Associations between methylenetetrahydrofolate reductase polymorphisms and hepatocellular carcinoma risk
An update meta-analysis and trial sequential analysis

Bin Feng Wang, MD, Miaomiao Ma, MD, Xiaojun Guo, BD, Yan Yan, PhD, Lang Li, MD

Abstract
Aim: To evaluate the associations between the methylenetetrahydrofolate reductase (MTHFR) single-nucleotide polymorphisms (SNPs) and hepatocellular carcinoma (HCC) with meta-analysis and trial sequential analysis.

Methods: PubMed, Embase, the Google Scholar, Wan fang database, VIP database, and China National Knowledge Infrastructure were extensively searched before April 2021. Odds ratios (ORs) and 95% confidence interval (95% CI) were calculated. Review Manager Version 5.3, STATA version 12.0 and TSA 0.9.5.10 Beta software were used.

Results: Nineteen studies with 6941 HCC patients and 9436 controls were finally included. The MTHFR rs1801133 (C677T) SNP was associated with increased HCC risk under heterozygote genetic model (OR = 1.10, 95% CI = [1.01, 1.20]). For Subgroup analysis, increased risks of HCC were detected in Mongoloid, Chinese. For MTHFR rs1801131 (A1298C) SNP, increased risk of HCC was only observed in Caucasians (allelic: OR = 1.86, 95% CI = [1.49, 2.31]; homozygote: OR = 3.39, 95% CI = [2.18, 5.27]), interesting decreased risk was detected in Mongoloid (recessive: OR = 0.30, 95% CI = [0.15, 0.58]; homozygote: OR = 0.41, 95% CI = [0.24, 0.72]). Sensitivity analysis indicated stability in our results. Publication bias was not detected based on Begg test and Egger test. Trial sequential analysis indicated further studies to confirm the associations in MTHFR C677T polymorphism.

Conclusion: The MTHFR rs1801133 SNP was associated with an increased risk of HCC in Mongoloid population especially in Chinese. Increased HCC risk is also observed in Caucasian population for the MTHFR rs1801131 SNP, and decreased risk of HCC is remarkably discovered in Mongoloid and Chinese subgroups, which need further validation.

Abbreviations: CI = confidence interval, HCC = hepatocellular carcinoma, MTHFR = methylenetetrahydrofolate reductase, ORs = odds ratios, SNP = single-nucleotide polymorphism.

Keywords: hepatocellular carcinoma, meta-analysis, methylenetetrahydrofolate reductase, polymorphism, trial sequential analysis

1. Introduction
Hepatocellular carcinoma (HCC) accounts for roughly 90% of all primary liver cancers, which is the sixth most common cancer and the second leading cause of cancer death worldwide.\(^{1–5}\) HCC is asymptomatic most of the time, and when symptoms appear patients are usually at the middle or late stage, which result in a high mortality.\(^{6–9}\) Therefore, early diagnosis based on related risk factors is of great significant to prevent HCC. HCC is a multifactorial disease due to the complex interactions between genetic and environmental factors. Genetic polymorphisms in HCC related genes such as toll-like receptor genes,\(^{10}\) PD-L1,\(^{11}\) matrix metalloproteinase-11 gene,\(^{12}\) have drawn increasing attention in the past decades. To filtrate predisposing gene polymorphisms is important to the early prevention of HCC.

Folate metabolism plays an important role in the DNA synthesis and methylation, which is crucial to the development of HCC. Methylenetetrahydrofolate reductase (MTHFR) is the key enzyme in folate and one-carbon metabolism, which can catalyze the 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. The 5-methyltetrahydrofolate is the predominant circulatory form of folate and serves as the methyl donor for the remethylation of homocysteine to methionine, which is the precursor of S-adenosylmethionine (SAMe), the principal biological methyl donor for methylation of DNA.\(^{13–18}\) Two functional single nucleotide polymorphisms (SNPs) in MTHFR were identified: the MTHFR rs1801133 (C677T) polymorphism (a C to T transition at nucleotide 677 at exon 4, resulting in an alanine-to-valine conversion in protein) and MTHFR rs1801131 (A1298C) polymorphism (a A to C transition at nucleotide 1298 at exon ten, causing a glutamate-to-alanine change in
protein\textsuperscript{[19,20]} Both of the 2 polymorphisms were reported to be associated with a lower MTHFR activity\textsuperscript{[21–28]} and the reduced enzymatic activity can promote or inhibit the occurrence of HCC by affecting DNA methylation and synthesis, which indicated these 2 polymorphisms could be associated with HCC risk.

Many studies were performed to discover the associations between the 2 MTHFR SNPs and HCC risk, however, the conclusions were inconclusive\textsuperscript{[13,29,30]} Former meta-analyses in 2014 and 2015 reported the MTHFR rs1801133 polymorphism was associated with an increased risk of HCC, but for the MTHFR rs1801131 polymorphism, no association was observed.\textsuperscript{[30,31]} Since then, controversial results emerged in different regions and ethnicities\textsuperscript{[13,29,32–34]} In order to reach a more accurate evaluation of these 2 polymorphisms and HCC risk, we performed an update meta-analysis with trial sequential analysis.

2. Methods

Based on the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-analyses) checklist\textsuperscript{[35]} we organized our update meta-analysis. Ethical approval was not necessary for the type of the study (meta-analysis).\textsuperscript{[36]}

2.1. Literature search

An extensive literature search for related studies regarding the associations between MTHFR polymorphisms and hepatocellular carcinoma risk was conducted on PubMed, Embase, the Google Scholar, Wan fang database, VIP database, and China National Knowledge Infrastructure before April 2021. We used the following keywords and MeSH terms: “Methylenetetrahydrofolate reductase” or “folate metabolism” or “one-carbon metabolism” or “MTHFR,” “polymorphism, single nucleotide” or “single nucleotide polymorphism” or “polymorphism” or “SNP,” and “carcinoma, hepatocellular” or “liver neoplasms” or “liver cancer.” No language restrictions were set in our search, furthermore, references of eligible studies were screened manually to identify potential relevant researches.

2.2. Inclusion and exclusion criteria

Studies included should meet the following inclusion criteria:
1. case-control studies or cohort studies;
2. studies on analyzing the associations between MTHFR polymorphisms and hepatocellular carcinoma risk;
3. hepatocellular carcinoma patients should be diagnosed by histopathology in the included study;
4. studies providing detailed genotype frequencies on case and control subjects.

The exclusion criteria were as follows:
1. reviews, comments and conference documents;
2. unclear diagnostic basis for case subjects;
3. animal research;
4. studies with insufficient data, especially without enough data for Hardy-Weinberg equilibrium;
5. duplicate publications.

2.3. Data extraction and quality assessment

Data from the potential eligible studies were independently retrieved by all the authors based on a pre-designed standard form. The following data were extracted: name of the first author, year of publication, country and region where the study was conducted, matching criteria, ethnicity, genotyping method, source of controls, genotype frequency in the cases and controls, quality score and results of Hardy-Weinberg equilibrium test. Ethnicity was categorized as Mongoloid, Caucasian. Hardy-Weinberg equilibrium (HWE) was evaluated for each study by Chi-Squared test in control groups for goodness of fit, and P < .05 was considered as a significant departure from HWE. Any disagreement was resolved by group discussion. The quality assessment for each eligible study was assessed based on the modified Newcastle-Ottawa quality assessment scale.\textsuperscript{[37]} Scores ranged from 0 to 10, with 0 as the lowest and 10 as the highest quality.

2.4. Statistics analysis

Odds ratio (OR) and 95% confidence intervals (CIs) were calculated to evaluate the strength of the association between MTHFR gene polymorphisms and hepatocellular carcinoma risk. Pooled ORs were performed for allelic model (rs1801133: T VS C; rs1801131: C VS A), recessive model (rs1801133: TT VS TC+CC; rs1801131: CC VS CA+AA), dominant model (rs1801133: TT+TC VS CC; rs1801131: CC VS CA+AA), heterozygote model (rs1801133: TC VS C; rs1801131: CA VS AA), homozygote model (rs1801133: TT VS CC; rs1801131: CC VS AA), respectively. Heterogeneity was evaluated by Q statistic (significance level of P < .1) and I\textsuperscript{2} statistic (greater than 50% as evidence of significant inconsistency). If the P value of heterogeneity test was more than .1 or I\textsuperscript{2} statistic less than 50%, the fixed-effect model was used to calculate the pooled OR, otherwise, the random-effect model was used. Sensitivity analysis was performed to detect the heterogeneity by omitting each study in each turn. In addition, subgroup analyses were stratified by HWE (Whether meet the Hardy Weinberg Equilibrium), Region (China, France, Italy, South Korea, USA, Brazil), ethnicity (Mongoloid, Caucasian), source of controls (Hospital based, Population based). The potential for publication bias was assessed with Begg funnel plot and Egg test. All the tests in this meta-analysis were conducted with Review Manager Version 5.3 and the STATA software (version 12.0; State Corporation, College Station, Texas, USA). All tests were two-sided and a P value of lower than .05 was considered as statistically significant.\textsuperscript{[38]}

2.5. Trial sequential analysis

Due to dispersed data and repeated significance testing, type I and type II errors are inevitable in traditional updated meta-analysis with new trials\textsuperscript{[19]} Bias from trials with low methodological quality, outcome measure bias, early stopping for benefit, and small trial bias may result in spurious P values, therefore, the trial sequential analysis which is a methodology that combines an information size calculation (cumulated sample sizes of all included studies) for an updated meta-analysis with the threshold of statistical significance was introduced in our analysis (http://www.ctu.dk/tools-and-links/trial-sequential-analysis.aspx). The trial sequential analysis software (TSA, version 0.9; Copenhagen Trial Unit, Copenhagen, Denmark, 2011) was used and the parameters were set as an overall type I error of 5%, a statistical test power of 80%, and a relative risk reduction of 20%.\textsuperscript{[40–42]} If the Z-curve crosses the trial sequential monitoring boundary line
or required information size is reached, a sufficient level of evidence has been acquired and no more further studies are needed, or else, further studies are essential.

### 3. Results

#### 3.1. The characteristics of the included studies

A total of nineteen articles were included and 6941 hepatocellular carcinoma patients and 9436 healthy controls were enrolled\(^{[13,29,32-34,43-56]}\) (The flow chat of literature selection was showed in Figure 1 and Table S1 (see Table S1, Supplemental Digital Content, which showed the flow chat of literature selection, http://links.lww.com/MD/G441). The characteristics of the included studies were showed in Table 1. Among the nineteen included articles, 20 studies were research regarding the associations between the MTHFR rs1801133 polymorphism (There were 2 different study groups from the article Yuan et al\(^{[49]}\) and hepatocellular carcinoma, and 8 studies were about the MTHFR rs1801131 polymorphism. For the MTHFR rs1801133 polymorphism, 5227 HCC patients and 6688 healthy controls were involved, which came from China, France, Italy, South Korea, USA and Brazil; as for the MTHFR rs1801131 polymorphism, 1714 HCC and 2775 healthy controls from

![Flow Diagram](image-url)
| Study          | Year | Country/region | Matching criteria                                                                 | Ethnicity   | Genotyping method                      | Controls source | Sample size | AA | AB | BB | Quality score | HWE  |
|---------------|------|----------------|------------------------------------------------------------------------------------|-------------|----------------------------------------|----------------|-------------|----|----|----|---------------|------|
| MTHFR C677T polymorphism |      |                |                                                                                   |             |                                        |                |             |    |    |    |               |      |
| Wang8         | 2018 | China-Hangzhou | Gender, Age                                                                       | Mongoloid   | TaqMan                                 | HB             | 731         | 80 | 121| 43 | 164           | 216  |
| Qiao32        | 2017 | China-Tianjin  | Gender, Age, BMI, Duration of HBV infection, HBV DNA, HCC family history          | Mongoloid   | PCR-DNA microarray-based assay         | HB             | 415         | 30 | 100| 74 | 40            | 120  |
| Jian33        | 2017 | China-Tianjin  | Gender, Age, HBVAg, HBV DNA, Child-Pugh stage, AFP, G677T-II, Drinking habit, Smoking habit | Mongoloid   | TaqMan                                 | HB             | 1275        | 168| 370| 188| 110           | 263  |
| Peres29       | 2016 | Brazil-Sao Paulo | Age, Gender, Alcoholic habit, Sm drinking habit                                    | Caucasian   | PCR-RFLP                               | PB             | 427         | 28 | 36 | 7  | 149           | 174  |
| Lei54         | 2016 | China-Enshi     | Gender, Age, Smoking habit, HCC family history, HBV infection, Drinking habit, BMI | Mongoloid   | PCR-RFLP                               | PB             | 250         | 19 | 38 | 64 | 12            | 32   |
| Zhang30       | 2015 | China-Shenyang  | Gender, Age, HBV infection, HCC infection                                         | Mongoloid   | PCR-Illumina Golden Gate platform      | PB             | 3000        | 440| 800| 260| 498           | 770  |
| Chang43       | 2014 | China-Taikin    | Gender, Age, BMI, Education, Smoking, Alcohol drinking, H. pylori Chagge status, HBsAg status, Anti-HCV status, Plasma AR1-albumin adduct levels | Mongoloid   | PCR-RFLP                               | PB             | 586         | 50 | 114| 30 | 135           | 199  |
| Xue55         | 2014 | China-Shanghai | Gender, Age, BMI, HCC infection                                                   | Mongoloid   | PCR-SNaPshot                            | PB             | 405         | 50 | 112| 43 | 50            | 111  |
| Cui44         | 2012 | China-Qingdao   | Gender, Age, Alcohol, BMI, Diabetes, Platelets count                               | Mongoloid   | PCR-SSCP                               | PB             | 987         | 52 | 179| 123| 121           | 325  |
| Couveret45    | 2012 | France-Paris    | Gender, Age, Alcohol, BMI, HCC family history, HBV infection, Drinking habit, BMI | Mongoloid   | PCR-DHPLC                              | PB             | 121         | 23 | 29 | 10 | 26            | 23   |
| Liu66         | 2012 | China-Tianjin   | Age, HBV infection status, AFP, Duration of HBV infection                          | Mongoloid   | TaqMan                                 | PB             | 333         | 39 | 85 | 57 | 37            | 64   |
| D’Amico47     | 2009 | Italy-Palermo   | Age, Duration of HBV infection                                                     | Caucasian   | PCR-RFLP                               | PB             | 188         | 30 | 37 | 27 | 56            | 28   |
| Fabris46      | 2009 | Italy-Udine     | Gender, Age, BMI, Smoking habit, Drinking habit, BMI                               | Caucasian   | TaqMan                                 | PB             | 301         | 22 | 30 | 13 | 69            | 113  |
| Kwak48        | 2008 | South Korea-Seongnam | Gender, Age, HBV infection, HCC family history, HBV infection, Drinking habit, BMI | Mongoloid   | TaqMan                                 | PB             | 257         | 32 | 46 | 18 | 64            | 106  |
| Yuan49        | 2007 | USA-Los Angeles | Gender, Age, Race/ethnicity, Level of education                                   | Mongoloid   | TaqMan                                 | PB             | 327         | 53 | 51 | 14 | 80            | 99   |
| Yuan49        | 2007 | China-Guangxi   | Gender, Age, Race/ethnicity, Level of education                                   | Mongoloid   | TaqMan                                 | PB             | 327         | 65 | 44 | 9  | 104           | 85   |
| Muts0         | 2007 | China-Taikin    | Gender, Age, Education, Income                                                    | Mongoloid   | TaqMan                                 | PB             | 585         | 50 | 114| 30 | 135           | 199  |
| Zhu51         | 2006 | China-Shanghai | Gender, smoking habit, Drinking habit, HBVAg, Anti-HCV, HCC family history         | Mongoloid   | TaqMan                                 | PB             | 1051        | 172| 226| 110| 173           | 268  |
| Ventura52     | 2005 | Italy-Foggia    | Age, BMI, Albumin, Quick, Creatinine, Haemoglobin, Platelet count                   | Caucasian   | TaqMan                                 | PB             | 72          | 7  | 986| 4394| 9  | 02  | 7  | 16 | 7 | 8  | 0.092         |
| Saftryg3      | 2004 | France-Paris    | Gender, Age                                                                       | Caucasian   | PCR-RFLP                               | PB             | 228         | 67 | 69 | 12 | 30            | 37   |
| MTHFR A1298C polymorphism |      |                |                                                                                   |             |                                        |                |             |    |    |    |               |      |
| Wang8         | 2018 | China-Hangzhou | Gender, Age                                                                       | Mongoloid   | TaqMan                                 | HB             | 731         | 181| 57 | 6  | 296           | 140  |
| Peres29       | 2016 | Brazil-Sao Paulo | Age, Gender, Alcoholic habit, Smoking habit                                       | Caucasian   | PCR-RFLP                               | PB             | 427         | 32 | 24 | 15 | 205           | 116  |
| Xue55         | 2014 | China-Shanghai | Gender, Age, BMI, HCC family history                                              | Mongoloid   | PCR-SNaPshot                            | PB             | 405         | 150| 52 | 3  | 152           | 44   |
| Cui44         | 2012 | China-Qingdao   | Gender, Age, BMI, Smoking status                                                  | Mongoloid   | PCR-SSCP                               | PB             | 267         | 253| 94 | 4  | 461           | 153  |
| Kwak48        | 2008 | South Korea-Seongnam | Gender, Age, HBV infection, HCC family history, HBV infection, Drinking habit, BMI | Mongoloid   | TaqMan                                 | PB             | 297         | 67 | 28 | 1  | 155           | 41   |
| Yuan49        | 2007 | USA-Los Angeles | Gender, Age, Race/ethnicity, Level of education                                   | Mongoloid   | TaqMan                                 | PB             | 549         | 159| 71 | 71 | 156           | 74   |
| Yuan49        | 2007 | China-Guangxi   | Gender, Age, Race/ethnicity, Level of education                                   | Mongoloid   | TaqMan                                 | PB             | 405         | 136| 101| 10 | 136           | 91   |
| Muts0         | 2007 | China-Taikin    | Gender, Age, Education, Income                                                    | Mongoloid   | TaqMan                                 | PB             | 588         | 135| 55 | 4  | 275           | 112  |

For MTHFR rs1801133 polymorphism, AA, AB and BB represent CC, CT and TT, respectively. For MTHFR rs1801131 polymorphism, AA, AB and BB refer to AA, AC and CC, HB = hospital based, HCC = hepatocellular carcinoma, HWE = Hardy-Weinberg Equilibrium, PB = population based, PCR-DHPLC = Polymerase Chain Reaction-Denaturing High Performance Liquid Chromatography, PCR-DNA microarray-based assay = Polymerase Chain Reaction with microarray-based assay, PCR-Illumina Golden Gate platform = Polymerase Chain Reaction based on Illumina Golden Gate platform, PCR-QIAamp DNA Blood Mini Kit = Polymerase Chain Reaction with QIAamp DNA Blood Mini Kit, PCR-RFLP = Polymerase Chain Reaction-Restriction Fragment Length Polymorphism, PCR-SNaPshot = Polymerase Chain Reaction with SNaPshot, TaqMan = Polymerase Chain Reaction with TaqMan probe

* P value for Hardy-Weinberg Equilibrium test in controls.
### Table 2

#### Pooled analysis and subgroup analysis of associations between MTHFR polymorphisms and HCC.

| Type of analysis | Number of the studies | Allelic/genic model | Dominant genic model | Recessive genic model | Heterozygote genic model | Homozygote genic model |
|------------------|------------------------|---------------------|----------------------|-----------------------|-------------------------|------------------------|
|                  |                        | OR [95% CI]         | OR [95% CI]          | OR [95% CI]           | OR [95% CI]             | OR [95% CI]            |
|                  |                        | P-meta-analysis     | P-meta-analysis      | P-meta-analysis       | P-meta-analysis         | P-meta-analysis       |
| MTHFR C677T      |                        |                     |                      |                       |                         |                        |
| polymorphism     |                        |                     |                      |                       |                         |                        |
| Pooled analysis  | 20                     | 1.05 [0.95, 1.17]   | .00068/R             | .330                   | 1.09 [0.96, 1.25]       | .00452/R              |
|                  |                        |                     |                      |                       |                         |                        |
| Subgroup analysis|                        |                     |                      |                       |                         |                        |
| Region           |                        |                     |                      |                       |                         |                        |
| China            | 12                     | 1.04 [0.94, 1.15]   | .00263/R             | .490                   | 1.11 [1.01, 1.21]       | .03444/F              |
| France           | 2                      | 0.87 [0.55, 1.39]   | .15515/R             | .560                   | 0.91 [0.59, 1.41]       | .19414/F              |
| Italy            | 3                      | 1.78 [0.77, 4.11]   | .00088/R             | .180                   | 1.71 [0.67, 4.33]       | .00681/R              |
| South Korea      | 1                      | 1.04 [0.73, 1.47]   | NA                   | .830                   | 0.93 [0.56, 1.57]       | NA                    |
| USA              | 1                      | 0.82 [0.59, 1.15]   | NA                   | .240                   | 0.76 [0.48, 1.20]       | NA                    |
| Brazil           | 1                      | 1.07 [0.73, 1.59]   | NA                   | .730                   | 1.11 [0.66, 1.86]       | NA                    |
| Entitcity        |                        |                     |                      |                       |                         |                        |
| Mongolia         | 13                     | 1.04 [0.94, 1.14]   | .00459/R             | .460                   | 1.09 [0.94, 1.09]       | .06414/F              |
| Caucasian        | 7                      | 1.18 [0.82, 1.72]   | .00081/R             | .380                   | 1.17 [0.78, 1.77]       | .00469/F              |
| Source of controls |                        |                     |                      |                       |                         |                        |
| Hospital Based   | 18                     | 1.04 [0.92, 1.17]   | .00070/R             | .570                   | 1.05 [0.91, 1.22]       | .00751/R              |
| Population Based | 2                      | 1.21 [1.04, 1.4]    | .0380/R              | .010                   | 1.43 [1.10, 1.86]       | .06580/R              |
| MTHFR A1298C     |                        |                     |                      |                       |                         |                        |
| polymorphism     | 8                      | 1.07 [0.78, 1.49]   | .00087/R             | .690                   | 1.08 [0.84, 1.39]       | .00171/R              |
|                  |                        |                     |                      |                       |                         |                        |
| Subgroup analysis|                        |                     |                      |                       |                         |                        |
| Region           |                        |                     |                      |                       |                         |                        |
| China            | 5                      | 0.83 [0.64, 1.09]   | .00074/R             | .190                   | 0.98 [0.70, 1.37]       | .00576/R              |
| South Korea      | 1                      | 1.27 [0.78, 2.08]   | NA                   | .350                   | 1.46 [0.84, 2.52]       | NA                    |
| USA              | 1                      | 1.92 [1.47, 2.62]   | NA                   | .000                   | 1.01 [0.80, 1.37]       | NA                    |
| Brazil           | 1                      | 1.74 [1.19, 2.53]   | NA                   | .004                   | 1.65 [0.99, 2.76]       | NA                    |
| Entitcity        |                        |                     |                      |                       |                         |                        |
| Mongolia         | 6                      | 0.88 [0.68, 1.14]   | .00373/R             | .340                   | 0.79 [0.52, 1.2]        | .00176/R              |
| Caucasian        | 2                      | 1.86 [1.49, 2.31]   | .0670/R              | .000                   | 1.25 [0.77, 2.02]       | .13579/R              |
| Source of Controls |                        |                     |                      |                       |                         |                        |
| Hospital Based   | 7                      | 1.11 [0.76, 1.66]   | .00089/R             | .600                   | 1.10 [0.81, 1.52]       | .00075/R              |
| Population Based | 7                      | 0.87 [0.67, 1.12]   | NA                   | .290                   | 0.97 [0.73, 1.39]       | NA                    |

EM = effect model, F = fixed effect model, HWE = Hardy Weinberg Equilibrium, NA = not available for one study, R = random effect model. Heterogeneity means $P$ value for heterogeneity; $P$ meta-analysis means $P$ value for meta-analysis.

Significant association with more than one study in each research group was bold and color red.
### 3.4.1 China

| Study or Subgroup | HCC Events | Control Events | Total Events | Weight | Odds Ratio M-H Fixed, 95% CI Year |
|-------------------|------------|----------------|--------------|--------|----------------------------------|
| Wang              | 121        | 201            | 322          | 7.5%   | 1.15 [0.81, 1.63] 2018           |
| Jiao              | 370        | 538            | 908          | 12.2%  | 0.92 [0.69, 1.23] 2017           |
| Qiao              | 100        | 130            | 230          | 3.1%   | 1.11 [0.65, 1.91] 2017           |
| Lei               | 38         | 57             | 95           | 1.5%   | 0.75 [0.32, 1.78] 2016           |
| Zhang             | 800        | 1240           | 2040         | 33.9%  | 1.10 [0.99, 1.38] 2015           |
| Chang             | 11 14 | 164            | 278          | 5.0%   | 1.55 [1.04, 2.30] 2014           |
| Xu                | 112        | 162            | 274          | 4.3%   | 1.01 [0.63, 1.62] 2014           |
| Cui               | 179        | 231            | 410          | 6.3%   | 1.28 [0.88, 1.88] 2012           |
| Liu               | 85         | 124            | 209          | 2.6%   | 1.26 [0.72, 2.19] 2010           |
| Mu                | 114        | 164            | 278          | 5.0%   | 1.55 [1.04, 2.30] 2007           |
| Yuanil            | 44         | 109            | 153          | 4.7%   | 0.83 [0.51, 1.34] 2007           |
| Zhu               | 226        | 398            | 624          | 13.8%  | 0.85 [0.64, 1.12] 2006           |
| Subtotal (95% CI) | 3518       | 4231           | 7749         | 100.0% | 1.11 [1.01, 1.22]           |

Total events: 2303

Heterogeneity: Chi² = 14.31, df = 11 (P = 0.22); I² = 23%
Test for overall effect: Z = 2.17 (P = 0.03)

### 3.4.2 France

| Study or Subgroup | HCC Events | Control Events | Total Events | Weight | Odds Ratio M-H Fixed, 95% CI Year |
|-------------------|------------|----------------|--------------|--------|----------------------------------|
| Couvert           | 29         | 52             | 81           | 30.0%  | 1.43 [0.65, 3.12] 2012           |
| Saffroy           | 69         | 136            | 205          | 70.0%  | 0.84 [0.46, 1.50] 2004           |
| Subtotal (95% CI) | 188        | 316            | 504          | 100.0% | 1.01 [0.63, 1.62]           |

Total events: 98

Heterogeneity: Chi² = 1.15, df = 1 (P = 0.29); I² = 13%
Test for overall effect: Z = 0.05 (P = 0.96)

### 3.4.3 Italy

| Study or Subgroup | HCC Events | Control Events | Total Events | Weight | Odds Ratio M-H Fixed, 95% CI Year |
|-------------------|------------|----------------|--------------|--------|----------------------------------|
| D'Amico           | 37         | 67             | 104          | 30.1%  | 2.47 [1.27, 4.78] 2009           |
| Fabris            | 30         | 52             | 82           | 57.5%  | 0.83 [0.45, 1.56] 2009           |
| Ventura           | 5          | 13             | 18           | 12.4%  | 1.05 [0.39, 3.13] 2005           |
| Subtotal (95% CI) | 132        | 309            | 441          | 100.0% | 1.35 [0.88, 2.07]           |

Total events: 72

Heterogeneity: Chi² = 5.62, df = 2 (P = 0.08); I² = 64%
Test for overall effect: Z = 1.40 (P = 0.16)

### 3.4.4 South Korea

| Study or Subgroup | HCC Events | Control Events | Total Events | Weight | Odds Ratio M-H Fixed, 95% CI Year |
|-------------------|------------|----------------|--------------|--------|----------------------------------|
| Kwak              | 46         | 78             | 124          | 100.0% | 0.87 [0.50, 1.50] 2008           |
| Subtotal (95% CI) | 46         | 146            | 192          | 100.0% | 0.87 [0.50, 1.50]           |

Total events: 46

Heterogeneity: Not applicable
Test for overall effect: Z = 0.51 (P = 0.61)

### 3.4.5 USA

| Study or Subgroup | HCC Events | Control Events | Total Events | Weight | Odds Ratio M-H Fixed, 95% CI Year |
|-------------------|------------|----------------|--------------|--------|----------------------------------|
| Yuanil            | 51         | 104            | 155          | 100.0% | 0.78 [0.48, 1.26] 2007           |
| Subtotal (95% CI) | 104        | 179            | 283          | 100.0% | 0.78 [0.48, 1.26]           |

Total events: 51

Heterogeneity: Not applicable
Test for overall effect: Z = 1.02 (P = 0.31)

### 3.4.6 Brazil

| Study or Subgroup | HCC Events | Control Events | Total Events | Weight | Odds Ratio M-H Fixed, 95% CI Year |
|-------------------|------------|----------------|--------------|--------|----------------------------------|
| Peres             | 36         | 64             | 100          | 100.0% | 1.10 [0.64, 1.89] 2016           |
| Subtotal (95% CI) | 64         | 174            | 238          | 100.0% | 1.10 [0.64, 1.89]           |

Total events: 36

Heterogeneity: Not applicable
Test for overall effect: Z = 0.35 (P = 0.73)

Test for subarous differences: Chi² = 3.77, df = 5 (P = 0.50), I² = 0%

**Figure 2.** Forest plot of the subgroup analysis of the MTHFR rs1801133 heterozygote genetic model (TC VS CC) and susceptibility to hepatocellular carcinoma in different regions.
China, South Korea, USA and Brazil were recruited. The quality assessment scale was showed in Table S2 (see Table S2, Supplemental Digital Content, which showed the quality assessment scale, http://links.lww.com/MD/G442) and the scores ranged from 6 to 9, which indicated the reliability of our included studies. The PRISMA checklist was attached as Table S3 (see Table S3, Supplemental Digital Content, which showed the PRISMA checklist, http://links.lww.com/MD/G443).

3.2. Meta-analysis results and heterogeneity analysis

Table 2 summarized the pooled and subgroup analysis of associations between the 2 MTHFR SNPs and HCC risk. In the pooled analysis of these 2 polymorphisms, significant association was only detected in heterozygote genetic model (OR = 1.10, 95% CI = [1.01, 1.20], Pmeta-analysis = 0.04; PHeterogeneity = 0.17, I2 = 23) for MTHFR rs1801133 polymorphism (Fig. 1), but for the MTHFR rs1801131 polymorphism, no significant association was found. In order to discover the potential associations and resource of heterogeneity, we conducted a comprehensive subgroup analysis stratiﬁed by HWE (In accordance with HWE or departure from HWE), Region (China, France, Italy, South Korea, USA, Brazil), Ethnicity (Mongoloid or Caucasian), and source of controls (Population based or Hospital based).

For the subgroup analysis stratified by HWE, no association was observed for the MTHFR rs1801133 polymorphism; significant association was detected in recessive genetic model (OR = 0.31, 95% CI = [0.14, 0.65], Pmeta-analysis = .002; PHeterogeneity = .03, I2 = 61) and heterozygote genetic model (OR = 0.36, 95% CI = [0.23, 0.55], Pmeta-analysis = .006; PHeterogeneity = .13, I2 = 44) for the Chinese subgroup for the MTHFR rs1801131 polymorphism (Fig. 2); as for the MTHFR rs1801131 polymorphism, significant associations were observed in recessive genetic model (OR = 1.11, 95% CI = [1.01, 1.21], Pmeta-analysis = .02; PHeterogeneity = .03, I2 = 44) and homozygote genetic model (OR = 0.36, 95% CI = [0.23, 0.55], Pmeta-analysis = .006; PHeterogeneity = .13, I2 = 44) for the Chinese (Fig. 3). For the MTHFR rs1801131 polymorphism, significant association was observed in the heterozygote genetic model (OR = 1.10, 95% CI = [1.00, 1.21], Pmeta-analysis = .04; PHeterogeneity = .24, I2 = 20) in Mongoloid subgroup (Fig. 4); but for the subgroup analysis stratified by ethnicity of the MTHFR rs1801131 polymorphism, significant associations were widely observed [Mongoloid: recessive genetic model (OR = 0.30, 95% CI = [0.19, 0.62], Pmeta-analysis = .004; PHeterogeneity = .82, I2 = 14) and heterozygote genetic model (OR = 1.11, 95% CI = [1.01, 1.21], Pmeta-analysis = .04; PHeterogeneity = .03, I2 = 44) for the Chinese (Fig. 3)].

![Forest plot of the subgroup analysis of the MTHFR rs1801131 homozygote genetic model (CC VS AA) and susceptibility to hepatocellular carcinoma in different regions.](http://links.lww.com/MD/G443)
CI = [0.15, 0.58], \( P_{\text{meta-analysis}} = .000; P_{\text{heterogeneity}} = .06, I^2 = 52 \), homozygote genetic model (OR = 0.36, 95% CI = [0.24, 0.55], \( P_{\text{meta-analysis}} = .000; P_{\text{heterogeneity}} = .21, I^2 = 31 \); Caucasian: allelic genetic model (OR = 1.86, 95% CI = [1.49, 2.31], \( P_{\text{meta-analysis}} = .000; P_{\text{heterogeneity}} = .67, I^2 = 0 \), homozygote genetic model (OR = 3.47, 95% CI = [2.24, 5.39], \( P_{\text{meta-analysis}} = .000; P_{\text{heterogeneity}} = .45, I^2 = 0 \) ) (Fig. 5). As for the subgroup analysis stratified by source of controls, no association was observed for the rs1801131 polymorphism and significant associations were found in allelic genetic model (OR = 1.21, 95% CI = [1.04, 1.40], \( P_{\text{meta-analysis}} = .010; P_{\text{heterogeneity}} = .93, I^2 = 0 \), dominant genetic model (OR = 1.43, 95% CI = [1.10, 1.86], \( P_{\text{meta-analysis}} = .007; P_{\text{heterogeneity}} = .68, I^2 = 0 \) ) and heterozygote genetic model (OR = 1.40, 95% CI = [1.07, 1.84], \( P_{\text{meta-analysis}} = .020; P_{\text{heterogeneity}} = .50, I^2 = 0 \) ) for the MTHFR rs1801133 polymorphism.

### 3.3. Sensitivity analysis and publication bias

The results of sensitivity analysis indicated that any single study had no significant effect on pooled OR of relationship between the MTHFR rs1801133 and rs1801131 polymorphisms and HCC risk (Fig. 6). Funnel plots for the MTHFR rs1801133 polymorphism under the heterozygote genetic model was symmetrical, implying no significant publication bias (Fig. 7) and the Egger linear regression test (\( P = .845 \)) also confirmed the negation of publication bias. But for the MTHFR rs1801131 polymorphism, because the number of studies included was less than 10, publication bias could not be assessed.

### 3.4. Trial sequential analysis

Trial sequential analysis was introduced to evaluate the pooled results of the MTHFR rs1801133 polymorphism and HCC risk under heterozygote genetic model (Fig. 8). Based on the sample size estimation, a sample size of 11259 was required to detect a plausible result for the association of the MTHFR rs1801133 polymorphism. In the present study, a sample size of 9412 has been tested using the heterozygote genetic model (TC VS CC), moreover, the Z curve line only cross the conventional boundary and do not cross the TSA boundary line, indicating that the cumulative evidence is inconclusive and further studies are required to confirm conclusion.
4. Discussion

Abnormal DNA synthesis and methylation caused by environmental or genetic factors play important role in the occurrence and development of HCC. A lower MTHFR activity will lead to the increased pool of 5,10-methylenetetrahydrofolate for thymidylate synthase and the decreased pool of 5-methyltetrahydrofolate for SAMe, which could favor optimal DNA synthesis, methylation and repair by reducing uracil mis incorporation and double strand breaks of DNA.\[58\] Functional researches have indicated that subjects with the mutant allele of these 2 polymorphisms showed lower MTHFR enzyme activities.\[59,60\] Previous meta-analysis reported an increased risk of HCC in the MTHFR rs1801133 polymorphism,\[31,61–63\] but the small sample size could bias the results, in addition, the influence of studies departure from Hardy Weinberg Equilibrium on the analysis was not discussed in the former studies. As for the MTHRF rs1801131 polymorphism, no association was detected in meta-analysis, but several late case-control studies reported the polymorphism was associated with HCC risk.\[13,29\] The important biological role of the 2 polymorphisms and the inconsistent conclusions from previous studies draw us to reevaluate the associations between MTHFR polymorphisms and HCC risk with comprehensive subgroup analysis and trial sequential analysis.

![Figure 5](image-url)

Figure 5. Forest plot of the subgroup analysis of the MTHFR rs1801131 homozygote genetic model (CC VS AA) and susceptibility to hepatocellular carcinoma in different ethnicities.

![Figure 6](image-url)

Figure 6. Sensitivity analysis of the pooled analysis of the MTHFR rs1801133 heterozygote genetic model (TC VS CC) and susceptibility to hepatocellular carcinoma.

![Figure 7](image-url)

Figure 7. Publication bias of the pooled analysis of the MTHFR rs1801133 heterozygote genetic model (TC VS CC) and susceptibility to hepatocellular carcinoma.
Our study included nineteen articles, involving 20 studies with 5227 HCC patients and 6688 healthy controls for the MTHFR rs1801133 polymorphism and 8 studies with 1714 HCC patients and 2775 healthy controls for the MTHFR rs1801131 polymorphism. The pooled meta-analysis results showed that the MTHFR rs1801133 polymorphism in the heterozygote genetic model was associated with a high risk of developing HCC. It implied the TC genotypes had a 10% increased risk of HCC compared to CC genotypes (OR [95% CI] = 1.10 [1.01, 1.20]). Moreover, the results of sensitivity analysis and publication bias also increased the reliability and stability of the association. However, the TSA results required more further large sample size studies to confirm the association. As for the MTHFR rs1801131 polymorphism, no association was discovered.

The differences in the genetic equilibrium of control group, region, ethnicity, source of controls may have an influence on the risk of developing HCC in a way of gene-environment interaction. Hence, we performed a comprehensive subgroup analysis based on the differences mentioned above. To test the Hardy-Weinberg Equilibrium in the control group is essential to reflect the homogeneity of selected population and reduce the bias in enroll research subjects. In the subgroup in accordance with HWE, a decreased risk of the MTHFR rs1801131 polymorphism under the recessive genetic model was observed (OR [95% CI] = 0.51 [0.29, 0.92]), indicating the CC genotype had a 49% decreased risk of HCC compared to CA/AA genotypes. Hospital based and Population based are the 2 main source of controls, the advantages of low selection bias and more randomization in Population based control group could generate more reliable and solid results. In the subgroup analysis stratified by source of controls, extensive increased risks of HCC in the MTHFR rs1801133 polymorphism under allelic (OR [95% CI] = 1.21 [1.04, 1.40]), dominant (OR [95% CI] = 1.43 [1.10, 1.86]), heterozygote (OR [95% CI] = 1.40 [1.07, 1.84]) genetic model were observed.

Geography information is an important environment variable for gene-environment interaction. Significant associations were observed for these 2 polymorphisms in Chinese group. The MTHFR rs1801133 polymorphism was associated with a high risk of HCC under dominant genetic model (OR [95% CI] = 1.11 [1.01, 1.21]) and heterozygote genetic model (OR [95% CI] = 1.11 [1.01, 1.22]). A decreased risk of HCC for MTHFR rs1801131 polymorphism was detected in recessive (OR [95% CI] = 0.31 [0.14, 0.65]) and homozygote (OR [95% CI] = 0.36 [0.24, 0.55]) genetic model. In addition, the same increased risk of HCC for the MTHFR rs1801133 polymorphism under the heterozygote genetic model was observed in Mongoloid population. But for the MTHFR rs1801131 polymorphism, interesting results were emerged. The decreased risk of HCC was detected in Mongoloid under the recessive (OR [95% CI] = 0.30 [0.15, 0.58]) and homozygote genetic model (OR [95% CI] = 0.36 [0.23, 0.55]). But the increased risk of HCC was observed in Caucasian under the allelic (OR [95% CI] = 1.86 [1.49, 2.31]).
and homozygote (OR [95% CI] = 3.47 [2.24, 5.39]) genetic model. As a brief summary, in Mongoloid especially in Chinese, an increased risk of HCC for the MTHFR rs1801133 and a decreased risk of HCC for the MTHFR rs1801131 were observed, nevertheless, an increased risk of HCC for the MTHFR rs1801131 in Caucasian was discovered.

The contrary risk associations in the Mongoloid and Caucasian populations of the MTHFR rs1801131 polymorphism arouse our great interests. After literature intensive reading, we found there are 2 ways of changed MTHFR enzyme activity on the HCC risk:

1. the reduced MTHFR enzymatic activity would result in reductive conversion of 5,10-methylenetetrahydrofolate into 5-methylentetrahydrofolate, next, a decreased level of S-adenosylmethionine lead to down-regulated DNA methylation and an increased risk of HCC occurrence;[64]

2. the reduced activity of MTHFR contribute to an accumulation of 5,10-methylenetetrahydrofolate, resulting in a lower dUMP/dTMP ratio, reduce the incidence of the incorrect incorporation of uracil into the DNA and double-strand DNA breaks, which can strengthen the ability of the DNA and finally lead to a lower HCC risk.[65,66]

We found the mutant 1298C allele could decrease the risk of HCC in Chinese and Mongoloid population, and the epidemic study reported that most cases of HCC occur in Asian,[67] particularly in East Asia with a very high incidence (over 20 cases/100000 population), which was a proof to our results. Anyway, larger studies are required to validate the associations.

Several limitations should be acknowledged in the present meta-analysis. Firstly, associations in relative small sample size in some subgroups should be interpreted with cautions such as the increased risk of HCC in Caucasian for the MTHFR rs1801131 polymorphism, the only 1 study in some subgroups (Brazil, South Korea, USA, Hospital based for MTHFR rs1801131 polymorphism); secondly, only English and Chinese literatures were retrieved, and missing of relevant studies in other language might bias our results; thirdly, for the type of our research (meta-analysis), the unreasonable data and bias in original studies could be the potential confounding factors; at last, the genotyping method were not uniform and could have an influence on the deviation of outcomes. All above, further studies with larger sample size from different regions and ethnicities are required to provide a more accurate association.

In conclusion, this meta-analysis indicates the TC genotype of the MTHFR rs1801133 polymorphism is associated with an increased risk of hepatocellular carcinoma (HCC) risk, in addition, the MTHFR rs1801133 polymorphism was associated with an increased risk of HCC in Mongoloid population especially in Chinese. As for the MTHFR rs1801131 polymorphism, increased HCC risk was observed in Caucasian population, and decreased risk of HCC was remarkably discovered in homozygous mutant CC genotypes in Mongoloid and Chinese subgroups. In the future, larger well-designed studies are warranted to verify these results.

Author contributions

Conceptualization: Lang Li.
Data curation: Binfenf Wang, Lang Li.
Formal analysis: Yan Yan.
Investigation: Miaomiao Ma, Yan Yan.

Methodology: Miaomiao Ma, Xiaojun Guo, Yan Yan, Lang Li.
Project administration: Lang Li.
Resources: Yan Yan.
Software: Binfenf Wang, Miaomiao Ma, Xiaojun Guo, Yan Yan.
Supervision: Binfenf Wang, Lang Li.
Validation: Binfenf Wang, Miaomiao Ma, Lang Li.
Visualization: Binfenf Wang, Lang Li.
Writing – original draft: Binfenf Wang, Lang Li.
Writing – review & editing: Binfenf Wang, Miaomiao Ma, Xiaojun Guo, Yan Yan, Lang Li.

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