Original Article

MicroRNA-21 and microRNA-146a identification in stool and its clinical significance in colorectal neoplasms

Hua Liu¹, Wentao Gong², Jie Lou³, Hui Ju¹, Xiaoyan Yin¹, Yao Liu¹, Zibin Tian¹

Departments of ¹Gastroenterology, ²Internal Medicine, The Affiliated Hospital of Qingdao University, Qingdao 266003, Shandong Province, China; ³Department of Gastroenterology, Weihai Central Hospital, Weihai 264400, Shandong Province, China

Received December 15, 2015; Accepted July 9, 2016; Epub August 15, 2016; Published August 30, 2016

Abstract: Colorectal carcinoma (CRC) is one of the deadliest killers worldwide and its processes of genesis include a sequence of molecular pathway from adenoma to cancer. Previous study showed that microRNAs played an important role in carcinogenesis. In this study, the different expressions of microRNA-21 (miRNA-21) and microRNA-146a (miRNA-146a) were investigated by quantitate real-time polymerase chain reaction (qRT-PCR) from the tissues of colorectal carcinoma (CRC), colorectal adenoma (CRA) and healthy controls as well as their matched stool samples. The relationship between the expression levels of miRNAs and clinicopathological features were analyzed. Then, the diagnostic values of stool-specific miRNAs for colorectal neoplasms were evaluated. As a result, the expression levels of miRNA-21 were confirmed to be significantly higher in stool and tissue samples of CRC and CRA patients than in healthy controls (P<0.01). The expression of miRNA-21 in CRCs was notably higher than in CRAs (P<0.05). High miRNA-21 expression in CRC patients was strongly associated with advanced TNM stage and lymph node metastasis. The expression level of miRNA-21 in CRA patients was correlated to histological types (P<0.05). There was a statistically positive correlation between miRNA-21 expression in CRC stools and matched tissues samples. Compared with healthy control subjects, the expression of miRNA-146a in CRC patients were decreased (P<0.01). No statistically significant differences were observed in miRNA-146a expression between CRA patients and healthy controls (P>0.05). The expression of miRNA-146a in CRCs was reduced compared with CRAs (P<0.01). Furthermore, the reduced expression of miRNA-146a in CRC patients was correlated with differentiation and TNM staging. Nevertheless, no significant correlation was found between miRNA-146a and clinicopathological features including age, gender, adenoma location, adenoma size, histology and CEA in CRA patients (P>0.05). MiRNA-21, miRNA-146a and combined expression levels in stool robustly distinguished (AUC = 0.877, 0.794, 0.878) CRC and (AUC = 0.769, 0.698, 0.761) CRA patients from controls. And they yielded AUC of 0.699, 0.815 and 0.729 in discriminating CRC patients from CRA patients. We conclude miRNA-21 and miRNA-146a in stool samples have potential values for early detection of colorectal cancer.

Keywords: microRNAs, colorectal neoplasms, tissue, stool, real-time polymerase chain reaction

Introduction

Colorectal cancer (CRC) is the common malignant tumor in digestive system with an increased incidence and mortality year by year. A majority of CRC originates from colorectal adenoma (CRA), a kind of precancerous lesion. The early diagnosis of Colorectal tumors plays a crucial role in reducing mortality and improving long-term prognosis. However, dissatisfied sensitivity and specificity of traditional test and examination methods for early detection in CRC and CRA have been showed in clinical practice. It’s great significance that searching for new biomarkers which possess higher sensitivity and specificity for early diagnosis and illness monitoring. MicroRNAs (miRNAs) are a type of noncoding single-chain small RNA and responsible for negative regulation of gene expression in post-transcriptional level. Results suggested that differential expression of miRNAs related to the occurrence and progression of CRC [1].

MiRNA-21 gene, which is located on chromosome 17q21.1, is one of the miRNAs that mankind discovers at the earlier stage. Some
MicroRNA in colorectal neoplasms

researches prove high expression of miRNA-21 in various kinds of tumors, which include pancreatic cancer, breast cancer, lung cancer, gastric cancer, etc [2, 3]. It has been reported that miRNA-146a, the corresponding gene located on chromosome 5q34, expresses highly in thyroid cancer and breast cancer nevertheless low in pancreatic cancer and gastric cancer [4]. Our study was designed to analyze the relationship between expression level of miRNA-21, miRNA-146a and clinical and pathological features, then discuss values of fecal miRNAs for early detection of CRC.

Materials and methods

Patients and tissue samples

150 CRC and 120 CRA patients’ feces and endoscopic biopsy tissue specimens were selected from the affiliated hospital of Qingdao university between January and June in 2015. Meanwhile, 98 age and gender matched (P>0.05) healthy physical examinees were recruited as controls. All of feces specimens were preoperative results and patients’ diagnoses had been verified by postoperative pathological results. Any radio-chemotherapy and immunotherapy weren’t applied to recruited volunteers. There weren’t any other basic diseases and primary diseases among selected patients and healthy examinees. The clinical stage (I-IV) of all CRC patients was done according to TNM staging system established by American Joint Committee on cancer (AJCC) and Union for International Cancer Control (UICC). Among controls, the age range was from 30 to 82 with a median of 63 years, examination results by colonoscopy were negative. Serum CEA, CA199 level and abdominal CT image suggested normal and all of them had no primary malignancy history. The course of collecting all the specimens acquired the patients’ informed-consent. Our research had been approved by ethics committee of the affiliated hospital of Qingdao university.

Sample disposal

Fresh tissue samples were snap-frozen in liquid nitrogen immediately after resection and stored at -80°C. All stool samples were collected in the morning with a 40 ml aseptic specimen cup. Prior to reaching the laboratory, the stool samples were kept at -20°C freezer for short-term and then transferred to -80°C freezer within 24 h for long-term storage.

RNA extraction and reverse transcription

Detection expression of miRNA-21 and miRNA-146a by means of RT-PCR: Total RNA was extracted from stool and tissue specimens by using routine TRIzol reagent. The concentration of RNA was detected by spectrophotometer. Then the RNA extracted was stored at -80°C and prepared for using. Using SYBR® Prime-Script™ miRNA RT-PCR Kit (TaKaRa, Bio, Kyoto, Japan) and 1 µg of the above RNA, cDNA was synthesized under the reactive condition of 37°C for 60 min, 85°C for 5 s. Then two-step real-time PCR was performed with reverse transcription products (cDNA) according to the instructions of LightCycler Real Time PCR Amplifier. The reactive condition: predenaturation at 95°C for 5 s, then incubated by 40 amplification cycles of 95°C for 5 s, 60°C for 30 s. Meanwhile, adding the tubes without PCR template as negative control, each specimen was repeated as the above steps for three times and average values were gained. After reaction, the threshold cycle of fluorescence (Ct) were calculated and to analyze expression level of miRNA in specimens by utilizing endogenous control U6. The relative expression was expressed by 2-ΔCT (ΔCT = CT_{target~gene} - CT_{reference~gene}).

Statistical analysis

Statistical analysis was performed by using the Statistical Program for Social Sciences (SPSS) software 18.0 (SPSS Incorporated, Chicago, IL, USA). The comparison of expression levels between tissue and stool specimens was made by method of Mann-Whitney U test and Kruskal-Wallis H test. P<0.05 was considered significant. Spearman test was utilized for analyzing the association of miRNA expression between

<table>
<thead>
<tr>
<th>miR-21</th>
<th>Normal</th>
<th>CRA</th>
<th>CRC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stool</td>
<td>0.133±0.142</td>
<td>0.346±0.093</td>
<td>0.393±0.083</td>
</tr>
<tr>
<td>Tissue</td>
<td>6.328±0.011</td>
<td>7.836±1.038</td>
<td>8.522±0.901</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>miR-146a</th>
<th>Normal</th>
<th>CRA</th>
<th>CRC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stool</td>
<td>0.373±0.043</td>
<td>0.316±0.038</td>
<td>0.286±0.031</td>
</tr>
<tr>
<td>Tissue</td>
<td>4.003±0.518</td>
<td>3.766±0.506</td>
<td>3.136±0.422</td>
</tr>
</tbody>
</table>
in tissue and stool. The evaluation of stool miRNA-21 and miR146a in diagnosis of colorectal cancer was assessed by receiver operating characteristics (ROC) analysis. Logistic Regre-
Expression Analysis was performed for evaluating the diagnostic Value of combined detection of fecal miRNA-21 and miR146a.

Results

Expression levels of miRNA-21 and miRNA-146a in tissue and stool

The expression level of miRNA-21 and miRNA-146a in each groups is shown in Table 1. The levels of miRNA-21 were higher in CRC and CRA tissue than in healthy control (P<0.01). Meanwhile, in contrast to healthy control, miRNA-21 in CRC and CRA patients’ stool expressed higher (P = 0.001, P<0.01). The level of miRNA-21 was significantly higher in CRC than in CRA (P = 0.01, P<0.01). The tissue and stool levels of miRNA-146a in CRC patients were lower than in healthy control (P<0.01). However the tissue and stool levels of miRNA-146a were not significantly different between CRA patients and the healthy control (P>0.05)

The correlation between the miRNA-21 and miRNA-146a expression levels and clinical features of colorectal tumors

As shown in Table 2, the over-expression of miRNA-21 in CRC tissue and stool was related to clinical stage (I+II, III+IV) and lymph node metastasis, whereas irrelevant to age, sex, tumor size, tumor location, differentiation degree, serum CEA and distant metastasis (P>0.05) (Table 2). MiRNA-21 expressed in CRA was relevant to pathological types. The more villiform ingredients CRA had, the higher level miRNA-21 expressed (Table 3). Decreased degree in miRNA-146a expression of CRC tissue and stool was associated with clinical stage (I+II, III+IV) and tumor differentiation. The degree in miRNA-146a was decreased along with the progressing of clinical stage and poor differentiation (Table 2). The low expression of miRNA-146a was not significantly correlated with clinical features, including sex, age, location, size, pathological type, serum CEA (P>0.05) (Table 2).

The value of stool miRNA-21 and miRNA-146a on diagnosing colorectal cancer

ROC curve analyses were performed with data from 150 CRC patients to evaluate the stool miRNA-21 and miRNA-146a diagnostic value to distinguish between CRC patients and healthy subjects, which showed AUCs (areas under the ROC curve) of 0.877 (95% CI: 0.810 to 0.972).
for miRNA-21, 0.794 (95% CI: 0.669 to 0.913) for miRNA-146a and 0.878 (95% CI: 0.779 to 0.965) for combination with miRNA-21 and miRNA-146a. At the optimal cutoff value of 1.4589 for miRNA-21, the sensitivity and specificity were 90.3% and 75.2%, and at the optimal cutoff value of 0.2691 for miRNA-146a, the sensitivity and specificity were 77.2% and 68.1%. The sensitivity and specificity of combined detection of miRNA-21 and miRNA-146a were 87% and 81.7% (Figure 1A, 1B, 1G). For the purpose of discussing the diagnostic value of colorectal adenoma (main precancerous changes of CRC) via detecting stool miRNA-21 and miRNA-146a, analyzing data from 120 patients’ stool specimen, ROC curve suggested AUCs of 0.769 (95% CI: 0.672-0.912) for miRNA-21, 0.698 (95% CI: 0.558-0.813) for miRNA-146a and 0.761 (95% CI: 0.654-0.912) for combination with miRNA-21 and miRNA-146a. At the optimal cutoff value of 1.4089 for miRNA-21, the sensitivity and specificity were 85.1% and 62.7%, and at the optimal cutoff value of 0.2679 for miRNA-146a, the sensitivity and specificity were 77.5% and 66.7%. The sensitivity and specificity of combined detection of miRNA-21 and miRNA-146a were 78.9% and 66.8% (Figure 1C, 1D, 1H). At the same time, we used ROC curve analysis to differentiate CRC from CRA. The results showed
that AUC was 0.699, 0.815 and 0.729 respectively, the sensitivity and specificity were 74.9% (79.7%), 88.5% (68.9%) and 78% (69.7%) (Figure 1E, 1F, 1I).

The correlations between miRNA-21, miRNA-146a expression in CRC tissue and in CRC patients' stool

The positive correlation between the expression of miRNA-21 in CRC tissue and in CRC patients' stool was observed by using Spearman rank correlation analysis ($r = 0.461$, $P<0.01$), however, no significant correlations of miRNA-146a.

Discussion

In recent years, the relationship between miRNA and cancer has become the research focus. Detecting aberrantly expressed miRNA in stool has emerged as a promising non-invasive approach to CRC screening [5-7]. In this study, it was shown that stool and cancer tissue miRNA-21 increased in CRC patients, which related to clinical stage and lymph node metastasis, but not to age, sex, size, location, differentiation degree and serum CEA level. Moreover, significant high level of miRNA-21 found in patients with advanced clinical stage and lymph node metastasis indicated that miRNA-21 may play some role in infiltration and metastasis of cancer cells. Nevertheless, some study suggested that expression level of miRNA-21 in CRC patients' serum was lower than those in healthy controls [8]. The discrepancy remains to be further validated because of only several samples put in that research and it is not clear whether the expression of serum and tissue is consistent. It is reported that the CRC size and degree of distant metastases were also concerned with expression level of miRNA-21 [9], which was inconsistent with the result we concluded. The possible reason was that relevant deficient sample amount. In addition, the research as well indicated overexpression of miRNA-21 in stool of CRAs and the difference was statistically significant. Our study also found miRNA-21 expressed higher obviously in CRC group than CRA group no matter in tumor tissue or stool. At the same time, there was a correlation between the increase of miRNA-21 in tumor tissue and stool. Therefore, miRNA-21 associated with tumor possibly derived from tumor tissue and secreted into stool to be detected. Thus, it's possible to distinguish between CRC and CRA patients by detecting stool miRNA-21 level. However, the research still showed the expression of miRNA of stool and tissue in some cases is not consistent. We speculate that it may be related to tumor size, activity, tumor invasiveness and individual differences, which need to be further explored. In addition, the present study first reported the correlation between expression level of miRNA-21 and clinical features of CRC by simultaneous detection of stool and tissue miRNA-21 level. The experimental results revealed that miRNA-21 expressed in CRA tissue and stool higher than healthy controls respectively and was relevant to pathological types. The more villiform ingredients CRA had, the higher level miRNA-21 expressed. Hence, it was indicated that miRNA-21 exerted its role on precancerous lesion of CRC and probably participated in the progression of CRA with malignant change.

The role of oncogene or anti-oncogene may be played by miRNA-146a in course of cancer genesis and progression. According to the present reports, the effects taken by miRNA-146a were inconsistent among different cancers. The research showed that the elevated expression of miRNA-146a were observed in cervical and thyroid cancer [10, 11], nevertheless, decreased expression in prostatic, pancreatic and gastric cancer [12, 13]. Meanwhile, abnormal expression of miRNA-146a also was verified in kinds of cancer cell lines [14, 15].
experiment, miRNA-146a deletion led to the regulation disturbance in NF-κB signal pathway, which further resulted in the genesis and progression of myeloid malignant tumors [16]. Some other researchs on miRNA-146a in vitro showed that high level of miRNA-146a may have inhibition of proliferation and inducing apoptosis for cancer cells by affecting NF-κB signal pathway [14, 17, 18]. Nowadays, the study on miRNA-146a mostly focused on single nucleotide polymorphism [19, 20] and rarely on level of expression and function. It's reported that low level of expression of miRNA-146a was found in tissues of colorectal malignant tumor [21]. In addition, the decreased expression of miRNA-146a in CRCs was associated with clinical stage (I+II, III+IV) and tumor differentiation. The lower-expression of miRNA-146a consists with more advanced clinical stage and poor differentiation. Further conclusion based on above is that miRNA-146a probably inhibits the invasion and differentiation of malignant tumor, and specific mechanism of which is still not certain. But the differential expression of miRNA-146a lacking of organ specificity blurs the clinical value for diagnosis, so that which needs further researches. Besides, our study showed that the decreased expression of miRNA-146a in CRC group was significant compared with the CRA group. However, there was rare relevance with tissue and stool based on further analyses for miRNA-146a expression, it needs further studies if detection results of stool miRNA-146a could distinguish between CRC and CRA or not. Some other research considered miRNA-146a expressed higher in CRC group [22], which was inconsistent with ours, and the reason may be the different origin of samples. Meanwhile, our study suggested the decreased expression of miRNA-146a in CRC group compared with the healthy controls was not statistically significant and irrelevant to clinical features. Up to the present, conclusion above had not been reported temporarily and need to be testified in further large sample studies.

At present, the most commonly used screening tests for CRCs are colonoscopy, fecal occult blood tests, fecal DNA, detection of serum CEA and CA-199, and fecal immunochemical tests. However, there was a lack of a low-cost, noninvasive screening method that has high sensitivity and specificity. Colonoscopy is invasive operation, which confines its application. However, other choices above also have its own limitation such as low specificity and sensitivity [23, 24]. ROC curve analysis showed that the stool miRNA-21 and miRNA-146a has high sensitivity and specificity to distinguish CRC and CRA. Therefore, stool miRNA-21 and miRNA-146a could contribute to early detection of colorectal neoplasms. In comparison with the invasive operation, such as colonoscopy, stool miRNA detection owned advantages of good compliance, easy collection, noninvasive and relatively high specificity and sensitivity and was used hopefully for early screen of colorectal tumor. Unfortunately, addition of miRNA-146a did not improve the differential power of miRNA-21 in discriminating CRC, CRA and healthy controls. Nevertheless, as the published researches, combined two or more miRNAs revealed a significant improved diagnostic efficiency. Hence it's hopefully to improve diagnostic sensitivity and specificity with screening more kinds of miRNAs and performing combined detection. Although differential expression of stool miRNAs possesses great value for diagnosis of colorectal tumor, there are certain limitations as the early-diagnosis biomarker. Previous studies have described the level of miRNAs in solid cancers, such as colorectal cancer, breast cancer, lung cancer, etc [25-27] and emphasized the organ and disease specificity regarding miRNA as independent biomarker. So it's worth to be questioned that the expression level of stool miRNA-21 and miRNA-146a is whether only associated with colorectal tumors themselves or the outcome responded by host immune system existing universally in the progression of all kinds of tumor. Our study discussed miRNA-21 and miRNA-146a expressed in each stage of colorectal tumor development, including CRA, stage of I+II and III+IV of CRC. If research will take the expression level of stool miRNAs in postoperative patients into account simultaneously, it will more powerfully demonstrate the great value of stool miRNAs for colorectal diagnosis. In addition, considering our specimen selected from the same area and race as well as small sample size, the diagnostic value needs further amounts of experiments and clinical practices to verify.

In a word, although detectable rate of colorectal tumor increases slightly, the present specif-
ic biomarker for early diagnosis of colorectal tumor is still unsatisfied. Our study showed miRNA-21 and miRNA-146a was of great value for early diagnosis, however further researches need to be done before the application of miRNA-21 and miRNA-146a detection to clinical practice.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Zibin Tian, Department of Gastroenterology, The Affiliated Hospital of Qingdao University, Qingdao 266003, Shandong Province, P. R. China. Tel: 86-532-82911302; Fax: 86-10-82911611; E-mail: tianzb@qdumh.qd.sd.cn

References

[20] Heo DS, Kim CW. MicroRNA-146a downregulates NF-kappaB activity via targeting TRAF6 and functions as a tumor suppressor having
MicroRNA in colorectal neoplasms

Associations of Single Nucleotide Polymorphisms in miR-146a, miR-196a, miR-149 and miR-499 with Colorectal Cancer Susceptibility. Asian Pac J Cancer Prev 2014; 15: 1047-1055.


