published in English language were selected in this meta-analysis, articles published in other
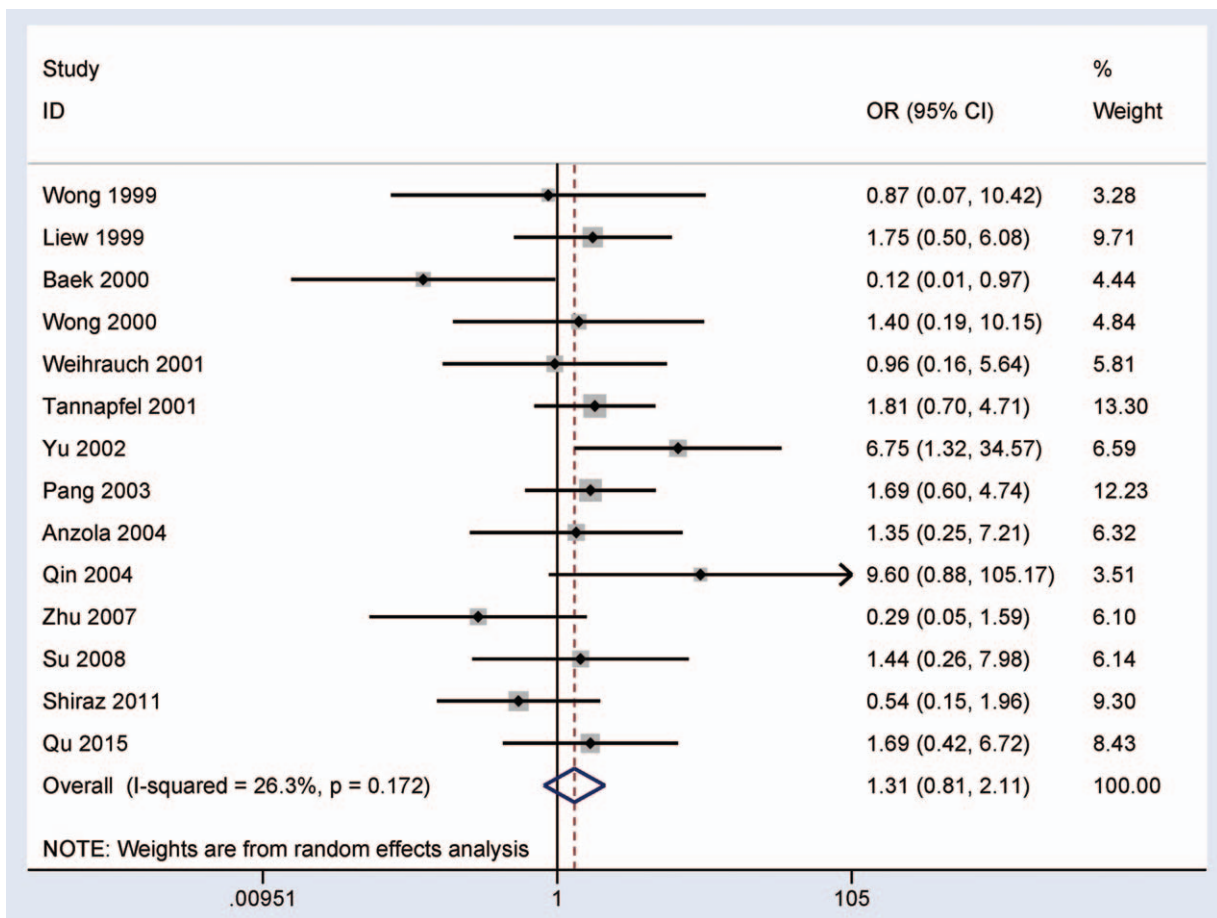
Figure 10. Forest plot of the association showing the pooled OR of *p16* promoter methylation with cirrhosis of hepatocellular carcinoma (cirrhosis [yes vs. no]: OR = 1.31, 95% CI = 0.81–2.11, *P* = .27). CI = confidence interval, OR = odds ratio.
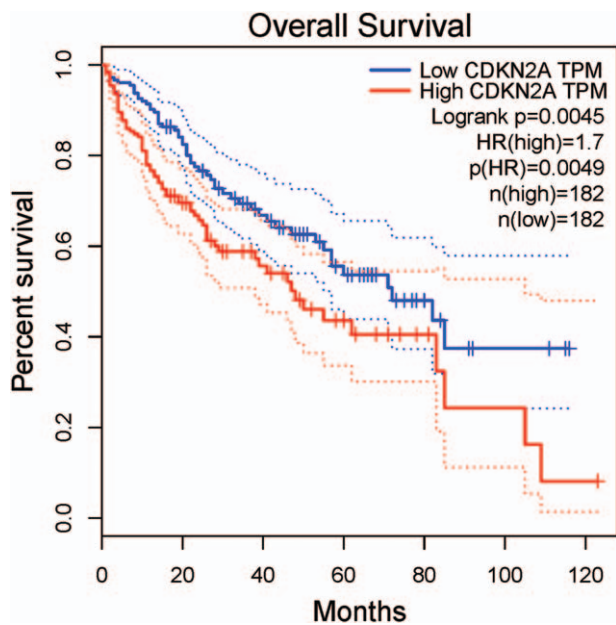
Figure 11. The correlation between *p16* expression and overall survival (OS) (*P* < .05), *p16* overexpression (red line) showed a poor OS of hepatocellular carcinoma. CI = confidence interval, OR = odds ratio.
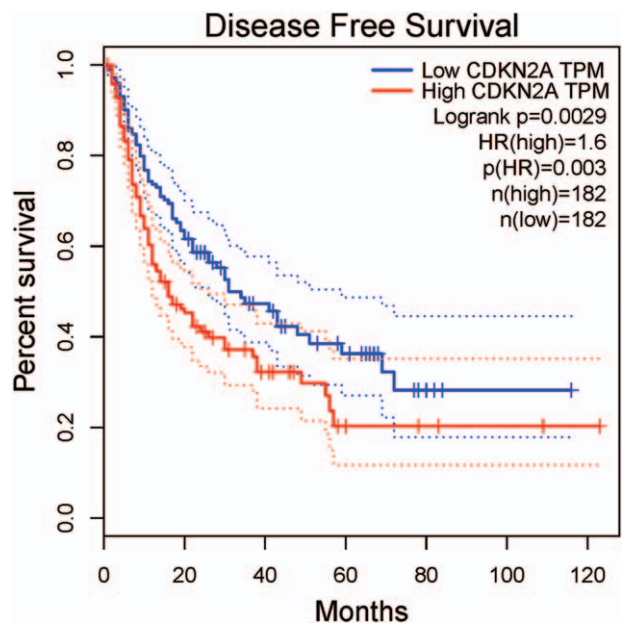
Figure 12. The correlation between *p16* expression and disease-free survival (DFS) (*P* < .05), *p16* overexpression (red line) showed a poor DFS of hepatocellular carcinoma. CI = confidence interval, OR = odds ratio.

languages and other styles such as conferences abstract were excluded because of insufficient information, which may result in a potential bias. Third, the studies of blood samples were fewer, which are essential to validate whether *p16* promoter methylation may be a noninvasive diagnostic biomarker.

In conclusion, our meta-analysis suggests that *p16* promoter has a higher methylation level in HCC than in chronic hepatitis and liver cirrhosis, higher in patients >50 years' old than in patients aged <50 years, and higher in HBV or HCV-positive HCC than in hepatic viruses-negative HCC. However, *p16* promoter methylation was not linked to sex, cirrhosis, tumor differentiation, clinical stage of patients with HCC. *p16* methylation or alteration was not associated with the prognosis of patients with HCC. Whereas *p16* expression was notably associated with a poor prognosis in OS and DFS. Further well-designed studies with large population are necessary to support our findings in the future.

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