Using circulating tumor DNA as a novel biomarker to screen and diagnose hepatocellular carcinoma: A systematic review and meta-analysis

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Abstract
Purpose: A meta-analysis was formulated to appraise the diagnostic accuracy of circulating tumor DNA (ctDNA) in hepatocellular carcinoma (HCC).

Materials and Methods: We enrolled all relevant studies published until September 2019. Four primary subgroups were investigated: the subgroup of quantitative or qualitative analysis of ctDNA, the subgroup of Ras association domain family 1 isoform A (RASSF1A) methylation in ctDNA and the subgroup of the combined alpha-fetoprotein (AFP) and ctDNA assay. We analyzed the pooled sensitivity (SEN), specificity (SPE), positive likelihood ratio (PLR), negative likelihood ratio (NLR), diagnostic odds ratio (DOR), and summary receiver operating characteristic (SROC) as well as the area under the curve (AUC).

Results: A total of 33 qualified articles with 4113 subjects were incorporated into our meta-analysis. The combined SEN, SPE, and DOR in quantitative studies were 0.722 (95% confidence interval (95% CI): 0.686-0.756), 0.823 (95% CI: 0.789-0.854), 18.532 (95% CI: 8.245-41.657), respectively, yielding an AUC of 0.880. For qualitative studies, the corresponding value was 0.568 (95% CI: 0.548-0.587), 0.882 (95% CI: 0.867-0.897), 10.457 (95% CI: 7.270-15.040) and 0.767, respectively. Detection of RASSF1A methylation yielded an AUC of 0.841, with a SEN of 0.644 (95% CI: 0.608-0.678) and a SPE of 0.875 (95% CI: 0.847-0.900). AFP combined with ctDNA assay achieved an AUC of 0.944, with a SEN of 0.760 (95% CI: 0.728-0.790) and a SPE of 0.920 (95% CI: 0.893-0.942).

Conclusion: Circulating tumor DNA displays a promising diagnostic potential in HCC. However, it is not independently sufficient and can serve as an assistant tool combined with AFP for HCC screening and detection.

Keywords
circulating tumor DNA, diagnostic accuracy, hepatocellular carcinoma, meta-analysis, methylation
1 | INTRODUCTION

Liver cancer, with over 841,000 patients globally, has currently become the second most frequent reason for tumor-related deaths.\(^1,2\) Hepatocellular carcinoma (HCC), as the most common pathologic subtype of primary liver tumors, occupies approximately 90% of all patients.\(^3,4,5\) The prognosis of untreated HCC patients is undesirable with a median survival of 2-14 months.\(^2,4,5\) Compelling observational data have demonstrated that earlier HCC detection and therapeutic interventions are conducive to boosting the overall survival of patients.\(^6\)

Currently, surgical intervention, such as partial hepatic resection and hepatic transplantation remain the primary therapeutic strategies for HCC patients. Indeed, if patients with early HCC that is currently hard to recognize and delineate could be accurately diagnosed, the 5-year survival rate for HCC patients who have received surgery would reach up to 90%.\(^7\) Unfortunately, a large proportion of HCC individuals are usually diagnosed at an advanced stage on account of the non-specific clinical symptoms and the limitations in detection methods, thus triggering that fewer than 30% of the patients are qualified for surgical treatment.\(^8,9\) Early screening for HCC has been conducted in several cohorts following the Asian-Pacific Association for the Study of the Liver guidelines, which advocates that HCC surveillance should be implemented for clinical subjects with liver cirrhosis and those with positive surface antigen of hepatitis B virus (HBsAg) by utilizing liver ultrasonography (US) and serum alpha-fetoprotein (AFP) test every 6 months.\(^10\)

Nevertheless, the diagnostic efficiency of AFP assay for HCC is not satisfactory, with a sensitivity (SEN) of 25%-65% and a specificity (SPE) lower than 82%, respectively.\(^11\) When liver US is applied for the detection of HCC nodules smaller than one cm, its SEN is approximately 60%.\(^12\) Additionally, the fluctuation in AFP levels is also associated with inflammation and liver disease type.\(^13\) For example, AFP levels may be enhanced in non-HCC conditions, including chronic liver diseases (such as liver cirrhosis and hepatic inflammatory), other tumors (such as intrahepatic cholangiocarcinoma and metastatic colon cancer) as well as pregnancy.\(^14-16\) Therefore, the detection of HCC with these methods remains suboptimal, it is imperative to develop additional biomarkers for early detection and diagnosis of HCC in a minimally invasive, convenient and accurate manner.

Accumulating evidence has indicated that the cumulation of genetic and epigenetic changes in liver tissue results in the tumorigenesis and development of HCC, which is intimately associated with the surrounding microenvironment.\(^17\) Recent progresses in the field have highlighted that the minimally invasive detection of circulating tumor DNA (ctDNA) confers a promising opportunity for the early screening and diagnosis of HCC. This assay, in conjunction with circulating tumor cells and circulating cell-free DNA, is termed “liquid biopsy”.\(^18\) Circulating tumor DNA is generally derived from apoptotic or necrotic tumor cells and further released into the circulation,\(^19\) which carries cancer-specific modifications in gene or epigenetics, including single nucleotide mutation,\(^20\) copy number aberration (CNA),\(^21\) and DNA methylation\(^19\) or 5-hydroxymethylcytosines.\(^16\) Quantitative alteration and qualitative alteration of ctDNA are primarily detected in HCC patients. The former is associated with measuring the quantity of ctDNA that is generally increased in HCC patients,\(^22\) and the latter refers to monitoring tumor-specific genetic aberrations. Specifically, with the booming development of next generation sequencing, a growing number of studies have been concentrating on the “methylation pattern” of ctDNA in HCC patients and demonstrated that tumor-specific alterations in methylation may represent a novel discriminatory tool for the screening, detection, and diagnosis of HCC. Thus, by deciphering the information of deoxyribonucleic acid derived from HCC patients’ circulation, clinicians can utilize this “liquid biopsy” technology to confer precise diagnosis and appropriate therapy for HCC patients.

Although a considerable number of studies have revealed the diagnostic efficiency of ctDNA for HCC, the results are very diverse partially ascribed to the discrepancies in study design and assay methods for ctDNA among studies. Thus, prior to its clinical utilization, a comprehensive analysis and evaluation of the diagnostic value of ctDNA in HCC is imminently required. Herein, we implemented a meta-analysis to objectively assess the diagnostic performance of ctDNA assays for HCC, which potentially confers guideline for technology improvement and clinical applications.

2 | MATERIALS AND METHODS

2.1 | Search strategy

All potentially relevant articles that were published up to September 2019 were retrieved and the following electronic databases were independently queried by two authors: PubMed, Web of Science, Embase, Cochrane Library, and China National Knowledge Infrastructure. The query terms were as follows: “circulating tumor DNA” OR “circulating DNA” OR “ctDNA” OR “plasma DNA” OR “serum DNA” OR “blood DNA” AND “liver cancer” OR “hepatocellular carcinoma” OR “liver neoplasms” OR “hepatic carcinoma” OR “liver tumor” AND “diagnosis” OR “sensitivity” OR “specificity” OR “accuracy”. The language of all articles was limited to English. We also manually screened the reference from the included articles and relevant reviews for enlarged retrieval.
2.2 | Inclusion and exclusion criteria

The publications that conformed to the following criteria were incorporated: (a) ctDNA indicators were used for the first diagnosis rather than the recurrent diagnosis of HCC; (b) the numerical value of SEN and SPE could be collected either directly from the papers or could be calculated in each study; and (c) specimens were extracted from peripheral blood. The exclusion criteria were as follows: (a) review, case report, letter or conference abstract; (b) the sample size of studies was less than 10; and (c) duplicate or overlapping publications that included the same population and gene. Two authors independently evaluated the eligibility of studies. Discrepancies were resolved via consensus.

2.3 | Data extraction

Two authors independently conducted data extraction from the included studies and further summarized the ultimate results. The information extracted from the incorporated publications was as follows: the first author's name, publication year, region/country, study design, participant characteristics (including sample size, control type), detection details (including source of specimens, sampling time, experimental

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**FIGURE 1** A PRISMA flow diagram of the literature search. CAN, copy number aberration; CNKI: China National Knowledge Infrastructure; ctDNA: circulating tumor DNA; HBV: hepatitis B virus; HCC, hepatocellular carcinoma; PRISMA, Preferred Reporting Items for Systematic Reviews and Meta-Analyses; SEN, sensitivity; SPE, specificity
methods, reference gene, cutoff values), diagnostic performance (including SEN and SPE, true positive (TP), true negative (TN), false positive (FP), and false negative (FN), positive likelihood ratio (PLR) and negative likelihood ratio (NLR), and diagnostic concordance).

2.4 Quality assessment

Based on the revised Quality Assessment of Diagnostic Accuracy Studies-2 (QUADAS-2), the risk of bias and concerns about applicability of all included publications were evaluated as “low risk”, “high risk” and “unclear risk” through four key domains including patient selection, index test, reference standard, and flow and timing.23 All the studies were independently assessed and rated by two authors. Divergence was discussed until an agreement was reached. If an article was evaluated to be of poor quality by two authors, it would be excluded.

2.5 Statistical analysis

We utilized RevMan Manager 5.3 and Meta-Disc 1.4 software to conduct this diagnostic meta-analysis. The pooled SEN and SPE, PLR, NLR, diagnostic odds ratio (DOR) and corresponding 95% confidence interval (95% CI) were calculated as evaluation indicators.24 Simultaneously, the summary receiver operating characteristic (SROC) curve and its corresponding area under the curve (AUC) value were formulated to evaluate the overall test accuracy.25-28 The closer the AUC value was to 1, the higher was the diagnostic efficiency.29,30 The AUC range of 0.5-0.7, 0.7-0.9, 0.9-1.0 corresponded to low, moderate or high accuracy, respectively.29,30 The Spearman correlation coefficient and its corresponding P value were used to identify the presence of the threshold effect. Generally, threshold effect was considered to exist when the P value was lower than 0.05. If heterogeneity resulted from non-threshold effect, we utilized the chi-square and I² test to evaluate the heterogeneity among the studies.31 The Deek's funnel plot was formulated to examine the existence of potential publication bias.32 A result with P value < .05 was considered to be statistically significant.

3 RESULTS

3.1 Study characteristics

Figure 1 shows a Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow diagram adapted from Moher et al34 depicting the retrieval strategy of databases to incorporate qualified publications. Initially, a total of 610 publications were queried through our search strategy. Eventually, 33 eligible articles22,35-66 published from 2000 to 2019 were incorporated into this diagnostic meta-analysis following the exclusion of duplicate studies, the examination of title and abstract as well as the comprehension of full text. Specifically, all the included studies consisted of quantitative analysis to measure ctDNA concentration (n = 5)22,56-59 and qualitative analysis to unravel tumor-specific single-gene methylation in ctDNA (n = 25),35-55,63-66 as well as both quantitative and qualitative analysis (n = 3).50-62 Among these 33 publications, 11 articles described the diagnostic role of circulating Ras association domain family 1 isoform A (RASSFIA) methylation in HCC37,39,40,48-50,52,60-62,65 and eight articles evaluated the diagnostic performance of ctDNA combined with AFP assay in HCC38,40,44,54,56 Nine articles assessed the diagnostic accuracy of AFP assay for HCC37,40-44,62-64. Our study enrolled a total population of 2268 HCC patients and 1845 control individuals (1318 patients with benign liver disorders and 527 healthy volunteers). The primary characteristics of all participants are summarized in Table 1. An overwhelming majority of participants were Asian (n = 3808), with the residual 160 participants from America and 145 individuals from Egypt. Twelve researches belonged to retrospective (n = 8) or prospective trial (n = 4), respectively, and the remaining publications (n = 21) did not definitely describe the study design. Among 19 studies with known time point of sampling, most of the samples were collected before treatment (n = 14) and ctDNA was obtained from plasma (n = 13), serum (n = 18), both plasma and serum (n = 2). The assay methods to measure the concentrations of ctDNA were real-time quantitative polymerase chain reaction (RT-qPCR) (n = 5), ultraviolet transilluminator (n = 1), enzyme-linked immunosorbent assay (ELISA) (n = 1) and droplet digital PCR DNA (DdPCR) (n = 1). For qualitative analysis of ctDNA, methylation-specific polymerase chain reaction (MSP) was the most common method used (n = 25).

3.2 Quality assessment

The quality assessment outcome of the eligible 33 publications is revealed in Figure 2 and Figure S1. A majority of the literature exhibited a moderate-high quality, indicating that the overall quality of the included studies was commonly robust. Nevertheless, 13 studies might generate an unknown risk of bias in index test because of insufficient information about predefined threshold. Additionally, 12 studies did not mention whether the patient selection was consecutive.
| First author, year | Country/Region | Study type | Control type | No. of HCC/BD/HC | Timing sample | Sample source | Detection methods | Assay indicators | Diagnostic concordance |
|-------------------|----------------|------------|--------------|-----------------|--------------|--------------|-------------------|------------------|------------------------|
| Kisiel, 2019      | USA            | Retrospective | LC           | 21/30/-        | During surgery | Plasma         | MSP Methylation (EMX1) | NA 76.0 100.0 100.0 | 16 0 5 20 |
| Wei, 2018         | China          | NA         | BD/HC        | 119/157/50     | Pre surgery   | Plasma         | MSP Methylation (SOCS3) | NA 28.6 95.2 95.2 | 34 10 10 197 |
| Dong, 2017        | China          | NA         | CHB and LC/HC | 98/165/80      | NA           | Serum         | MSP Methylation (RASSF1A) | NA 52.0 93.1 93.1 | 16 4 8 2 |
| Mansour, 2017     | Egypt          | NA         | HCV infection | 45/40/-        | NA           | Serum         | MSP Methylation (RASSF1A) | 8 copies/µL 86.7 72.5 72.5 | 53 10 10 197 |
| Hu, 2017          | China          | NA         | LC, CHB      | 80/80/-        | Pre treatment | Serum         | MSP Methylation (UBE2Q1) | NA 66.3 [53.8] 57.5 [87.5] | 53 10 10 197 |
| Huang, 2015       | China          | NA         | CHB          | 190/120/-      | Pre treatment | Serum         | MSP Methylation (RASSF1A) | NA 64.2 [80.9] 89.8 [93.4] | 53 10 10 197 |
| Li, 2014          | China          | NA         | CHB          | 136/46/-       | Pre treatment | Serum         | MSP Methylation (IGFBP7) | NA 65.4 82.6 82.6 | 93.4 93.4 |
| Kuo, 2014         | Taiwan         | NA         | BD           | 39/-/34        | During surgery | Plasma         | MSP Methylation (HOXA9) | >0.88 | 93.3 93.3 |
| Liu, 2014         | China          | NA         | CHB          | 309/217/-      | During surgery | Plasma         | MSP Methylation (MT1M) | NA 69.8 83.8 83.8 | 122 122 |
| Kuo, 2013         | China          | NA         | CHB          | 309/217/-      | During surgery | Plasma         | MSP Methylation (MT1G) | NA 69.8 83.8 83.8 | 122 122 |
| Zhang, 2013       | China          | NA         | CHB          | 309/217/-      | During surgery | Plasma         | MSP Methylation (MT1G) | NA 69.8 83.8 83.8 | 122 122 |

(Continues)
| First author, year | Country/Region | Study type | Control type | No. of HCC/BD/HC | Timing sample | Sample source | Detection methods | Assay indicators | Cutoff value | SEN (%) | SPE (%) | PLR | NLR | Diagnostic concordance |
|--------------------|----------------|------------|--------------|------------------|--------------|---------------|-------------------|-----------------|-------------|---------|---------|-----|-----|------------------------|
| Sun, 2010          | Hong Kong      | Retrospective | LC/HC        | 35/16/12         | NA           | Plasma        | RT-qPCR          | Methylation (LMNB1) | NA          | 80.0    | 82.0    | 4.444 | 0.244 | 0.810                  |
| Hu, 2010           | China          | Retrospective | HC           | 35/-/10          | Pre surgery  | Serum         | MSP               | Methylation (RASSF1A) | NA          | 40.0    | 100.0   | Infinity | 0.600 | 0.533                  |
| Chang, 2008        | China          | NA          | LC           | 26/-/16          | NA           | Plasma        | MSP               | Methylation (RASSF1A) | NA          | 26.9    | 81.3    | 1.459 | 0.899 | 0.476                  |
| Zhang, 2007        | Taiwan         | Prospective  | HC           | 50/-/50          | Closest to diagnosis | Serum | MSP | Methylation (P16) | NA | 44.0 | 96.0 | 11.00 | 0.583 | 0.700 |
|                       |                |             |              |                  |              |               |                   |                 |            |         |         |      |      | 22 | 2 | 28 | 48 |
| Zhang, 2007        | Taiwan         | Prospective  | HC           | 50/-/50          | Closest to diagnosis | Serum | MSP | Methylation (P16) | NA | 22.0 | 100.0 | Infinity | 0.780 | 0.610 |
|                       |                |             |              |                  |              |               |                   |                 |            |         |         |      |      | 11 | 0 | 39 | 50 |
| Wang, 2006         | China          | NA          | LC           | 328/-/-          | NA           | Serum         | MSP               | Methylation (GSTP1) | NA          | 50.0    | 62.5    | 1.333 | 0.800 | 0.525 |
| Yeo, 2005          | Hong Kong      | NA          | HC           | 40/-/-          | Pre surgery  | Plasma        | MSP               | Methylation (RASSF1A) | NA          | 42.5    | 100.0   | Infinity | 0.575 | 0.540 |
| Lin, 2005          | China          | NA          | BD/HC        | 64/15/20         | Pre- and post-surgery | Serum | MSP | Methylation (p16) | NA | 76.6 | 100.0 | 0.234 | 0.848 |
| Chu, 2004          | Korea          | NA          | LC           | 46/23/-         | NA           | Serum         | MSP               | Methylation (p16NK4a) | NA | 47.8 | 82.6 | 2.747 | 0.632 | 0.594 |
| Wong, 2003         | Hong Kong      | Prospective  | BD/HC        | 45/30/20        | Pre-, intra- and post-surgery | Serum/ plasma | MSP | Methylation (p16NK4a) | NA | 31.1 | 100.0 | Infinity | 0.689 | 0.674 |
| Wong, 2000         | Hong Kong      | Prospective  | BD/HC        | 25/35/20        | Pre surgery  | Serum/plasma  | MSP               | Methylation (p16) | NA | 60.0 | 100.0 | Infinity | 0.400 | 0.594 |
| Gai, 2018          | Hong Kong      | NA          | HBV carriers and LC/HC | 40/29/30        | NA           | Plasma        | DdPCR             | ctDNA            | 370 copies/mL | 93.0 | 60.0 | 2.325 | 0.117 | 0.674 |
| Huang, 2012        | China          | NA          | HC           | 72/-/41         | Pre treatment | Plasma        | RT-qPCR       | ctDNA            | 18.2 ng/mL | 90.2 | 90.3 | 9.299 | 0.109 | 0.903 |
| Yang, 2011         | China          | case control | HBV infection/HC | 60/21/29        | Pre treatment | Plasma        | RT-qPCR       | hTERT           | 1.87 × 10^6 copies/ul | 64.0 | 90.0 | 6.400 | 0.400 | 0.903 |
| Dong, 2008         | China          | NA          | LC, CH and AH/HC | 117/152/40      | Pre surgery  | Plasma        | ELISA          | TGF-b1          | 1.2 μg/L | 89.7 | 91.1 | 10.08 | 0.113 | 0.755 |
| Ren, 2006          | China          | NA          | LC/HC        | 79/20/20        | Pre surgery  | Plasma        | Ultraviolet transilluminator | ctDNA          | 36.6 ng/mL | 51.9 | 77.5 | 2.307 | 0.113 | 0.906 |

Abbreviations: AH, acute hepatitis; CDO1, Cysteine dioxygenase 1; CH, chronic hepatitis; CHB, Chronic hepatitis B; CLD, chronic liver diseases; DdPCR, droplet digital PCR; ELISA, enzyme-linked immunosorbent assay; EMX1, empty spiracles homeobox 1; FN, false negative; FP, false positive; GPC-3, glypican-3; HBV, hepatitis B virus; HCV, hepatitis C virus; IGFBP7, insulin-like growth factor-binding protein 7; LC, liver cirrhosis; LMNB1, Lamin B1; MSP, methylation-specific polymerase chain reaction; NA, not applicable; NLR, negative likelihood ratio; No. of HCC/BD/HC, number of hepatocellular carcinoma/benign liver diseases/healthy controls; RASSF1A, Ras association domain family 1 isoform A; PLR, positive likelihood ratio; RT-qPCR, real-time quantitative polymerase chain reaction; SEN, sensitivity; SPE, specificity; TGR5, G-protein-coupled bile acid receptor Gpbar1; TN, true negative; TP, true positive.

The SEN and SPE, PLR and NLR, diagnostic concordance, TP, TN, FP and FN of the ctDNA combined with AFP for HCC detection are presented in [1].

A limit of detection (LOD) of 5%.

Methylation index.
3.3 | Diagnostic accuracy

3.3.1 | Diagnostic value of quantitative and qualitative analysis of ctDNA for HCC

The quantitative detection of ctDNA discriminated HCC patients from control individuals with a SEN of 0.722 (95% CI: 0.686-0.756) and a SPE of 0.823 (95% CI: 0.789-0.854) (Figure 3A,B). The numerical value of PLR, NLR and DOR was 4.208 (95% CI: 2.526-7.009), 0.264 (95% CI: 0.145-0.483), 18.532 (95% CI: 8.245-41.657), respectively. This also corresponded to the SROC curve with an AUC of 0.880 (Figure 5A), indicating a higher level of moderate overall accuracy to differentiate HCC patients from control subjects. Among the included quantitative studies, there was significant heterogeneity (SEN: $I^2 = 94.3\%$, $P = .000$; SPE: $I^2 = 88.3\%$, $P = .000$; DOR: $I^2 = 80.7\%$, $P = .000$) and the Spearman correlation coefficient was 0.283 ($P = .460$), indicating that heterogeneity among studies was derived from nonthreshold effects.

Similarly, in the qualitative analysis group associated with tumor-specific single-gene methylation, a SEN of 0.568 (95% CI: 0.548-0.587), a SPE of 0.882 (95% CI: 0.867-0.897) and a DOR of 10.457 (95% CI: 7.270-15.040) were revealed (Figure 4C,D). The value of PLR, NLR and DOR was 4.525 (95% CI: 2.757-7.426) and 0.439 (95% CI: 0.345-0.557) and the DOR was 12.550 (95% CI: 7.262-20.126). The AUC for RASSF1A was 0.841 (Figure 5D), indicating that ctDNA RASSF1A methylation can be considered as a potential HCC diagnostic biomarker with a higher level of moderate overall accuracy.

3.3.2 | Diagnostic value of ctDNA combined with AFP assay for HCC

Initially, we evaluated the diagnostic efficiency of AFP assay in HCC. The AFP test yielded an AUC of 0.638, with a SEN of 0.478 (95% CI: 0.447-0.509) and a SPE of 0.840 (95% CI: 0.809-0.867) (Figure S2). The value of PLR, NLR and DOR were 3.368 (95% CI: 1.913-5.929), 0.611 (95% CI: 0.506-0.738), and 6.284 (95% CI: 3.109-12.700), respectively. Furthermore, the combination of ctDNA and AFP assay yielded an AUC of 0.944 (Figure 5C), with a SEN of 0.760 (95% CI: 0.728-0.790) and a SPE of 0.920 (95% CI: 0.893-0.942) (Figure 4A,B). This corresponded to a PLR of 9.469 (95% CI: 5.178-17.313), an NLR of 0.234 (95% CI: 0.154-0.357) and a DOR of 54.864 (95% CI: 19.980-150.66), highlighting that compared with the ctDNA assay or AFP test alone, the detection of ctDNA integrated with AFP could distinguish HCC patients from control individuals with a remarkably increased high level of accuracy (Table 2).

3.3.3 | Diagnostic value of circulating RASSF1A methylation for HCC

In the qualitative analysis of ctDNA, circulating RASSF1A promoter methylation is the most frequently detected epigenetic change in HCC. Thus, we also estimated the diagnostic efficacy of RASSF1A methylation in discriminating HCC patients from controls. In the 11 studies describing circulating RASSF1A methylation, the pooled SEN and SPE was 0.644 (95% CI: 0.608-0.678) and 0.875 (95% CI: 0.847-0.900), respectively (Figure 4C,D). The pooled PLR and NLR was 4.525 (95% CI: 2.757-7.426) and 0.439 (95% CI: 0.345-0.557), respectively, and the DOR was 12.550 (95% CI: 7.262-20.126). The AUC for RASSF1A was 0.841 (Figure 5D), indicating that ctDNA RASSF1A methylation can be considered as a potential HCC diagnostic biomarker with a higher level of moderate overall accuracy.
3.4 Subgroup analysis and meta-regression analysis

Subgroup analysis was performed based on different covariates: region (Asia vs non-Asia), sample size (≥100 vs <100), control type (benign disease vs healthy controls), sample source (plasma vs serum), assay methods (RT-qPCR vs other methods in the quantitative study; MSP vs other methods in the qualitative studies) and methylation gene location (RASSF1A vs other gene targets in the qualitative studies) (Table 3). For the quantitative analysis of ctDNA, subgroup analyses based on sample source revealed that compared with sample collected from serum, sampling from plasma achieved an increased diagnostic accuracy in discriminating HCC from control subjects, with SEN of 0.777 (95% CI: 0.731-0.819) vs 0.654 (95% CI: 0.598-0.708), and SPE of 0.846 (95% CI: 0.805-0.880) vs 0.773 (95% CI: 0.703-0.834) as well as an AUC of 0.902 vs 0.843, respectively. Another subgroup analysis associated with control type showed that the SEN, SPE and AUC for quantitative ctDNA assay to distinguish HCC patients from healthy subjects was 0.775 (95% CI: 0.731-0.814), 0.843 (95% CI: 0.804-0.877) and 0.895, respectively. While the corresponding indicators to discriminate HCC from benign liver diseases were much lower at 0.697 (95% CI: 0.657-0.735), 0.817 (95% CI: 0.780-0.851) and 0.868, respectively. Similarly, in term of subgroup analysis related to control type in the included qualitative analysis of
ctDNA, studies using healthy controls were characterized with more satisfactory diagnostic efficiency compared with those utilizing subjects with benign liver disorders, displaying SEN of 0.604 (95% CI: 0.563-0.644) vs 0.556 (95% CI: 0.533-0.579) and SPE of 0.938 (95% CI: 0.916-0.955) vs 0.852 (95% CI: 0.831-0.872), respectively. These results highlighted a more robust capability of the ctDNA assay to differentiate HCC patients from healthy individuals than from benign patients. We also performed the meta-regression analysis to further explore the source of heterogeneity. As has been revealed in Table 4, the parameter of “control type” potentially was the primary source of heterogeneity in the qualitative analysis group (P = .022). None of parameters might generate significant heterogeneity in the quantitative analysis group (both P > .05).

3.5 Publication bias

We examined the potential publication bias of the incorporated articles by performing Deek’s funnel plot asymmetry test. Our results revealed that no significant publication bias existed in the group of quantitative analysis (Figure 6A, P = .114), in the group of qualitative analysis (Figure 6B, P = .725), in the group of ctDNA combined with AFP assay (Figure 6C, P = .079), or in the group of RASSF1A methylation detection (Figure 6D, P = .449).

4 DISCUSSION

HCC is a high-grade malignant neoplasm with undesirable prognosis and high mortality, which is largely attributable to its low early diagnostic rate. Therefore, it is essential to disclose novel and effective biomarkers for the detection and diagnosis of early-stage HCC. Applying novel molecular technologies to liquid biopsies has advanced our understanding of the effect of ctDNA detection on HCC diagnosis. In this diagnostic meta-analysis, we aimed to incorporate these published results for the first time and systematically estimate the diagnostic accuracy of ctDNA for HCC.

In our meta-analysis, compared with the group of quantitative analysis, the group of qualitative analysis yielded a lower SEN (0.568 vs 0.722) and AUC (0.787 vs 0.880), and that was probably because some genetic loci selected for test were predominantly expressed in non-HCC individuals. However, the SPE (0.882) of the qualitative group was superior to that of the quantitative group (0.823). Notably, we specifically concentrated on RASSF1A methylation in the qualitative analysis of ctDNA RASSF1A is a well-acknowledged tumor suppressor and is continually inactivated by promoter hypermethylation in HCC. It has the capability to trigger autophagy defects to facilitate oxidative stress and genome instability, thus accelerating tumorigenesis. We revealed that RASSF1A methylation discriminated HCC patients from control individuals with a SEN of 0.644 and a SPE of 0.875, contributing to an improvement of AUC from...
0.787 to 0.841. These results indicate that circulating tumor DNA \textit{RASSF1A} methylation can serve as a potential biomarker to screen HCC.

Our results also showed that AFP, as the most frequently used biomarker for HCC diagnosis, exhibited an unsatisfactory diagnostic performance on account of a low SEN of merely 0.478, which was relatively lower than the result of Farinati et al (the SEN was 0.540).\textsuperscript{68} Thus, quantitative or qualitative analysis of ctDNA was more sensitive and feasible, and the diagnostic accuracy of ctDNA was superior to the AFP assay alone (the AUC was merely 0.638). Additionally, the combined detection of ctDNA and AFP assay resulted in a remarkably increased diagnostic accuracy with a SEN of 0.760 and a SPE of 0.920 as well as an AUC of 0.944 in discriminating HCC from control individuals. This encouraging result highlights that the combined AFP and ctDNA assay for diagnosing and evaluating HCC can generate much more favorable accuracy than does either method on its own and that ctDNA detection potentially develops into a novel auxiliary tool for AFP in the screening and detection of HCC.

Furthermore, we also analyzed the DOR to evaluate the diagnostic accuracy in each group. The discriminatory test performance would be considered satisfactory when the numerical value of DOR was higher than 10.\textsuperscript{25} In our results, the DOR for quantitative and qualitative ctDNA assay to distinguish HCC cases from control subjects was 18.532 and 10.457, respectively. The DOR of \textit{RASSF1A} methylation detection of ctDNA (12.550) was slightly higher than that of qualitative ctDNA assay (10.457). While the DOR for AFP assay to discriminate HCC and controls was much lower at 6.284. The DOR was dramatically improved to 54.864 when utilizing the combined detection of ctDNA and AFP, indicating a powerful capability of integrating ctDNA analysis with AFP to exactly screen and diagnose HCC.

In our report, the value of PLR in quantitative and qualitative detection of ctDNA was 4.208 and 4.378, respectively, manifesting that HCC cases have an approximately four to five fold higher chance of being ctDNA assay-positive in comparison with control individuals. Compared with the quantitative detection of ctDNA, the qualitative detection of ctDNA displayed a higher NLR (0.489), implying that the probability for cases with negative qualitative assay results to have HCC is 48.9%. Thus, a negative ctDNA test result should be explained prudently when single-gene methylation is independently utilized to screen and detect HCC. Nevertheless, addition of AFP statistically boosted the overall accuracy and robustness, with a PLR of 9.469 and an NLR of 0.234.

Publication bias was not revealed in our meta-analysis by formulating Deek’s funnel plot. Furthermore, a meta-regression analysis was performed to explore the potential source
of heterogeneity, thus demonstrating that in these quantitative studies, none of the parameters (such as sample source, sample size, control types and assay methods) represented a primary source of heterogeneity. Heterogeneity might have arisen because of additional reasons, including enrolled patients’ age, tumor size, lymph node invasion, lesion metastasis, TNM staging and discrepancies in the surgical protocol, which failed to be evaluated in this study on account of partial deficiency of the data or illegible details. Furthermore, the covariate of “control types” potentially exerted certain influence on heterogeneity in the qualitative analysis group. Therefore, further large clinical trials should reasonably select control individuals to boost the diagnostic performance of ctDNA in HCC.

Notably, several limitations deserve to be discussed in our meta-analysis. Firstly, in spite of the thorough literature search, we did not incorporate several valuable articles because we failed to access their full texts. Moreover, a relatively smaller number of publications were incorporated into the quantitative group, thus potentially diminishing the statistical significance. Thirdly, some bias was potentially generated in this analysis because we merely included English-language articles. Ultimately, we failed to include some covariates that were not depicted in these included studies, such as neoplasm size, lymph node invasion, lesion metastasis, and TNM staging of tumors. Therefore, more large-scale prospective clinical researches that delineate the diagnostic value of ctDNA detection for HCC are needed to further identify the conclusions of this meta-analyses.
### TABLE 3  Subgroup analysis of diagnostic performance of ctDNA assay for HCC

| Analysis                  | Group     | Subgroup | SEN (95% CI)     | SPE (95% CI)     | DOR (95% CI)    | AUC       |
|---------------------------|-----------|----------|------------------|------------------|----------------|-----------|
| Quantitative analysis     | Control type | HC       | 0.775 (0.731-0.814) | 0.843 (0.804-0.877) | 21.320 (6.848-66.377) | 0.895     |
|                           |           | BD       | 0.697 (0.657-0.735) | 0.817 (0.780-0.851) | 16.015 (6.334-40.496) | 0.868     |
|                           | Sample size | ≥100     | 0.693 (0.652-0.732) | 0.864 (0.829-0.895) | 20.501 (6.323-66.466) | 0.887     |
|                           |           | ≤100     | 0.848 (0.773-0.906) | 0.672 (0.580-0.756) | 15.676 (7.740-31.750) | 0.855     |
|                           | Sample source | Plasma   | 0.777 (0.731-0.819) | 0.846 (0.805-0.880) | 23.762 (6.321-89.324) | 0.902     |
|                           |           | Serum    | 0.654 (0.598-0.708) | 0.773 (0.703-0.834) | 10.632 (6.199-18.236) | 0.843     |
|                           | Assay method | RT-qPCR  | 0.693 (0.647-0.736) | 0.817 (0.765-0.862) | 17.568 (8.502-36.304) | 0.873     |
|                           |           | Other methods | 0.775 (0.717-0.827) | 0.828 (0.780-0.870) | 18.307 (2.271-147.58) | 0.875     |
| Qualitative analysis      | Region    | Asian    | 0.554 (0.534-0.575) | 0.886 (0.869-0.900) | 9.883 (6.672-14.639) | 0.767     |
|                           |           | Other areas | 0.744 (0.672-0.808) | 0.842 (0.769-0.900) | 15.206 (7.798-29.653) | 0.860     |
|                           | Control type | HC       | 0.604 (0.563-0.644) | 0.938 (0.916-0.955) | 22.151 (14.827-33.093) | 0.893     |
|                           |           | BD       | 0.556 (0.533-0.579) | 0.852 (0.831-0.872) | 6.990 (4.661-10.483) | 0.740     |
|                           | Sample size | ≥100     | 0.557 (0.533-0.580) | 0.880 (0.862-0.897) | 10.196 (6.694-15.529) | 0.770     |
|                           |           | ≤100     | 0.541 (0.508-0.574) | 0.911 (0.890-0.929) | 9.878 (5.667-17.218) | 0.802     |
|                           | Sample source | Plasma   | 0.516 (0.476-0.555) | 0.934 (0.909-0.953) | 11.476 (5.024-26.212) | 0.718     |
|                           |           | Serum    | 0.586 (0.563-0.609) | 0.861 (0.841-0.880) | 10.170 (6.782-15.251) | 0.800     |
|                           | Assay method | MSP      | 0.553 (0.533-0.574) | 0.885 (0.869-0.900) | 9.483 (6.433-13.980) | 0.750     |
|                           |           | Other methods | 0.767 (0.694-0.829) | 0.848 (0.773-0.906) | 20.130 (10.035-40.381) | 0.908     |
|                           | Methylation gene location | RASSF1A | 0.644 (0.608-0.678) | 0.875 (0.847-0.900) | 12.550 (7.826-20.126) | 0.841     |
|                           |           | Other gene location | 0.535 (0.511-0.559) | 0.886 (0.867-0.904) | 10.031 (6.266-16.058) | 0.750     |

Abbreviations: 95% CI: 95% confidence interval; AUC: area under the curve; BD, benign live diseases; ctDNA, circulating tumor DNA; DOR, diagnostic odds ratio; HC, healthy controls; HCC, hepatocellular carcinoma; MSP, methylation-specific polymerase chain reaction; RT-qPCR, real-time quantitative polymerase chain reaction; SEN, sensitivity; SPE, specificity.
In summary, we performed the first integrated meta-analysis on the overall diagnostic accuracy of circulating tumor DNA assays in HCC. The diagnostic performance of quantitative and qualitative analysis of ctDNA was superior to the classical HCC biomarker AFP. Specifically, ctDNA RASSF1A methylation potentially serves as an effective diagnostic biomarker.

### TABLE 4  Meta-regression of impacts of study features on diagnostic value of ctDNA for HCC

| Analysis          | Covariates     | Coefficient | SE    | P value | RDOR (95% CI) |
|-------------------|----------------|-------------|-------|---------|---------------|
| Quantitative      | Control type   | 1.649       | 0.959 | 0.184   | 5.20 (0.25-109.94) |
|                   | Sample size    | 0.541       | 1.129 | 0.665   | 1.72 (0.05-62.41)   |
|                   | Sample source  | 0.264       | 1.227 | 0.843   | 1.30 (0.03-64.73)   |
|                   | Assay method   | 0.679       | 1.108 | 0.583   | 1.97 (0.06-67.02)   |
| Qualitative       | Region         | -0.779      | 0.589 | 0.196   | 0.46 (0.14-1.53)    |
|                   | Control type   | 1.146       | 0.473 | 0.022   | 3.15 (1.20-8.27)    |
|                   | Sample size    | 0.401       | 0.385 | 0.307   | 1.49 (0.68-3.28)    |
|                   | Sample source  | 0.109       | 0.421 | 0.798   | 1.11 (0.47-2.64)    |
|                   | Assay method   | -0.756      | 0.671 | 0.269   | 0.47 (0.12-1.85)    |
|                   | Methylation gene location | 0.289       | 0.416 | 0.493   | 1.34 (0.57-3.13)    |

Abbreviations: 95% CI: 95% confidence interval; ctDNA: circulating tumor DNA; HCC, hepatocellular carcinoma; RDOR: relatively diagnostic odds ratio; SE: standard error.

### FIGURE 6  Funnel plots to evaluate the publication bias for (A) the quantitative detection subgroup; (B) the qualitative detection subgroup; (C) the subgroup of ctDNA combined with AFP assay; and (D) the RASSF1A methylation detection subgroup. AFP, alpha-fetoprotein; ctDNA, circulating tumor DNA; DOR, diagnostic odds ratio; ESS, effective sample sizes

## 5  CONCLUSION

In summary, we performed the first integrated meta-analysis on the overall diagnostic accuracy of circulating tumor DNA
for HCC. Notably, because of deficiency of robustness, the ctDNA assay cannot be utilized as an independent diagnostic tool. The combined assays of ctDNA and AFP yielded a higher level of discriminatory power in HCC detection. Therefore, quantitative and qualitative analysis of ctDNA can be used as a complementary strategy integrated with AFP assay for the early detection and diagnosis of HCC. Larger sample studies are needed to further confirm our conclusions and to make the ctDNA approach more sensitive and specific.

CONFLICTS OF INTEREST
The authors declare that there are no conflicts of interest.

AUTHOR CONTRIBUTIONS
PG Cao, ZY Zhang designed/planned the study and wrote the paper. ZY Zhang, P Chen, H Xie performed the computational modeling, acquired and analyzed clinical data. H Xie and P Chen performed the imaging analysis. ZY Zhang, H Xie, P Chen, PG Cao participated in discussion of related data.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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