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Diagnostic accuracy of high resolution melting analysis for detection of *KRAS* mutations: a systematic review and meta-analysis

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Increasing evidence points to a negative correlation between *KRAS* mutations and patients' responses to anti-EGFR monoclonal antibody treatment. Therefore, patients must undergo *KRAS* mutation detection to be eligible for treatment. High resolution melting analysis (HRM) is gaining increasing attention in *KRAS* mutation detection. However, its accuracy has not been systematically evaluated. We conducted a meta-analysis of published articles, involving 13 articles with 1,520 samples, to assess its diagnostic accuracy compared with DNA sequencing. The quality of included articles was assessed using the revised Quality Assessment for Studies of Diagnostic Accuracy (QUADAS-2) tools. Random effects models were applied to analyze the performance of pooled characteristics. The overall sensitivity and specificity of HRM were 0.99 (95% confidence interval [CI]: 0.98–1.00) and 0.96 (95%CI: 0.94–0.97), respectively. The area under the summary receiver operating characteristic curve was 0.996. High sensitivity and specificity, less labor, rapid turn-around and the closed-tube format of HRM make it an attractive choice for rapid detection of *KRAS* mutations in clinical practice. The burden of DNA sequencing can be reduced dramatically by the implementation of HRM, but positive results still need to be sequenced for diagnostic confirmation.

KRAS, a critical gene in the development of many cancers, is one of three members of a family of genes (*KRAS*, *NRAS* and *HRAS*) that encode small guanine nucleotide-binding proteins. It is a key element of cell-signaling pathways, such as mitogen-activated protein kinase (MAPK), Janus kinase/signal transducers and activators of transcription (JAK–STAT) and phosphatidylinositol-3 kinase (PI3K) pathways and plays an important role in a variety of cellular processes, including proliferation and apoptosis[1]. The protein encoded by *KRAS* is a key signal transducer for a variety of cellular receptors, including the epidermal growth factor receptor (EGFR). *KRAS* mutations have been associated with poor prognosis in different tumor types, including pancreatic cancer (~65%), colon cancer (~40%), lung cancer (~20%) and ovarian cancer (~15%)[2]. While having some utility as a genetic marker for diagnostic and prognostic purposes, *KRAS* mutation status has great value in assisting with EGFR-targeted therapy decisions because of its strong association as a negative predictor of responses to monoclonal antibody based therapies in colon cancer, and as a marker of resistance to small-molecule tyrosine kinase inhibitors in non-small-cell lung cancer (NSCLC)[1,3,4]. These findings not only make *KRAS* a strong predictor of clinical resistance to EGFR-targeted therapies but also demonstrate the significant diagnostic and prognostic implications of *KRAS* mutation status in a broad range of clinical settings. Therefore, the demand for *KRAS* mutational analysis as a predictive marker has increased rapidly. Prior to treatment with EGFR inhibitors in colorectal cancer (CRC), *KRAS* testing has become mandatory in the European Union[5] and is recommended in the United States[6].

A number of laboratory methods have been utilized to detect mutation status in the *KRAS* gene, most of which fall under the categories of DNA sequencing, single-strand conformation polymorphisms, allele-specific PCR, denaturing high performance liquid chromatography, denaturant gradient gel electrophoresis, array/strip analysis and high resolution melting analysis (HRM)[1,7–9]. All of these laboratory methods have been successfully applied to clinical *KRAS* mutation testing, and each has its unique feature. Although DNA sequencing, including direct DNA sequencing and pyrosequencing, is considered to be the "golden standard" for known/unknown mutation scanning[10], its relatively low sensitivity or limits of detection may not be optimal for clinical settings.

HRM is a simple, PCR-based method for detecting DNA sequence variation by measuring changes in the melting of a DNA duplex[11]. To follow the transition of double-stranded DNA (dsDNA) to single-stranded (ssDNA), intercalating dyes such as LC Green and LC Green Plus, ResoLight, EvaGreen and SYTO 9 were employed[12]. These dyes emit more strongly when bound to dsDNA than ssDNA, namely, the fluorescence intensity decreases as two strands of the dsDNA melt apart. The level of fluorescence intensity vs. temperature is plotted, which is known as a melt curve. The melting temperature at which 50% of the DNA is in the double stranded state can be approximated by taking the derivative of the melting curve[13]. The distinctive melting curve can be used to detect DNA sequence variants without the need for any post-PCR handling. Advantages of the method include a rapid turn-around time, a closed-tube format that greatly reduces contamination risk, high sensitivity and specificity, low cost and, unlike other methods, no sample processing or separations after PCR[14]. Furthermore, HRM is a nondestructive method. Therefore, subsequent analysis of the sample by other methods, such as DNA sequencing or gel-electrophoresis can still be performed after HRM[13]. Due to the advantages mentioned above, HRM might be an attractive choice for the detection of *KRAS* mutations. However, the accuracy of HRM for the detection of *KRAS* mutations has not been systematically assessed. Thus, we conducted this meta-analysis to assess accuracy of HRM for the detection of *KRAS* mutations.

Results

Literature search outcome. A total of 288 records were retrieved after an independent search of the scientific literature by reviewers. One hundred and thirty-four records were excluded because of duplicates and 126 records were excluded after reviewing of the title and abstract. Twenty-eight full-text papers were deemed to be potentially relevant and were examined in detail. Fifteen full-text papers were excluded for the reasons described in Figure 1. Finally, 13 studies[5,10,15–25] met the inclusion criteria and were included in this meta-analysis. These articles were divided into 15 'units' for statistical analysis according to the specimen source.

Study characteristics and quality assessment. The main characteristics of the eligible studies are summarized in Table 1. A total of 13 studies with 1,520 samples were included in our meta-analysis study. Disease types included colorectal cancer (CRC) or colon cancer (CC; n=9), non-small cell lung cancer (NSCLC; n=3) and pancreatic cancer (PA; n=1). The average sample number was 101 (range 28 to 200). The most common specimen source was formalin-fixed and paraffin-embedded tissues (FFPE; n=7), followed by fresh frozen tissues (FF; n=6). All studies used DNA extraction kits from Qiagen. The most frequently used dye was Syto9 (S9; n=7), followed by Resolight (RL; n=7). Amplicon lengths varied from 59 bp to 247 bp, with an average length of 143 bp. The total volume (μL) of HRM was 9 μL (n=1), 10 μL (n=8), 20 μL (n=3), 25 μL (n=1) and 50 μL (n=1), while one study did not report the total volume of HRM. All the eligible studies used DNA direct sequencing as the reference standard. The number of false positives and false negatives obtained from each eligible study varied greatly. The number of false positives ranged from 0 to 22 while the number of false negatives ranged from 0 to 2.

We assessed the quality of the eligible studies using the wildly accepted revised Quality Assessment for Studies of Diagnostic Accuracy (QUADAS–2) tools[26]. The domains of patient selection, index test and reference standard, both in risks of bias and applicability concerns, were labeled as "low risk". The flow and timing domain was labeled as "unclear risk".

Diagnostic accuracy and threshold analysis. The Spearman correlation coefficient was 0.150 (P=0.593), which indicated an absence of a threshold effect. Therefore, we pooled the sensitivity, specificity, positive likelihood ratio (PLR), negative likelihood ratio (NLR) and diagnostic odds ratio (DOR) by the data from the eligible studies. The overall diagnostic sensitivity was 0.99 (95% CI: 0.98–1.00) and the overall specificity was 0.96 (95%CI: 0.94–0.97; Figure 2). PLR, NLR and DOR were three other commonly used test indicators for diagnostic tests[27]. The greater the value of PLR, the more likely the test result was associated with the disease, while the lower the value of NLR, the more likely the result was associated

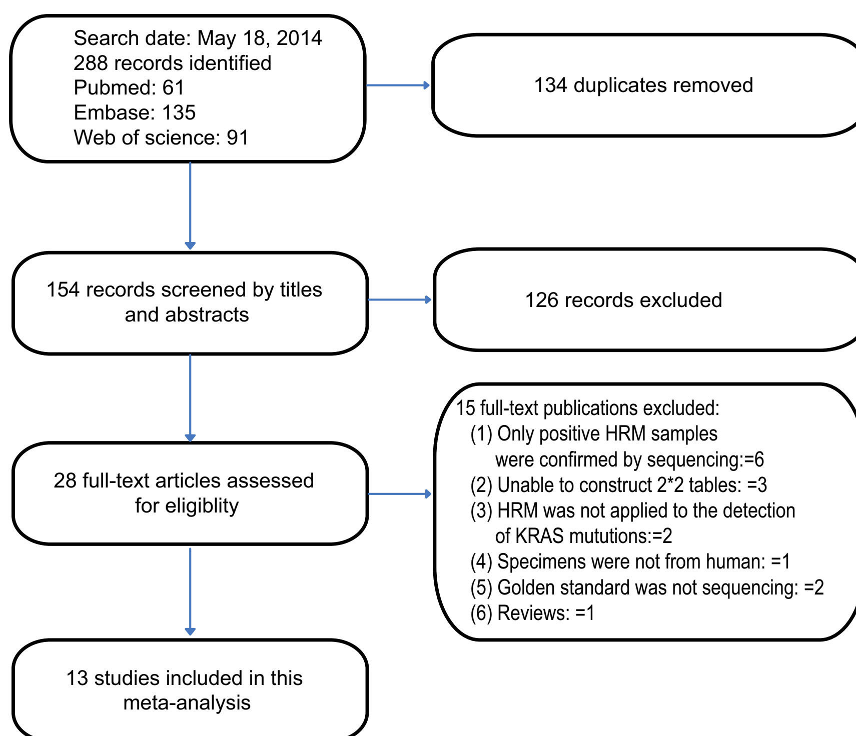

Figure 1 | Flowchart describing the systematic literature search and study selection process.

Table 1 | Characteristics of the 13 eligible studies in the meta-analysis

Study	Country	Year	Disease	Number	Specimen source	Instrument	Dye	AL	Total volume (μL)	TP	FP	FN	TN
Krupuy et al.[15]	Australia	2006	NSCLC	30	FF	RG	S9	92, 189	20	9	0	0	21
Do et al.[16]	Australia	2008	NSCLC	200	FFPE	RG	S9	92	20	25	0	0	175
Simi et al.[17]	Italy	2008	CRC	116	FF	RG	S9	167	10	50	0	0	66
Fassina et al.[18]	Italy	2009	NSCLC	77	CS	LC480	RL	172	NR	9	0	0	68
Ma. et al.[19]	China	2009	CRC	100	FFPE	LC480	RL	170	10	61	0	1	38
Whitehall et al.[20]	Australia	2009	CRC	160	FFPE	LC480	S9	80–92	10	54	0	2	104
Whitehall et al.[20]	Australia	2009	CRC	140	FF	RG	S9	80–92	10	40	4	0	96
van Eijk, R. et al.[23]	Netherlands	2010	CRC	92	FFPE	LS96	S9	166	10	52	5	0	35
van Eijk, R. et al.[23]	Netherlands	2010	CRC	28	FF	LS96	S9	166	10	14	1	0	13
Franklin et al.[22]	USA	2010	CC	118	FFPE	LC480	RL	NA	10	42	22	0	54
Bennani et al.[21]	Morocco	2010	CRC	56	PE	LC480	RL	143	10	17	7	0	32
Zhang et al.[25]	China	2011	PC	50	FFPE	LC480	RL	59, 163	20	19	0	0	31
Solassol et al.[24]	France	2011	CRC	131	FF	LC480	RL	247	50	47	0	0	84
Krol. et al.[5]	Netherlands	2012	CRC	125	FF	LC480	RL	189	25	39	2	0	84
Akiyoshi et al.[10]	Japan	2013	CRC	97	FFPE	LS96	LS	92	9	51	3	2	41

AL: Amplicon length; NSCLC: non-small cell lung cancer; CRC: colorectal cancer; CC: colon cancer; PC: pancreatic cancer; FF: fresh frozen tissue; FFPE: formalin-fixed and paraffin-embedded; CS: cytologic slides; PE: paraffin-embedded; RG: Rotorgene6000; LS96:lightScanner96; S9: Syto 9; RL: Resolight; LS: LightScanner Master Mix; NR: not reported; TP: true-positive; FP: false-positive; FN: false-negative; TN: true-negative.

with absence of the disease. DOR is defined as the odds of true positives vs. that of false positives. The value of DOR ranged from 0 to infinity, higher values indicating better discriminatory test performances[28]. Figures 3a–b show that HRM has a high PLR (27.24, 95%CI: 11.06–67.14) and a low NLR (0.03, 95% CI: 0.02–0.05), indicating HRM's excellent ability to both confirm and exclude the presence of *KRAS* mutations. In addition, the value of DOR was 1,121.36 (95%CI: 503.10–2,499.41), further indicating HRM is an effective method for *KRAS* mutation scanning (Figure 3c). The I^2 test for heterogeneity demonstrated greater heterogeneity for specificity (I^2 = 88.3%, P= 0.00) and PLR (I^2 =87.7%, P=0.00). The summary receiver operating characteristic (sROC) curve, which is intended to present the relationship between sensitivity and specificity across all included studies in the context of a meta-analysis[29], is shown in Figure 3d. The sROC curve from our data showed that the Q value was 0.97, while the area under the curve (AUC) was 0.996.

Meta-regression analysis and publication bias. The results of multivariate meta-regression analysis (Table 2) demonstrated that there was no statistical significance between eligible studies. The subgroup analysis was not executed because the source of heterogeneity was not found. In our study, the Deek's Funnel Plot Asymmetry Test demonstrated that publication bias was not significant (P=0.56; Figure 4).

Discussion

Since it was first introduced for genotyping in 2003[30], HRM has been used to detect mutations such as *EGFR*[31], *KIT*[32], *BRAF*[33], *BRCA*[34], *TP53*[35] and *KRAS*[15]. The present meta-analysis found 13 published studies on the diagnostic accuracy of HRM for detection of *KRAS* mutations. The results of the overall sensitivity and specificity indicate a very high level of overall diagnostic accuracy for HRM. In addition, no publication bias was found in the present meta-analysis, indicating that the results of the present meta-analysis are reliable. Therefore, we concluded that HRM is a very sensitive and specific method for scanning *KRAS* mutations. The AUC under the sROC curve is a global indicator for assessing the diagnostic performance of an index test[36]. The present meta-analysis found that the AUC was close to 1 (0.996), also indicating that HRM is an effective method for *KRAS* mutation scanning. Similar results were obtained in a recently published study[28] designed to evaluate overall accuracy of HRM for rapid detection of *BRAF* mutations. Overall sensitivity and specificity values in that study were 0.99 (95%CI = 0.99–1.00) and 0.99 (95%CI = 0.96–1.00), respectively. Results from the two studies showed that HRM is a robust method for mutation scanning for both *KRAS* and *BRAF*.

However, there are discordant results between HRM and DNA sequencing in seven[5,10,19–23] of the 13 included studies. The total number of false positives was 44, which may be explained by different

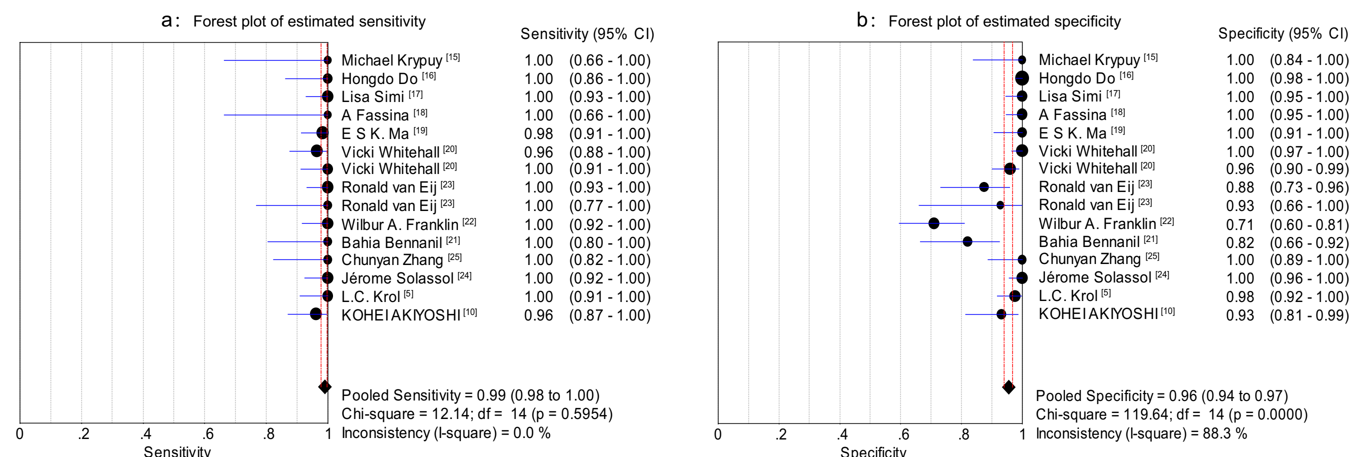

Figure 2 | Forest plots of estimated sensitivity (a) and specificity (b) for HRM with 95%CI. Each solid circle represents an eligible study. The size of the solid circle reflects the sample size of each eligible study. Error bars represents 95%CI.

Figure 3 | Forest plots of estimated PLR (a), NLR (b) and DOR (c) for HRM with 95%CI, and sROC (d). Each solid circle represents an eligible study. The size of the solid circle reflects the sample size of each eligible study. Error bars represents 95%CI.

limits of detection or sensitivity for the two methods. HRM can detect mutations in samples containing ~1% to 10% cells with mutated DNA, as opposed to direct sequencing, which requires the presence of mutated DNA in at least 10% to 30% of the sample[37]. Some clinical samples that are low in tumor cells or DNA may be labeled as positive by HRM but negative by direct sequencing, which results in a false positive. The total number of false negatives was five, which may be explained by greater amplicon length and lower GC content[38].

Considerable heterogeneity was observed in the pooled value of specificity and PLR. Though a multivariate meta-regression analysis was performed, we could not find the source of heterogeneity. This heterogeneity may be related to additional factors, such as the sequence length, GC content and the presence of substances such as DMSO or betaine[38–40]. However, this information is hard to collect and quantitatively analyze.

Though the accuracy of HRM is very high, several factors have to be taken into consideration when implementing HRM techniques into routine clinical practice, such as PCR specificity, sample type, length of the amplicon, GC content, dye, instrument, and melting analysis software. Melting is performed directly after PCR and different heterozygotes may produce melting curves so similar to each other that, although they clearly vary from homozygous variants, they are not differentiated from each other. Therefore, specific amplification of the interested targets is critical, requiring careful choice of primers and optimized temperature cycling[41].

Both FF and FFPE can be used for *KRAS* mutation scanning; however, a higher sensitivity was obtained when fresh tissues were used for HRM, because DNA may degenerate during sample processing and storage[14]. A head-to-head comparison study[24] showed that the agreement for the mutational status of *KRAS* between FF and matched FFPE specimens was low. Therefore, the authors suggested that fresh frozen specimens (FF) are favored when possible. When only FFPE samples are available, the risk of DNA degeneration should be prevented by using large amounts of template DNA or by performing multiple amplifications. Mutations in a long amplicon complicate the analysis. Hence, shortening the length of the amplicons can make melting curve differences between mutant and wild-type alleles more obvious and, in turn, easier to distinguish[42]. For example, for PCR products below 400 bp, sensitivity and specificity were 100%, decreasing to 96.1% sensitivity and 99.4% specificity if the PCR products were between 400 bp and 1000 bp[14]. Some studies showed that a lower GC content might have an association with false negative results[11,38]. Besides, different dyes are variably effective. For example, LCGreen Plus detects heterozygotes better than Syto 9, which is better than EvaGreen, which is better than SYBR Green I[9]. Instruments also have an impact on the sensitivity and specificity of HRM, because instruments have differences in their ability to resolve shape and absolute temperature differences, explained by their signal-to-noise ratios, acquisition rates, data density and software[8,12,14]. Also, it should be noted that although HRM may prove to be a helpful screening method for *KRAS* mutations, the requirement for confirmation by direct sequencing or other methods is necessary, especially in a diagnostic context.

Several limitations must be pointed out in our present meta-analysis despite the analysis showing high sensitivity and specificity. Firstly, though a meta-regression had been performed, we still could not find the source of the heterogeneity. Secondly, the sample size (1,520) included in our meta-analysis is relatively small, which might have some effect on the overall accuracy of the HRM. Lastly,

Table 2 | Results of the multivariable meta-regression model for the characteristics with backward regression analysis (Inverse variance weights; variables were retained in the regression model if P<0.05)

Variables	*Coeff.*	*Std.Err.*	*P-value*	*RDOR*	*[95%CI]*
Cte.	4.238	6.4547	0.5405	---	---
S	−0.472	0.3554	0.2417	---	---
Disease	0.612	1.1813	0.6262	1.85	0.09–38.44
Number	0.011	0.0180	0.5818	1.01	0.96–1.06
Specimen source	−0.151	0.6377	0.8222	0.86	0.17–4.43
Instrument	−0.702	1.0401	0.5299	0.50	0.03–7.19
Dye	−0.640	0.5565	0.2728	0.53	0.16–1.77
Lengths	0.009	0.0107	0.4266	1.01	0.98–1.04
Total volume	0.018	0.0648	0.7903	1.02	0.86–'1.20

Cte: Constant Coefficient; S: Statistic S; RDOR: Relative diagnostic odds ratio.

sufficient information about other factors was not collected, which may affect the accuracy of HRM, such as the sequence length, GC content and the presence of substances such as DMSO or betaine.

In summary, high sensitivity and specificity, less labor, a rapid turn-around and the closed-tube format of HRM make it an attractive choice for rapid detection of *KRAS* mutations in clinical practice. The DNA sequencing burden can be reduced dramatically by the implementation of HRM, but positive results still need to be sequenced for diagnostic confirmation.

Methods

Literature search strategy. Medline (using PubMed as the search engine), Web of science and Excerpta Medica Database (EMBASE) were searched to identify relevant publications published in English until May 18, 2014. The search words (in Title/Abstract fields) were used: 'high resolution melting analysis' or 'high resolution melting analyses' or 'high resolution fluorescent melting analysis' or 'high resolution amp icon melting analysis' or 'HRM' or 'HRMA' or 'HRMCA' **AND** 'mutation' or 'mutations' **AND** '*KRAS*' or '*K-RAS*' **AND** 'sequence' or 'sequencing'. We also performed a manual search for additional eligible studies in the reference lists of the articles identified.

Inclusion and exclusion criteria. The following inclusion criteria were used: 1) HRM was applied to the detection of *KRAS* mutations in humans; 2) DNA sequencing was used as a reference standard; and 3) sensitivity and specificity of HRM were reported or could be calculated from the provided data. The exclusion criteria were as follows: 1) studies were performed using only HRM; 2) only positive HRM samples were confirmed by sequencing; 3) the reference standard was not DNA sequencing; 4) the samples were cell lines or artificially created sequences; 5) they were unable to construct 2×2 tables; 6) the articles were reviews, letters, comments, and conference abstracts because of limited data; and 7) publications were identified as duplicates.

Data extraction and quality assessment. Two reviewers (YPL and HYW) independently extracted relevant data from each eligible study. The following data were collected: author's name, country, disease type, specimens source, reference standard, instruments used, dye used, lengths of amplicon, number of samples, and number of samples with the indicated results (TP, FP, FN, and TN), and 0.5 was added to all cells to handle studies with empty cells. We assessed the quality of the eligible studies using the widely accepted revised Quality Assessment for Studies of Diagnostic Accuracy (QUADAS–2) tools. Disagreements between reviewers were resolved by discussion or by consensus including a third author.

Statistical analysis. The outcome parameters were overall sensitivity, specificity, positive likelihood ratio (PLR), negative likelihood ratio (NLR), diagnostic odds ratio (DOR) and their corresponding 95% confidence intervals (CIs) by a random-effects

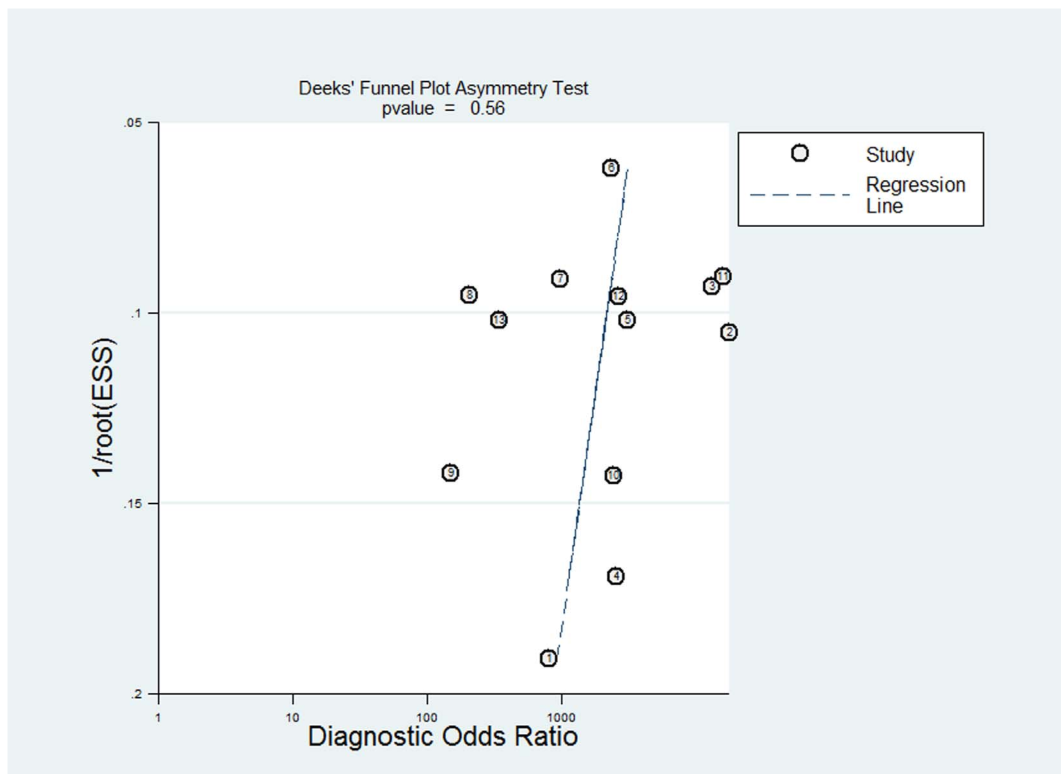

Figure 4 | Deek's Funnel Plot Asymmetry Test for the assessment of potential publication bias. Each solid circle represents a study in this meta-analysis. The publication bias was not significant (P=0.56).

model. Forest plots were used to describe the pooled sensitivity and specificity, as well as heterogeneity of eligible studies.

The degrees of heterogeneity were explored with a chi-square test of heterogeneity (Q Cochran's Q statistical) and inconsistency index (I-square), which was determined using Meta-Disc (version 1.4) software[43]. Taking into account the low statistical power of these tests, significant heterogeneity was defined as a Q test P value of <0.10, or an I^2 measure >30%.

The summary receiver operating characteristic (sROC) curves and area under the curve (AUC) were applied to demonstrate the overall diagnostic performance of HRM. In addition, the Spearman correlation coefficient was used to verify if the heterogeneity in meta-analysis could be explained by a threshold effect; a threshold effect was defined as a positive correlation (P<0.05). Deek's Funnel Plot Asymmetry Test was applied to determine the presence of publication bias using STATA 12.1 software (Stata Corp., College Station, Texas, USA.)[44].

Meta-regression analysis. Meta-regression analysis was performed to explore the factors of heterogeneity using Meta-Disc (version 1.4) software. We applied a multivariable regression model and used a backward stepwise algorithm with the covariates including disease type, specimen source, instrument, dye, length of PCR products, and total volume of HRM; variables were retained in the regression model if P<0.05.The subgroup analysis was performed if factors of heterogeneity could be found.

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Author contributions

Conceived and designed the experiments: Y.P.L., W.L.F., Q.H. Performed the experiments: H.Y.W., X.Y., Q.H.X. Analyzed the data: D.C., Y.P.L. Wrote the manuscript: Y.P.L., H.Y.W.

Additional information

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