RESEARCH ARTICLE

Limited Diagnostic Value of microRNAs for Detecting Colorectal Cancer: A Meta-analysis

Xuan-Jun Zhou, Zhao-Gang Dong, Yong-Mei Yang, Lu-Tao Du, Xin Zhang, Chuan-Xin Wang*

Abstract

Background: MicroRNAs have been demonstrated to play important roles in the development and progression of colorectal cancer. Several studies utilizing microRNAs as diagnostic biomarkers for colorectal cancer (CRC) have been reported. The aim of this meta-analysis was to comprehensively and quantitatively summarize the diagnostic value of microRNAs for detecting colorectal cancer. Methods: We searched PubMed, Embase and Cochrane Library for published studies that used microRNAs as biomarkers for the diagnosis of colorectal cancer. Summary estimates for sensitivity, specificity and other measures of accuracy of microRNAs in the diagnosis of colorectal cancer were calculated using the bivariate random effects model. A summary receiver operating characteristic (SROC) curve was also generated to summarize the overall effectiveness of the test. Result: Thirteen studies from twelve published articles met the inclusion criteria and were included. The overall sensitivity, specificity, positive likelihood ratio, negative likelihood ratio and diagnostic odd ratio of microRNAs for the diagnosis of colorectal cancer were 0.81 (95%CI: 0.79-0.84), 0.78 (95%CI: 0.75-0.82), 4.14 (95%CI: 2.90-5.92), 0.24 (95%CI: 0.19-0.30), and 19.2 (95%CI: 11.7-31.5), respectively. The area under the SROC curve was 0.89. Conclusions: The current evidence suggests that the microRNAs test might not be used alone as a screening tool for CRC. Combining microRNAs testing with other conventional tests such as FOBT may improve the diagnostic accuracy for detecting CRC.

Keywords: Colorectal cancer - microRNAs - diagnostic - meta-analysis

Asian Pac J Cancer Prev, **14 (8)**, 4699-4704

Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide. In 2008, over 1.2 million people worldwide were diagnosed with CRC, causing nearly 608,700 deaths (Jemal et al., 2010). CRC is curable if detected at an early stage. The 5-year survival rate for CRC patients are strikingly different by stage, ranging from greater than 93% for stage I disease to less than 8% for stage IV disease (O'Connell et al., 2004). Given improved survival rates seen with stage I and IV CRC, it is necessary to develop a screening test by which the cancer can be diagnosed at an early stage. To date, several screening methods for the early detection of CRC are available, including fecal occult blood testing (FOBT), stool DNA test and colonoscopy (Levin et al., 2008; Mandel 2008). However, none of them has been established as an effective screening tool. The convenient and inexpensive FOBT has the limitation of low sensitivity and requires meticulous dietary restriction (Collins et al., 2005). The stool DNA test has acceptable sensitivity for CRC but widespread application is limited by the labor-intensive sample handling process and high cost (Lansdorp-Vogelaar et al., 2010). Colonoscopy is a reliable screening tool for CRC. However, many patients delay or completely avoid colonoscopy because of its invasive nature and high cost. Thus, new approaches that can complement and improve the current CRC screening methods are urgently needed. MicroRNAs are a functional class of non-protein-coding RNA molecules with 18-24 nucleotides that negatively modulate the activity of specific mRNA targets. Studies have demonstrated that microRNAs play important roles in the multistep carcinogenesis process through the dysregulation of oncogenes and tumor suppressor genes (Zhang et al., 2007). Aberrant expression of microRNAs is also found in CRC tissue, blood and feces (Ng et al., 2009; Huang et al., 2010; Koga et al., 2010; Pu et al., 2010; Kalimutho et al., 2011; Kanaan et al., 2012; Wang et al., 2012; Wang et al., 2012; Wu et al., 2012; Giraldez et al., 2013; Wang et al., 2013a; Wang et al., 2013b). In recent years, an increasing number of studies utilizing microRNAs in blood or tissue samples as diagnostic biomarkers for CRC have been reported. Meanwhile, several studies also evaluated the feasibility of using microRNAs from fecal specimens as screening biomarkers for CRC. The results of these studies are variable even with

*Department of Clinical Laboratory, Qilu Hospital, Shandong University, Jinan, China *For correspondence: cxwang@sdu.edu.cn*

some encouraging information. Therefore, we performed the present meta-analysis to analyze the diagnostic accuracy of microRNAs for CRC.

Materials and Methods

Search strategy and study selection

A systematic literature search in PubMed, Embase and Cochrane Library for articles published up to February 28, 2013 was performed to achieve the accessible original articles that focused on the diagnostic value of microRNAs for CRC. No start data limit was applied. The search terms used were "colon cancer" OR "colorectal cancer" OR "rectal cancer" AND "microRNA". A manual search with a reference list of all the relevant publications was also performed.

Two investigators (Xuanjun Zhou and Zhaogang Dong) independently inspected all the article titles and abstracts to identify those studies that likely reported the diagnostic value of microRNAs for CRC and then retrieved the full texts of these published articles to determine whether they were exactly eligible. Disagreements between two investigators were resolved by consensus. Inclusion criteria for the primary studies were as follows: (1) the study must be published in English; (2) all the participants involved in the study must have been confirmed by standard test (such as colonoscopy or histopathologic analysis); (3) studies evaluated the diagnostic value of microRNAs for detecting human CRC; (4) sufficient data should be included to reconstruct the diagnostic 2×2 contingency table of microRNAs.

Data extraction and quality assessment

The following data were extracted and filled onto standardized data forms: (a) first author, (b) publication year, (c) study of state, (d) specimen, (e) total sample size including numbers of cases and controls, (f) assay method, (g) microRNA expression signature, (h) the diagnostic test results. The methodological quality of each study was assessed by QUADAS tool, which is a tool for the quality assessment of studies of diagnostic accuracy included in systematic reviews (Whiting et al., 2003). The QUADAS tool is structured as a list of 14 items, which should be answered with 'yes' 'no', or 'unclear'. When a specific item was fulfilled, a score of '1' was given, '0' if this item was unclear and '-1' if the item was not achieved. The same two reviewers (Xuanjun Zhou and Zhaogang Dong) extracted the data and assessed the study quality independently, and disagreements were resolved by consensus.

Statistical Methods

Standard methods recommended for meta-analysis of diagnostic studies were used (Deville et al. 2002). Based on the 2×2 contingency table, we extracted the numbers of participants with true-positive (TP), false positive (FP), true-negative (TN) and false-negative (FN) results from every included study. The chi-square and inconsistency index (I^2) were performed to detect statistically significant heterogeneity within studies (Higgins et al., 2003). Heterogeneity was considered significant when I^2 was greater than 50%. Subgroup analysis and meta-regression were performed to investigate the heterogeneity within the included studies. A bivariate random effects model was used to calculate the pooled sensitivity, specificity and other related indexes across studies (Reitsma et al., 2005). A summary receiver operating characteristic (SROC) curve, which based on the sensitivity and specificity of each included study, was constructed. The area under the SROC curve (AUC) represents an analytical summary of test performance. An AUC close to 0.5 indicates a poor test whereas an AUC of 1.0 indicates that a test can accurately distinguish cases from non-cases. Meta-DiSc statistical software version 1.4 (Unit of Clinical Biostatistics, Ramony Cajal Hospital, Madrid, Spain) was used for all the above statistical analyses. Concerning the publication bias for meta-analyses of diagnostic studies, we explored the potential publication bias through Deeks' funnel plots (Deeks et al., 2005). These statistical analyses were undertaken using STATA 11.0 (Stata Corporation, College Station, TX). All statistical tests were two sided, and $p < 0.05$ was considered statistically significant.

Results

Characteristics and quality of the included studies

The article selection process used in this study is summarized in Figure 1. A total of 13 studies from 12 English language articles met the inclusion criteria and were included in the present meta-analysis. The main clinical characteristics of the included studies, along with QUADAS scores, are presented in Table 1. Overall, the 13 selected studies included 1,512 individuals and the sample size varied from 32 to 316 individuals with a median size of 116 individuals. The included studies originated from 6 countries or regions (including United States, Italy, Spain, Japan, Hong Kong and China) and were published from 2009 to 2013. The categories of specimens included colorectal tissue (2 studies, 15.4%), plasma (7 studies, 53.8%), serum (1 study, 7.7%) and feces (3 studies, 23.1%). Six studies (Ng et al., 2009; Pu et al., 2010; Kalimutho et al., 2011; Kanaan et al., 2012; Wang et al., 2012; Wu et al., 2012) evaluated a single microRNA as the diagnostic biomarker, while the other seven studies (Huang et al., 2010; Koga et al., 2010; Kanaan et al., 2012; Wang et al., 2012; Giraldez et al., 2013;Wang et al., 2013a; Wang et al., 2013b) focused on multiple microRNAs for detecting CRC. In total, 23 microRNAs (miR-1, miR-7, miR-15b, miR-17, miR-18a, miR-19a, miR-19b, miR-20a, miR-21, miR-29a, miR-31, miR-92a, miR-92, miR-93,

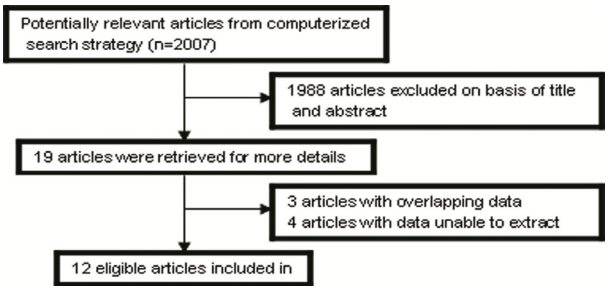

Figure 1. Flow Chart of Selection Process for Eligible Articles

Table 1. Main Characteristics of 13 Studies Included in Meta-analysis

First author	Publication year	Location	Specimen	Total sample size	Tp	Fp	Fn	Tn	Assay method	threshold	microRNA profile	QUADAS scores
Ng et al., 2009	2009	Hong Kong	plasma	140	80	15	10	35	qRT-PCR	Yes	miR-92	11
Koga et al., 2010	2010	Japan	feces	316	146	25	51	94	qRT-PCR	Yes	miR-17-92 cluster#, miR-21, miR-135	10
Pu et al., 2010	2010	China	plasma	140	89	22	14	15	qRT-PCR	Yes	miR-221	8
Huang et al., 2010	2010	China	plasma	159	83	9	17	50	qRT-PCR	Yes	miR-29a, miR-92a	9
Kalimutho et al., 2011	2011	Italy	feces	75	26	5	9	35	qRT-PCR	No	miR-144*	11
Giraldez et al., 2013	2012	Spain	plasma	95	33	11	9	42	qRT-PCR	No	miR19a, miR19b, miR15b	9
Wang et al., 2012	2012	China	plasma	148	75	4	15	54	qRT-PCR	Yes	miR-29a, miR-92a, miR-760	9
Kanaan et al., 2012	2012	America	tissue	32	16	2	0	14	qRT-PCR	No	miR-1, miR-31, miR-133a, miR-135b	9
Kanaan et al., 2012	2012	America	plasma	40	18	2	2	18	qRT-PCR	Yes	miR-21	12
Wang et al., 2012	2012	China	serum	71	28	10	4	29	qRT-PCR	Yes	miR-21	9
Wang et al., 2013a	2012	China	tissue	58	31	4	2	21	qRT-PCR	No	miR-92a, miR-375, miR-424	9
Wu et al., 2012	2012	Hong Kong	feces	189	63	27	25	74	qRT-PCR	Yes	miR-92a	9
Wang et al., 2013b	2013	China	plasma	49	18	3	4	24	qRT-PCR	NO	miR-409-3p, miR-7, miR-93	9

#miR-17-92 cluster included miR-17, miR-18a, miR-19a, miR-19b, miR-20a and miR-92a

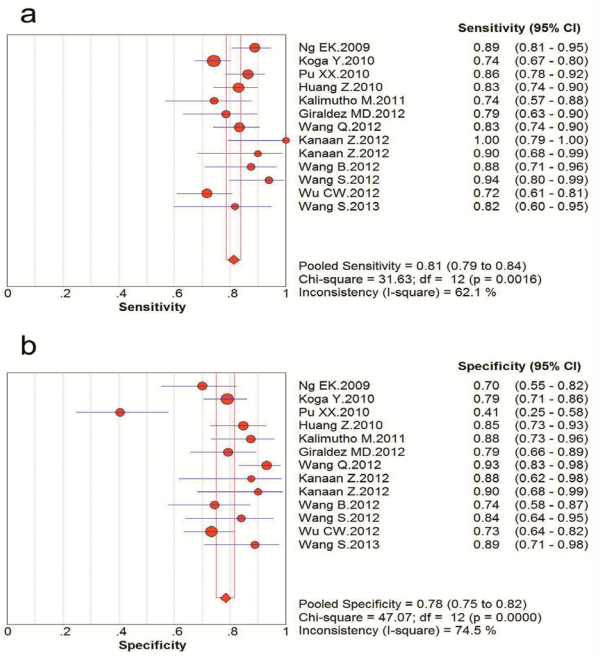

Figure 2. Forest Plot of Estimates of Sensitivity (a) and Specificity (b) for microRNAs on Detection of CRC for All Studies. The point estimates of sensitivity and specificity for each study are shown as solid circles and the size of each solid circle indicates the sample size of each study. Error bars are 95% confidence intervals

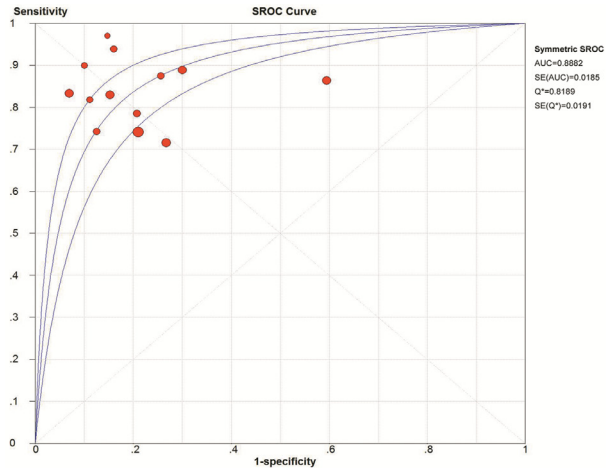

Figure 3. Summary Receiver Operating Characteristic (SROC) Curve for microRNAs on Detection of CRC for All Studies. Solid circles represent each study included in the meta-analysis. The size of each solid circle indicates the size of each study. The regression SROC curve summarizes the overall diagnostic accuracy

miR-133a, miR-135a, miR-135b, miR-144*, miR-221, miR-375, miR-409-3p, miR-424, miR-760) using as diagnostic biomarkers involved in this meta-analysis. Quantitative reverse transcription PCR (q RT-PCR) was used for microRNAs detection in all 13 studies and eight studies (Ng et al., 2009; Huang et al., 2010; Koga et al., 2010; Pu et al., 2010; Kanaan et al., 2012; Wang et al., 2012; Wang et al., 2012; Wu et al., 2012) mentioned the threshold of the diagnostic biomarkers.

The methodological quality of each study was assessed according to QUADAS guidelines. Of the 13 included studies, four (Ng et al., 2009; Koga et al., 2010; Kalimutho et al., 2011; Kanaan et al., 2012) had QUADAS score ≥ 10, and the other nine (Huang et al., 2010; Pu et al., 2010; Kanaan et al., 2012; Wang et al., 2012; Wang et al., 2012; Wu et al., 2012; Giraldez et al., 2013; Wang et al., 2013a; Wang et al., 2013b) had a QUADAS score < 10. Most studies (10/13) recruited a group known to have the target disorder and a group of healthy controls, which may lead to spectrum bias. Eight studies (Ng et al., 2009; Koga et al., 2010; Pu et al., 2010; Kanaan et al., 2012; Wang et al., 2012; Wang et al., 2012; Wang et al., 2013a; Wang et al., 2013b) used histopathologic analysis as the standard test, whereas the remaining five (Huang et al., 2010; Koga et al., 2010; Pu et al., 2010; Kalimutho et al., 2011; Wu et al., 2012; Giraldez et al., 2013) used histopathologic analysis and colonoscopy as the standard test. One study (Pu et al., 2010) did not report the uninterpretable index test results. All studies clearly stated that the results of the standard test were interpreted blind to the results of index test. However, it was unclear whether the index test results were interpreted without knowledge of the results of the standard test in most studies (Huang et al., 2010; Koga et al., 2010; Pu et al., 2010; Kanaan et al., 2012; Wang et al., 2012; Wang et al., 2012; Wu et al., 2012; Giraldez et al., 2013; Wang et al., 2013a; Wang et al., 2013b). It may cause review bias.

Table 2. Summary Results for Diagnostic Accuracy of microRNAs

	CRC vs. non-CRC	Blood-based microRNAs	Feces-based microRNAs
Number of studies	13	8	3
Sensitivity (95% CI)	0.81 (0.79-0.84)	0.85 (0.82-0.88)	0.73 (0.68-0.78)
I2 (p)	62.1 % (0.0016)	0.0 % (0.8015)	0.0 % (0.9000)
Specificity (95% CI)	0.78 (0.75-0.82)	0.78 (0.73-0.82)	0.78 (0.73-0.83)
I2 (p)	74.5 % (0.0000)	83.3 % (0.0000)	46.2 % (0.1559)
PLR (95% CI)	4.14 (2.90-5.92)	4.27 (2.42-7.56)	3.36 (2.39-4.73)
I2 (p)	80.2 % (0.0000)	87.0 % (0.0000)	42.2 % (0.1773)
NLR (95% CI)	0.24 (0.19-0.30)	0.21 (0.16-0.26)	0.34 (0.28-0.41)
I2 (p)	51.2 % (0.0168)	0.0 % (0.6267)	0.0 % (0.6495)
DOR (95% CI)	19.15 (11.65-31.48)	21.19 (10.94-41.05)	9.90 (6.17-15.89)
I2 (p)	64.3 % (0.0008)	63.8 % (0.0072)	24.6 % (0.2656)
AUC	0.89	0.91	0.785

CRC, colorectal cancer, non-CRC, individuals without colorectal cancer, PLR, positive likelihood ratio, NLR, negative likelihood ratio, DOR, diagnostic odds ratio, AUC, area under the SROC curve

Table 3. Mata-regression of Potential Heterogeneity Within the Included Studies

Covariates	Coefficient	SE	RDOR (95% CI)	*p* value
Single microRNA	-0.724	0.5937	0.48 (0.12-1.91)	0.2572
QUADAS scores≥10	0.169	0.5855	1.18 (0.31-4.57)	0.7798
Sample size≥100	-0.781	0.5302	0.46 (0.13-1.55)	0.1788

Diagnostic accuracy

Figure 2 presents the forest plots of pooled sensitivities, specificities of microRNAs in the diagnosis of CRC. The sensitivity ranged from 0.72 to 1.00 and the specificity ranged from 0.41 to 0.93. The pooled sensitivity and specificity calculated by the bivariate random effects model were 0.81 (95%CI: 0.79-0.84) and 0.78 (95%CI: 0.75-0.82), respectively. The overall positive and negative likelihood ratios were 4.14 (95%CI: 2.90-5.92) and 0.24 (95%CI: 0.19-0.30), respectively. The pooled diagnostic odds ratio (DOR) was 19.15 (95%CI: 11.65-31.48). Figure 3 presents the SROC curve of microRNAs, and the AUC was 0.89. The summary results for diagnostic accuracy of microRNAs are listed in Table 2. The between-study heterogeneity was assessed by I^2 index. The I^2 of sensitivity, specificity, positive likelihood ratio (PLR), negative likelihood ratio (NLR) and DOR were 62.1 % (p = 0.0016), 74.5 % (p = 0.0000), 80.2 % (p = 0.0000), 51.2 % (p = 0.0168), and 64.3 % (p = 0.0008), respectively, indicating high statistical heterogeneity among studies.

A subgroup analysis was also conducted regarding the clinical specimen used in the studies. Because there were only two studies using tissue, we did not summarize the diagnostic accuracy of tissue-based microRNAs tests. Since measurements of microRNAs obtained from plasma or serum were strongly correlated (Mitchell and others 2008), the diagnostic ability of microRNAs for detecting CRC was determined between blood-based studies and feces-based studies. The subgroup analysis results are also listed in Table 2. For the blood-based studies, the pooled sensitivity, specificity, PLR, NLR and DOR were 0.85 (95%CI: 0.82-0.88), 0.78 (95%CI: 0.73-0.82), 4.27 (95%CI: 2.42-7.56), 0.21 (95%CI: 0.16-0.26), and 21.19 (95%CI: 10.94-41.05), respectively. Moreover, for the feces-based studies, the pooled sensitivity, specificity, PLR, NLR and DOR were 0.73 (95%CI: 0.68-0.78), 0.78 (95%CI: 0.73-0.83), 3.36 (95%CI: 2.39-4.73), 0.34 (95%CI: 0.28-0.41), and 9.90 (95%CI: 6.17-15.89),

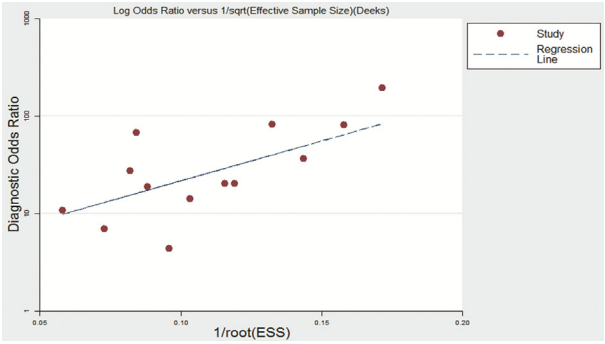

Figure 4. Assessment of the Potential Publication bias of the 13 Included Studies. The funnel graph plots the log of the diagnostic odds ratio (DOR) against the 1 / root (effective sample size). The dotted line indicates the regression line. The result of the test showed that there was a significant small study bias (p = 0.035)

respectively. The AUC was 0.91 for blood-based tests and 0.785 for feces-based tests. Thus, compared with the feces-based microRNAs tests, the blood-based microRNAs tests had a higher level of diagnostic accuracy. The subgroup analysis for feces-based studies showed no significant heterogeneity, whereas the heterogeneity for blood-based studies was still apparent in specificity, PLR and DOR.

Meta-regression and publication bias

A meta-regression was performed to investigate the potential heterogeneity within the included studies. We used 3 covariates in the present meta- regression: (1) single or multiple microRNAs used as diagnostic biomarkers; (2) sample size (≥100 or < 100); (3) QUADAS scores (≥ 10 or < 10). The outcomes of the regression are shown in Table 3. In the present study, none of the above covariates were found to be the significant source of heterogeneity (all $p > 0.05$).

Since publication bias is concerned for meta-analyses of diagnostic studies, we explored the potential publication bias through Deeks' funnel plots. The Deeks' test indicated that there was a significant small publication bias (p = 0.035) (Figure 4).

Discussion

MicroRNAs are small non-coding RNA molecules that play a crucial role in the development and progression of CRC (Corte et al., 2012). During the past few years, relevant studies have been carried out to assess the diagnostic use of microRNAs for CRC. However, the exact role of microRNAs for detecting CRC still needs to be analyzed. Hence, we performed a comprehensive meta-analysis on the use of microRNAs in detecting CRC.

We included 13 studies with a total participant population of 1,512. Calculated with the bivariate random effects model, utilizing microRNAs as biomarkers for detecting CRC yielded an overall sensitivity of 85 % and an overall specificity of 78 %. The AUC represents an overall summary measure of the SROC curve and the test's overall ability to accurately distinguish cases from non-cases. In this meta-analysis, the AUC was 0.89, indicating a relatively high level of diagnostic accuracy. The DOR is a single indicator of diagnostic performance, which ranges

from 0 to infinity (Glas et al., 2003). The higher value of a DOR indicates greater diagnostic accuracy. A DOR of 1.0 shows that the test can not distinguish individuals with the disease from those without it. In the present meta-analysis we found that the pooled DOR was 19.15, also indicating that the overall accuracy of microRNAs test for detecting CRC is high.

Moreover, likelihood ratios (LRs), which combine the stability of sensitivity and specificity to provide an omnibus index of test performance, are considered to be more clinically meaningful than the SROC curve and DOR (Gallagher 1998). A PLR greater than 10 or a NLR less than 0.1 generates large and often conclusive changes from pre-test to post-test probability. In the present meta-analysis, a pooled PLR of 4.14 (95%CI: 2.90-5.92) suggests that individuals with CRC have about 4.14-fold higher chance of being tested positive using microRNAs compared with individuals without CRC. A pooled NLR of 0.24 (95%CI: 0.19-0.30) means that the probability of the individuals having CRC is 24 % when the microRNAs test is negative. Neither the PLR nor the NLR alone was adequate to confirm or rule out the diagnosis of CRC.

We also performed subgroup analysis regarding the clinical specimen used in the studies. Our meta-analysis showed that using blood microRNAs as biomarkers for detecting CRC yielded an overall sensitivity of 85 % and an overall specificity of 78 %. The AUC was 0.91 and DOR was 21.19, also indicating a relatively high level of diagnostic accuracy. In terms of feces-based microRNAs tests, the overall sensitivity was 73 %, specificity was 78 %, and AUC was 0.785, indicating a moderate level of diagnostic accuracy. Moreover, AUCs were used in the current meta-analysis to compare diagnostic accuracies between blood-based microRNAs tests and feces-based microRNAs tests. Our data showed that the microRNAs detection had a higher accuracy in blood than in feces, which suggested that microRNAs in blood may be better diagnostic biomarkers for detecting CRC. The pooled PLR was 4.27 (95%CI: 2.42-7.56) for blood-based microRNAs tests and 3.36 (95%CI: 2.39-4.73) for feces-based microRNAs tests. Neither of them was high enough for clinical purposes. The NLR values of blood-based tests and feces-based tests were 0.21 (95%CI: 0.16-0.26) and 0.34 (95%CI: 0.28-0.41), respectively, which were also not low enough to rule out CRC.

Because of the limited studies, we did not summarize the diagnostic performance of tissue-based microRNAs tests. Although the tissue-based microRNAs test might have higher diagnosis accuracy for CRC than blood-based microRNAs test, its invasive and inconvenient nature may still hinder its wide application.

There were 23 microRNAs used as diagnostic biomarkers involved in our meta-analysis. These microRNAs biomarkers may provide new insight into the early detection of CRC. Among them, miR-92a was found to be used as the biomarker in five studies (Huang et al., 2010; Koga et al., 2010; Wang et al., 2012; Wu et al., 2012; Wang et al., 2013a). Zhou et al. demonstrated that the high expression of miR-92a was closely associated with advanced clinical stage, lymph node metastases, distant metastasis, and poor overall survival in CRC (Zhou et al., 2013). MiR-21 was used as the biomarker in three studies (Koga et al., 2010; Kanaan et al., 2012; Wang et al., 2012). And it was also the most frequent microRNA to be studied in CRC. Multiple studies showed that high expression of miR-21 was associated with advanced disease and worse outcome (Schetter et al., 2008; Schetter et al., 2009; Liu et al., 2011). However, most reported microRNAs for the diagnosis of CRC had also been reported in other human malignancies. It is important to identify the microRNAs patterns that are specific to CRC in the future.

The overall results of this meta-analysis showed high statistical heterogeneity among studies. In order to explore the possible sources of heterogeneity, we performed subgroup analysis and meta-regression. The outcomes of the regression did not show any statistical significance. However, the subgroup analysis result showed that the heterogeneity across blood-based studies was still apparent in specificity, PLR and DOR.

There were also some limitations in our meta-analysis. First, the presence of spectrum bias might lead to the overestimation of diagnostic accuracy of microRNAs. Second, the Deeks' test indicated a significant small publication bias. The potential reasons might be attributed to that small studies with positive results may be published easier than studies with negative results. Because of the relatively small number of relevant studies for the statistical analysis, it is still difficult to make a definitive conclusion about the diagnostic accuracy of microRNAs for CRC. In addition, most included studies were unclear about whether the index test results were interpreted without knowledge of the results of the standard test, which may cause review bias. But the review bias may not be important because the detection of microRNAs test is quantitative (Whiting et al., 2004).

In conclusion, our meta-analysis demonstrated that microRNAs may be potential novel biomarkers for detecting CRC. Moreover, the microRNAs in blood had higher diagnostic accuracy than in feces, which provides important evidence for the further development of noninvasive method for diagnosing CRC in the future. However, the microRNAs test may not be used alone as a screening tool for CRC. Combining microRNAs with other conventional tests such as FOBT may improve the diagnostic capability for detecting CRC.

Acknowledgements

This work was supported by National Natural Science Foundation of China (No. 81271916) and Shandong Province Natural Science Foundation of China (No. ZR2010H004). The author(s) declare that they have no competing interests.

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