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The Diagnostic Value of Stool-Based MicroRNA-135b in the Early Detection of Colorectal Cancer and the Potent Neuro Function of MiR-146a

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Abstract. Background: Detecting microRNA (miRNA) in stool is a non-invasive approach for colorectal cancer (CRC) screening. This study aims to assess the diagnostic performance of stool-based microRNA-135b in detection of colorectal cancer and the role of miR-146a. Methods: To identify eligible studies that are well suited for diagnostic value of miR-135b in stool of colorectal cancer patients, we have evaluated 3 papers after systematic literature search of public database. The sensitivity and specificity were used to plot the summary receiver operator characteristic (SROC) curve and calculate the area under the SROC curve (AUC). The between-study heterogeneity was evaluated by Q test and I2 statistics. Importantly, we have also identified microRNA (miRNA) gene expression patterns by expression profiling. Moreover, we execute the pathway analysis to find the function of miR-146a. Results: A total of 3 studies from 19 articles were included for the meta-analysis according to the inclusion criteria. The overall analysis showed that microRNA-135b has a relatively good diagnostic performance in colorectal cancer, with a sensitivity of 0.685, a specificity of 0.813 and a partial AUC of 0.658. In stool samples, level of microRNA-135b was 2.21 fold change higher in subjects with CRC ($P < 0.0001$). Moreover, our study proved that miR-146a involved in the neuro and immune function. Interpretation: In conclusion, stool-based microRNA-135b shows a moderate level of overall diagnostic accuracy in diagnosis of CRC, which may present as auxiliary means for the diagnosis of colorectal cancer compare with colonoscopy. Further large-scale prospective studies are necessary to validate their potential applicability (miR-135b and miR-146a) combined with FBT methods in human cancer diagnosis.

Keywords: miRNA, colorectal cancer, meta-analysis, gene expression, biomarkers, non-invasive.

1. Introduction

Colorectal cancer was currently the fourth most common malignant cancer in the worldwide, accounting for 8% of all cancer deaths(Link et al., 2010). Risk factors to cancer incidence have been changed such as decreased smoking and red meat consumption and increased use of aspirin(Meyerhardt, 2011). Importantly, the introduction and dissemination of early detection tests, and improvements in treatment decreased the incidence rate(Du M et al., 2019). However, effective diagnostic tests in the very early stage of colorectal cancer have not been found, whereas, new biomarkers showed good potential in early detection(Ahmed et al., 2013).

microRNAs are short (~22nucleotides) non-coding RNA transcripts and high-quality biomarkers, which are stable, having a unique expression profile and being accessible to measurement by quantitative PCR and microarray (Hu et al., 2011). Studies have shown that miRNAs often act by regulating protein expression during cellular processes such as growth, development, and differentiation at the transcriptional, posttranscriptional, or translational level and also produce protein or amino acid chains(Berindan-Neagoe et al., 2014; Chai et al., 2017).

Currently, miRNA-135b expression is often found up-regulation in colorectal cancer. High expressions of CEA and CA-199 in pre-operative serum samples are associated with miR-135b (Faltejskova et al., 2012). It might inhibit or promote neoplastic growth and invasion, apoptosis, metastasis and angiogenesis through pathways regulation. Studies by Nicola Valeri have found that miRNA-135b act as downstream effector of oncogenic pathways and promotes cancer in colon

cancer(Zhou et al., 2012). Zhou et al. found that targeting MTSS1, miR-135b at least partially promotes the growth and invasion of CRC cells.(Zhou et al., 2012b). Study by Kottorou identified that the degree of malignancy may particularly correlated with microRNA-135b levels (Kottorou et al., 2018).The potential of microRNA-135(microRNA-135b) as a biomarker for CRC detection has been studied in several studies. However, the results regarding to the diagnostic abilities were inconsistent.

On the other hand, both miR-135b and miR146a regulate lipid cells differentiation (Li et al., 2016; Kopke et al., 2015). Through sequence alignment blasting found that mature sequences of miR-146a and miR-135b are highly conservative(Kopke et al., 2015).MiR-135b may be involved in the regulation of fatty acid carbon chain extension, while MiR-146a promotes adipocyte differentiation by targeting Runx1t1 and Smad4 in its early period (Yongdong, P, 2014).Studies have also shown that miR-135b and miR-146a target the CaSR and reduce its expression in colorectal tumors, reducing the antiproliferative and prodifferentiating actions of calcium (Zekri et al., 2016).

Two studies suggest that miR-135 and miR-146 are both over expressed and differentiated expressed in both mouse and human (Valeri et al., 2014).Both these two miRNAs was correlated with miR-214. Although no study have been directly proved the correlation between miR-146 and miR-135, large proofs support the coexpression levels of these miRNAs (Valeri et al., 2014). The experiment reveals the miR-146a actually take part in the neuro function in cellar pathway (Li et al., 2018; Nguyen et al., 2018). There is reason to believe that miR-135b also have function in neuro. In fact, Study by Zhang Y, Xing H,et.al. proved that miR-135b has a neuroprotective role via targeting of β-site APP-cleaving enzyme (Zhang et al., 2016).

Therefore, we conducted the present meta-analysis to systematically assess the diagnostic value of microRNA-135b for CRC. This article aims to found a consistently reported result for microRNA-135b and evaluate the over whole diagnostic ability of miR-135b and miR-146a and their potential functions.

2. Materials and methods

2.1 Meta analysis-Literature search

This meta-analysis was conducted according to guidelines for meta-analysis evaluating diagnostic tests [28]. Eligible studies published up to 20 May 2019 were selected for meta-analysis by conducting a systematic literature search of public databases including PubMed, Embase, Web of Science, Medline, Science Direct databases, Google scholar without language limitation.

The following retrieval strategy was used: (microRNA-135[TITLE] OR miRNA-135[TITLE] OR miR-135[TITLE]) AND ((colorectal cancer [Title/Abstract]) OR (colorectal cancer[Title/Abstract]) OR (CRC[Title/Abstract]) OR (colorectal tumor) OR (colorectal carcinoma) OR(colon[Title/Abstract])) AND (stool OR fecal OR feces).

ROC analysis using literature searching items (‘colorectal tumor’ OR ‘colorectal cancer’ OR ‘colorectal cancer’ OR ‘CRC’ OR (colorectal carcinoma)) AND (‘microRNA-135’ OR ‘miRNA-135’ OR‘miR-135’)AND (‘diagnosis’ OR ‘sensitivity’ OR ‘specificity’ OR ‘ROC curve’) AND (‘case-control’ OR ‘paired’ OR ‘comparative’ OR ‘pair-wise’OR ‘compared with’).

In addition, we also searched reference lists of eligible articles on this topic manually to obtain additional sources.

2.2 Meta analysis-Inclusion and exclusion criteria

Eligible studies included in this meta-analysis have to fulfill the following criteria: (1) studies regarding the diagnostic potential of stool-based miRNAs for colorectal cancer; (2) studies with a gold reference standard for the colorectal cancer diagnosis;(3) studies with sufficient data for

construction of two-by-two tables [i.e., true positive (TP), false positive (FP), true negative (TN) and false negative (FN)].

Exclusion criteria were: (1) publications unrelated to the diagnostic values ;(2)No important results regarding microRNA-135b;(3)studies with duplicate data reported in other studies; (4)letters, editorials, case reports.

2.3 GEO datasets

In order to find the function of miR-146a, we searched the PUBMED database and found the dataset with GEO accession number GSE 54177

(https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE54177).

2.4 Statistical Methods

META analysis was complimented using R from the package of mada. The fixed effect model was calculated using univariate method and bivariate model using random effect model was under the hypothesis of correlation between sensitivity and specificity. Both methods show different sides of the ROC summary analysis and under grounded conditions.

2.5 Results

Meta-Analysis of miR-135b

According to the inclusion and exclusion criteria, six studies were found to be related to microRNA-135b within past five years, in which all results showed up-regulated in CRC or advanced adenoma and tumor. (Shown by table 1), four authors were included and partly articles reported the ROC results.

Table 1. Six Studies related to diagnostic microRNA-135b within past five years

Article	Relevant Citations	Year	Sample	miRNA levels	Design	ROC	Refs
Koga et al	67	2010	Stool	Up	CRC VS Normal	YES	[9]
Wu et al	9	2014	Stool	Up	CRC VS Normal	YES	[10]
Ng et al	NO	2012	Stool	Up	Advanced Neploma vs Adenoma/control	NO	[7]
Wu et al	5	2011	Stool	Up	Polyps patients vs Normal	YES	[11]
Phua et al	NO	2013	fecal	Up	CRC vs Normal mucosa	NO	[8]
Phua et al	NO	2014	Blood	Up	Tumor vs Normal	NO	[12]

According to the selection criteria, three studies were chosen as the final dataset. These three articles all have good qualities and an appropriate sample size under case-control study design.

The datasets were extracted from selected literatures, according to table 3, these three study results have a similarity in the same author of Wu and a big difference with Koga. These important diagnostic markers of ROC, such as CI (confidence interval); SEN, SPE, PLR, NLR, DOR. PLR showed a particularly higher value of 8.58 in Koga's study compared with Wu's study, however, the NLR were 0.575 which are 0.272 and 0.249 bigger in Wu's study,respectively. Estimation of DOR comes to be quite high in Koga's study, reaching at 14.922.

Table 2. Comparison of microRNA-135b ROC raw dataset and predicted results from literature

First author	Case/Cont rolNum.		Case/Control Mean(yr)		Speci men	SEN(%)/Pre **S**EN(80%CI)	SPE (%)/Pre SPE(80%CI)	TP	F N	FP	TN
Koga	90	113	63.0	60.0	Fecal	45.7/45.6	95.0/94.7	41	49	6	107
etal.	90	113	63.0	60.0	Fecal	(39.0,52.3)	(91.3,96.8)	41	49	6	107
Wu et	47	47	---	---	Stool	78.5/78.7	69.3/70.2	37	10	14	33
al.	47	47	---	---	Stool	(70.2,85.3)	(61.1,78.0)	37	10	14	33
Wu et	104	109	66.8	60.4	Stool	78.0/77.9	68.0/67.9	81	23	35	74
al.	104	109	66.8	60.4	Stool	(72.3,82.6)	(61.9, 73.3)	81	23	35	74

The results of test for equality of sensitivities showed a X2 value of 26.6909,and a p-value of 1.6e-06.Similarly,the test results for equality of specificity are X2 value of 27.5548 and p-value of 1.04e-06.Forest plot of these three studies' sensitivity and specificity are shown in figure 1.

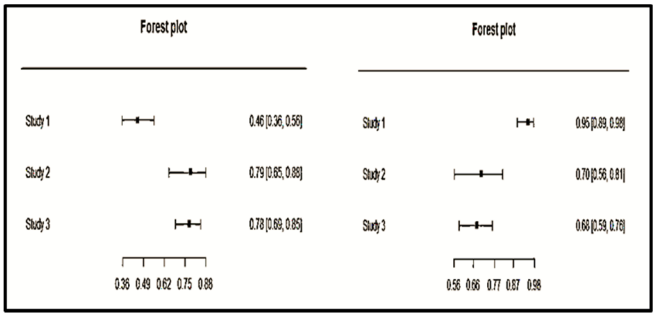

Figure 1. Forest plot of sensitivity (left) and specificity (right) regarding of selected three studies.

As regard to the test statistics of Cochran's Q and Higgins' I^2 ,the heterogeneity results were calculated and reveal no heterogeneity .(Cochran's Q: 1.527 , p = 0.466; Higgins' I^2:>0.0) . Summary forest plot of LOG OR were found to be grounded which results suggest that the summary log OR is 2.21 and proved that up-regulated microRNA-135b to be a hazard factor of CRC.

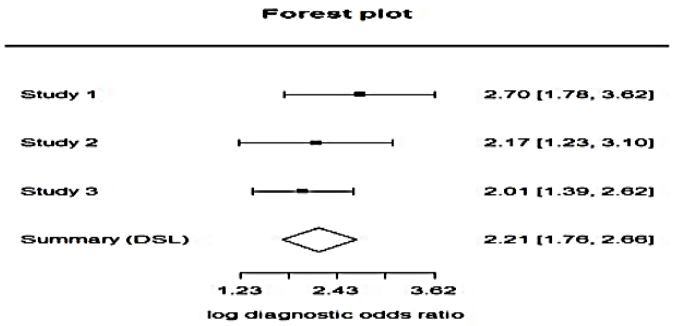

Figure 2. Summary forest plot of LOG OR.

Under the hypothesis of (positive) correlation between sensitivities and false positive rates, the summary ROC plot using bivariate model can be calculated and the results turn out to have a partial AUC (area under the curve.) of 0.658 and a sensitivity of 0.685, also a specificity of 0.813 with a good fitness.(logLik=6.936,AIC=-3.872 ,BIC=-4.913)

2.6 Comparison of the expression levels in several miRNAs under the APC gene

After searching the PUBMED database, we found the dataset with GEO accession number GSE 54177. Study showed that miR-135b over expression triggered by APC loss. RNA was extracted from fresh frozen tissues from tumors coming from APC loss mice and normal matched tissues. The original expression of miRNAs of 11 individuals showed the distribution difference among patients. The distribution among the same group performs the similar. In the first row of 6 samples, the distribution generally presents central high trend which means the expression of some microRNAs are quite high whereas most of them are relatively low except for the 5th sample. On the other hand, the distribution of the Cancer APC group presents mostly high expressed situation which means some miRNAs which is low expressed in normal group can be high expressed in cancer group.

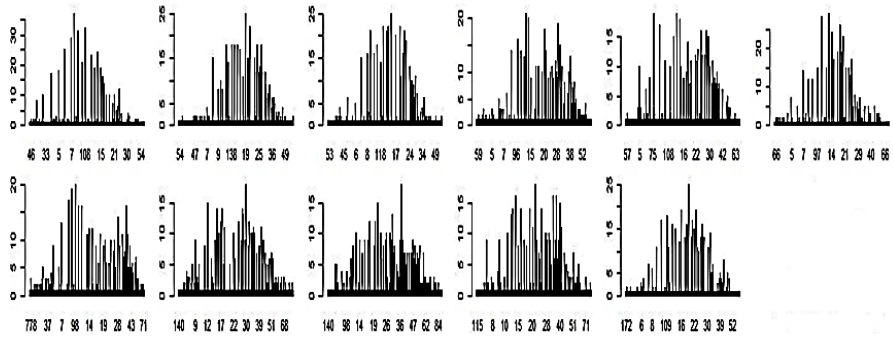

Figure 3. Overview of the miRNAs expression in 11 samples.

The mean value of the original expression level in Normal APC and cancer group shows that miR-720,miR-21,miR145,miR-143,miR-429,miR-94,miR-1937b,miR-194,miR-31,miR-99b,miR-96,miR-75,miR-78,let-7a,let-7b,et.al. are the highly expression genes compared in normal and cancer groups.

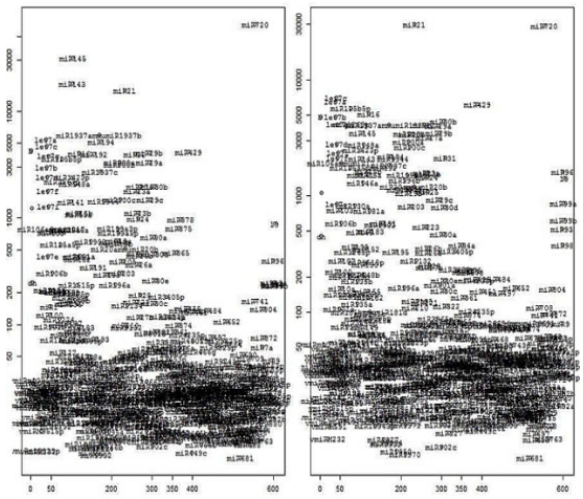

Figure 4. the mean value of the miRNAs in two groups(normal group (left) and cancer group (right)).

Further analysis in Fig 8 showed that the mean value difference in APC group(left),compared with the mean value difference in AOMDSS group(right), the most significant expression levels differs in miRNAs (miR-145,miR-143,miR-720,miR-21).

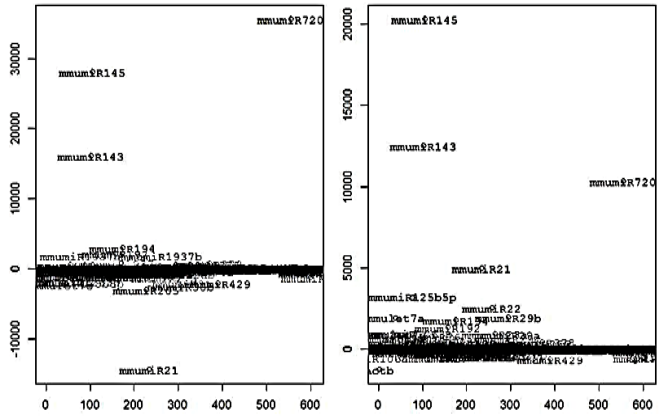

Figure 5. The mean value difference of the miRNAs in Normal APC vs. Cancer APC(left), Normal AOM-DSS vs. Cancer AOM-DSS(right)

In table 5, it shows the list of significantly different expressed miRNAs between cancer group and normal group.In the table, miR-146a,miR-135b,miR-145,miR-378 are all in the list.

Table 5. Selected MiRNAs in Normal APC vs. Cancer APC Group.

miR-666-3p	miR-668	miR-669a	miR-669i	miR-669j	miR-670
miR-34b-5p	miR-34c	miR-365	miR-370	miR-375	miR-376b
miR-1958	miR-1959	miR-1960	miR-1961	miR-1964	miR-1965
miR-15a	miR-28	miR-290-5p	miR-291a-3p	miR-291b-3p miR-292-3p	
miR-181a	miR-181b/d	miR-181c	miR-183	miR-184	miR-186
miR-99a	Rpl19	miR-142-5p	miR-143	miR-145	miR-146a
miR-338-3p	miR-339-3p	miR-340-3p	miR-340-5p	miR-342-3p	miR-342-5p
miR-1196	miR-1197	miR-1198	miR-1199	miR-122	miR-1224
miR-873	miR-875-5p	miR-877	miR-878-5p	miR-879	miR-883a-3p
miR-466l	miR-467a	miR-467c	miR-135b	miR-105	miR-106a/17
miR-2135	miR-2137	miR-2138	miR-511	miR-710	miR-717
miR-140	miR-136	miR-31	miR-335-3p	miR-466c-5p miR-466d-3p	
miR-1946a	miR-1948	miR-320	miR-323-3p	miR-20a/b	miR-135b
miR-M55-1	miR-m59-2	miR-m88-1	miR-M1-3	miR-M1-7-3p miR-M1-7-5p	
miR-671-3p	miR-673-3p	miR-674	miR-678	miR-682	miR-683
miR-376c	miR-378	miR-380-3p	miR-380-5p	miR-383	miR-19a
miR-1967	miR-196a	miR-196b	miR-1983	miR-199a-3p miR-30a	
miR-296-5p	miR-29c	miR-300	miR-301a	miR-302b	miR-18a
miR-187	miR-1892	miR-1894-5p	miR-1895	miR-1898	miR-218
miR-3472	miR-3475	miR-34a	miR-216b	miR-217	miR-1191
miR-1186	miR-1186b	miR-1187	miR-760	miR-761	miR-764-3p
miR-93	miR-744	miR-759	miR-599	miR-137	miR-139-5p
miR-10a	miR-10b	miR-590-5p	miR-1941-5p	miR-1943	miR-1945
miR-720	miR-741	miR-1941-3p	miR-132	miR-133a	miR-134
miR-466d-5p	miR-466k	miR-128	miR-467g	miR-590-3p	miR-01-3
miR-2132	miR-2134	miR-127	miR-1	miR-100	miR-652
let-7b	let-7e	let-7i	miR-696	miR-701	miR-34b-3p
miR-688	miR-694	miR-695	miR-455	miR-463	miR-1956
miR-425	miR-429	miR-450b-3p	miR-206	miR-207	miR-149
miR-19b	miR-200c	miR-205	miR-551b	miR-574-5p	miR-15b
miR-30e	miR-542-3p	miR-547	miR-193	miR-1937c	miR-96
miR-1904	miR-191	miR-192	miR-125b-5p	miR-126-3p	miR-337-5p
miR-125a-3p	miR-125a-5p	miR-125b-3p	miR-27b	miR-483	miR-1195
miR-223	miR-25	miR-27a	miR-467e	miR-467f	miR-488
miR-2146	miR-216a	miR-1194	miR-871	miR-2139	miR-493
miR-770-3p	miR-770-5p	miR-802	miR-323-5p	miR-101a	miR-499
miR-467h/669d miR-468		miR-471	miR-708	miR-214	miR-501-5p
miR-335-5p	miR-2141	miR-485	miR-464	miR-194	
miR-107-1-3p	miR-107-1-5p miR-108-2-3p		miR-108-2-5p	miR-21-1	
			.1		

In Fig 6, the performance of miR-135b is inferior to the expression level of miR-146a.Compared with different expression levels of miR-378 in two groups, the performance of miR-145 is better.

Fig 6 Comparison of the expression level of microRNAs(miR-135b,miR-135b,miR-146a, miR-146a,miR-378,miR-145)

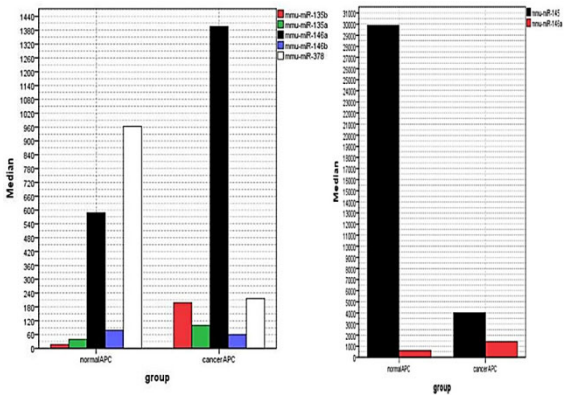

Figure 6. The potential neuro function of miR-146a

Previous study experiment reveals the miR-146a actually take part in the neuro function in cellar pathway. Through searching database, no GEO datasets has been found about miR-135b. PathView analysis performs of donation data GEO43802, in which GM23061HT29-vector was used as reference and GM23062 GM23063 as samples. The samples in GSM923061-923063 are gene expression microarrays (affymetrix U133 Plus 2 platform) which contain the expression of transcripts/ coding genes. Hsa03008 take part in the process of ribosome synthesis .hsa04623 take part in the cytosolic DNA-sensing pathway. Further analysis identified that miRNA-146a regulates the neuro and immune system related pathways.

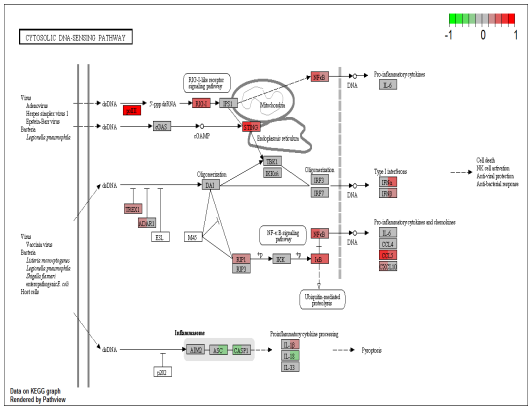

Figure 7. hsa04623 in the pathway of cytosolic DNA-sensing.

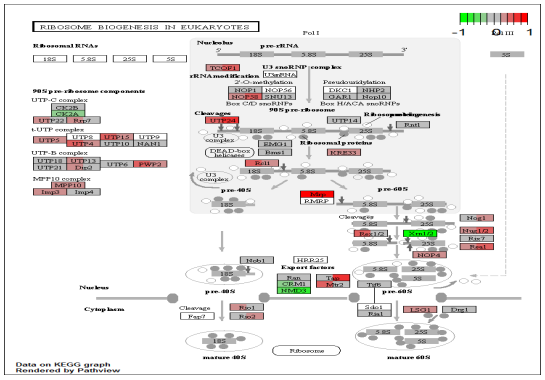

Figure 8. hsa03008 in the pathway of ribosome biogenesis in eukaryotes.

3. Discussion

As a early diagnostic biomarker,current studies about early diagnostic value of microRNA-135 in colorectal cancer showed a good result in stool samples. However, large sample experiments are needed to prove its diagnostic value in even early stage and its underlying mechanisms.

In fact, the study by Williams MD, Xian L proved that early detection of precursor lesions in fecal metabolome in hmga1 transgenic mice with polyposis showed that lipids in colon cancer can diagnose phase | colon cancer but also colon tumor(Williams et al., 2016). Study by Ishikawa H, Yamada H,et.al. indicated that stability of serum high-density lipoprotein-microRNAs and miR-135 a and miR-223 present in high-density lipoprotein(Ishikawa et al., 2017).

Study by showed that miR-135b up-regulation is common to sporadic and inflammatory bowel disease-associated human CRCs and correlates with tumor stage and poor clinical outcome.Inhibition of miR-135b in CRC mouse models reduces tumor growth controlling genes involved in proliferation, invasion and apoptosis(Nunez et al., 2013).

These facts strongly proved that miR-135b in serum may be a very potent diagnostic biomarker in the very early stage of colorectal cancer.

Therefore,miR-135b present in lipoprotein which part of lipid especially for phospholipid can diagnose phase | colon cancer. Previous studies have provide lots of proofs about this microRNA, for example, Aslam MI et al. suggest that expression levels of hsa-microRNA-135b correlate with expression of APC proteins in colorectal tissue which reveals its role in tumor initiation(Aslam et al., 2015). Furthermore, Valeri N et al. prove that microRNA-135b as a key downstream effector of oncogenic pathways and a potential target for CRC treatment(Valeri et al., 2014b).Studies show that miR-135b over-expression is triggered in mouse and humans by APC loss, PTEN/PI3K pathway deregulation and by SRC over-expression formation and progression and promotes tumor trans(Valeri et al., 2014b). Also, miR-135b up-regulation is common to sporadic and inflammatory bowel disease-associated human CRCs and correlates with tumor stage and poor clinical outcome. Inhibition of miR-135b in CRC mouse models reduces tumor growth controlling genes involved in proliferation, invasion and apoptosis. Khatri R et al. hold that MicroRNA-135b and its circuitry networks as potential therapeutic targets in colorectal cancer(Khatri and Subramanian, 2013). Studies have also identified that the calcium-sensing receptor (CaSR) mediates the antitumorigenic effects of calcium against colorectal cancer (CRC)(Fetahu et al., 2016;Yongdong,P.,2014). miR-135b and miR-146a both targets CaSR(Fetahu et al., 2016).Therefore, mechanism about this microRNA-135b in diagnosis and therapy is needed to reveal.

Our study searched all the stool-based studies about microRNA135b,which might be a promising non-invasive biomarker in detecting colorectal cancer. The results showed a 2.21 fold change in CRC and a 0.658 area under curve, which provide conclusive results about this microRNA and prove its potentiality in diagnosis of CRC. Further studies regarding this miroRNA are needed.

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